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EDITORIAL

DECISION-MAKING PROCESS AS GUIDE TO THE MANAGEMENT OF NON-CARIOUS CERVICAL LESIONS WITH AND WITHOUT PAINFUL SYMPTOMATOLOGY

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The aim of this study is to develop a decision-making path for the management of non-carious cervical lesions (NCCLs) associated with or without dentin hypersensitivity (DH). This will allow to limit or delay invasive approaches identifying the causes that produced them. The need for this review is because there are no clear guidelines in the current literature for the treatment of NCCLs. Usually, the selection of the best therapy option is postponed to clinical judgment which can be influenced by a patient's demands (aesthetic, symptomatologic reasons or worsening of pre-existing NCCL). To establish a therapeutic plan the young dentist should be able to distinguish the NCCLs that need to be monitored over time from those in need of early treatment. Indeed, the experience of the dentist and the compliance of the patient play a decisive role for the success of the therapy.

An electronic search was conducted in the PubMed and MEDLINE databases with different combination of the following search terms: non-carious and non-carious cervical lesions, treatment, restoration, therapy, management, rehabilitation. The “OR” and “AND” operating factor were used for the search. The analysis was restricted to articles written in English and to the review as article type. Only studies that addressed the treatment of dentinal hypersensitivity and treatment strategies of non-carious cervical lesions were selected. A total of forty-four studies were included in this review.

Non-carious cervical lesions (NCCLs) involve the cervical third of the dental crown with loss of hard tissue. These lesions are identified in very high percentages (60-70%) in patients aged 20-64 years and it is a common idea that the number and expression of

cervical lesions increase with the age of the subjects (1-3). Moreover, the cervical area of buccal surfaces of canines and premolars of both arches represents the most commonly affected site (4).

According to the literature it is not possible to identify a unique causal factor, but the authors seem to agree on the assumption of NCCLs being a multifactorial condition unrelated to carious disease and with unclarified etiology.

The aetiology of tooth surface cervical lesions is usually a product of more than two mechanisms working synergistically, sequentially or alternately. Abrasion, erosion and possibly abfraction have all been associated with the genesis of NCCLs; however, abrasion seems to be the most cited among the causal factors. Depending on the prevalence and intensity of one of the causal factors there

Key words: NCCL; cervical lesions; dentine hypersensitivity; dental pain

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will be clinical lesions of different morphology (5, 6). This cervical enamel wear might be associated with several outcomes: dentine hypersensitivity (DH), plaque retention, caries incidence, structural integrity, and pulp vitality (5, 7). In particular, DH is a clinical discomfort described as pain. The preservation of teeth in absence of any other clinical symptomatology throughout the life of the patient represents a unique challenge for the dentist.

NCCLs and dentine hypersensitivity

Often NCCLs are associated with dentine hypersensitivity (DH), but not all DHs are expressions of NCCLs, and not all NCCLs are DH-related (7). An international workshop describes DH as follows: "Dentine hypersensitivity is characterized by short, sharp pain arising from exposed dentin in response to stimuli, typically thermal, evaporative, tactile, osmotic or chemical and which cannot be ascribed to any other dental defect or pathology" (8).

The hydrodynamic theory explains the link existing between the NCCLs and DH: the painful symptomatology advances when, after the loss of the enamel, there is a patency of the dentinal tubule that determines a variation of intratubular fluid pressure due to the dripping of this fluid into the oral cavity and the production of new liquid at the same time. However, dentin exposure, even if a necessary condition, it is not sufficient to achieve the painful situation that will result only from a disequilibrium between the intensity of causal factors and the responsive ability of the host (9). In fact, when etiologic factors act slowly with non-aggressive chronic effects, the pulpo-dentinal complex can organize the reparative events with production of sclerotic dentin that can lead to the reduction of the lumen of dentinal tubules until their occlusion. In this case, the NCCL is painless and, if it does not affect esthetics, the diagnosis could also be occasional. On the other hand, if the etiologic factors act rapidly and deeply and/or in additive manner no time will be available to set up a dentin reparative defense, and painful symptomatology will be the main reason to require a dental visit. For this reason, not all the exposed dentins are responsible for DH, the choice to add a mechanical barrier does not always represent

the most appropriate solution for all cases of NCCLs.

It is not questionable that the most appropriate treatment plan for the patient is the identification and elimination of causes (causal therapy) with the help of compliance from the patient. On the other hand, the restoration treatment, that should be seen as a therapy to recover what has been lost, has been extensively discussed in the literature, suggesting different procedures to treat NCCLs, therefore today the choice of the best strategy to pursue is postponed to clinical judgment. Rocha et al. (10) proposed that the application of an adhesive system in a frictional mode and the mechanical removal of dentin surfaces with a bur (with further loss of hard tissue) improved retention rates of resin composite restorations in NCCLs, however, according to the author, the clinical evidence was insufficient to support this conclusion over longer-term follow-ups as the majority of included studies evaluated the outcomes only up to 24 months.

Regarding the long-term results, Scotti et al. (11) emphasized that the experience of the operator and the type of adhesive used were basic requirements for the long-term success of composite restorations for NCCLs and to avoid recurrent caries, discoloration of margins and detachment of restorations.

Regarding the dentine hypersensitivity treatment of non-carious cervical lesions, the meta-analysis performed by Moraschini et al. underlined that dentinal tubule occlusion and nerve desensitization provided the best outcomes for treatment of dentine hypersensitivity both for in-office and for at-home treatments. The results suggested that among in-office treatments, dentinal tubule occlusion (whether chemical or physical) and nerve desensitization provide the best outcomes for treatment of dentin hypersensitivity. For at-home treatments, only chemical occlusion of dentin tubules and nerve desensitization showed a greater treatment efficacy than placebo and the difference was statistically significant (12).

On the same topic, the longitudinal *in-vivo* study performed by Lopes et al. tried to evaluate the effectiveness of dentine hypersensitivity treatments by applying laser-therapy of different strengths and doses and used alone or in conjunction with Gluma

Desensitizer. Up to the 18-month evaluation, the study results showed no statistical difference among the several protocols applied (13).

The decision-making strategies

The first necessary step to organize a decision-making algorithm for NCCL therapy is to identify the diagnostic process to detect the etiologic factors. Other factors should also be taken into consideration, such as the shape of the lesion and understanding the needs of the patient. The patient may complain of the painful symptomatology and, in this case, the dentist should be sure that the NCCL is responsible for the referred pain and exclude that it is an aesthetic discomfort which negatively influences the lifestyle of the patient. In some cases, the NCCL may result as an occasional finding. In addition, also the diversity of therapeutic solutions proposed in the literature may influence the clinical decision (5, 14).

NCCL can be diagnosed through detailed medical history, presumptive diagnosis, or by visual

and tactile examination, and clinical diagnosis. The latter involves the examination of the buccal, lingual and palatal surfaces of all teeth by a probe tip under magnification. The tip of the probe must be held in a vertical position, slightly inclined to touch the surface of the tooth and advanced forward the gingival sulcus, until reaching the bottom, beyond the cemento-enamel junction (CEJ). If the probe detects some irregularity at the cervical enamel and at the CEJ it is considered an NCCL (9).

To help the dentist identify the possible etiological factors, a detailed anamnesis should be combined with some questions regarding the dietary and behavioral habits of the patient, such as:

- drinking citrus juices, wine, carbonated drinks and sugary drinks;
- holding the above drinks in their mouth for longer;
- consuming highly acidic foods such as fruit or yogurt;
- oral hygiene habits (incongruous tooth brushing,

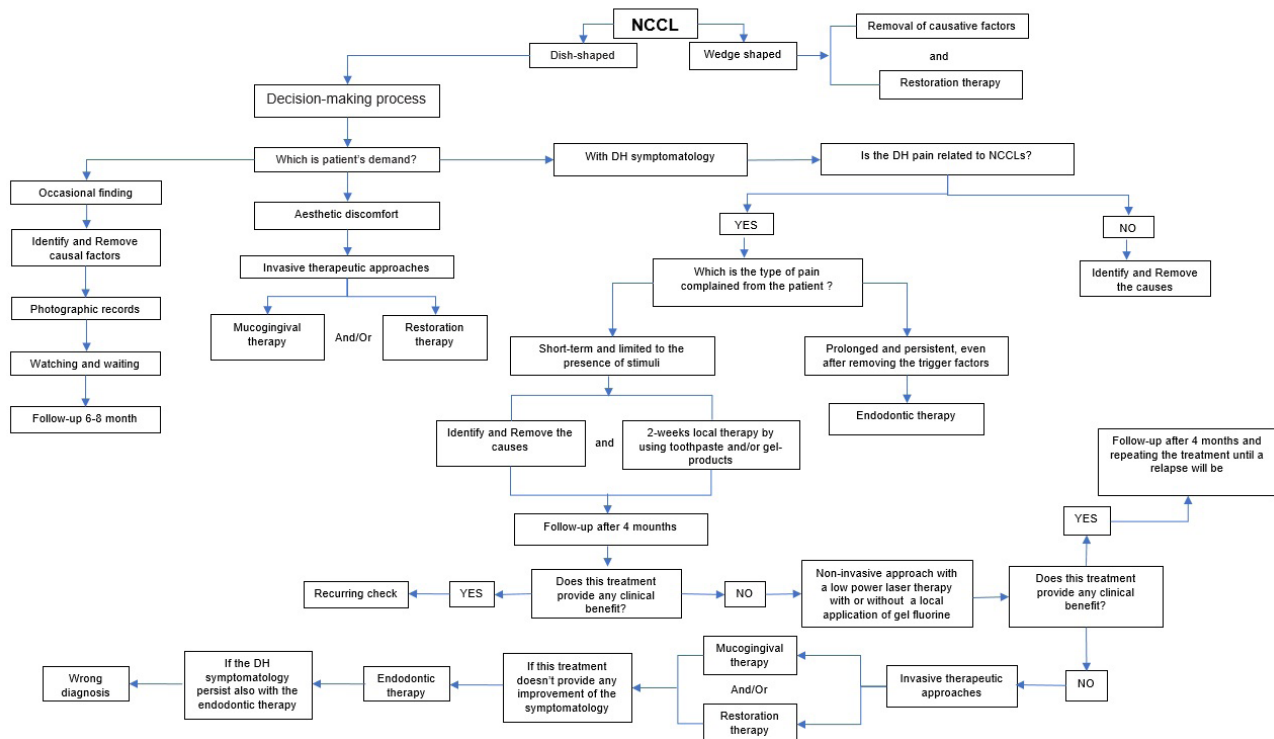


Fig. 1. Flow chart for a decision-making process of NCCL therapy with and without DH.

tooth brushes with hard bristles, excessive pressures and excessive scrubbing around the cervical areas);

- usual consumption of drugs that may influence the salivary swab power or that may reduce salivation;
- acid reflux by gastro-esophageal reflux disease or provoked vomiting (15).

In the presence of NCCL diagnosis, associated or not with DH symptomatology, the compliance of the patient is mandatory for the success of any treatment. Moreover, the patient should be advised to change his/her usual dietary habits and lifestyle.

In dentistry, as well as in medicine, the rule of aiding the physiological processes of repair by limiting or delaying restoration procedures may be also valid in this instance. According to this philosophy, many authors think that the most valid choice is the "Watch and Wait" approach and delay the restoration intervention (16). Several factors influence the choice of the best therapeutic approach for each single case, such as the NCCL shape. In presence of narrow, deep and wedge-shaped cervical lesions (recalling abrasive etiological factors), the ideal area for bacterial biofilm difficult to remove by means of the usual oral hygiene maneuvers, the removal of causative factors and initiation of a dental restoration therapy is required. In the presence of large cervical lesions with a smooth appearance and a concave bottom (recalling erosion etiological factors), a multistep approach needs to be planned to achieve a restorative outcome (6, 17).

The preferences of the patient are another important factor that influences the choice between an early restoration treatment and "Watch & Wait" approach. In fact, the restoration approach should be preferred if the reason for the visit of the patient is due to aesthetic discomfort; differently if the diagnosis of NCCLs is an occasional discovery during a visit required for other reasons.

If the visit is due to painful symptomatology linked to dentine hypersensitivity, it is necessary to firstly assess the clinical evidence of NCCL and to rule out other pathologies or conditions that could support the referred pain, such as dental fractures, carious lesions, reduction of vertical size of dental crown

wear, palate-gingival grooves, periodontal pockets, gingival inflammation and post-bleaching sensitivity. Once ascertained that the dentine hypersensitivity pain is related to NCCLs, the type of pain should be investigated. If the pain is prolonged and persistent, even after removing the trigger factors, it would be advisable to address the decisional plan towards the endodontic therapy; on the other hand, if the pain is short-lived and restricted only to time of stimuli (tactile, thermal or chemical), lifestyle habits need investigating and suggestions on how to prevent pain by modifying and changing negative dietary and/or lifestyle habits as well as how to manage eventual concomitant orthodontic treatments (18-21). Moreover, the treatment option varies depending on the localization of the dentin cervical exposure. The NCCL localized only on specific teeth requires local therapy that must be performed in-office while, if the lesions have a generalized involvement, both in-office and at-home therapy must be performed (7).

Moreover, a two-week local therapy is required to control the hypersensitivity symptoms and different local products with different activities can be used: fluorine or casein phosphopeptide-amorphous calcium, phosphate in toothpaste which promotes the occlusion of the dentinal tubule avoiding dripping of the fluid; desensitizing agent such as GLUMA[®], containing hydroxyethyl methacrylate and glutaraldehyde, that promotes the seal of dentin tubules by coagulation of the dentin fluid proteins within the tubules; potassium nitrate paste used to block the neural transmission to the pulp, or alternatively using associated activities of the combination of toothpaste and in gel products (22).

To ensure long-term stability of the effects of repair therapy for DH associated with NCCLs, the preventive management program includes follow-up every four months and repeating the same therapy protocol if there is a DH recurrence. If during follow-up the symptoms disappear, thus improving the quality of life of the patient, no further treatment will be required, but only regular checkups. However, if the symptomatology remains unchanged with no pain relief or a worsening of the quality of life of the patient, a more advanced treatment for DH in-office must be initiated (23).

In this last case, a non-invasive approach using low power laser therapy is recommended. It can be used alone to further the formation of reparative dentin or in association with a local application of gel fluorine to further allow a major and deeper precipitation of calcium fluoride in the dentinal tubule. If this treatment provides a clinical benefit, a follow-up is planned every 4 months and the treatment can be repeated; if there is a relapse, any in-office procedures must be followed by the use of desensitizing toothpaste twice a day as at-home procedure (9, 24). If a non-aggressive approach does not provide an adequate symptomatic advantage or long-term improvement, a more invasive procedure has to be preferred after appropriate advanced diagnosis. Mucogingival surgery and/or dental restoration could be the most appropriate therapeutic choices for NCCLs not responsive to non-invasive approaches. These therapeutic solutions could represent the first therapeutic choice for NCCLs without DH, but responsible of aesthetic discomfort. Only in extreme cases, when the symptomatology persists or even increases after the restorative procedures, endodontic therapy is preferred.

If these therapeutic solutions resolve DH symptomatology, the patient should be checked up every 4 months, controlling the compliance of patients to an adequate lifestyle which should prevent further problems. In the eventuality that endodontic therapy does not make any improvement for the painful symptomatology experienced by the patient, an advanced diagnosis excluding pain reflex, periodontal pain or atypical facial pain will need to be reconsidered (Fig.1) (7, 14).

CONCLUSIONS

The management of NCCLs can be extremely challenging for the dentist, because the choice of the best therapy must depend on the clinical judgment of the treating clinician. The success of the therapy is strongly influenced by a careful assessment of the etiological factors from the medical history, by the level of experience of the dentist and the compliance of the patient who should be educated on how to adopt an appropriate.

Restoration therapy is not always the most suitable solution to NCCL resolution, therefore to establish a therapeutic plan, the neophyte dentist should be able to distinguish the NCCLs that need to be monitored over time while avoiding further tissue loss with the aggravation of clinical symptoms or the genesis of iatrogenic damage from those that are associated with DH symptoms and need early treatment. The aim of this paper is to establish a flowchart for a simple decision-making process intended for NCCL therapy and the treatment of DH correlated to NCCL. The flowchart will help the neophyte dentist in the etiological diagnosis of NCCLs and in the choice of the most appropriate therapeutic strategy for each single case.

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EDITORIAL

MESENCHYMAL STEM CELLS AND IL-37: A POWERFUL COMBINATION

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Mesenchymal stem cells (MSCs) are able to exert immunomodulatory and anti-inflammatory actions. Thanks to these properties, MSCs may be a promising alternative approach for the treatment of inflammatory disease. Important cytokines involved in inflammation are those included in the IL-1 family. Interleukin-37 (IL-37) is one of the member able to suppress both innate and adaptive immunity. Recently, it was found that MSCs and their derivatives can modulate IL-37, and MSCs expressing IL-37 seem to have an enhanced therapeutic efficacy.

Inflammation starts as a protective response in the organism, acting in host defences against infectious agents and injury, but it is also involved in the pathophysiology of different chronic disorders. In this way, cells of the innate and adaptive immune system and inflammatory mediators participate in acute and chronic inflammation involved in the development of different diseases, including neurodegenerative disorders (5).

Interleukin (IL) 37 was discovered and characterized recently as an immunosuppressive factor (1). IL-37 is a member of the IL-1 family, the members of which participate, with different activities, in the modulation of innate and adaptive immune responses and inflammation. Indeed, the members are divided into effectors and regulators on the basis of the IL-1 consensus sequence and the

signaling receptor chain. In particular, among the 11 members, IL-37 is an anti-inflammatory cytokine, whereas IL-1 α , IL-1 β , IL-18, IL-33, IL-36 α , IL-36 β , and IL-36 γ possess stimulatory activity. On the contrary, IL-1Ra, IL-36Ra, and IL-38 act as receptor "antagonists" (2). IL-37 can inhibit excessive immune responses, specifically, it is capable of suppressing both innate and adaptive immunity in different ways. IL-37 can suppress the innate immune response through the reduction of pro-inflammatory cytokine production, such as IL-6, IL-8, TNF- α and IFN- γ , and the other members of the IL-1 family, including IL-1 α , IL-1 β , IL-1Ra and IL-37, can also inhibit chemokines (1). IL-37 is also able to inhibit inflammasome activation (2). Furthermore, IL-37 can inhibit dendritic cell functions and reduce the inflammation mediated by T cells (1).

Key words: mesenchymal stem cells; IL-37; inflammation

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IL-37 expression increases after stimulation with LPS, different TLR ligands and pro-inflammatory cytokines (1, 3). The IL-37 anti-inflammatory activity depends on the inhibition of pro-inflammatory pathways, such as STATs, p38 MAPK and NF- κ B (1). Moreover, IL-37 binding to IL-18R α inhibits immune response and allergic inflammation (4).

Given its activity, IL-37 plays a role in autoimmune and inflammatory disorders, as demonstrated by its altered expression in different diseases such as inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis and psoriasis (6).

Mesenchymal stem cells (MSCs)

MSCs are able to exert immunomodulatory and anti-inflammatory actions; for this reason, they may be a good approach for the treatment of different disorders. MSCs are a class of adult multipotent stem cells with self-renewal capability. According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy the minimal criteria to define human MSCs are: (i) MSCs are plastic-adherent in standard culture conditions; (ii) MSCs must express CD105, CD73 and CD90, while they are negative for CD45, CD34, CD14 or CD11b, CD79 α or CD19 and HLA-DR; (iii) MSCs must be able to differentiate toward osteoblasts, adipocytes and chondroblasts in *in vitro* culture (7). However, MSCs, other than toward the mesodermal lineage, may differentiate also toward ectoderm lineage, including neurocytes, and endoderm lineages. MSCs may be isolated from many sources such as bone marrow, adipose tissue, dental tissues and umbilical cord (8).

The therapeutic benefits exerted by MSCs depend on their multidifferentiation capacity, but are also due to the protective factors that they are able to produce, known as *secretome*. Indeed, MSCs are able to secrete different molecules that included cytokines, chemokines, lipid mediators, growth factors, as well as exosomes and microvesicles. The secretome is known to be able to exert protective effects, including anti-apoptotic, anti-inflammatory, and immunomodulatory effects (9, 10). The anti-inflammatory cytokines secreted by MSCs include TGF- β , IL-10, IL-1Ra, but MSC secretome may

vary, based on the microenvironment during *in vitro* culture (10, 11). Thanks to the release of molecules that exert immunoregulatory and anti-inflammatory functions, MSCs may modulate different cell types in the innate and adaptive immune systems. In particular, MSCs may suppress the proliferation and actions of T and B lymphocytes, natural killer and dendritic cells; however, the proliferation of regulatory T cells may be induced (12).

MSCs and IL-37 combination

A novel therapeutic strategy against inflammatory disease may be to combine the properties of MSCs and IL-37, improving their efficacy. Zhao et al. found that umbilical cord-derived mesenchymal stem cells (UCMSCs) expressed the isoform d of IL-37 (13). Wang et al. (14) evaluated the administration of MSCs transduced with an adenovirus vector expressing IL-37b (MSC-IL37b) in mice with colitis induced by dextran sulfate sodium (DSS). In these mice the intraperitoneal administration of MSC-IL37b significantly reduced the loss of body weight and the histological score. However, the histological scores of the MSC-administered mice were also significantly lower compared to those of the PBS-treated mice. In addition, colon length was significantly longer in MSC-IL-37b-administered mice compared to the other groups that were treated with PBS, MSCs or MSCs with the control adenovirus not expressing IL-37. MSC-IL37b transfection augmented the percentage of regulatory T cells (Tregs) among splenic CD4⁺ T lymphocytes compared to all the other groups, but also MSC-treated mice showed a significant difference compared to mice treated with PBS. MSC-IL37b increased also the percentage of myeloid-derived suppressor cells (MDSCs) among total splenic mononuclear cells compared to mice treated with PBS and MSCs with the control adenovirus not expressing IL-37, but not compared to those treated with MSCs. Furthermore, MSC-IL37b transplantation was able to increase the IL-2⁺ cells among splenic CD4⁺ T cells, while reducing the IFN- γ ⁺ cells compared to all groups except mice treated with MSCs. In conclusion, IL-37b improved the anti-inflammatory effects of MSCs in mice with colitis through the increase of the percentage of

Tregs and MDSCs and modulating the production of cytokines. MSCs shows a partial activity, but IL-37 and MSCs seem to act in a synergistic way (14).

van Geffen et al. (15) studied the protective effect of IL-37 on MSC-based cartilage formation in a microenvironment that mimics the one present in osteoarthritis (OA). In this study, MSCs were transduced with IL-37, in order to induce its overexpression, and after 10 days of chondrogenic differentiation, MSCs were treated with IL-1 β or OA synovium-conditioned medium (OAS-CM) in order to induce a pro-inflammatory microenvironment. The results showed that both IL-1 β and OAS-CM significantly decreased the amounts of sulfated glycosaminoglycans in differentiating MSCs, but IL-37 increased their content. Moreover, IL-37 significantly reduced the expression of pro-inflammatory cytokines such as IL-1 β , IL-6, and IL-8, but also matrix metalloproteinase 3 (MMP3) gene and protein expression in IL1 β - and OAS-CM-treated cells. IL-37 induced an increase of Col2:Col1 ratio, indicating an improvement of hyaline cartilage formation. All together these results indicated that IL-37 improved stem cell-based cartilage repair (15).

Other reports showed that administration of MSCs or of their secretome may increase the release of IL-37. Umbilical cord blood MSCs (UCB-MSCs) showed immunomodulatory properties in lipopolysaccharide (LPS)-induced inflammation in macrophages. In particular, UCB-MSCs were co-cultured with phorbol 12-myristate 13-acetate (PMA)-activated human THP-1 cells and UCB-MSCs enhanced the LPS-stimulated expression of IL-10 and IL-37 through the PI3K/Akt pathway (16).

Recently, our group evaluated the ability of conditioned medium (CM) derived from human periodontal ligament stem cells (hPDLSCs) cultured under hypoxic conditions (H-hPDLSCs-CM) infused intravenously to improve the progression of experimental autoimmune encephalomyelitis (EAE). The pre-conditioning of the cells in hypoxic condition was reported to enhance their protective actions (11). H-hPDLSCs-CM treatment reduced the clinical and histologic disease score, reducing the inflammatory cell infiltration and demyelination. Moreover, in EAE mice treated with H-hPDLSCs-

CM the increased expression of IL-37 was associated with the suppression of pro-inflammatory cytokines. As a consequence, the regulation of oxidative stress, autophagic, and apoptotic markers was found in mice with EAE treated with hPDLSCs-CM (17).

CONCLUSION

These data indicate that MSCs and IL-37 may act synergistically to reduce inflammation. Recent studies also indicate the ability of MSCs and their derivatives to induce IL-37 production, indicating another explanation of the anti-inflammatory and immunomodulatory effects of MSCs. For these reasons, MSCs and IL-37 may represent a new strategy to counteract inflammatory-based diseases.

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DIFFERENTIAL EXPRESSION OF TOLL-LIKE RECEPTOR 13 AND RIBOSOMAL PROTEIN L29 IN INFLAMMATORY LUNG AND BRAIN

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Viral dsRNA acts as the paramount pathogen-associated molecular pattern on infection and orchestrates inflammation or immune cascades of the host's tissues. The comparative effects or mechanisms of inflammation or immunity in different organs on viral infections are critical in immunology or virology. To outline the organ-based molecular mechanisms of inflammation or immunity on viral infection, we challenged mice with the viral mimic poly(I:C) and quantified inflammatory cytokines Il-1b and TNF- α in the brain and lung tissues. As cytokines showed differential expression, transcriptome screenings of mouse lung and brain tissues were analyzed. We identified 629 differentially expressed genes (DEGs) in lung and 137 DEGs in brain tissues with a few overlapping genes. Most of those DEGs were interferon-stimulated genes (ISGs) that are involved in the anti-viral defense mechanisms. The expression patterns of viral dsRNA stimulated genes, and consequently, their association with different molecular mechanisms of inflammation and immunity were specific to the organs. The effects of viral mimic were higher in the lung than in the brain in terms of the number of DEGs and ISGs. Interestingly ribosomal protein L29 (Rpl29), a cell surface heparin-binding protein, was upregulated in the brain and downregulated in the lung. The contrasting expression of Rpl29 gene might be responsible for tissue-specific inflammatory responses in lung and brain tissue on virus infection. In addition, the upregulation of Tlr13, a dsRNA and bacterial 23s rRNA receptor, in the poly(I:C)-stimulated mouse lungs suggests its important role in lung inflammatory responses. It is likely that the combined effects of these genes orchestrate the organ-specific inflammatory or immune responses. Our findings would be beneficial to explore new insights in inflammation and immunity against many critical viral diseases.

Viruses can cause numerous lung and brain diseases, and exacerbate inflammatory conditions of various diseases including asthma and chronic

obstructive and pulmonary diseases. Some viruses encompass double-stranded RNA (dsRNA) either as genetic materials or as intermediates during RNA

Key words: inflammation; immune response; RNA sequence; poly(I:C); mouse

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synthesis in the replication cycle (1). Viral dsRNA is recognized in host cells as a pathogen-associated molecular pattern (PAMP) by different cell surface or cytosolic pattern recognition receptors (PRRs) and activates the inflammatory and immune system upon infection (2). Toll-like receptors (TLRs), which are multi-dynamic receptors having the leucine-enriched portion and essential for recognition of a large extent of ligands, can regulate cell signaling via connecting different related receptors using different adaptor proteins (3). The viral dsRNA stimulates interferons (IFNs) and IFN-stimulated genes (ISGs) via activating TLRs and other PRRs including melanoma differentiation-associated gene 5 and retinoic acid-inducible gene-I (RIG-I) (1). 2'-5'-oligoadenylate synthetase (OAS) stimulates latent RNase upon recognition of the foreign RNA, which slices viral or host RNA and thereby inhibits viral replication and produces additional pathogen (PAMPs) RIG-I like receptors (RLR) (4). PRR signals are transduced for transcription factor activity through mitochondrial antiviral-signaling protein (MAVS) and triggers phosphorylation of IFN regulatory factors 3 or 7 (IRF3/7), or phosphorylation and ubiquitination of κ B inhibitor (1). Phosphorylation, dimer formation, nuclear translocation, binding and interaction of IRF3/7 or NF- κ B to specific promoters trigger inflammatory cytokines, IFNs and some of the ISGs expression (5). These inflammatory cytokines, IFNs, and ISGs primarily orchestrate viral infection-induced inflammation or immunity.

Poly(I:C) shows stimulatory effects on the organism's immune system as a viral infection and has been used as an immune stimulant or viral mimic in different *in vitro* or *in vivo* studies (6). TLR3, melanoma differentiation-associated gene 5 and RIG-I activation by poly(I:C) stimulates a lot of signaling molecules which in turn activate NF- κ B and IRF3 pathways in different cells (7). The microRNA-22 is overexpressed in glial cells upon Japanese encephalitis virus (JEV) infection or poly(I:C) treatment and plays anti-inflammatory roles by reducing IFN- β , IL-1 β or TNF- α through decreasing NF- κ B or IRF3 activities targeting MAVS (7). Poly(I:C) downregulates microRNA-22 expression in endothelial cells and

its mimetic upregulation results in inflammation in both endothelial cells and mouse lung targeting endothelial barrier protein VE-cadherin (8). Lung bronchial and alveolar endothelial and epithelial cells possess higher TLR3 that are further induced upon viral infection (9). As an immune-privileged site, the brain shows a lower response to pathogens (10). Therefore, we hypothesized that inflammatory and immune responses of different organs to viral dsRNA might be different and tissue-specific.

Numerous studies on viral infections have been carried out to elucidate molecular mechanisms orchestrating inflammation and immune response, however, the majority emphasized on a specific pathway or molecular mechanism. RNA sequencing, a modern technology, allows us to investigate different molecular mechanisms in cells or organs with unprecedented details. Besides these, studies on the comparative immunization effects of pathogenic dsRNA among different organs are unavailable. Therefore, we investigated gene expression of inflammatory brain and lung tissues of poly(I:C)-treated mice using mRNA sequencing to outline the tissue specificity and the mechanisms of inflammatory or immune responses against viral infection. Our study has produced comprehensive information regarding the expression of genes involved in inflammation or immunity that would be beneficial for many immunology or virology studies.

MATERIALS AND METHODS

Animals

Eight C57BL/6 male mice (7 weeks old) were taken from Laboratory Animal Center, Huazhong Agricultural University, Wuhan, China. Mice were randomly divided into treatment and control groups (n=4 in each) after being maintained for about one week for adaptation and were treated with 100 μ g of poly(I:C) potassium salt (Sigma-Aldrich, St. Louis, Missouri, USA) in 100 μ l normal saline (NS) and 100 μ l NS/mouse respectively through intraperitoneal injection. After the treatment period of 24 hours, the mice were sacrificed and whole brains and lungs were collected following institutional guidelines. The tissues of control and treated mice were flash frozen using liquid nitrogen immediately and stored

at -80°C followed by RNA extraction.

Animal handling and experimental procedures were approved by the Animal Care and Use Committee of Hubei Province, China and carried out in accordance with guidelines developed by the China Council on Animal Care and Protocol. The ethics approval number was HZAHMD-2016-037.

RNA isolation

Total RNA extraction from lung and brain tissues was executed using the reagent RNAiso Plus (Takara Bio Inc. Kusatsu, Shiga, Japan) following the manufacturer's instructions. The RNA concentration was assessed using NanoDrop 2000 (Thermoscientific, Wilmington, DE19810, USA). The quality of RNA was assured by Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA), and RNA having integrity number more than seven was considered as qualified for the next step of the study.

Quantitative reverse transcriptase polymerase chain reaction (qPCR)

Reverse transcription was performed using 1 µg total RNA as a template with PrimeScript™ RT reagent Kit (Cat. #RR047A, TaKaRaFa) according to the manufacturer's protocol. Light Cycler-96 RT-PCR detection system (Roche) was used to perform qPCR using SYBR Premix Ex Taq™ II (Cat. #RR820A, TaKaRa). The information on primers is enlisted in Table I. Expression of a specific gene was standardized to that of the control sample and fold change was calculated using the $2^{-\Delta\Delta C_t}$ method. The qPCR thermal cycles were 95°C for 300 s followed by 45 cycles (95°C for 15 s, 58°C for 30 s) and 72°C for 30 s.

Library preparation and sequencing

Twelve high-quality strand-specific cDNA libraries were constructed as per the manufacturer's recommendations using 1 µl total RNA/sample and RNA Library Prep Kit 'NEBNext Ultra™' for Illumina (NEB, USA). Briefly, Enrichment of mRNA was executed using oligo-T magnetic beads. Following mRNA fragmentation, first stranded cDNA was synthesized using random hexamers within NEBNext First-Strand Synthesis Reaction Buffer (5X). Then, second strand cDNA chain was synthesized using RNase H, DNA polymerase I, buffer fluid and dNTPs and purified with AMPure XP

system (Beverly, CA, USA). The purified double chain cDNA was then subjected to end repairment and addition of adaptor followed by enrichment of the desired fragment size using the ampure XP beads. The cDNA libraries were obtained by PCR enrichment, and the quality of the libraries was assured using the Agilent Bioanalyzer 2100 system. Index-coded libraries of each sample were then clustered on the cBot Cluster Generation System and sequenced on an Illumina platform, which produced PE150bp reads. The sequences and metadata files (twelve files) of our experiment were stored in the Sequence Read Archive (SRA), NCBI (Bio project ID: PRJNA 486617, accession numbers SRR7716754, SRR7716755, SRR7716752, SRR7716753, SRR7716750, SRR7716751, SRR7716748, SRR7716749, SRR7716756, SRR7716757, SRR7716746, SRR7716747).

Differential expression analysis

Quality reads were differentiated by removing lower quality reads, reads with poly-N or adaptor sequences through the software Perl Scripts. Mapping of these quality reads was performed to *Mus musculus* reference genome GRCm38. The reads having a perfect match or only one mismatch were considered for the next step of analysis and annotation based on the mouse reference genome. Hisat2-StringTie- Edge R pipeline was utilized to map reads with the reference genome, assemble transcript and differential expression, respectively. Fragments per kilobase of transcript per million (FPKM) was used to normalize the gene expression. The $P < 0.05$ and $|\log_2 \text{fold change}| \geq 1$ was set as the threshold for significant differential expression.

KOG and GO enrichment analysis

DEGs were merged in Eukaryotic Orthologous Groups (KOG) updated database to compare with paralog and ortholog proteins and to classify them into different clusters (<http://www.ncbi.nlm.nih.gov/KOG>). GO-seq R tool was used for Gene Ontology (GO) annotation and enrichment analysis of DEGs from both the tissues based on Wallenius non-central hypergeometric distribution.

Interferome analysis

We utilized interferome v2.01 (<http://www.interferome.org/interferome>), a database resource of type-I, II and III IFN regulated genes and identified the

ISGs in the DEGs of both the tissues.

KEGG pathway enrichment analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database is a valuable resource to understand the molecular functions in depth and coordination of the different biological systems of the cells, organism or ecosystem from large-scale sequencing data (<http://www.genome.jp/kegg/>). The KOBAS tool was utilized to analyze the KEGG pathway enrichment of DEGs generated in our experiment.

Statistical analysis

Each experiment was performed a minimum three times, having similar results. The GraphPad Software Prism 6 (San Diego, CA, USA) was used to analyze the qPCR results and the values were represented as mean $\pm$ standard deviation. Data were compared with two-tailed unpaired Student's *t*-test. For all tests, $P < 0.05$ was considered significant.

RESULTS

Cytokines Il-1b and TNF- α expression analysis

To observe the inflammatory effect of viral dsRNA on different organs confirmed by many scientists (11, 12), we treated mice with the viral mimic poly(I:C) and analyzed the mRNA expression of Il-1b and TNF- α cytokines through qPCR in the brain and lung tissues. The lung tissues showed much higher expression of Il-1b (3.56-fold) and TNF- α (1.93-fold) than that of brain tissues (1.32- and 1.54-fold, respectively) (Fig. 1 a-d). These results indicated the different inflammatory conditions in these two tissues upon poly(I:C) stimulation. The lung tissues were more responsive and showed higher inflammation than those of the brain on viral infection.

Overview of transcriptome analysis

To explore the molecular mechanisms of viral dsRNA-induced differential and tissue-specific inflammation, the lung and brain tissues of poly(I:C)-treated mice were subjected to transcriptome analysis. Illumina HiSeq Xten Genome Analyzer platform was used to sequence library with paired-end 150 base pair tag ranging depth from 22,109,370

- 40,391,272 read pairs and the sequencing obtained total 346.27 M clean reads or 103.57 Gb quality data with Q30 base percentage was over 89.84% (Table II). Mapping of these reads to the mouse reference genome, *M. musculus* GRCm38 revealed that approximately 40-74 million reads were mapped to reference sequences, accounting for 91.25-95.17% of total reads (Table II). We identified the DEGs using a $P \leq 0.05$, fold change ≥ 2 and $FDR \leq 0.05$ as thresholds. We obtained 629 (236 up and 393 downregulated) DEGs out of 15447 transcripts in the lung (Fig. 2a) and 137 (92 up and 45 downregulated) DEGs out of 14911 transcripts in the brain (Fig. 2b) due to poly(I:C) treatment compared to control.

We also obtained 51 new genes in treated lung and 17 new genes in treated brain tissues with a minimal known function that were expressed differentially in response to poly(I:C). There were 15 upregulated and 05 down-regulated DEGs overlapped between the brain and lung tissues (Fig. 2, c-d). Only one DEG was upregulated in the brain but downregulated in the lung, whereas no DEGs overlapped between the brain downregulated, and lung upregulated DEGs (Fig. 2, e-f). These results indicated that the effect of virus infection is higher in the lung compared to the brain. The majority of genes activated in lung and brain tissues were different and tissue-specific.

Gene functional annotation/KOG and GO enrichment analysis

The KOG class analysis indicated that 372 out of 629 DEGs in the lung and 72 out of 137 DEGs in the brain tissues were involved in different subclasses of four main KOG classes (Fig. 3 a-b). There were 12 genes in the lung, and 3 genes in brain tissues with unknown function expressed differentially. DEGs from both tissues belonged to almost all classes, but in maximum cases, the frequency and rate of DEG assignment varied between the two tissues. Notably, the differences in the number of DEG involvement was markedly higher in transcription, cell division, cell cycle control, chromosome partitioning, defense mechanisms, signal transduction, cytoskeleton, lipid transport and metabolism and posttranslational modification, protein turnover, chaperones KOG classes. The GO classification analysis revealed

that 122 out of 137 DEGs in brain tissues and 577 out of 629 DEGs in lung tissues were associated with 3 GO classes; cellular component, biological process and molecular function (Fig. 4a-b). 101 (70 up- and 31 down-regulated) DEGs of brain and 494 (166 up and 328 downregulated) DEGs of lung tissues were functionally associated with the subclasses of biological process. Although the rate of common DEGs originated from both tissues was only 2.61%, they shared some common and vital GO terms of the biological process including response to virus, defense response to virus, innate immune response and immune system process (Table III). The GO-terms cell cycle, cell division, mitotic nuclear division, adaptive immune response, T-cell receptor signaling pathway, inflammatory response, chromosome segregation, positive regulation of IFN-gamma secretion, neutrophil chemotaxis, and T-cell activation were different between the two types of tissues considering the significant participation of DEGs (Table III). The number of DEGs assigned to different GO subclasses also varied between these two types of tissues. These results confirmed that the viral dsRNA mimic poly(I:C) showed organ-specific effects on inflammation and immune responses in the lung and brain tissue of mouse, and stimulated DEGs are responsible for this organ-specific effect.

Interferome analysis

We merged these DEGs in the interferome database and found that 481 out of 577 in lung and 98 out 122 genes in brain tissue were ISGs. 158 and 28 ISGs were stimulated by IFN type-I, 35 and 9 by type-II and 288 and 61 by both IFN type-I and II in lung and brain tissue, respectively, and no ISGs were stimulated by IFN type-III (Fig. 5a-b). In lung tissue, 35 DEGs were ISGs out of 37 that contributed to the GO-class innate immune response and 3, 3 and 29 genes were stimulated by IFN type I, II and their combined effect, respectively. In brain tissue, all DEGs involved in the same go-class were ISGs, and out of 17, 1 gene was stimulated by type-I and the rest of the ISGs were stimulated by the combined effect of type-I and II IFNs (Fig. 5c-d). These results pinpointed that viral dsRNA-induced physiopathological mechanisms, including inflammation

and immunity, were mainly controlled by IFN type-I and II and respective ISGs.

Pathway enrichment analysis

Pathway enrichment analysis delineated that 238 of 629 DEGs in lung and 52 of 137 DEGs in brain tissue were significantly involved in different KEGG pathways (Table IV). Therefore, it was revealed that the genes stimulated by poly(I:C) were mostly involved in different pathways in the brain and lung and control many pathophysiological mechanisms. Among these pathways, the DEGs of lung tissues were assigned to hematopoietic cell lineage, primary immunodeficiency, T-cell receptor signaling, *Staphylococcus aureus* infection, measles, P53 signaling, HTLV-I infection, cell cycle, cytokine-cytokine receptor interaction, cell adhesion molecules (CAMs) and leukocyte transendothelial migration (Table IV). Whereas, the DEGs of brain tissues were assigned significantly to measles, herpes simplex infection, influenza A, hepatitis C and CAMs pathways (Table IV). Some pathways were similar, but most of them were different between the two tissues. The number and type of DEGs differ within the same pathway also in two tissues. These results also indicated that poly(I:C) is an excellent viral mimetic; its effects are specific to organs and higher in lung tissues.

qPCR validation of RNA sequence data

Six DEGs per tissue were randomly selected (3 up-regulated and 3 down-regulated) for qPCR justification of the RNA sequencing data. The gene expression was normalized with that of the control sample, and Gapdh was used as internal control. The genes Irf7, Tlr13, Itifm3, Chchd1, Rpl29, and Ppara selected from lung DEGs showed differential expression in qPCR with the average log₂fc value of 4.18, 1.40, 1.11, -1.64, -1.09 and -1.00, respectively (Fig. 6a). Similarly, the genes Irf7, Ifitm3, Rpl29, Chchd1, Atpaf1, and Carns selected from brain DEGs expressed differentially in qPCR examination maintaining trend as RNA sequencing with the average log₂fc of 2.808, 1.73, 1.14, -1.67, -1.39 and -1.20, respectively (Fig. 6b). All selected genes were significant to their control in expression ($P \leq$

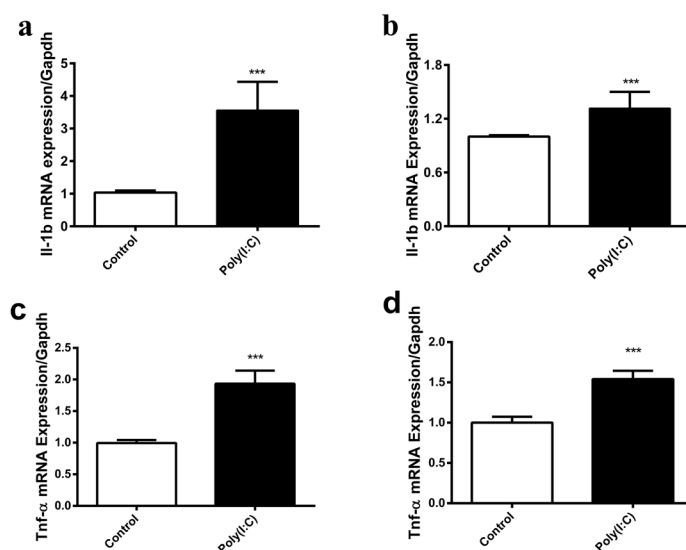


Fig. 1. *Il-1b* and *TNF-α* were upregulated in both poly-treated lung and brain tissues. The relative mRNA levels of cytokines *Il-1b* and *TNF-α* in poly(I:C)-treated lung (**a** and **c**) and brain (**b**, and **d**) tissues compared to those of controls was quantified by qPCR, respectively. *Gapdh* was used as internal control. All qPCR expression data are representative of at least three independent experiments with three replication. (***) $P < 0.0001$.

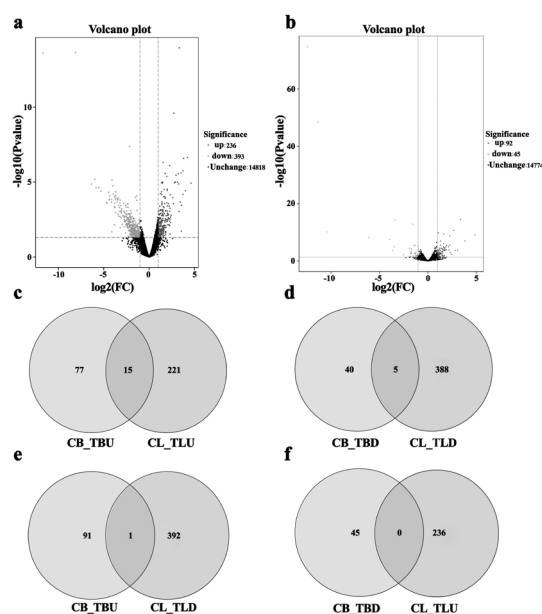


Fig. 2. Differentially expressed genes in lung and brain tissues in response to poly(I:C) treatment. Volcano plot of DEGs in lung (**a**) and brain (**b**) tissues. Each point in the volcano plot represents a gene. The black dots represent non-differentially expressed genes, the dots on the left of the black dots represent downregulated and the dots on the right represent upregulated genes. **c-d**) Venn diagram of overlapped upregulated DEGs (**c**) and downregulated DEGs (**d**) between lung and brain tissue. Venn diagram of overlapped DEGs that were upregulated in brain and downregulated in lung tissues (**e**) and Venn diagram of overlapped DEGs that were downregulated in the brain and upregulated in the lung tissues (**f**). CB_TBU and CB_TBD indicate up- and down-regulated DEGs in brain tissues of ploy(I:C)-treated mice compared to those of controls, respectively; CL_TLU and CL_TLD indicate up- and down-regulated DEGs in lung tissues of ploy(I:C)-treated mice compared to those of controls.

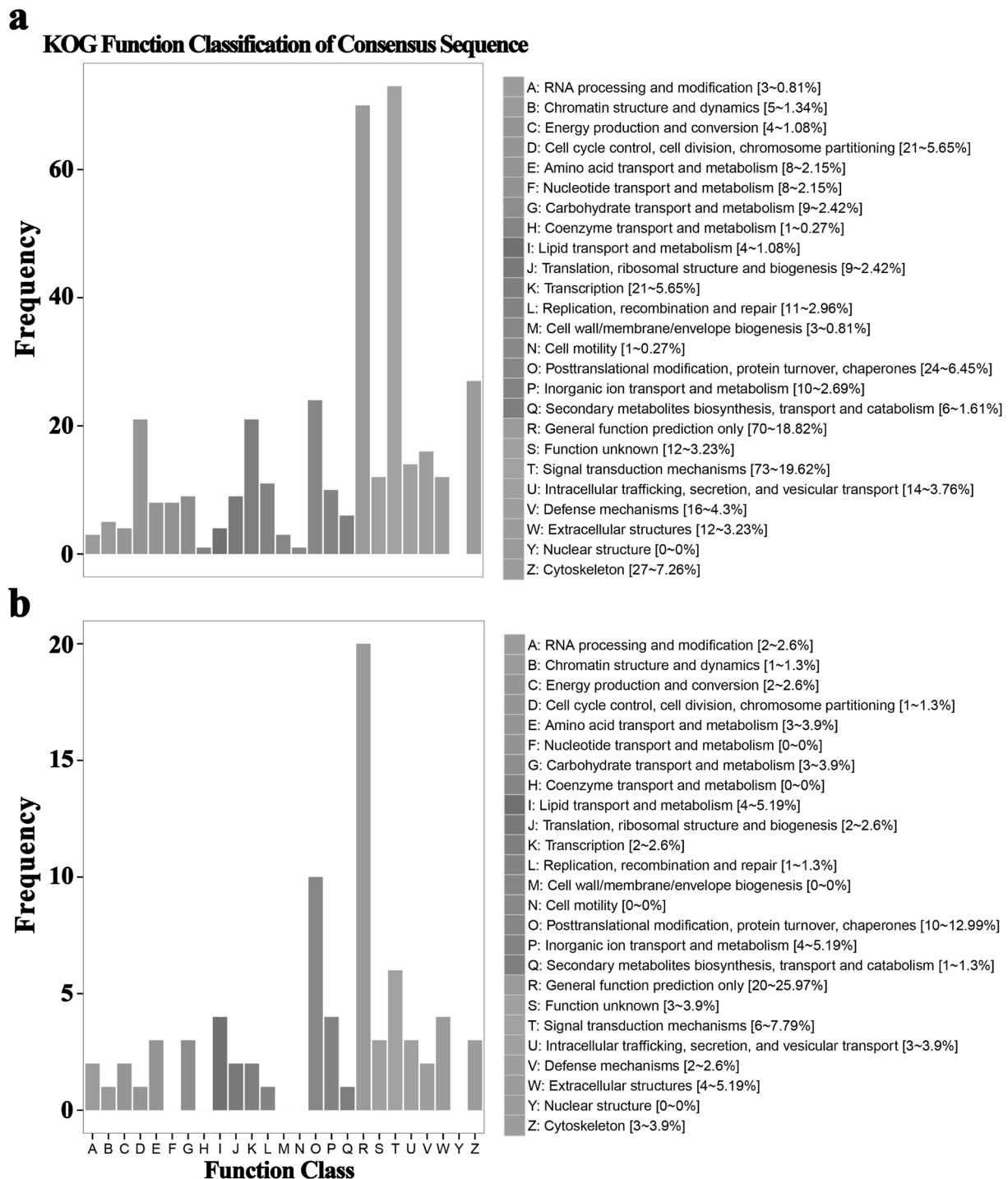


Fig. 3. KOG functional classification of poly(I:C)-stimulated DEGs from lung and brain tissues. Association of DEGs with different eukaryotic orthologous groups in lung (a) and brain (b) tissues. Y-axis represent the number of DEGs and X-axis represent different groups of KOG.

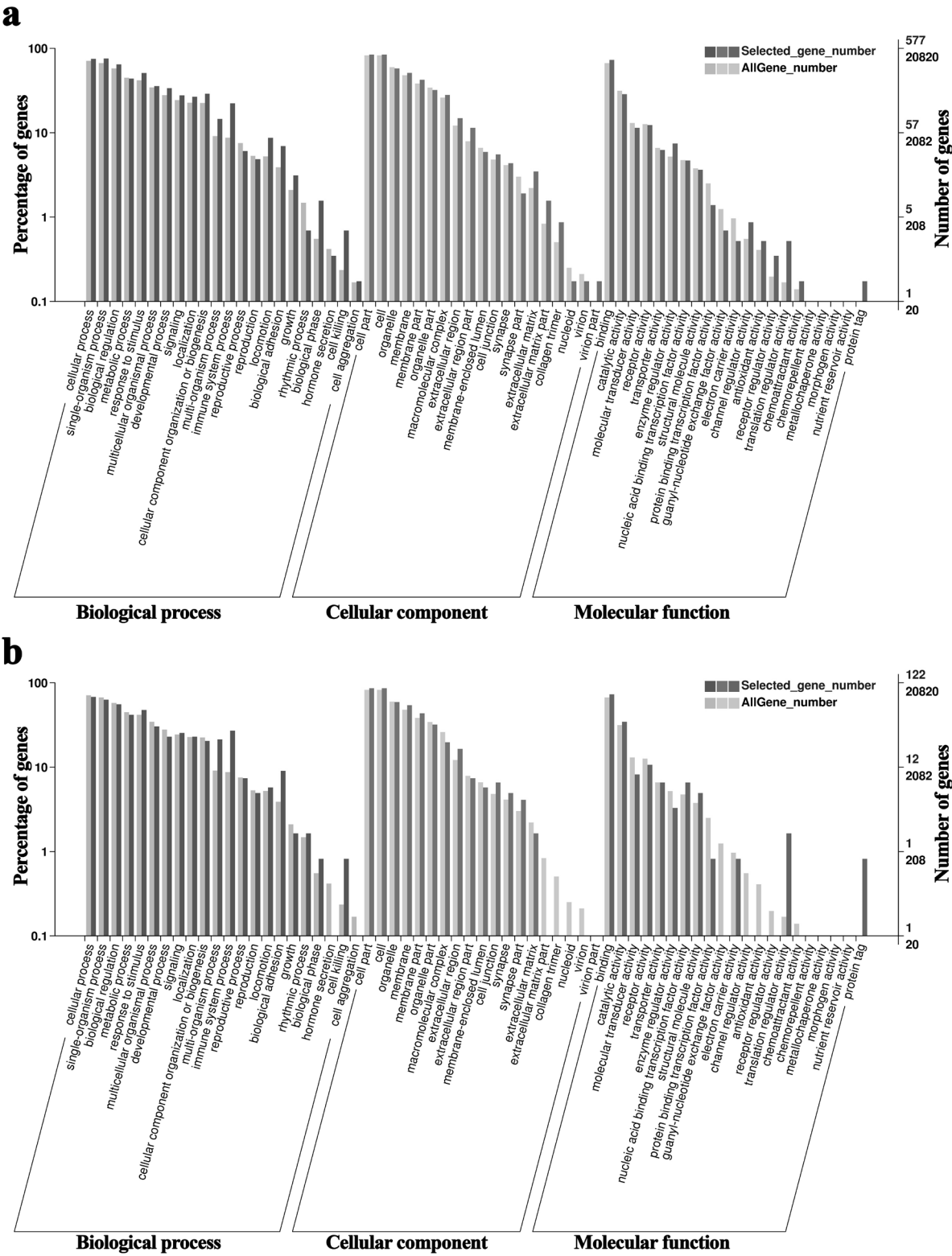


Fig. 4. GO functional classification of DEGs from lung (a) and brain (b) tissue compared to that of controls. Deep color bars represent the selected gene and light color bars represent the all gene of respective GO classes. Y-axis (right) represents the number and Y-axis (left) represents the percentage of DEGs involved in different GO classes.

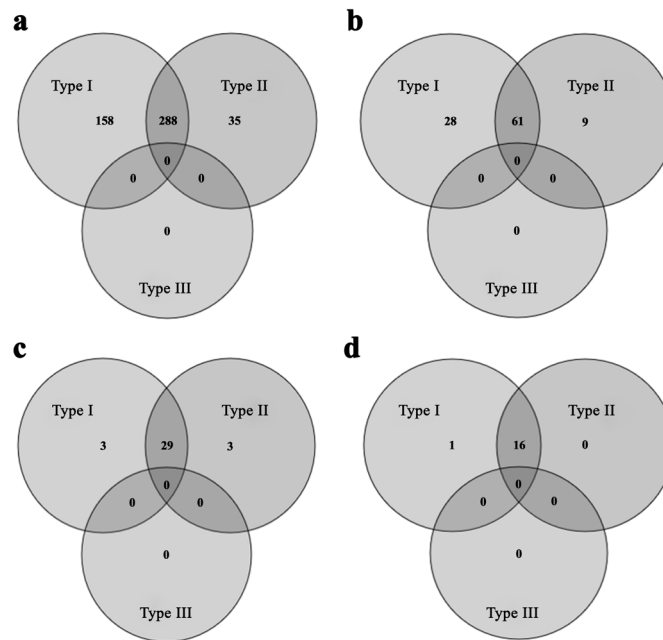


Fig. 5. Venn diagram showing IFN-stimulated genes among the DEGs of poly(I:C)-treated mice lung and brain tissues. **a)** Among the 577 GO enriched lung DEGs, 481 genes were ISGs out of which 158 were responsive to IFN type-I, 35 were responsive to type-II and 288 were responsive to both type-I and II. **b)** Among the 122 GO enriched brain DEGs, 98 genes were ISGs out of which 28 were responsive to IFN type-I, 9 were responsive to type-II and 61 were responsive to both type-I and II. **c)** Out of 37 lung DEGs of the GO-term innate immune response, 35 genes were ISGs and the number of genes responsive to IFN type-I, type-II and combination of type-I and II were 3, 3 and 29, respectively. **d)** All 17 brain DEGs of the GO-term innate immune response were ISGs and the number of genes responsive to IFN type-I and combination of type-I and II were 1 and 16, respectively.

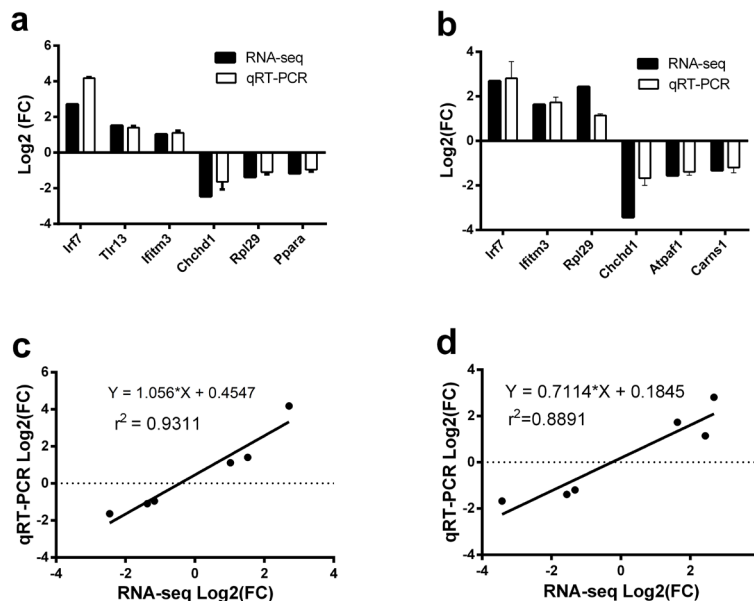


Fig. 6. Quantitative reverse transcriptase PCR validation of DEGs obtained by RNA sequencing. Six DEGs from both lung and brain tissue were selected randomly and quantified by qPCR. The expression patterns of a gene obtained from RNA sequence and qPCR were mostly similar in trends (log₂fc) in both lung (**a**) and brain tissues (**b**). The Pearson's coefficients of differential expression pattern of selected DEGs obtained by these two techniques were 0.9311 for lung (**c**) and 0.8891 for brain (**d**) tissues. All qPCR expression data are representative of at least three independent experiments.

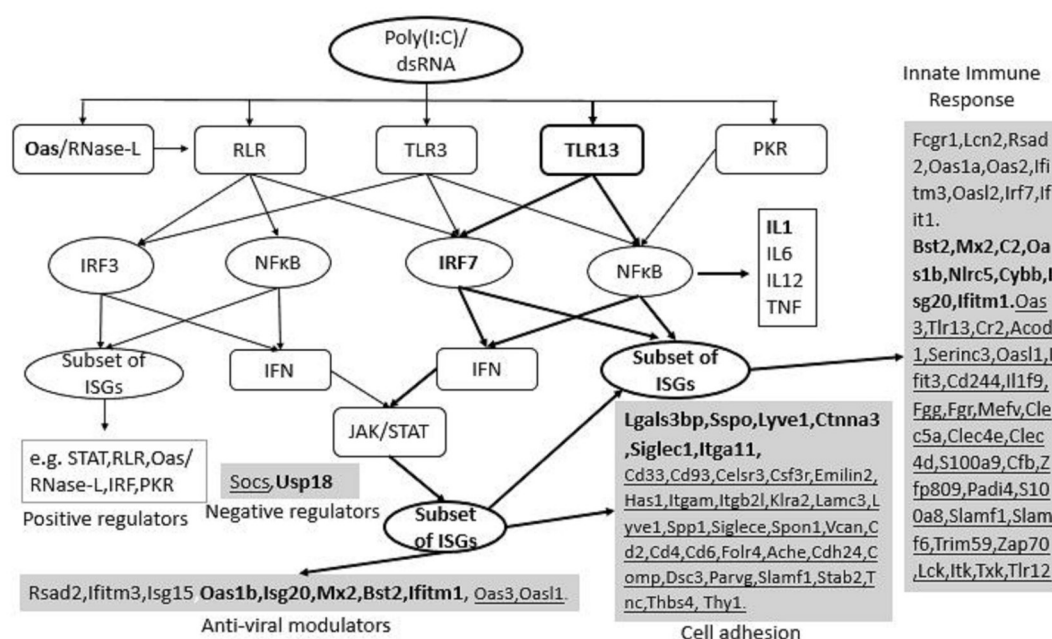


Fig. 7. Schematic diagram of poly(I:C) induced inflammatory and immune responses in lung and brain tissues of mouse. Upon recognition of poly(I:C), different types of receptors including TLR, RLR, protein kinase receptor (PKR) and OAS promote nuclear translocation of IRF3, 7 and NF-κB, which in turn stimulate the production of inflammatory cytokines, IFNs, and a subset of ISGs. IFNs stimulates more ISGs production via activating JAK-STAT pathways that orchestrate viral infection and subsequent processes including anti-viral modulation, cell adhesion, inflammation, and immune responses in cells and tissues. In this figure, the name of the genes are in normal font applicable for both, bold font for brain and underlined for lung tissues only.

0.001). Although the expression of these genes was not precisely the same by RNA sequence profile and qPCR, the trend was similar (Fig. 6a-b). Pearson's correlation coefficients of gene expression of lung and brain tissue by qPCR and RNA sequence were 0.9311 and 0.889, respectively, which demonstrated a rich correlation between the two techniques (Fig. 6c-d).

DISCUSSION

In the current study, firstly we confirmed the higher inflammatory condition of lung tissues than that of the brain through the differential expression of cytokines Il-1b and TNF-α. Then, we performed whole genome transcriptome screening of brain and lung tissues of the mice at 24 hours after poly(I:C) stimulation to underpin the tissue specificity of inflammatory or immune responses of those tissues

upon viral infection. We treated the mice with 100 μg of poly(I:C) through an intra-perineal injection that was enough to stimulate inflammation and was not lethal as mentioned by many scientists (13). We selected the treatment period of 24 hours to see the late acute responses of viral infection contrary to many other experiments performed to stimulate inflammatory cascades for a shorter period (13). We identified 629 (236 up, 393 down) out of 15447 transcripts in lung and 137 (92 up, 45 down) out of 14911 transcripts in brain tissues that were differentially expressed upon poly(I:C) treatment compared to the respective controls. There were only 15, and 5 overlapped DEGs showed over and down expression in both tissues, respectively. We found no genes in common showing upregulation in the lung but downregulation in the brain, whereas only one gene 'Rpl29' that showed different

Table I. List of primers used qPCR.

Gene	Forward sequence (5'>3)	Reverse sequence (5'>3)
Gapdh	AAATGGTGAAGGTCGGTGTGAAC	TGAAGGGGTCGTTGATGGC
Il-1beta	GTGTGGATCCAAAGCAATAC	GTCTGCTCATTCATGACAAG
TNF- α	CGAGTGACAAGCCTGTAGCC	ACAAGGTACAACCCATCGGC
Irf7	ACAGGGCGTTTTATCTTGCG	TACTGCAGAACCTGTGTGGG
Tlr13	TGCTCGGAAACCTACCCAAG	GAAGGAGAGCTGGCACTTGT
Rpl29	GATGCAGGCCAACAATGCAA	CTTAGGCTTCGGTTGGCAGA
Chchd1	GCGAACCCGGGAAAACCTA	CCTGAGCCCTGGAAGAACAAT
Ifitm3	CAGCCCCCAAACCTACGAAAGA	AAGTGTGAAGGTTTTGAGCGT
Ppara	CTGGGCAAGAGAATCCACGA	CGTCTTCTCGGCCATACACA
Atpaf1	TTCAGCTCTTCTACGCCACC	TCTTGTTCTGGGCACGTTT
Carns1	GGACCTGGATGGAAAGGAAGA	AGAGATAGGAAGGTGCTGGGA

Table II. Summary of RNA sequence data.

Serial no.	Sample ID	Total Reads	Mapped Reads	Mapping Ratio (%)	GC (%)	Q30 (%)
1	CB1	73231154	67577364	92.28	50.46	89.84
2	CB2	61310792	56692963	92.47	50.81	90.14
3	CB3	45923884	41933836	91.31	52.13	90.38
4	CL1	47123162	43471233	92.25	51.14	90.32
5	CL2	80782544	74707270	92.48	50.75	90.2
6	CL3	53444796	49909911	93.39	50.5	94.14
7	TB1	44218740	40348945	91.25	51.82	90.45
8	TB2	61682056	57053177	92.50	51.11	90.38
9	TB3	70375198	65228770	92.69	50.59	90.16
10	TL1	60058990	57155336	95.17	50.75	94.24
11	TL2	45921130	43396016	94.50	52.14	94.47
12	TL3	48470216	45588296	94.05	51.36	94.37

Sample ID: The uniform number of the sample for the project; CB: control brain; TB: treated brain; CL: control lung; TL: treated lung; clean reads: number of clean reads obtained; clean bases: clean base obtained; Q30 (percentage): The percentage of the base with a mass value greater than or equal to 30; GC (percentage): Sample GC content.

Table III. GO functional clusters (BP) of DEGs (fold change ≥ 2 and $P \leq 0.05$) of brain and lung tissue linked to immunity and inflammation.

GO class	Tissue	DEGs	Common name of involved gene
Innate immune response	Brain	17	<i>Fcgr1, Lcn2, Rsad2, Oas1a, Oas2, Ifitm3, Oasl2, Irf7, Ifit1, Bst2, Mx2, C2, Oas1b, Nlrc5, Cybb, Isg20, Ifitm1.</i>
	Lung	37	<i>Fcgr1, Lcn2, Rsad2, Oas1a, Oas2, Ifitm3, Oasl2, Irf7, Ifit1, Oas3, Tlr13, Cr2, Acod1, Serinc3, Oasl1, Ifit3, Cd244, Il1f9, Fgg, Fgr, Mefv, Clec5a, Clec4e, Clec4d, S100a9, Cfb, Zfp809, Padi4, S100a8, Slamf1, Slamf6, Trim59, Zap70, Lck, Itk, Txk, Tlr12</i>
	Brain	18	<i>Oas2, Oasl2, Fcgr1, Ifitm3, Irf7, Ifit1, Lcn2, Rsad2, Skap1, Oas1b, Lime1, Mx2, Nlrc5, Bst2, C2, H2-Q7, Ifitm1, Isg20, Oas2, Oasl2, Fcgr1, Ifitm3, Irf7, Ifit1, Lcn2, Rsad2, Skap1, Oas3, Oasl1, Clec4d, Clec4e, Clec5a, Cd244, Cd300lb, Cd300lf, Cd300ld, Fgr, Mefv, S100a8, S100a9, Acod1, Cfb, Cr2, Fgg, Ifit3, Il1f9, Lirb4a, Pirb, Padi4, Serinc3, Tlr13, Cd3e, Cd4, Cd8a, Cd8b1, Itk, Slamf6, Sla2, Txk, Camk4, Il2ra, Lat, Lrmp, Kcnn4, Prkcb, Prkcq, Ptpn22, Slamf1, Themis, Tlr12, Zap70</i>
Defense response to virus	Brain	14	<i>Oas1a, Oas2, Oasl2, Ifitm3, Ifit1, Isg15, Rsad2, Oas1b, Mx2, Nlrc5, Bst2, Ifitm1, Isg20, Slfn8</i>
	Lung	15	<i>Oas1a, Oas2, Oasl2, Ifitm3, Ifit1, Isg15, Rsad2, Oas1g, Oas3, Oasl1, Ifit1b12, Ifit3, Ifit3b, Serinc3, Cd8a</i>
Neg. reg. of viral genome rep.	Brain	8	<i>Rsad2, Ifitm3, Isg15, Oas1b, Isg20, Mx2, Bst2, Ifitm1.</i>
	Lung	5	<i>Rsad2, Ifitm3, Isg15, Oas3, Oasl1,</i>
Cell adhesion	Brain	6	<i>Lgals3bp, Sspo, Lyve1, Ctnna3, Siglec1, Itga11</i>
	Lung	29	<i>Cd33, Cd93, Celsr3, Csf3r, Emilin2, Has1, Itgam, Itgb2l, Klra2, Lamc3, Lyve1, Spp1, Siglece, Spon1, Vcan, Cd2, Cd4, Cd6, Folr4, Ache, Cdh24, Comp, Dsc3, Parvg, Slamf1, Stab2, Tnc, Thbs4, Thy1.</i>
	Brain	0	
Inflammatory response	Lung	27	<i>Mefv, S100a8, S100a9, Acod1, Ccr1, C5ar1, Cyp26b1, Fpr1, Fpr2, Fpr3, Itgb2l, Il1b, Il1f9, Ltb4r1, Mmp25, Pla2g7, Spp1, Tlr13, Cd27, Camk4, Ccl25, Lat, Prkcq, Tbx2r, Tlr12, Tusc2, Zap70</i>

	Brain	0	
Signal transduction	Lung	43	Tlr13, Fgg, Cmkrlr1, C5ar1, Cxcr2, Nfam1, Cxcr1, Ltbr4r1, Adrala, Celsr3, Ccr1, (Lphn3, Adgrl3), Fpr3, Fpr2, Nrnx2, Pilra, Slpr4, Fpr1, Hrh2, Il18rap. Down: Ptger4, Grem1, Bcl11b, Lat, Cd83, Gpr174, Rgs5, Pacsin1, Tbx2r, Ackr4, Edaradd, Sox9, Crhr2, Camk4, P2ry10, Rasal1, Racgap1, Acap1, Wnt10a, Ccr9, Rasal3, Gpr132, Tlr12
Leukocyte migration	Brain	0	
	Lung	6	Fpr1, Fpr2, Fpr3, Il1b, Mmp9, Ccl25 .
Neutrophil chemotaxis	Brain	0	
	Lung	11	S100a8, S100a9, Cxcr2, Csf3r, C5ar1, Itgam, Il1b, Spp1, Trem1, Trem3, Ccl25 .

Highlighted genes were downregulated and DEGs in italic fonts under same GO class between two tissues were overlapped.

expression between the lung and brain. Maximum DEGs stimulated in experimental tissues were different and might be responsible for organ-specific inflammatory and immune responses. The DEGs from both tissues were assigned to some common and important GO-terms including innate immune response, immune system process, response to IFN and negative regulation of viral genome replication. Most of the DEGs of the same GO-term were different between the two tissues along with some common DEGs. GO-classification of DEGs also revealed that the genes convoluted in GO classes including inflammatory responses, signal transduction, neutrophil chemotaxis, leukocyte migration, cell cycle, cell division, T-cell activation and adaptive immune response were differentially expressed in lung tissues but were not observed in brain tissues. According to the KOG functional analysis, DEGs from both brain and lung tissues varied widely in their involvement in the various functional classes as signal transduction, transcription, lipid metabolism, cytoskeleton, cell division and in defense mechanism in term of frequency and rate.

KEGG pathway statistics showed that the maximum DEGs in brain and lung tissues were correlated with inflammation and immunity-related

pathways. The DEGs of lung tissues were significantly involved in KO-terms of cytokine-cytokine receptor interaction, HTLV-I infection, T-cell receptor signaling pathway, measles, CAMs, herpes simplex infection, phagosome, cell cycle, *Staphylococcus aureus* infection, leukocyte trans-endothelial migration and primary immunodeficiency, whereas that of brain tissues were involved in KO-terms of measles, herpes simplex infection, influenza A, hepatitis-C, CAMs and phagosome. In both lung and brain tissues, some common pathways were regulated by different DEGs including some common DEGs. The DEGs of KO-term CAMs were entirely different between these two tissues. These variations of gene involvement in common pathways and stimulation of different pathways between the two tissues indicated that the molecular mechanisms of controlling inflammation or innate immunity of lung and brain tissue to viral dsRNA are different.

The variation in the number of DEGs might be relevant to the sensitivity and susceptibility of the lung to microbial infections and to the blood-brain barrier (BBB) of the brain tissue that prevents the infectious agent from entering into this organ. As reported by many scientists, the lung is a sensitive and primary target site for microbial infection

Table IV. KEGG pathway enrichment of DEGs of lung and brain tissue.

DEGs of Lung Tissue			DEGs of brain tissue		
Pathway description	DE Gs	P-value	Pathway description	DE Gs	P-value
Hematopoietic cell lineage	20	1.82E-12	Measles	6	0.00046
Primary immunodeficiency	10	1.88E-07	Herpes simplex infection	7	0.000547
T cell receptor signaling pathway	16	1.89E-07	Influenza A	6	0.001279
<i>Staphylococcus aureus</i> infection	11	1.22E-06	Hepatitis C	5	0.002259
Measles	15	5.19E-05	Cell adhesion molecules (CAMs)	5	0.005
p53 signaling pathway	9	0.000307	Phagosome	5	0.007017
HTLV-I infection	20	0.000469	Graft-versus-host disease	2	0.051089
Cell adhesion molecules (CAMs)	14	0.00059	Allograft rejection	2	0.056262
Cell cycle	12	0.000606	<i>Staphylococcus aureus</i> infection	2	0.058026
Cytokine-cytokine receptor interaction	19	0.000686	-		
Leishmaniasis	7	0.003704	-		
Glycine, serine and threonine metabolism	5	0.008129	-		
Intestinal immune network for IgA production	5	0.016964	-		

(13). Lung bronchial and alveolar endothelial and epithelial cells are usually richer in TLR3 than other cells (9). In primary human lung microvascular endothelial cells, poly(I:C) disrupts endothelial barrier and allow more PAMPs to enter the cells which, in turn, lead to further IFN or inflammatory cytokine production including TNF- α , IL6, and IL8 (14). However, in the brain, IFN- β expression is

higher in microglia or astrocytes and IFN signaling is useful for brain microvascular endothelial cells to reduce permeability and proper BBB functioning (9). The brain might show a lower response to poly(I:C) because of the presence of some anti-inflammatory factors, lack of conventional draining lymphatics, antigen-presentation or presence of lower MHC molecules in addition to BBB (10).

Some scholars reported that microRNA-22 plays an anti-inflammatory role in glial cells in response to poly(I:C) and JEV virus infection, whereas its overexpression increased inflammation in human umbilical cord endothelial cells (7, 8). The findings of many scientists support that mechanisms of controlling inflammation in different cells or organs are different (7-9, 14). All of these findings supported our results. Ultimately, our study suggested that both the organs are responsive to viral dsRNA but the brain tissues might be more stable than lung tissues, and mechanisms of controlling inflammation and immune responses to viral infection are tissue specific.

Many recent studies emphasized on genes that activated immediately following viral stimulation in the different types of cells. The dsRNA receptor TLR3 is the highly noticeable gene upon poly(I:C) stimulation, which in turn activated transcription factors NF- κ B and activator protein (AP)-1, and enhanced proinflammatory cytokines like IL6, IL12, TNF- α , and IFN- $\alpha/\beta/\gamma$ (1). Another study also confirmed that NF- κ B translocation starts at 3 hours, reach maximal condition at 12 hours and disappears within 24 hours post poly(I:C) stimulation in NIT-1 B-cells. Some scientists reported that increased expression of IL6 and IL-1 β returns to the normal level within 24 hours in rat brain (15). Scientists also elucidated that IFN- α and IFN- β were induced in mouse lymphoid organs at 1 to 6 hours after poly(I:C) stimulation and then reduced gradually to normal level (13). Our study did not represent any differential expression in most of these genes and cytokines except Il-1b in the lung as our data represented the snapshot at 24 hours of poly(I:C) stimulation. We found 3.56-fold increased expression of Il-1b in lung and 1.32-fold in brain tissues compared to the respective controls. The reason for deference in Il-1b expression in the brain between RNA-seq and qPCR is the fold change cut-off value (fold change=2) of analysis. It was confirmed by Interferome analysis that most of the DEGs of poly(I:C)-immunized lung and brain tissues were ISGs and stimulated by IFN- $\alpha/\beta/\gamma$ which is in accordance with other studies. DEGs regulate inflammation, signal transduction, and stimulate defense mechanisms of the innate

immune system against viruses (16). The results of this study also revealed that the IFN- $\alpha/\beta/\gamma$ are the important modulators that regulate ISG expression and inflammatory or immune responses in viral infections. Some studies identified IFNs as primary cytokines which orchestrate immune mechanisms in viral infection (1). The results of our study agreed with these findings.

ISG expression on viral infection depends on IFNs production that in turn depends upon viral load, virulence, and cell type and defense mechanisms of the target organ. The anti-viral defense and immunity depend on ISG expression that regulates pathogen recognition, IFN desensitization, and inhibition of viral entry and replication (5). In our study, different ISGs along with overlapped ISGs of Lcn2, Rsad2, Oas1a, Oas2, Ifitm3, Oas12, Irf7, and Ifit1 contributed to the innate immune response in the lung and brain tissue, as also described by other scientists (17-19). In the lung, viral entry inhibitor protein Trim59, JAK-STAT signaling pathway inhibitor Socs1 and Asb5 and viral replication inhibitor Zap70 were downregulated, but profound inflammatory effector Il-1b and TNFs9 and genes responsible for negative regulation of viral genome replication including Rsad2, Ifitm3, Isg15, Oas3 and Oas11 were upregulated in this study. The Mmp3, Mmp8, Mmp9, and Mmp25 were overexpressed in the lung and are responsible for leukocyte migration and emphysema development (9). In brain tissue, upregulated ISG Usp18 promote IFN desensitization; Mx2 and Ifitm1 restrict entry of virus and viral particles; and Rsad2, Ifitm3, Isg15, Oas1b, Isg20, Mx2, Bst2, and Ifitm1 arrest viral replication by repressing viral protein translation (5). In addition, Oas1b was induced only in the brain and Oas1g, Oas3 and Oas11 were only induced in lung tissues of mice in our study. The differential expression of OAS family member might also be connected with the organ-based inflammatory or immune responses. According to the reports of some scientists, about 99% of OAS genes are highly expressed in response to viral RNA and modulate immunity of infected organisms which is also in accordance with our findings (20). According to available data and annotated DEGs, proposed mechanisms of poly(I:C)-induced inflammation

and/or immunity are partly demonstrated in Fig. 7. Altogether, our findings showed that the effect viral dsRNA on ISGs activation are different, and are higher in the lung than brain tissue, which in turn regulate innate immune responses or pathophysiology of viral infection in an organ-specific manner.

In this study, the orphan receptor Tlr13 was stimulated by synthetic dsRNA in the lung but not in the brain tissues. We confirmed the overexpression of Tlr13 through qPCR from independent experiments. Tlr13 was assigned to different vital immune and inflammation-related GO-terms including innate immune response, immune system process, inflammatory response and signal transduction. In addition to Tlr13, the lung-specific DEGs including *Mefv*, *S100a8*, *S100a9*, *Acod1*, *Ccr1*, *C5ar1*, *Cyp26b1*, *Fpr1*, *Fpr2*, *Fpr3*, *Itgb2l*, *Il-1b*, *Il1f9*, *Ltb4r1*, *Mmp25*, *Pla2g7*, *Spp1*, *Cd27*, *Camk4*, *Ccl25*, *Lat*, *Prkcq*, *Tbxa2r*, *Tlr12*, *Tusc2*, and *Zap70* were associated with inflammatory responses. Numerous recent studies obtained similar results in viral infection (18, 21-26). In lung tissue, the upregulated genes *Il-1b* and *TNFsf14* and downregulated genes *Ltb*, *Lck*, *Chek1*, *Prkcq*, and *Lat* were responsible for inflammation and were involved in the NF- κ B pathway (18, 27). About 47 genes involved in GO-term signal transduction, 29 in cell adhesion, 6 in leucocyte migration, 11 in neutrophil chemotaxis were differentially expressed only in the lung on viral dsRNA stimulus. The number of DEGs assigned to different GO-terms or KEGG pathways were also markedly higher in the lung than the brain tissue. Shi et al. reported that Tlr13 could recognize vesicular stomatitis virus and stimulate inflammatory or immune responses (26). The PRR *Oas3* and Tlr13 along with *Cr2*, *Acod1*, *Serinc3*, *Oasl1*, *Ifit3*, *Cd244*, *Il1f9*, *Fgg*, *Fgr*, *Mefv*, *Clec5a*, *Clec4e*, *Clec4d*, *S100a9*, *Cfb*, *Zfp809*, *Padi4*, *S100a8*, *Slamf1*, *Slamf6*, *Trim59*, *Zap70*, *Lck*, *Itk*, *Txk*, and *Tlr12* genes were differentially expressed only in lung tissue in this study and were involved in GO-term innate immune response. Especially the Tlr13 can sense several bacterial ribosomal RNA (23S) along with viral RNA and activate innate immunity against entire bacteria and virus through inducing different cytokines in mice (26, 28, 29). Therefore,

the lung-specific enhancement of Tlr13 might be one of the reasons behind the higher inflammatory conditions in the lung compared to those of the brain. Unfortunately, this receptor is absent in human (29).

Rpl29 was the only gene that showed different expression between the lung and brain tissues having differential inflammatory conditions. Its expression was almost negatively correlated with that of *Il-1b* and *TNF- α* in both tissues. These results indicated that Rpl29 might have an anti-inflammatory role and its tissue-specific different expression might be responsible for differential inflammatory responses of the lung and brain tissues upon virus infection. One study mentioned that Rpl29 regulates angiogenesis, cell proliferation, and cell differentiation (30), however, there is no reference on the inflammatory effect of this gene to date. For the first time, we identified the association of Rpl29 with inflammation in response to viral dsRNA. Our study delineated that the inflammatory or immune responses are higher in the lung than the brain or the lung is more sensitive to viral dsRNA than the brain tissue. Although, most of the inflammation or immunity regulatory genes were different between two experimental tissues. It is more likely that Rpl29 and Tlr13 may play a role in the organ-specific inflammatory and immune responses. Our study would not exclude the roles of other genes.

The comparative gene expression analysis of poly(I:C)-stimulated mouse lung and brain tissues have produced comprehensive information about inflammation and immunity. Our data uncover some facts that the efficacy of viral infection is organ specific and more prominent in lung tissue compared to that of the brain. IFNs and/or ISGs are principal modulators of viral infection-induced inflammation or immunity. Our study suggested that the different expressions of Tlr13 and Rpl29 might be responsible for organ-specific inflammatory or immune responses in viral infection along with other genes including some OAS and ISGs. Tlr13 is a PRR of viral and bacterial RNA and its expression pattern was similar to *Il-1b* and *TNF- α* in the lung tissues. Rpl29 showed different expression between inflammatory brain and lung tissues of mice. Therefore, we primarily focused on these two genes

among the other DEGs. Further studies based on these findings would be beneficial to outline a new insight into viral infection-induced inflammation or the immune responses.

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FOXA1 PROMOTES PROLIFERATION, MIGRATION AND INVASION BY TRANSCRIPTIONAL ACTIVATING KRT7 IN HUMAN GASTRIC CANCER CELLS

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Gastric cancer (GC) is the fourth leading cause of gynecological malignancies worldwide. 5-fluorouracil (5-FU)-mediated chemotherapy is the adjuvant treatment for patients with GC following surgical resection. Many studies have indicated the cancer-type specific roles of forkhead box protein A1 (FOXA1) and keratin 7 (KRT7) in human malignancies. However, the potential mechanism underlying the involvement of FOXA1 and KRT7 in the pathogenesis and chemoresistance of GC are still not entirely clear. In our study, gain- and loss-of-function experiments proved that FOXA1 promoted cell proliferation, migration and invasion in AGS and SGC-7901 cells. Consequently, KRT7 was identified to be transcriptional activated by FOXA1 using Dual luciferase reporter assay. Our results also indicated that FOXA1 exerted its functions in enhancing viability and invasion of AGS and SGC-7901 cells through activating KRT7. Finally, interference of FOXA1 or KRT7 increased the chemosensitivity of AGS and SGC-7901 cells to 5-fluorouracil (5-Fu) treatment by suppressing cell proliferation. In conclusion, these data indicate that FOXA1 promoted proliferation, migration, invasion, and decreased chemosensitivity of GC cells to 5-Fu treatment through transcriptional activator KRT7. The present study provides a novel therapeutic strategy for the enhancement of efficacy in GC treatment and provides important insights into the molecular mechanism underlying 5-FU-mediated chemoresistance.

Gastric cancer (GC) is the fourth most common malignancy and the second leading cause of cancer-related death worldwide (1, 2). Remarkable progress has been achieved in the diagnose and therapeutic strategies for GC, however, the 5-year overall survival rate has not improved significantly over time. The poor prognosis of GC is associated with the lack of accurate diagnostic methodology and significant symptoms for identification in early stages (1, 3). Previous studies have reported that

molecular targeted therapy could be broadly defined as the transfer of genetic material to cure GC, or at least to improve the clinical status of patients (3-5). Thus, investigating and understanding the molecular mechanism underlying GC progression has important implications for GC prevention and treatment.

Forkhead box protein A1 (FOXA1, also known as HNF3 α) is a member of the FOX transcription factor family and is considered as a prognosis marker in various cancers (6-9). Previous studies have revealed

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the function of FOXA1 in breast cancer, liver cancer and prostate cancer (7, 9). Several studies have demonstrated the important role of FOXA1 in GC progression and development (10-12). For example, FOXA1 was highly expressed in GC tissue, and deficiency of FOXA1 suppressed GC cell proliferation and enhanced GC apoptosis accompanied with a decreased level of Yes-associated protein (10). Moreover, the enhanced FOXA1 expression was associated with lymph node metastasis in early GC (11). However, FOXA1 was identified as a tumor suppressor targeted by miR-1290 (13) and exhibited as an inhibitor of epithelial mesenchymal transition in human GC (12). Considering the role of FOXA1 in GC progression is still controversial, in the present study the underlying mechanism of FOXA1 in GC was analyzed.

Keratin 7 (KRT7) is one of the intermediated filament proteins and is mainly expressed in epithelium and epithelial tumors (14). In GC, KRT7 was identified as a target of long non-coding antisense RNA KRT7-AS (lncRNA KRT-AS) and proved to be involved in GC progression (15). Importantly, Sano et al. found that KRT7 was transcriptional regulated by FOXA1 in esophageal squamous cell carcinomas (16). Nevertheless, the association between FOXA1 and KRT7 in GC still remains largely unknown. On this basis, the aim of the present study is to expatiate the role of FOXA1 and KRT7 in the progression and development of GC and explore the underlying mechanism, which may provide potential biomarkers and therapeutic evidence for GC.

MATERIALS AND METHODS

Cell culture and chemosensitivity assay

The human gastric adenocarcinoma cancer cell lines AGS and SGC-7901 were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Invitrogen, Carlsbad, USA) and 100 U/mL penicillin/streptomycin (Invitrogen). Cells were cultured in a 5% CO₂ atmosphere at 37°C. All experiments were performed using an 80% confluent cell monolayer.

The cell lines were seeded in a 96-well plate overnight with different treatments. The 5-fluorouracil solutions (5-Fu, Sigma-Aldrich, St. Louis, USA) were freshly prepared and added to medium to obtain the final concentrations of 0, 1, 2, 4, 8, 16 µM, respectively. After 48 h, cell viability was performed.

Cell transfection

Small interfering RNAs (siRNA) for FOXA1 (siFOXA1) or KRT7 (siKRT7), the corresponding control RNA (si-NC), pcDNA3.1-FOXA1 overexpression vector (FOXA1) or the control vector (pcDNA), were purchased from GenePharma (Shanghai, China). After being seeded for 24 h, cells were transfected using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. Cells were collected at 48 h after transfection. The sequences of siFOXA1 and siKRT7 are listed in Table I.

qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and was quantified using a Nanodrop ND-100 spectrophotometer (ThermoFisher, Waltham, MA). cDNA were then synthesized using PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Otsu, Japan). qRT-PCR analysis was conducted using SYBR Green PCR Master Mix (Takara) on a Bio-Rad Real-Time PCR instrument (Bio-Rad, Hercules, USA). The expression level of FOXA1 or KRT7 was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using comparative 2^{-ΔΔC_t} method. Each experiment was performed in triplicate and the primer sequences are listed in Table I.

MTT assay

After transfection or 5-Fu treatment, cells were subsequently added with 15 µL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (final concentration, 0.5 mg/mL, Sigma) and incubated at 37°C for 4 h. Next, purple formazan crystals dissolved in DMSO were added into each well and the optical density was detected at 490 nm using a microplate reader (Bio-Tek, Winooski, USA).

Colony formation assay

Cell colonies were fixed with methanol for 30 min

and dyed using 0.5% crystal violet for 10 min. The colony forming cells were photographed by a microscope (Olympus, Tokyo, Japan) and the total number was counted.

Wound-healing assay

Cells were seeded in 6-well plates and incubated 24 h before transfection. On reaching ~80% confluence, cell monolayer was scratched with a 10 μ L Eppendorf tip. After 24 h, the wound gaps were analyzed by an Olympus microscope (10 $\times$ 10) and the migration rate was calculated as follows: Original width-width after migration/Original width. The data were obtained from three independent experiments.

Transwell assay

For invasion and migration detection, Transwell assay was performed using Transwell chambers (Corning, New York, USA) following the manufacturer's protocol. A density of 5×10^4 cells per well were plated in the upper chamber without serum and then the chamber was placed in the lower chamber filled with RPMI 1640 medium with 10% FBS. After incubation for 24 h, non-invading cells were removed with a cotton swab, and the invaded cells were fixed with 4% paraformaldehyde and subsequently stained with 0.1% crystal violet. The invasive capacity was quantified by nine independent fields/per chamber captured by an Olympus microscope (40 $\times$ 10). Three independent experiments were performed in triplicate.

Western blot

Cells were lysed using RIPA lysis buffer (Sigma) for 30 min and the protein concentration was determined using a BCA detecting kit (Beyotime Biotechnology, Nanjing, China). After separating by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDA-PAGE), the proteins were transferred onto a PVDF membrane (EMD Millipore, Billerica, USA). Next, after blocking, the membranes were incubated with primary antibodies Mouse anti-KRT antibody (Abcam, Cambridge, UK; dilution rates of 1:500), Mouse anti-FOXA1 antibody (Abcam; dilution rates of 1:500) or Mouse anti-GAPDH antibody (Abcam; dilution rates of 1:1000) at 4°C overnight. Membranes were then incubated with secondary antibody goat anti-Mouse IgG HRP (Abcam, dilution rates of 1:2000) for 2 h at room temperature. Detection was visualized with enhanced chemiluminescence detection system (Pierce, Rockford, USA) and the intensities of bands were quantified using Image Lab™ Software (Bio-Rad).

Dual-luciferase reporter assay

Genomic DNA fragment of KRT7 promoter element was synthesized by Beijing Genomics Institute BGI (Beijing, China) and subcloned into pGL3-luciferase reporter plasmid (Promega, Madison, USA). AGS or SGC-7901 cells were co-transfected with pGL3-KRT7 construct and FOXA1 overexpression plasmid (FOXA1), FOXA1 siRNA (siFOXA1) or their corresponding

Table I. siRNAs and primers for qRT-PCR used in the study.

Name	Primer	Sequence (5'-3')
siFOXA1	Sense	5'-GAACAGGCACUGCAAUACUCGCCUU-3'
	Anti-sense	5'-AAGGCGAGUAUUGCAGUGCCUGUUC-3'
siKRT7	Sense	5'-UGGAGGACUUCAAGAAUAAUU-3'
	Anti-sense	5'-AAUUAUUCUUGAAGUCCUCCA-3'
FOXA1	Forward	5'-TACACACCTTGGTAGTACGCC-3'
	Reverse	5'-UUUGCCUUUAGGAUUCUAG-3'
KRT7	Forward	5'-CGGCATCATCGCTGAGGTCAA-3'
	Reverse	5'-GCCTGGAGGGTCTCAAACCTTGG-3'
GAPDH	Forward	5'-GGAGCGAGATCCCTCCAAAAT-3'
	Reverse	5'-GGCTGTTGTCATACTTCTCATGG-3'

controls using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested with a lysis buffer (Promega) 48 h after transfection. The dual-luciferase reporter assay kit (Promega) was used and Renilla and Firefly luminescence were measured. Experiments were carried out in three independent replicates.

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences version 16.0 (SPSS 16.0; SPSS Inc., Chicago, USA). The data were expressed as Mean $\pm$ standard deviations (SD) of three independent experiments. Statistical analyses among groups were performed with one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered as statistically significant.

RESULTS

FOXA1 promoted proliferation, migration and invasion of GC cell lines

To detect the role of FOXA1 in GC progression, gain- or loss-of-function experiments were performed. FOXA1 pcDNA plasmid or siFOXA1 was constructed and transfection efficiency was verified using qRT-PCR method ($P < 0.001$). MTT assay demonstrated that cell viability was promoted by FOXA1 overexpression but suppressed by FOXA1 siRNA in AGS and SGC-7901 cell lines in a time-dependent manner ($P < 0.001$, Fig. 1B). Colony formation assay showed that cell proliferation was enhanced by FOXA1 overexpression compared with control group, but suppressed by siFOXA1 compared with siNC in AGS and SGC-7901 cells ($P < 0.001$, Fig. 1C). Wound-healing assay showed that cells transfected with FOXA1 pcDNA exhibited a lower rate of open wound compared with pcDNA group, but cells transfected with siFOXA1 showed a higher rate of open wound in AGS and SGC-7901 cell lines ($P < 0.01$, $P < 0.001$, Fig. 1D). Besides, the invasive ability of AGS and SGC-7901 cell lines were dramatically increased by FOXA1 overexpression and declined by interference of FOXA1 as compared to the corresponding control groups, respectively ($P < 0.001$, Fig. 1E). Totally, FOXA1 enhanced cell proliferation, migration and invasion in GC.

FOXA1 positively regulated the expression of KRT7

Previous study has reported that KRT7 was transcriptional activated by FOXA1 in esophageal squamous cell carcinomas (16). Herein, we found that FOXA1 overexpression increased the mRNA level of KRT7 compared with control group, while FOXA1 knockdown by siFOXA1 decreased KRT7 mRNA expression level compared with the corresponding control groups in AGS and SGC-7901 cell lines ($P < 0.001$, Fig. 2A). Next, we analyzed the effects of FOXA1 on the activity of KRT promoter using dual luciferase reporter assay. pGL3 -KRT7 was generated and co-transfected with FOXA1 pcDNA or siFOXA1 in AGS and SGC-7901 cells, respectively. As shown in Fig. 2B, the luciferase activities of cells co-transfected with pGL3 -KRT7 and FOXA1 overexpression plasmid were dramatically enhanced as compared to the control group in AGS and SGC-7901 cell lines ($P < 0.001$, Fig. 2B). However, the luciferase activities of cells co-transfected with pGL3 -KRT7 and si-FOXA1 were reduced as compared to the control group in AGS and SGC-7901 cell lines ($P < 0.001$, Fig. 2B). Consistently, as shown in Fig. 2C, the protein level of KRT7 was significantly increased by FOXA1 overexpression, while decreased by transfecting with FOXA1 siRNA in AGS and SGC-7901 cells ($P < 0.01$, $P < 0.001$, Fig. 2C). Taken together, these data demonstrated that FOXA1 positively regulated the expression of KRT7 in GC cells.

FOXA1 promoted GC cell proliferation through transcriptional activating KRT7

To determine whether FOXA1 exerts its functions in GC cells by targeting KRT7, AGS and SGC-7901 cells were co-transfected with FOXA1 overexpression vector and si-KRT7. MTT and colony formation assay exhibited that the FOXA1 overexpression-induced enhancement of viable and proliferative abilities of AGS and SGC-7901 cells were significantly attenuated by interference of KRT7 expression in AGS and SGC-7901 ($P < 0.01$, $P < 0.001$, Fig. 3A and B). Thus, these results suggested that FOXA1 promoted GC cell proliferation by enhancing KRT7 expression.

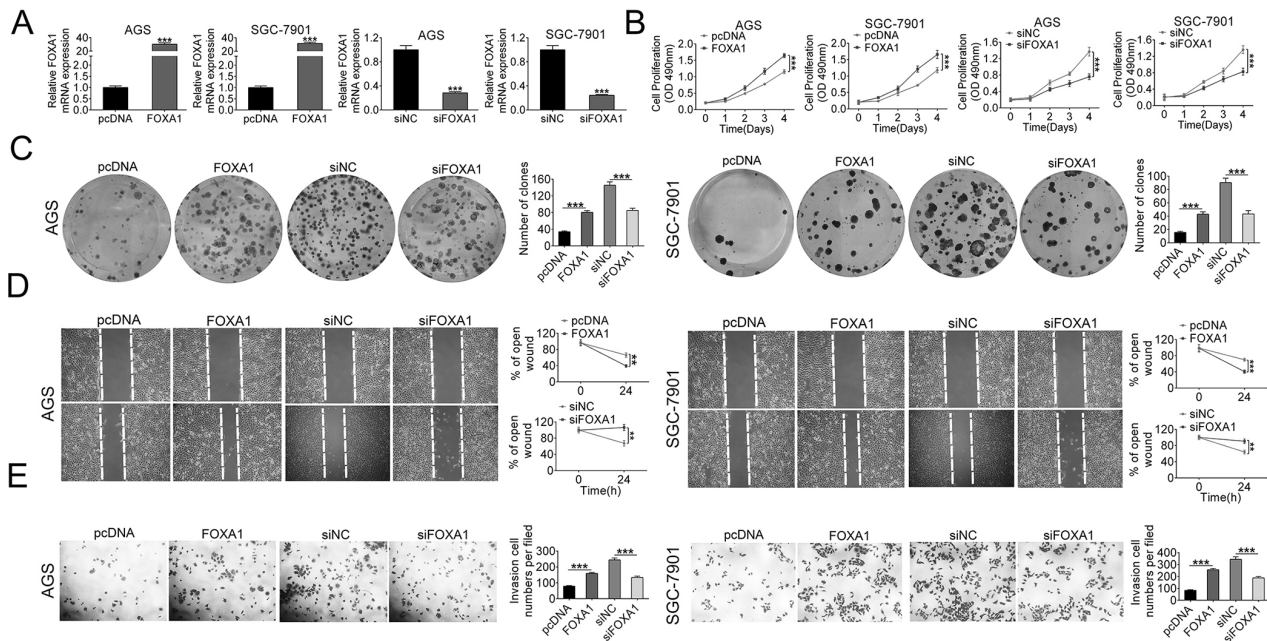


Fig. 1. FOXA1 regulated GC cell proliferation and invasion. AGS or SGC-7901 cell lines were transfected with FOXA1 overexpression plasmid, FOXA1 siRNA (siFOX1) or their corresponding controls. **A)** The expression of FOXA1 mRNA level was detected by qRT-PCR to verify the transfection efficiency of FOXA1 overexpression plasmid or siFOX1 in GC cell lines. **B)** The effect of FOXA1 on cell proliferation was analyzed by MTT assay in GC cell lines. **C)** The effect of FOXA1 on colony formation was measured by colony formation assay in GC cell lines. **D)** The effect of FOXA1 on rate of open wound was analyzed by wound-healing assay. **E)** The effect of FOXA1 on the invasive ability of GC cells was determined by transwell assay. Data were presented as mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$ represent statistically difference.

FOXA1 promoted GC cell migration and invasion through transcriptional activating KRT7

Furthermore, to verify whether KRT7 was involved in the effect of FOXA1 on GC cell migration and invasion, wound-healing and Transwell assay was performed. As shown in Fig. 4A and B, the promotion of migratory and invasive abilities induced by FOXA1 overexpression were abrogated by KRT7 knockdown in AGS and SGC-7901 cells ($P < 0.001$). Altogether, the effects of FOXA1 on enhancing cell migration and invasion were dependent on the activation of KRT7 in GC cells.

FOXA1 or KRT7 knockdown improved chemosensitivity of GC cells to 5-Fu treatment

Understanding the molecular mechanism underlying the chemoresistance to 5-Fu is important for providing novel therapeutic strategy for GC (17). AGS and SGC-7901 cells that transfected with

FOXA1 overexpression or si-FOXA1 were treated with 5-Fu for 48 h at various concentrations (1, 2, 4, 8 and 16 μ M, respectively), and MTT assay was performed. As shown in Fig. 5A, cell viability was decreased by 5-Fu treatment in a dose-dependent manner, and siFOX1 aggravated the suppressive effects of 5-Fu in AGS and SGC-7901 cells ($P < 0.05$, Fig. 5A), which suggested that silencing of FOXA1 enhanced GC cell chemosensitivity to 5-Fu treatment.

Moreover, cells were treated with 5-Fu for 48 h at the concentration of 1, 2, 4, 8 and 16 μ M and co-transfected with FOXA1 overexpression plasmid and KRT7 siRNA. The result showed that cell viability was decreased by 5-Fu treatment in a dose-dependent manner, but was reversed by overexpression of FOXA1 in AGS and SGC-7901 cells, particularly, the FOXA1 overexpression-induced enhancement of cell viability was

eliminated by KRT7 knockdown in AGS and SGC-7901 cells treated with 5-Fu ($P < 0.05$, $P < 0.01$, $P < 0.001$, Fig. 5B), suggesting that FOXA1 enhanced GC cell chemosensitivity to 5-Fu treatment by activating KRT expression. These investigations demonstrated that interference of FOXA1 or

KRT7 enhanced GC cell chemosensitivity to 5-Fu treatment by suppressing cell viability.

DISCUSSION

Despite great enhancements achieved in

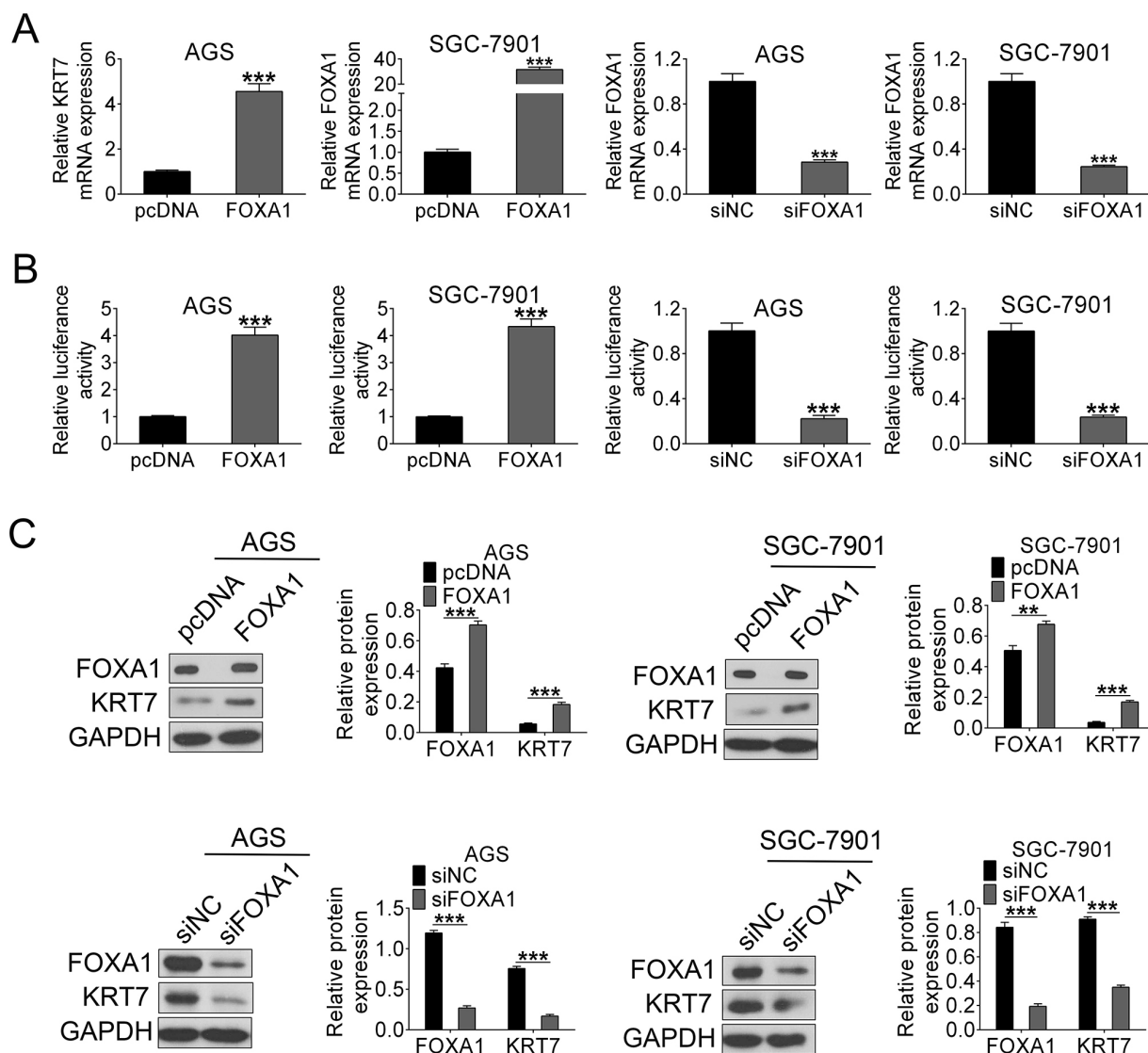


Fig. 2. FOXA1 positively regulated the expression of KRT7. **A)** The effect of FOXA1 on the KRT7 mRNA level was detected by qRT-PCR in GC cell lines. **B)** Luciferase activities of AGS and SGC-7901 cells that co-transfected with pGL3 plasmid containing KRT7 promoter and FOXA1 overexpression plasmid or siFOXA1 were analyzed by dual-luciferase reporter assay. **C)** The effect of FOXA1 on KRT7 protein level was detected by western blot in AGS and SGC-7901 cells. Data were presented as mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$ represents statistically difference.

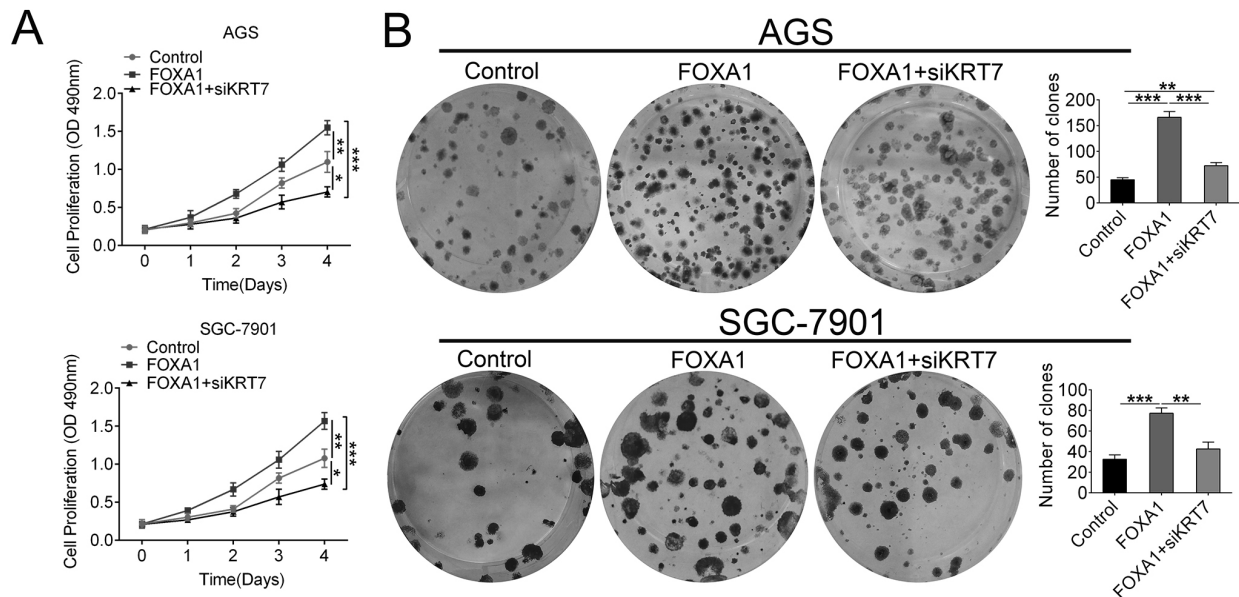


Fig. 3. FOXA1 enhanced GC cell proliferation via transcriptional activating KRT7. AGS or SGC-7901 cell lines were transfected with FOXA1 overexpression plasmid FOXA1 or co-transfected with KRT siRNA. **A)** The effect of FOXA1 overexpression and KRT7 knockdown on GC cell viability was detected by MTT assay. **B)** The function of FOXA1 overexpression and KRT7 knockdown on GC cell proliferative ability was analyzed by colony formation assay. Data were presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ represent statistically difference.

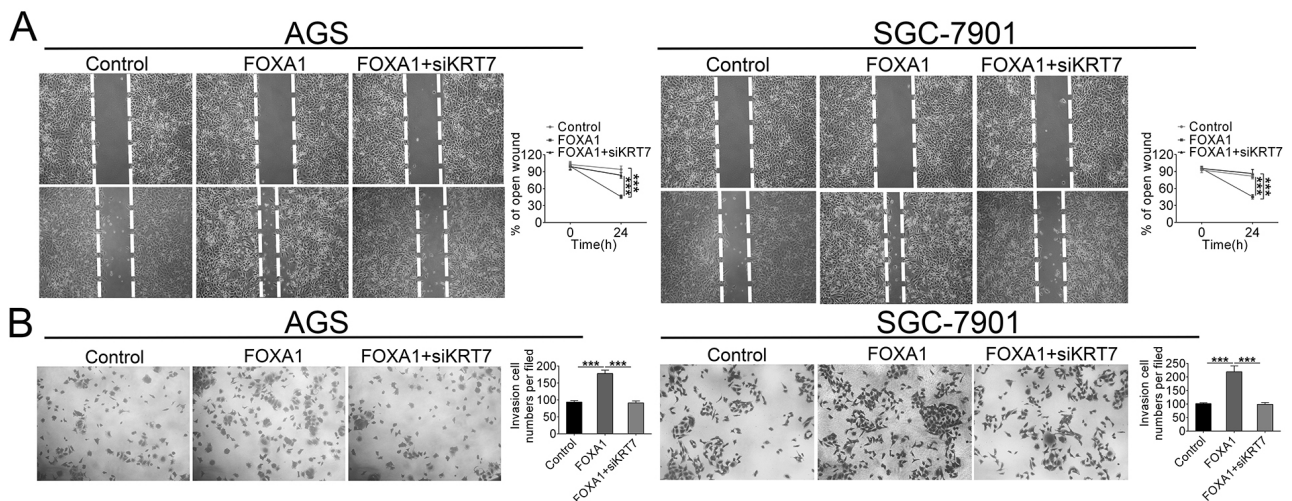


Fig. 4. FOXA1 enhanced GC cell migration and invasion through transcriptional activating KRT7. AGS or SGC-7901 cell lines were transfected with FOXA1 pcDNA plasmid or co-treated with KRT siRNA. **A)** The effect of FOXA1 overexpression and KRT7 knockdown on GC cell migration was detected by wound-healing assay. **B)** The role of FOXA1 overexpression and KRT7 knockdown on GC cell invasive ability was analyzed by Transwell assay. Data were presented as mean $\pm$ SD. *** $P < 0.001$ represents statistically difference.

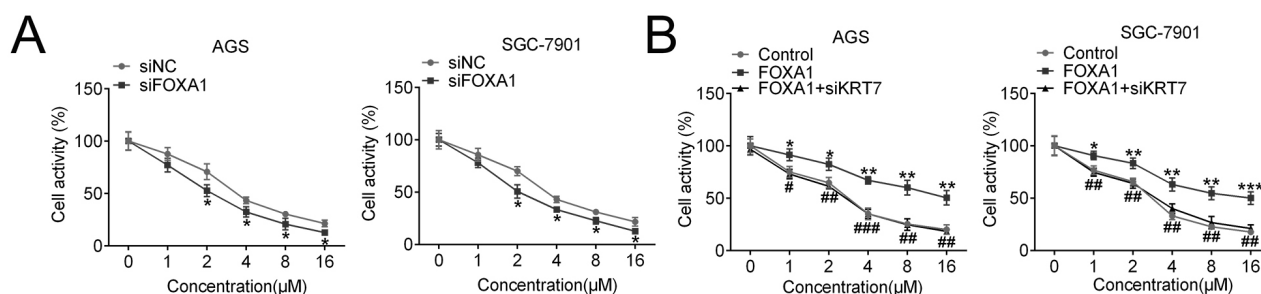


Fig. 5. FOXA1 decreased GC cell chemosensitivity to 5-Fu by targeting KRT7 in GC cells. **A)** AGS and SGC-7901 cells that transfected with FOXA1 overexpression or si-FOXA1 were treated with 5-Fu for 48 h at various concentrations (1, 2, 4, 8 and 16 μ M, respectively), and MTT assay was performed. * $P < 0.05$ represents statistically difference compared with control groups. **B)** AGS or SGC-7901 cells that treated with 5-Fu for 48 h at different concentrations of 0, 1, 2, 4, 8 and 16 μ M were co-transfected with FOXA1 pcDNA and KRT7 siRNA, and subsequently analyzed by MTT assay. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ represent statistically difference compared with control groups, # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ represent statistically difference compared with FOXA1 group.

therapeutic method for GC in the past decades, the incidence and mortality rates of GC still remain high (1, 3). Thus, exploring new molecular mechanism is vital for GC treatment. Herein, we found FOXA1 enhanced cell proliferation, migration and invasion in AGS and SGC-7901 cell lines, suggesting its potential role in GC progression and development.

Acting as a transcriptional regulator, FOXA1 plays critical roles in the development of various tissues, particularly, it exerts important functions in a variety of human cancers (18, 19). FOXA1 is considered to be a good prognosis in cancers due to its highly expressed level in prostate cancer and breast cancer (7, 19). Recently, several studies focus on the role and molecular mechanism of FOXA1 in the modulation of GC. FOXA1 expression level was dramatically elevated in GC tissues compared with normal tissues (10, 20), and highly expressed FOXA1 was reported to be notably associated with lymph node metastasis in GC (11). These investigations suggested that FOXA1 acts as an oncogene of cancer. Controversially, Lin et al. found that FOXA1 acts as a cancer suppressor (13). In a previous study, FOXA1 was a downstream factor targeted by miR-1290, which promoted cell proliferation and migration in GC (13). Differently, FOXA1 was highly expressed in GC cell lines but lightly expressed in GC samples (12). Here, FOXA1 was

highly expressed and promoted cell proliferation and invasion in GC cell lines, indicating its oncogenic role in GC cells. FOXA1 was also proved to exert oncogenic or tumor suppressive functions in other cancers (18). Thus, our further analysis are needed to be performed to determine the effect of FOXA1 in GC *in vivo*.

The underlying mechanism of FOXA1 in GC was explained in previous study, which indicated that the strengthened UGT2B15 level increased FOXA1 expression, and the downstream factors were associated with Hipp-YAP or Yes-signalling pathway (10, 20). Our study indicated that FOXA1 enhanced cell proliferation, migration and invasion through regulating KRT7 expression via activating its promoter in AGS and SGC-7901 cell lines. Consistently, previous study has also reported that KRT7 was transcriptional activated by FOXA1 in esophageal squamous cell carcinomas (16). KRT7 was also proved to be involved in the progress and development of GC (15, 21, 22). Besides, Huang et al. (15) suggested that lncRNA KRT-AS is activated in GC and participates in cancer cell progression by increasing KRT7 expression. In accordance with these findings, our present study proved that FOXA1 act as an oncogene through transcriptionally up-regulating KRT7 in GC cells. 5-FU-mediated chemotherapy is the adjuvant

treatment for patients with GC following surgical resection. (17). However, a number of patients with GC develop 5-FU resistance, which is a major challenge for the effective treatment of GC. (23). Therefore, to investigate the molecular mechanisms underlying GC cell chemoresistance to 5-FU, AGS and SGC-7901 cells that transfected with FOXA1 overexpression or si-FOXA1 were treated with 5-Fu for 48 h at various concentrations (1, 2, 4, 8 and 16 μ M, respectively), and MTT assay was performed. The results showed that silencing of FOXA1 or KRT7 increased cell chemosensitivity to 5-Fu by suppressing viability in AGS and SGC-7901 cells. The mechanism may be associated with the structure of 5-FU, which is similar to pyrimidine, a nucleoside required for DNA replication(24). Therefore, 5-FU primarily affects nucleoside metabolism and may be incorporated into RNA and DNA molecules. Consequently, 5-FU enhances cell-cycle arrest at the G1 phase and/or apoptosis in cancer cells (25).

In summary, this is the first evidence demonstrating that FOXA1 promotes GC cell proliferation, migration and invasion through activation of KRT7. Knockdown of FOXA1 or KRT7 enhances GC cell chemosensitivity to 5-Fu. These results have therapeutic implications and may be exploited for further treatment of GC.

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MICROGLIA M1/M2 POLARIZATION CONTRIBUTES TO ELECTROMAGNETIC PULSE-INDUCED BRAIN INJURY

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The development of electronic technology has attracted attention on the biological effects of electromagnetic fields (EMFs) and electromagnetic pulse (EMP). It remains controversial whether EMP irradiation is neurotoxic or beneficial for recovery from injuries such as cerebral ischemia. Microglia is innate immune cells in the brain, exhibiting either neurotoxicity or neuroprotection effect during various central nervous system diseases, depending on their activation into a classical (M1) or alternative (M2) phenotype, respectively. The Toll-like receptor-4 (TLR4), myeloid differentiation factor 88 (MyD88) and nuclear factor kappa B (NFκB) pathway is important for microglia activation. In this study, we investigated the effect of EMP on neuronal apoptosis and microglia polarization *in vivo* and *in vitro*, using an EMP of 400 kV/m and 1 hertz for 200 pulses. Short EMP irradiation (≤ 24 h) resulted in microglial conversion from the resting to the M1-type state, activation of the TLR4/MyD88/NFκB pathway, higher levels of inflammatory cytokines including interleukin (IL)-6, IL-1β and tumor necrosis factor-α, as well as neuronal apoptosis induction. In contrast, long EMP irradiation (3 days) resulted in microglial activation into the M2-type, decreased apoptosis and inflammatory mediator production, and increased levels of the neuroprotective effectors IL-10, transforming growth factor beta, and brain-derived neurotrophic factor. EMP induces both neuronal damage and neuronal recovery by influencing the switch of M1/M2 polarization and the TLR4/MyD88/NFκB pathway.

The development of electronic technology has led to increased exposure to electromagnetic fields (EMFs). The biological effects of EMFs on tissues may differ depending on wave frequency, carried

energy and heat production (1). An electromagnetic pulse (EMP) is a pulsed EMF distribution, which can be used as a medical device or as a weapon, depending on the amount of energy it carries (2). The central

Key words: electromagnetic pulse; microglia; apoptosis; TLR4/MyD88/NFκB pathway

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nervous system (CNS) is sensitive to electromagnetic radiation, and EMF exposure results in higher rates of headaches, sleep disorders and chronic fatigue (3). Animal experiments have suggested that EMPs lead to neuronal damage, oxidative stress, inflammatory reactions, neuronal apoptosis and excessive release of excitatory neurotransmitters (4). However, the underlying mechanism responsible for the effect of EMP on the CNS remains elusive.

Microglia is innate immune cells of the CNS. They are activated when cell debris or invading pathogens bind to special pattern recognition receptors on their surface. Activated microglia is characterized by a classical (M1) and an alternative (M2) activation phenotype. Microglia can alter their phenotypes in response to micro-environmental disturbances. M1 microglia overactivation and uncontrolled central inflammation are implicated in the pathogenesis and progression of various diseases, including cerebral ischemia-reperfusion injury and Alzheimer's disease (AD) (5). On the contrary, M2 is the major phenotype present during hematoma removal and functional recovery after hemorrhagic stroke (6). Our previous studies have confirmed that repeated EMP exposure leads to neuronal morphological changes, increased apoptosis and upregulation expression of an activated microglial marker named Iba1 (ionized calcium binding adaptor molecule 1). However, the impact of EMP on microglia polarization is still largely unclear (7).

In the current study we investigated the effect of EMP exposure on neuronal apoptosis and microglia polarization at different time points. We also sought to clarify roles of Toll-like receptor-4 (TLR4), myeloid differentiation factor 88 (MyD88), and nuclear factor kappa B (NF κ B) pathway, all of which are important for microglial activation (8) during EMP exposure.

MATERIALS AND METHODS

Animals

Male adult Sprague-Dawley rats, weighing 200-220 g, were purchased from the Experimental Animal Centre of the Fourth Military Medical University (Xi'an, China). The experimental protocol was designed based on the Guidelines for Animal Experimentation of the Second

Affiliated Hospital of Air Force Medical University, and was approved by the Ethics Committee of the same University.

Cell culture

The N9 microglial cell line was acquired from the Chinese Academy of Sciences, and the HT22 cell line was acquired from the Xuzhou Medical College (Xuzhou, China), both of which have been tested by short tandem repeat analysis, as well as morphological observation, to verify their identities. Dulbecco's Modified Eagle medium (DMEM), Iscove's Modified Dulbecco's medium (IMDM) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TLR4 signaling inhibitor CLI-095 (C15H17CIFNO4S) was purchased from InvivoGen (San Diego, CA, USA). N9 microglia were cultured in IMDM containing 10% FBS, while HT22 cells were cultured in DMEM containing 10% FBS, both at 37°C with 5% CO₂ in a humidified culture incubator. Cells were cultured in 6-well plates, with medium replaced every 3 days.

EMP exposure

EMP exposure was conducted using the all-solid-state nano-second (ns) generator as previously described (9). Briefly, an EMF pulse was delivered through a spark gap generator and transmitted into a giga-hertz (Hz) transverse electromagnetic cell. This system was set to deliver an EMP of 400 kV/m peak intensity, 10 ns rise time, 350 ns pulse width, 1 Hz, for a total of 200 pulses. Pulse waveform was monitored by the Tektronix 7000 B oscilloscope (Tektronix, Beaverton, OR, USA). No significant changes in body temperature before and after EMP exposure were observed. For both sham and EMP exposure, cells were placed in the upper chamber of a Perspex™ water bath maintained at 37°C, by circulating heated water through a lower closed chamber. No EMP was delivered for sham exposure.

To evaluate the effects of EMP on cerebral cortical neurons and microglia *in vivo* and *in vitro*, male rats or N9 microglia, respectively, were randomly divided into four groups (n = 6 rats or 6 wells of cells in each group): control (sham exposure); EMP12h, tested 12 h after exposure; EMP24h, tested 24 h after exposure; EMP3d, tested 3 days after exposure. Apoptosis was assessed by protein levels of cleaved caspase-3, B-cell lymphoma-2 (Bcl-2), and

Bcl-2-associated X (Bax). Changes in microglial M1/M2 polarization after EMP exposure were examined by real-time PCR, Western blot and immunofluorescence staining (IF) of M1 microglial marker inducible nitric oxide synthase (iNOS) and M2 microglial marker arginase 1 (Arg1). To explore the TLR4/MyD88/NF κ B pathway, we performed Western blot for TLR4, MyD88 and inhibitor of kappa B (I κ B- α) proteins. Additionally, we tested the mRNA levels of inflammation-related factors including tumor necrosis factor alpha (TNF- α), interleukin (IL)-6, IL-1 β , IL-10, brain-derived neurotrophic factor (BDNF) and transforming growth factor beta (TGF- β).

To investigate the effects of EMP in the presence of the TLR4 inhibitor CLI-095, we cultured N9 microglia in the presence or absence of 1 μ M CLI-095 for 12 h, with or without EMP exposure (10). Four groups were analyzed (n = 6 wells, each): control (no EMP exposure); CLI (no EMP exposure); EMP (400 kV/m, 200 pulses); and CLI+EMP (EMP exposure and incubation with CLI-095 for 12 h). After EMP exposure, M1 and M2 microglial markers and mRNA levels of inflammation-related factors were analyzed as above.

To examine whether TLR4 inhibition could alleviate EMP-induced injury, we co-cultured the N9 cells with HT22 neurons using Transwell chambers (Corning Incorporated, Corning, NY, USA). N9 cells (1×10^5 cells per well, sham- or EMP-exposed) were placed on the porous upper part of the Transwell chamber in IMDM, and HT22 neurons (1×10^5 cells per well, not exposed) on the lower part in DMEM, in a 6-well plate. Co-cultured cells were treated by CLI-095 as in Experiment 2, and HT22 neurons were then collected to for an apoptotic rate assay.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the rat brain or N9 microglia with Trizol reagent (Thermo-Fisher, Waltham, MA, USA) following the manufacturer's protocol. 5 μ g of total RNA was used for first strand cDNA synthesis using the cDNA synthesis kit (Thermo-Fisher). The PCR conditions were as follows: denaturation at 95°C for 2 min; 45 cycles of 94°C for 5 s, 60°C for 10 s, and extension at 72°C for 10 min. The final results were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as the fold change to the target gene over GAPDH. The primers used are listed as follows: iNOS sense 5'-ACC ATG GAG CAT CCC AAG TA-

3', anti-sense 5'-CAG CGC ATA CCA CTT CAG C-3'; Arg1 sense 5'-CCT ATG CGT CAT TTG GGT GG-3', anti-sense 5'-TAC ACG ATG TCC TTG GCA GA-3'; IL-1 β sense 5'-AGG CTG ACA GAC CCC AAA AG-3', anti-sense 5'-CTC CAC GGG CAA GAC ATA GG-3'; IL-6 sense 5'-CCC AAC TTC CAA TGC TCT CCT-3', anti-sense 5'-AGG TTT GCC GAG TAG ACC TC-3'; TNF- α sense 5'-GCC CAG ACC CTC ACA CTC-3', anti-sense 5'-CCA CTC CAG CTG CTC CTC T-3'; IL-10 sense 5'-CCT GCT CTT ACT GGC TGG AG-3', anti-sense 5'-TGT CCA GCT GGT CCT TCT TT-3'; TGF- β 1 sense 5'-AAG AAG TCA CCC GCG TGC TA-3', anti-sense 5'-TGT GTG ATG TCT TTG GTT TTG-3'; BDNF sense 5'-ACC ATC CTT TTC CTT ACT ATG-3', anti-sense 5'-TCT TCC CCT TTT AAT GGT CAA-3'; GAPDH sense 5'-CTG CAC CAC CAA CTG CTT AC-3', anti-sense 5'-CAG AGG TGC CAT CCA GAG TT-3'.

Western blot

Western blot analysis was performed as described previously (11). Briefly, the cortex was removed from rats to extract total protein. Blots were probed with the following primary antibodies: rabbit anti-cleaved caspase-3 (17 kDa; 1:200; Abcam, Cambridge, MA, USA), rabbit anti-iNOS (1:500; Abcam), rabbit anti-Arg1 (1:1000; Abcam), rabbit anti-MyD88 (1:200; Abcam), mouse anti-I κ B- α (1:500, Novus Biologicals), rabbit anti-TLR4 (1:100; Abcam), mouse anti-Bcl-2 and anti-Bax (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA), and GAPDH (1:1000; Cowin Biosciences, Taizhou, Jiangsu, China). Followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:5000; Cowin Biosciences), the signal was quantified by densitometry and the relative protein expression was shown as the ratio to GAPDH.

Apoptotic rate

HT22 cells were collected and centrifuged at 1000 rpm for 5 min, washed twice with ice-cold phosphate buffered saline (PBS), followed by resuspension in binding buffer (1×10^6 cells/mL) as described previously (12). For detection of apoptotic cells, 5 μ L of FITC-conjugated anti-annexin-V staining solution and 2 μ L of propidium iodide solution were added to 100 μ L of cell suspension in binding buffer. After thorough mixing, cells were incubated for 15 min at 20-25°C in dark, and the apoptotic

rate was measured within 1 h using flow cytometry (BD, Franklin Lakes, NJ, USA).

IF staining

Brain sections were fixed for 20 min, incubated in PBS for 5 min, blocked for 60 min with bovine serum albumin, and incubated overnight at 4°C with rabbit primary antibodies against iNOS (1:200; Abcam) and Arg1 (1:500; Abcam). Sections were washed with PBS for 3 times, followed by incubation with Cy3-conjugated goat anti-rabbit secondary antibody (1:500; Cowin Biosciences) for 30 min and stained with DAPI (Beyotime Institute of Biotechnology, Shanghai, China). Images were acquired with laser confocal scanning microscope (Olympus Corp., Shinjuku, Tokyo, Japan).

Statistical analysis

Values were calculated as mean $\pm$ standard deviation (SD). Results from difference experimental groups were analyzed by one-factor analysis of variance, with Tukey's

multiple comparison tests. $p < 0.05$ was considered statistically significant difference.

RESULTS

Effect of EMP exposure in vivo on neuronal apoptosis

At 12 and 24 h after EMP exposure, the expression of cleaved caspase-3 and Bax in rat brains increased compared to the control group, while the expression of Bcl-2 decreased ($p < 0.05$). However, 3 days after EMP exposure, the expression of cleaved caspase-3 and Bax decreased and the expression of Bcl-2 increased, compared to the 12-h and 24-h EMP groups ($p < 0.05$; Fig. 1, a-c).

Modulation of microglial polarization after EMP exposure in vivo and in vitro

Microglia exhibited dynamic polarization after EMP exposure, switching from the classically-activated M1 phenotype to the alternatively-activated

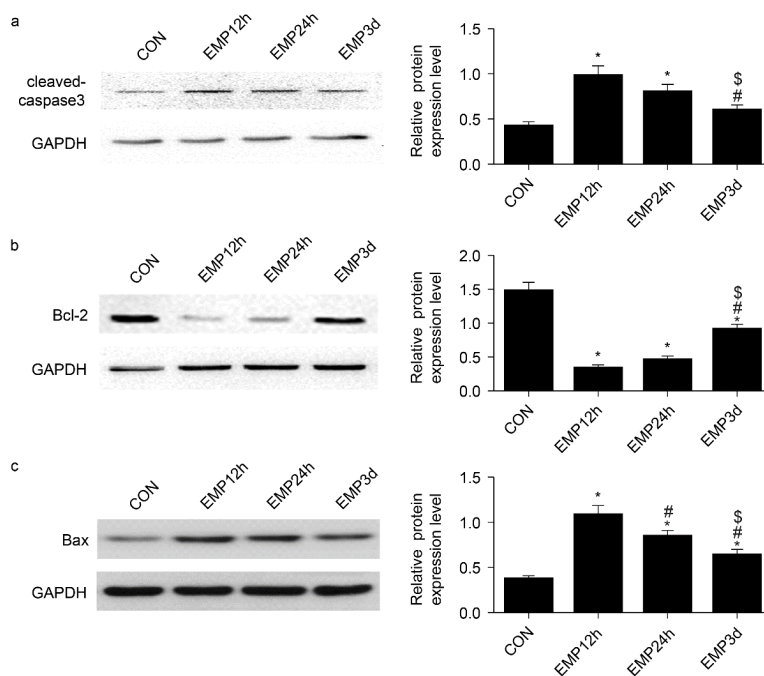


Fig. 1. Neuronal apoptosis at different time points after electromagnetic pulse (EMP) exposure in vivo. **a-c)** Western blot analysis and quantification of cleaved caspase-3 (**a**), Bcl-2 (**b**), and Bax (**c**) expression levels ($n = 6$). The gels/blots were cropped for illustration purposes. The data are presented as means $\pm$ SD. * $p < 0.05$ compared to the control group; # $p < 0.05$ compared to the 12-h EMP-exposed group; \$ $p < 0.05$ compared to the 24-h EMP-exposed group. Con, control group (not exposed); EMP12h, 12-h EMP-exposed group; EMP24h, 24-h EMP-exposed group; EMP3d, 3-d EMP-exposed group.

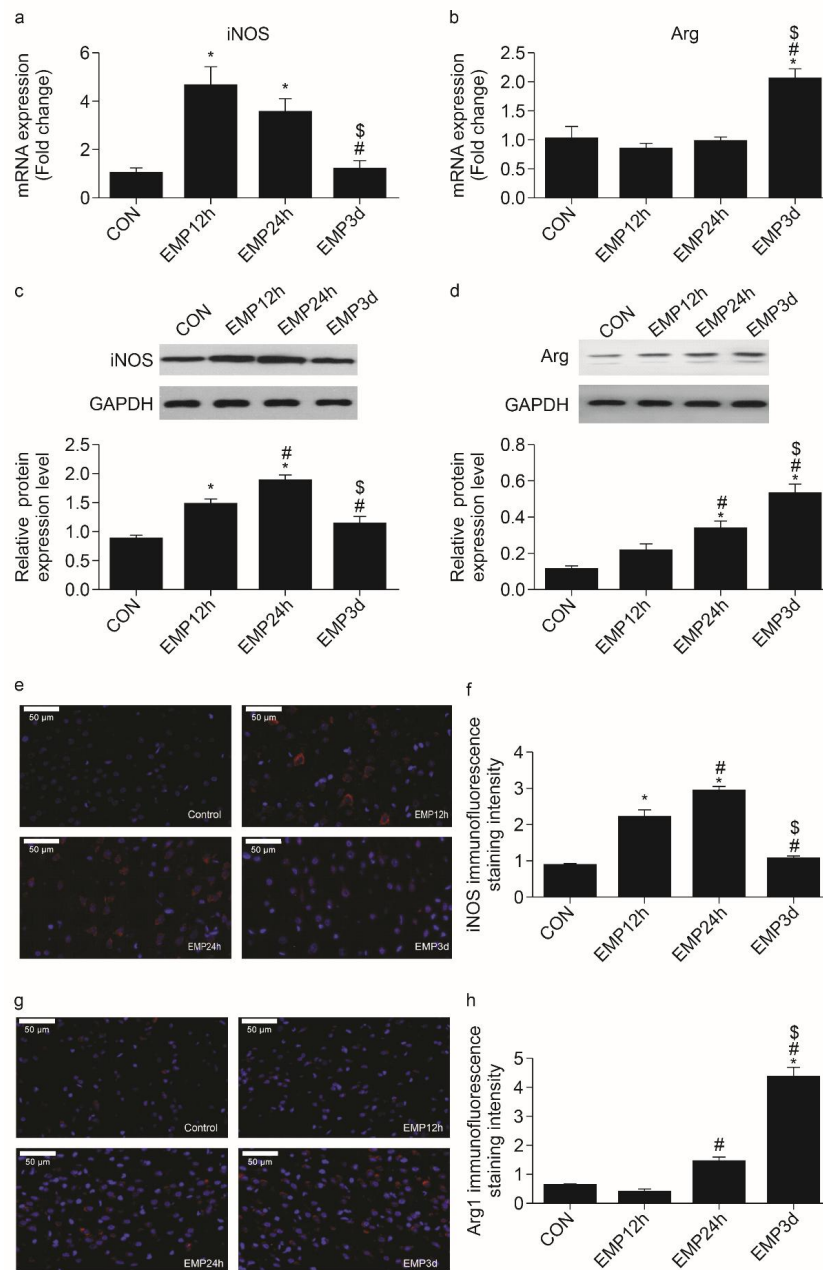


Fig. 2. Switch of microglial M1/M2 polarization at different time points after electromagnetic pulse (EMP) exposure in vivo. **a-d**) Effects of EMP exposure on the mRNA (a, b) and protein (c, d) levels of the M1 marker, iNOS (a, c), and the M2 marker, Arg1 (b, d), at different time points ($n = 6$). The gels/blots were cropped for illustration purposes. **e**) Representative rat brain sections immunostained for the M1 microglial marker iNOS (red) and DAPI (blue). Scale bars = 50 μ m. **f**) Quantification of immunofluorescence intensity of iNOS staining in each experimental group. **g**) Representative rat brain sections immunostained for the M2 microglial marker Arg1 (red) and DAPI (blue). Scale bars = 50 μ m. **h**) Quantification of immunofluorescence intensity of the Arg1 staining in each experimental group. The data are presented as means $\pm$ SD. * $p < 0.05$ compared to the control group; # $p < 0.05$ compared to the 12-h EMP-exposed group; \$ $p < 0.05$ compared to the 24-h EMP-exposed group. Con: control group (not exposed); EMP12h: 12-h EMP-exposed group; EMP24h: 24-h EMP-exposed group; EMP3d: 3-d EMP-exposed group.

M2 phenotype. Analyses with qRT-PCR and Western blot showed significantly higher expression levels of M1-microglial marker iNOS in the 12- and 24-h EMP exposure groups than the control group ($p < 0.05$). In contrast, the 3-day EMP exposure group showed lower iNOS expression than the 12- or 24-h EMP groups ($p < 0.05$; Fig. 2, a and c), while expression of M2-microglial marker Arg1 was higher in the 3-day EMP exposure group than the other groups ($p < 0.05$; Fig. 2, b and d).

The M1 and M2 microglia were identified in brain sections using iNOS and Arg1 labelling, respectively (Fig. 2, e and g). Significant increased numbers of iNOS-positive microglia were observed in the 12- and 24-h EMP groups compared to the

control group ($p < 0.05$), which was then significantly decreased in the 3-day EMP group ($p < 0.05$; Fig. 2f). On the contrary, Arg1-positive microglia increased significantly in the 3-day EMP group compared to the other groups ($p < 0.05$; Fig. 2h). We repeated the experiment *in vitro* using the N9 microglial cell line, and obtained consistent results with our *in vivo* study (Fig. 3, a-d).

EMP effect on the TLR4/MyD88/NF κ B signaling pathway

To evaluate the effect of EMP on the TLR4/MyD88/NF κ B signaling pathway, we analyzed protein expressions of TLR4, MyD88 and I κ B- α in the brains of EMP-exposed and control rats. When I κ B is

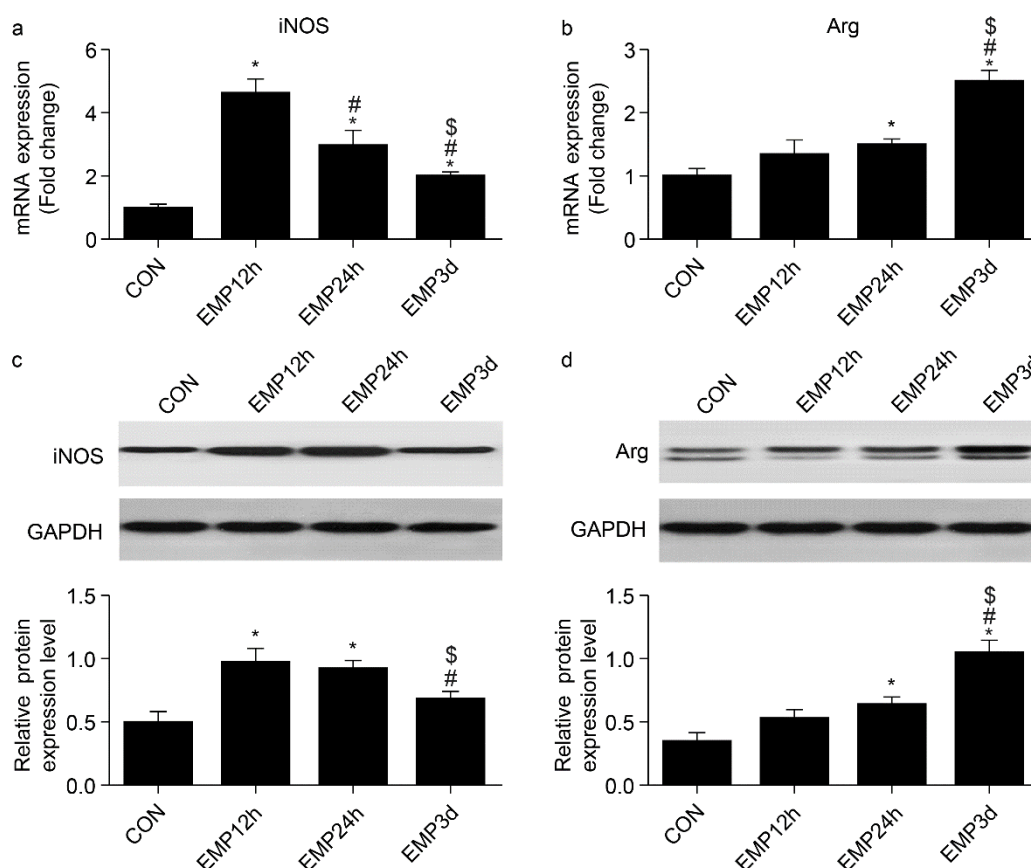


Fig. 3. Switch of microglial M1/M2 polarization at different time points after electromagnetic pulse (EMP) exposure in cultured N9 microglia. **a-d**) Effects of EMP on the mRNA (a, b) and protein (c, d) levels of the M1 marker iNOS (a, c) and the M2 marker Arg1 (b, d) at different time points ($n = 6$). The gels/blots were cropped for illustration purposes. The data are presented as means $\pm$ SD. * $p < 0.05$ compared to the control group; # $p < 0.05$ compared to the 12-h EMP-exposed group; \$ $p < 0.05$ compared to the 24-h EMP-exposed group. Con: control group (not exposed); EMP12h: 12-h EMP-exposed group; EMP24h: 24-h EMP-exposed group; EMP3d: 3-d EMP-exposed group.

phosphorylated, NF κ B is activated and translocates into the nucleus to trigger transcription of genes involved in inflammatory response (13). We found that TLR4 and MyD88 protein levels were increased, while I κ B- α level was evidently decreased, in the 12- and 24-h EMP groups compared to control ($p < 0.05$; Fig. 4, a-c). In contrast, the 3-day EMP exposure restored the levels of TLR4 and MyD88 to control values ($p < 0.05$; Fig. 4, a-c).

As TLR4/MyD88/NF κ B pathway is important for inflammatory signal transduction (14), we also

measured the mRNA levels of several key pro-inflammatory and anti-inflammatory molecules produced by microglia. The results showed that EMP exposure for 12 and 24 h increased the mRNA levels of M1 microglia-related molecules, such as IL-1 β , IL-6 and TNF- α , while a 3-day EMP exposure restored them to control values ($p < 0.05$; Fig. 4, d-f). The mRNA levels of anti-inflammatory M2-related molecules IL-10, TGF- β and BDNF were significantly increased after short term EMP exposure, and even more robustly so after long EMP exposure ($p < 0.05$; Fig. 4, g-i).

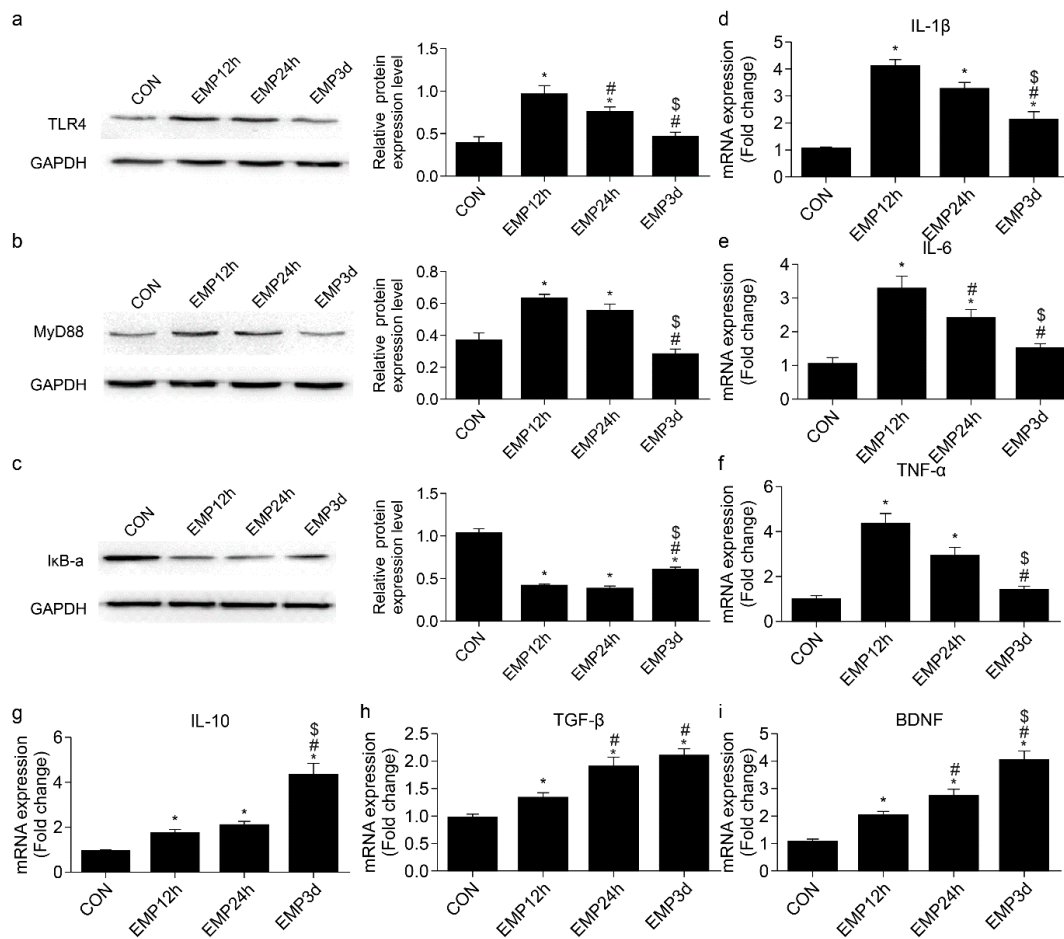


Fig. 4. Effects of electromagnetic pulse (EMP) exposure on the TLR4/MyD88/NF κ B signaling pathway and the expression of pro- and anti-inflammatory factors in vivo. **a-c)** Western blot analysis and quantification of the protein levels of TLR4 (a), MyD88 (b), and I κ B- α (c) at different time points after EMP exposure, as indicated ($n = 6$). The gels/blots were cropped for illustration purposes. **d-f)** Relative mRNA levels of the pro-inflammatory molecules IL-1 β , IL-6, and TNF- α at different time points after EMP exposure ($n = 6$). **g-i)** Relative mRNA levels of the anti-inflammatory molecules IL-10, TGF- β , and BDNF at different time points after EMP exposure, as indicated ($n = 6$). The data are presented as means $\pm$ SD. * $p < 0.05$ compared to the control group; # $p < 0.05$ compared to the 12-h EMP-exposed group; \$ $p < 0.05$ compared to the 24-h EMP-exposed group. Con: control group (not exposed); EMP12h: 12-h EMP-exposed group; EMP24h: 24-h EMP-exposed group; EMP3d, 3-d EMP-exposed group.

EMP effect on microglial polarization and neuronal apoptosis upon inhibition of TLR4 *in vitro*

To investigate the effect of EMP in the presence of the TLR4 inhibitor CLI-095, we cultured N9 microglia in the presence or absence of CLI-095 for 12 h, with or without EMP exposure. Consistent with our previous results, iNOS mRNA and protein levels were significantly higher in EMP-exposed cells than control ($p < 0.05$, Fig. 5, a, c, e). Although CLI-095 did not change iNOS level in control cells, it partially restored EMP-elevated iNOS level in the CLI+EMP group to control values ($p < 0.05$; Fig. 5, a, c, e). In contrast, there was no significant difference in the mRNA and protein levels of Arg1 among the experimental groups ($p > 0.05$; Fig. 5, b, d, f).

We also analyzed the mRNA levels of IL-1 β , IL-6, TNF- α , IL-10, TGF- β and BDNF. We found that the pro-inflammatory mRNAs were higher in the EMP than the control group, while CLI-095 reduced their levels in the EMP-exposed cells ($p < 0.05$; Fig. 6, a-c). Similarly, the mRNA levels of TGF- β and BDNF were also higher in the EMP than the control

group, while the addition of CLI-095 in EMP-exposed cells significantly increased the mRNA levels of IL-10 and BDNF ($p < 0.05$; Fig. 6, d-f). In the presence of CLI-095, TGF- β level was lower in the CLI+EMP than the EMP group ($p < 0.05$; Fig. 6e).

To examine whether TLR4 inhibition could alleviate EMP-induced injury, we co-cultured the N9 cells with HT22 neurons. We analyzed the apoptosis rate of HT22 cells by flow cytometry after 12 h of co-culture with or without EMP exposure, in the presence or absence of CLI-095. We found that the neuronal apoptotic rate was higher in the EMP than the control and CLI-treated groups, while CLI-095 negated the effect of EMP exposure in the CLI+EMP group ($p < 0.05$; Fig. 6g, 6h).

DISCUSSION

In this study, we have demonstrated that short EMP exposure (≤ 24 h) leads to M1-type microglia polarization, TLR4/MyD88/NF κ B signaling pathway activation, inflammatory cytokine

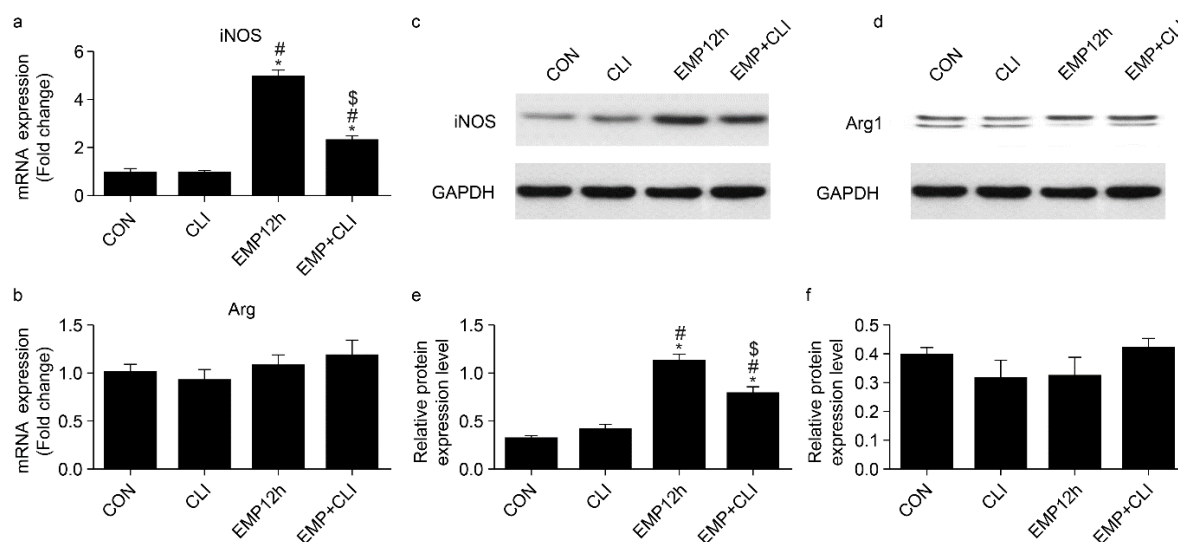


Fig. 5. Effects of electromagnetic pulse (EMP) exposure on microglial polarization in the presence of TLR4 inhibitor CLI-095 in cultured N9 cells. **a, b**) Relative mRNA expression of the M1 marker iNOS (a) and the M2 marker Arg1 (b) after EMP exposure and treatment with CLI-095, as indicated ($n = 6$). **c, d, e, f**) Effects of EMP and CLI-095 on M1 marker iNOS and M2 marker Arg1 protein levels ($n = 6$). The gels/blots were cropped for illustration purposes. The data are presented as means $\pm$ SD. * $p < 0.05$ compared to the control group; # $p < 0.05$ compared to the CLI-095-treated group; \$ $p < 0.05$ compared to the EMP-exposed group. Con: control group (not exposed); CLI: CLI-095-treated group (not exposed); EMP: EMP-exposed group; CLI+EMP: EMP-exposed group treated with CLI-095.

upregulation and neuronal apoptosis. In contrast, long-term EMP exposure (3 days) induces self-repair mechanisms, including microglia conversion to the M2-type, downregulation of inflammatory cytokines, and concomitant upregulation of neuroprotective

effectors, leading to recovery of the apoptosis rate.

EMPs are high-voltage, short EMF pulses, characterized by a spectral bandwidth ranging from extremely low frequency up to 1.5 GHz and extremely fast rise time. The biological effects of EMFs have

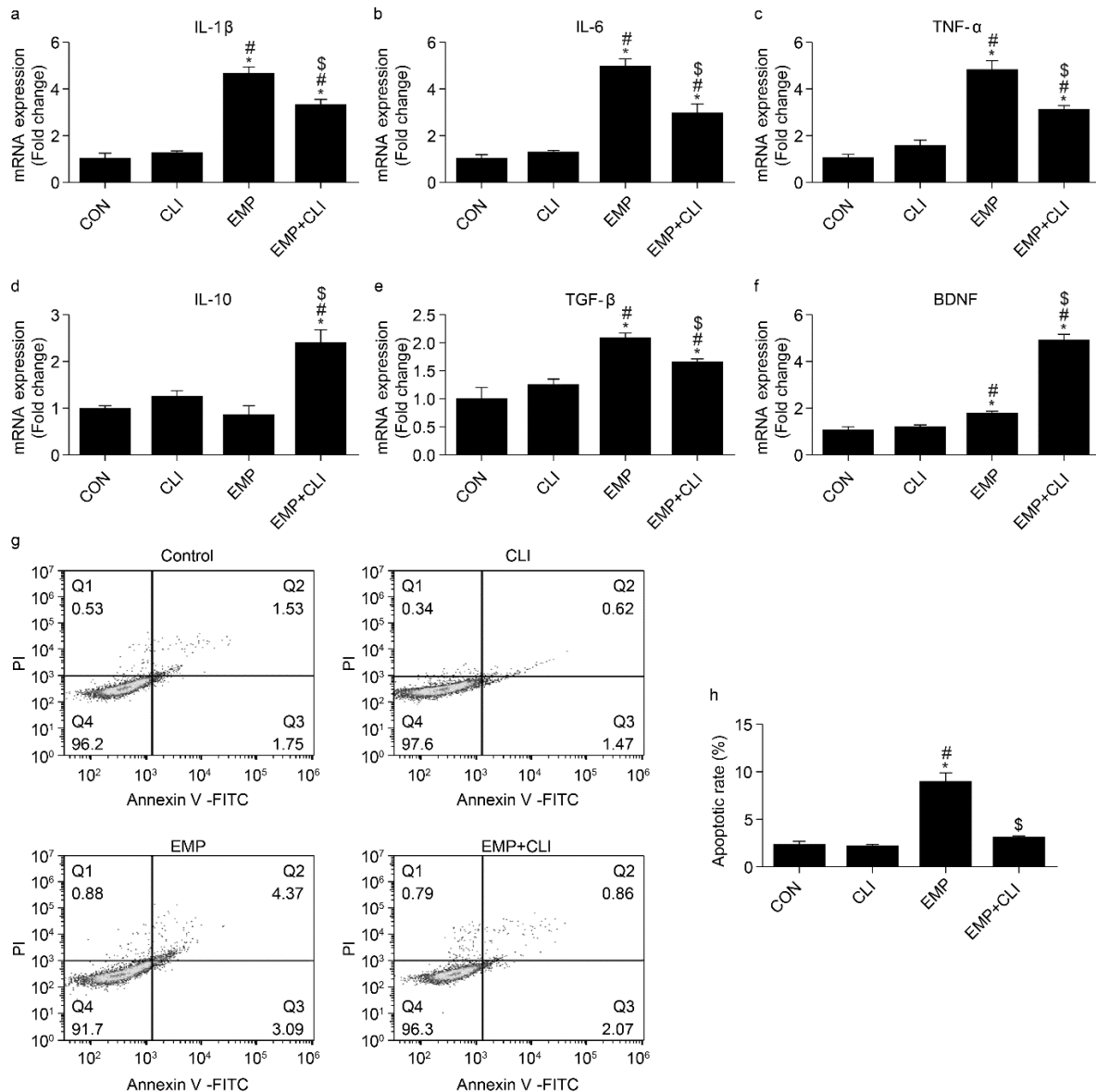


Fig. 6. Effects of electromagnetic pulse (EMP) exposure and CLI-095 treatment on the expression of pro- and anti-inflammatory factors and apoptosis in co-cultured N9 and HT22 cells. **a-c)** Relative mRNA levels of the pro-inflammatory molecules IL-1 β , IL-6, and TNF- α ($n = 6$). **d-f)** Relative mRNA levels of the anti-inflammatory molecules IL-10, TGF- β , and BDNF ($n = 6$). **g)** Flow cytometry analysis of co-cultured cells in each experimental group, as indicated. **h)** Quantification of the apoptotic rate in each experimental group, as indicated. The data are presented as means $\pm$ SD. * $p < 0.05$ compared to the control group; $^{\#}p < 0.05$ compared to the CLI-095-treated group; $^{\S}p < 0.05$ compared to the EMP-exposed group. Con: control group (not exposed); CLI: CLI-095-treated group (not exposed); EMP: EMP-exposed group; CLI+EMP: EMP-exposed group treated with CLI-095.

a cumulative effect, and EMF exposure has been reported to correlated with neurodegenerative diseases such as AD (15, 16). EMP causes neuronal organelle impairments, and long-term EMP exposure induces β -amyloid ($A\beta$) deposition and cognitive dysfunction, through induction of oxidative stress and overexpression of the amyloid precursor proteins (17, 18). However, a clinical study showed that low-frequency, low-energy EMP treatment after arthroscopic surgery resulted in faster and complete short-term functional recovery, which was also maintained at 3-year follow-up (19). Moreover, it has been demonstrated that this treatment protects the heart against ischemia/reperfusion injury (20). The effects of low-frequency, low-energy EMP against hypoxic damage have also been demonstrated in SH-SY5Y and in PC12 cells, where EMP mediated a significantly reduced productions of reactive oxygen species and pro-inflammatory cytokines (21). Taken together, these results suggest that the biological effects of EMP are related to the intensity, frequency and pulse duration of the fields, and a strong EMP can cause serious tissue damage. In this study, we showed that low-intensity EMP results in less damage and self-repair. Moreover, exposure to low-frequency, low-energy EMP could be beneficial for tissue healing.

A state of high M1/M2 ratio has been associated with the pathological processes of ischemic stroke, intracerebral hemorrhage (ICH) and traumatic brain injury, as M1 cells produce excessive amounts of pro-inflammatory cytokines, which subsequently induce neuronal apoptosis and influence neurogenesis (22). M2 microglia, on the other hand, exhibit a strong phagocytic capacity for apoptotic and necrotic cells, and positively regulate neurogenesis through the production of neurotrophic factors (23). Therefore, M2 cells are important in maintaining CNS homeostasis under pathological conditions.

The regulation of M1 to M2 polarity transition has been considered a potential target for the treatment of some CNS diseases. AD is characterized by the accumulation of $A\beta$, which induces M1 activation and subsequent synthesis of pro-inflammatory factors, including $TNF-\alpha$, nitric oxide and superoxide (24, 25). Nevertheless, microglia have

also been involved in the phagocytosis of $A\beta$, thus acting in a neuroprotective manner. However, it has been difficult to identify their specific roles in the pathogenesis of AD (26). The anti-inflammatory drug minocycline has been reported to be beneficial if used following disease onset, but detrimental if given at later stages of AD (27, 28). Thus, switching the M1/M2 ratio would a key step to clarify the role of microglial polarization at different stages of the disease. Our experiments, focusing on EMP exposure and its effects on activated microglia phenotype and neuronal apoptosis, may warrant further studies on EMP treatment, by aiming for the microglial transition from M1 to M2.

Microglia expresses TLR4, which contributes to inflammatory response after CNS damage, such as cerebral ischemia and ICH (29, 30). TLR4 knockout resulted in higher numbers of M2 and lower numbers of M1 microglia, leading to improved performance in behavioral tests (31). TLR4 promotes microglial polarization toward the M1 phenotype, thus inducing neuroinflammation. Our results are consistent with these findings: after short EMP irradiation, the M1 microglia-mediated inflammatory cytokine production is associated with TLR4 signaling, as the TLR4 antagonist downregulated the expression of M1 phenotype biomarkers and alleviated the production of inflammatory cytokines. However, direct evidence is lacking regarding the potential relationship between the increased late-stage expression of M2 markers and the TLR4 signaling pathway. M2 microglia subtypes M2a, M2b, M2c and M2d can be induced by different environmental factors (32). M2b microglia, which are induced by agonists of IL-1R and the TLRs, express the suppressor of cytokine signaling 3 (SOCS3), which negatively regulates TLR4 signaling (33). Therefore, M2b microglia and SOCS3 may be associated with the microglial polarization switch from the M1 to M2 state, following long-term EMP exposure.

In conclusion, short-term EMP exposure induces microglial activation toward the M1-type, thus promoting neuro-inflammation and neuronal apoptosis through the TLR4/MyD88/NF κ B pathway. On the other hand, long-term EMP exposure activates microglia toward the M2-type, which reduces

inflammatory response and promotes expression of anti-inflammatory and repair mediators, leading to recovery of neuronal apoptosis. Further research on the regulation of microglial M1/M2 polarization, particularly to determine the causal link between microglial M1/M2 polarization and observed changes in molecular factors, would be useful for developing EMP-based clinical therapies.

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OVEREXPRESSION OF miR-145-5p ALLEVIATED LPS-INDUCED ACUTE LUNG INJURYYL. YU¹, G. YU², ZY. DING¹, SJ. LI¹ and QZ. FANG¹

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Acute lung injury (ALI) is a disease with high incidence and no effective therapeutic treatments. miR-145-5p has been reported to be aberrantly expressed in lung injury tissues, suggesting a potential role in the progression and development of ALI. To validate this hypothesis and explore the underlying mechanism, a mouse model of ALI was established using lipopolysaccharide (LPS). Hematoxylin and eosin (H&E) staining verified the successful establishment of mouse model with ALI. Levels of interleukin (IL)-1 β , IL-6, tumor necrosis factor α (TNF- α) and myeloperoxidase (MPO) were detected by both enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry. Mouse type II alveolar epithelial cells (AT II) were isolated and treated with LPS. miR-145-5p was significantly down-regulated both in mice with acute lung injury and LPS-induced AT II cells. Dual luciferase assays confirmed miR-145-5p could target and regulate Toll Like Receptor 4 (TLR4). Further analysis showed that miR-145-5p overexpression decreased the expression levels of IL-1 β , IL-6 and TNF- α in LPS-induced AT II cells. miR-145-5p overexpression also blocked the LPS-induced activation of nuclear factor kappa B (NF- κ B) pathway and reactive oxygen species (ROS) accumulation in AT II cells. Finally, in ALI mouse model, miR-145-5p overexpression alleviated lung tissue injury, decreased the expression levels of IL-1 β , IL-6 and TNF- α and reduced MPO activity. In conclusion, miR-145-5p participated in the progression and development of ALI by decreasing the production of pro-inflammatory cytokines, inhibiting NF- κ B pathway and suppressing ROS accumulation, shedding light on miR-145-5p as a potential therapeutic target for the treatment of ALI.

Acute lung injury (ALI) is a disease with high incidence and no effective therapeutic strategies, which threatens the life of patients (1). The pathogenic incidence and mortality are relatively high worldwide, accounting for approximately 190,600 cases in the United States per year, including 74,500 deaths (3). The pathogenic mechanisms are diverse and complicated, including sepsis, pneumonia, chest trauma, (1). ALI is characterized by the disruption of alveolar barrier and inflammation response of lung tissues (2). Usually, permeability increase of alveolar

capillary membrane, excessive lung inflammation, edema, and apoptosis of alveolar epithelial cell are observed as clinical tissue alterations and biomarkers in patients (1). Inhibition of inflammatory response and cell apoptosis have been reported to improve the prognosis of patients with ALI (5, 6). As lipopolysaccharide (LPS) exhibited the ability to induce the apoptosis of alveolar epithelial cells (7, 8), it was utilized to generate ALI mouse model in this study (9, 10). Previous studies indicated excessive apoptotic type II alveolar epithelial cells

Key words: acute lung injury; miR-145-5p, lipopolysaccharide; NF- κ B; reactive oxygen species

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(AT II) could damage the epithelial barrier and lead to ALI (4), and were therefore used in our *in vitro* experiment.

As small noncoding RNA molecules containing 18-22 nucleotides, miRNAs play vital roles in post-transcriptional regulation by targeting 3'UTR of target genes (11). Accumulating evidence shows many miRNAs were aberrantly expressed in patients suffering from ALI, suggesting miRNAs might participate in its progression and development (12). Lu et al. reported there were 12 miRNAs differentially expressed in patients with ALI after severe acute pancreatitis (SAP) (12). Neudecker et al. concluded translocation of miR-223 from neutrophils to pulmonary epithelial cells alleviated ALI through inhibiting the expression of poly (adenosine diphosphate-ribose) polymerase-1 (PARP-1) (13). Fang et al. found miR-1246 down-regulation could inhibit pulmonary endothelial cell apoptosis and alleviate ALI by targeting angiotensin-converting enzyme 2 (ACE2) (8). Xiao et al. revealed miR-429 was involved in inflammatory response, and inhibition of miR-429 could attenuate lung tissue injury (14). Interestingly, miR-145-5p was also identified as an important miRNA in lung injury and was decreased in lung injury tissues in influenza pneumonia model (15), indicating its potential role in the progression and development of ALI. To validate this hypothesis, a mouse model of ALI was established, and AT II cells were isolated. Cells and mice were treated with LPS. In this study, the expression level, downstream targets, and the signaling pathway of miR-145-5p were explored in the context of ALI *in vitro* and *in vivo*, with the aim to reveal the potential pathogenic mechanisms.

MATERIALS AND METHODS

Animals and cells

Twenty-four mice (Beijing Vital River Laboratory Animal Technology Co., Ltd) aged 7-8 weeks were divided into two groups: control and ALI groups (n=12 in each group). Mice in the ALI group were injected intratracheally with 10 mg/kg LPS and the same concentration of phosphate-buffered saline (PBS) was injected into mice in the control group. 5 mg/kg miR-

145-5p mimic was intravenously injected into ALI mice via the tail vein. After being anesthetized with 100 mg/kg sodium pentobarbital 24 h post-injection, the mice were sacrificed. In each group, three mice were used to measure wet/dry weight ratio of lung, three mice were used to evaluate tissue damage, and the other 6 mice were used for molecular biological analysis.

Mouse type II alveolar epithelial cells (AT II) were isolated according to a previous study (16). Briefly, the cells were maintained using DMEM medium containing 10% FBS, 100 U/ml penicillin and streptomycin (Gibco, USA) at 37°C in a humidified 5% CO₂ atmosphere. AT-II cells were treated with 2μg/ml of LPS for 12 h.

Hematoxylin and eosin (H&E) staining

After LPS treatment, lung tissues were fixed and embedded in 4% paraformaldehyde and paraffin, respectively. The embedded tissues were cut into 5-μm sections and stained with hematoxylin for 5 min and eosin for 1 min. Stained sections were evaluated under a light microscope.

RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR)

Trizol (Invitrogen, Carlsbad, California, USA) was used to extract total RNA. Reverse Transcriptase Kit and PrimeScript RT Master Mix Kit (Sigma-Aldrich, USA) were used to obtain miRNAs and cDNA, respectively. qRT-PCR was performed using SYBR Premix Ex Taq TM II kit (Takara, Dalian, China). The primer sequences were: miR-145-5p: 5'-GTCCAGTTTTCCCAGGAATCCCT-3' (17); TLR4_F: 5'-ACCTGTCCCTGAACCCTATGAA-3'; TLR4_R: 5'-CTTCTAAACCAGCCAGACCTTG-3' (18). Interleukin-1β (IL-1β)_F: 5'-TGAAATGCCACCTTTTGACAG-3'; IL-1β_R: 5'-CCACAGCCACAATGAGTGATAC-3' (19). Interleukin-6 (IL-6)_F: 5'-CTGCAAGAGACTTCCATCCAG-3'; IL-6_R: 5'-AGTGGTATAGACAGGTCTGTTGG-3' (19); Tumor necrosis factorα (TNF-α)_F: 5'-GGCAGGTCTACTTTGGAGTCAT-3';

TNF-α_R: 5'-CAGAGTAAAGGGGTCAGAGTGG-3' (19). *GAPDH* was utilized as an internal control. Data were analyzed using the 2^{-ΔΔCt} method.

Protein level of myeloperoxidase (MPO), IL-6, IL-1β and TNF-α in lung tissues of ALI mice were assessed by

enzyme linked immunosorbent assay (ELISA) according to the manufacturers' instructions (R&D Systems, Minneapolis, MN, USA).

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

MTT assays were used to detect cell proliferation ability. Cells were inoculated into 96-well plates at a concentration of 1×10^4 cells per well, then 24 h post-seeding, 10 μ l of MTT (ThermoFisher Scientific, USA) was added into each well. Cells were then incubated for 4 h at 37°C in a humidified 5% CO₂ atmosphere. The absorbance was measured at a wavelength of 490 nm using a microplate reader (Thermo Fisher Scientific, USA).

Cell transfection

miR-145-5p mimic, miR-145-5p inhibitor and corresponding controls (NC mimic and NC inhibitor) were synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). 6×10^5 cells were inoculated into each well and maintained at 37°C in a humidified 5% CO₂ for 24 h. Then, miR-145-5p mimic, inhibitor and controls were respectively transfected into cells using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA). Cells were processed for subsequent experiments 48 h post-transfection.

Luciferase reporter assay

Downstream target gene of miR-145-5p was determined using luciferase reporter assay. Wild type (WT) and mutant types of 3'-untranslated region of *TLR4* were constructed into the pmiRGLO vector (Promega, Madison, WI, USA). miR-145-5p mimic, NC mimic and pmiRGLO were then transfected into HEK-293T cells, respectively, and 48 h post-transfection, relative luciferase activities were measured and Renilla luciferase activity was utilized to normalize luciferase activity.

Western blot

RIPA buffer (Pierce, Rockford, IL, USA) was used to lyse cells and extract total proteins. Protein concentration was measured using BCA kit (Pierce, Rockford, IL, USA); 30 μ g of protein were separated by a 12% SDS-PAGE and then transferred onto PVDF membranes. After blocking with 4% non-fat milk for 1 h, the PVDF membranes were incubated with anti-TLR4 (1:500, Abcam), anti-p-p65 (1:500, Abcam), anti-p65 (1:500, Abcam), anti-p-IkBa

(1:500, Abcam), anti-IkBa (1:500, Abcam) or anti-GAPDH (1:1000, Abcam) antibodies for 12 h at 4°C. GAPDH was used as an internal control and the blots were visualized using ECL Plus reagents (Thermo Fisher Scientific, USA).

Immunofluorescence

Immunofluorescence was used to determine the translocation of p65 from cytoplasm to nuclei. AT II cells were inoculated into six-well plates and maintained for 24 h, and were then fixed using 4% paraformaldehyde (PFA) and blocked by 2% bovine serum albumin for 1 h at 25°C. The cells were probed with anti-p65 (1:200, Abcam) for 12 h at 4°C and with fluorescent-labeled secondary antibodies (1:500, Abcam), after which they were stained with DAPI at 25°C for 5 min and observed under a laser confocal microscopy (Carl Zeiss, Oberkochen, Germany).

Reactive oxygen species detection

The reactive oxygen species (ROS) accumulation level was detected by dichlorofluorescein-diacetate (DCFH-DA) kits (Sigma-Aldrich) according to manufacturers' instructions. Cells were cultured in six-well plates for 24 h and then incubated with DCFH-DA (10 μ M) for 30 min at 37°C. After removal of the excess, cells were washed three times using 1xPBS buffer. A fluorescence microscope was used for image acquisition. FACS Calibur flow cytometry (BD Bioscience, San Jose, CA, USA) was used to quantify the fluorescence intensity on FL-1 channel.

Immunohistochemistry

The expression level of MPO in lung tissues was determined using immunohistochemistry. Lung tissues were firstly fixed using 10% formaldehyde and embedded in 4% paraffin. Then embedded tissues were cut into 5- μ m sections, followed by incubation with anti-MPO antibody (1:100, Abcam) for 12 h at 4°C and with goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP) at 25°C for 1 h. The stained sections were observed under light microscopy (Olympus, Japan).

Statistical analysis

SPSS statistics software 22.0 (Chicago, IL, USA) was used for statistical analysis. Data were represented as mean $\pm$ standard deviation (SD). Student's *t*-test was used to analyze the differences between the two groups. One-way ANOVA was used to analyze the differences

among multiple groups. A p -value < 0.05 was considered statistically significant.

RESULTS

miR-145-5p expression was decreased in ALI mice and LPS-induced AT II cells

A mouse model was established, and lung tissue injury and lung wet/dry weight ratio were assessed. HE results showed more serious neutrophil infiltration and lung tissue injury were observed in the ALI group than that in controls (Fig. 1A). Consistently, the lung injury score and wet/dry weight ratio in ALI mice were higher compared to those in control mice ($***p < 0.001$, Fig. 1A). In addition, the activity and expression levels of MPO in ALI mice were higher ($***p < 0.001$, Fig. 1, B-C). Expression levels of IL-1 β , IL-6 and TNF- α were significantly elevated in ALI mice (Fig. 1D), indicating the successful establishment of an ALI mouse model.

AT II cells were isolated and treated with LPS, and the miR-145-5p levels were significantly decreased in ALI mice and in LPS-induced ATII cells compared to those in the control group, respectively ($***p < 0.001$; Fig. 1, E-F). In addition, the viability of LPS-induced ATII cells was inhibited (Fig. 1G). Taken together, these results indicate that miR-145-5p was downregulated in mice of ALI and that LPS-induced ATII cells.

TLR4 was a target gene of miR-145-5p

miRNAs have been reported to exert functions through regulating the expression of target genes. To explore the potential target genes of miR-145-4p, we used the database of TargetScan (<http://www.targetscan.org>), miRanda (<http://www.microrna.org/microrna/getExprForm.do>) and miRDB (<http://mirdb.org/>), and the bioinformatics results predicted TLR4 was a potential target of miR-145-5p. Putative binding sequences between TLR4 and miR-145-5p are shown in Fig. 2A. We subsequently cloned luciferase reporter constructs. miR-145-5p mimic significantly increased the relative luciferase activity in cells transfected with WT TLR4 3'-UTR compared to the control group ($***p < 0.001$, Fig. 2A). miR-145-5p was overexpressed or knocked

down by transfecting miR-145-5p mimic or inhibitor constructs, respectively ($***p < 0.001$, Fig. 2B). miR-145-5p mimic significantly reduced TLR4 expression and miR-145-5p inhibitor significantly elevated TLR4 level ($***p < 0.001$, Fig. 2, B-C). These results demonstrated that TLR4 was targeted and negatively regulated by miR-145-5p.

miR-145-5p inhibited pro-inflammatory cytokine production in LPS-induced AT II cells

The following analysis suggested that miR-145-5p mimic exhibited no significant effects on the mRNA expression levels of IL-1 β , IL-6 and TNF- α ($p > 0.05$), and LPS increased expression levels of these markers. However, miR-145-5p mimic significantly decreased the mRNA levels of IL-1 β , IL-6 and TNF- α ($*p < 0.05$) in LPS-treated AT II cells (Fig. 3A). Interestingly, miR-145-5p inhibitor showed contrary results in LPS-treated AT II cells ($**p < 0.01$, Fig. 3A). ELISA results verified miR-145-5p mimic down-regulated the protein levels of IL-1 β , IL-6 and TNF- α ($**p < 0.01$) and miR-145-5p inhibitor increased the protein levels of IL-1 β , IL-6 and TNF- α ($*p < 0.05$) in LPS-treated AT II cells (Fig. 3B). Collectively, our results revealed that miR-145-5p reduced pro-inflammatory cytokine production in LPS-induced AT II cells.

miR-145-5p inhibited LPS-induced activation of NF- κ B pathway in AT II cells

NF- κ B signaling pathway plays an important role in the process of inflammation, therefore, the effects of miR-145-5p on NF- κ B signaling pathway were determined. LPS was found to elevated the protein levels of p-p65 and p-I κ Ba compared to the control group, while miR-145-5p mimic decreased such LPS-induced p-p65 and p-I κ Ba upregulation in AT II cells ($**p < 0.01$, Fig. 4A). In addition, p65 was expressed in cytoplasm in the NC-mimic group, while p65 was localized in nuclei upon LPS treatment (Fig. 4B). It is worth mentioning that p65 protein was observed in cytoplasm upon miR-145-5p mimic transfection and LPS treatment simultaneously in AT II cells (Fig. 4B). To sum up, the results obtained from this section suggested that LPS induced translocation of p65 from cytoplasm to nuclei in AT II cells, while

miR-145-5p mimic inhibited such translocation, suggesting miR-145-5p inhibited LPS-induced activation of NF- κ B pathway in AT II cells.

miR-145-5p decreased LPS-induced ROS accumulation in AT II cells

ROS production was higher in the LPS-treated group than that in the control group. Interestingly, ROS amount was lower in the miR-145-5p mimic + LPS-treated group compared to that in the LPS-treated group (Fig. 5A). Flow cytometry results confirmed the relative fluorescent intensity in LPS-treated cells was elevated ($***p < 0.001$, Fig. 5B), and the relative fluorescent intensity in LPS-induced AT II cells was reduced upon miR-145-5p mimic transfection ($***p < 0.001$, Fig. 5B). These results indicated miR-145-5p decreased LPS-induced ROS accumulation in AT II cells.

miR-145-5p alleviated lung injury in ALI mice

The role of miR-145-5p *in vivo* was further explored, and it was observed that the level was significantly up-regulated in ALI mice treated with miR-145-5p mimic ($***p < 0.001$, Fig. 6A). H&E staining exhibited miR-145-5p mimic alleviated neutrophil infiltration and lung tissue injury in ALI mice compared to the control group (Fig. 6B). The score of lung injury, lung wet/dry weight ratio and the levels of IL-1 β , IL-6, TNF- α were decreased in ALI mice treated with miR-145-5p mimic ($*p < 0.05$, Fig. 6, C-D). In addition, both ELISA and immunohistochemistry results showed that MPO activity was inhibited post-miR-145-5p mimic treatment in ALI mice as compared to the control ($**p < 0.01$, Fig. 6E-F), indicating miR-145-5p could alleviate lung injury in mice with ALI.

DISCUSSION

ALI is a disease with high incidence and mortality (3), and accumulating efforts have been devoted to underlying molecular mechanisms with the aim to reveal new therapeutic targets. In this study, we firstly established a mouse model of ALI using LPS. Various schemes could be utilized to induce ALI, such as oleic acid, acid aspiration, intravenous bacteria, hyperoxia, bleomycin, cecal

ligation, peritonitis, saline lavage and puncture (20). LPS was considered as the optimal candidate as LPS-induced ALI mice shared similarities with acute respiratory distress syndrome (ARDS), including the inflammatory response of neutrophils and up-regulation of cytokines in lung tissues (20). We discovered LPS induced serious neutrophil infiltration and lung tissue injury and the expressions of IL-1 β , IL-6, TNF- α and MPO were elevated in ALI mice. These results suggested LPS treatment successfully established a mouse model of ALI.

Many miRNAs are involved in the progression of ALI (21-23), among which miR-145-5p was decreased in lung injury tissues (15), suggesting its potential role in the progression of ALI. We found miR-145-5p was down-regulated in the ALI mouse model and cell model, which were consistent with the conclusions obtained by Tan et al. (15), but were completely incompatible to those found by Lee et al. (24), who concluded miR-145-5p level was increased in microvesicles from bronchoalveolar lavage fluid in LPS-induced ALI. These contradictory results might be explained by the different samples applied in various studies.

To explicate the underlying mechanisms, we detected the production amount of pro-inflammatory cytokines. We concluded that miR-145-5p overexpression blocked the levels of IL-1 β , IL-6, TNF- α and MPO both *in vitro* and *in vivo*. In addition, NF- κ B pathway activation and ROS accumulation caused by LPS in AT II cells could also be inhibited by miR-145-5p overexpression. To sum up, our results confirmed miR-145-5p inhibited pro-inflammatory cytokine production, suppressed NF- κ B pathway activation and decreased ROS accumulation in LPS-induced AT II cells. NF- κ B was a common signaling pathway mediating lung inflammation, and LPS-induced NF- κ B activation was associated with lung neutrophilia and epithelial permeability in lung disease (25). Previous studies reported ROS was produced and oxidized phospholipids were formed during ALI (26). Based on our results and previous studies, we hypothesized that miR-145-5p might alleviate lung injury through inhibiting pro-inflammatory cytokine production, suppressing NF- κ B pathway

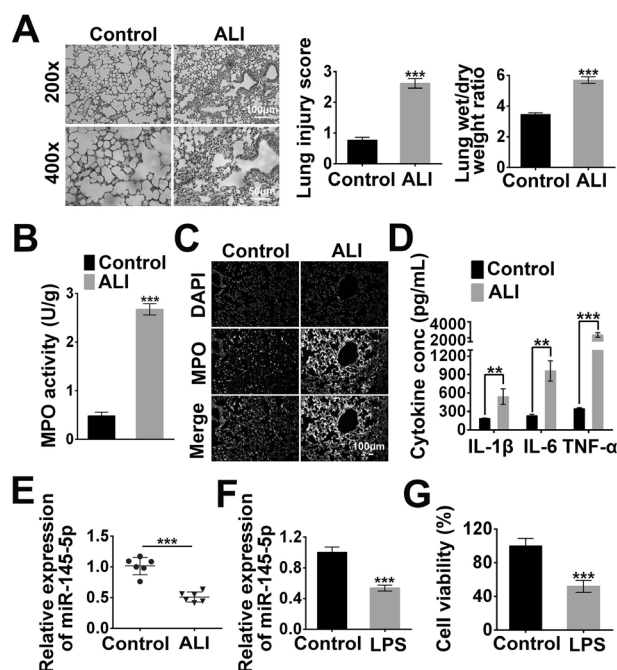


Fig. 1. miR-145-5p expression was decreased in mice of acute lung injury (ALI) and LPS-induced AT II cells. **A)** HE staining and lung wet/dry weight ratio were determined to assess lung injury. **B)** ELISA assay was used to detect the activity of MPO in ALI and control groups. **C)** Immunohistochemistry was performed to confirm the expression of MPO in ALI and control groups. **D)** ELISA assay was performed to detect the expression levels of IL-1 β , IL-6 and TNF- α . **E)** qRT-PCR measured miR-145-5p level in mice of ALI. **F)** qRT-PCR measured the level of miR-145-5p in AT II cells induced by LPS. **G)** Cell viability was determined in AT II cells treated with LPS. (** $p < 0.01$; *** $p < 0.001$).

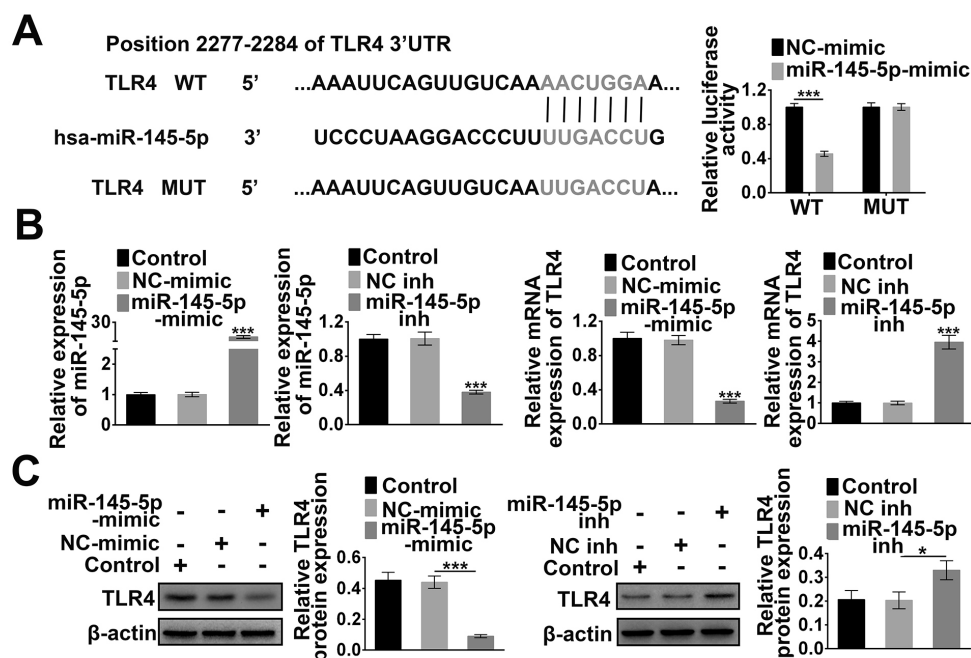


Fig. 2. TLR4 was identified as a target gene of miR-145-5p. **A)** Putative binding sequences between miR-145-5p and TLR4 were listed and luciferase reporter assay was performed. **B)** mRNA level of TLR4 was negatively regulated by miR-145-5p. **C)** Protein levels of TLR4 were negatively regulated by miR-145-5p. (** $p < 0.01$; *** $p < 0.001$).

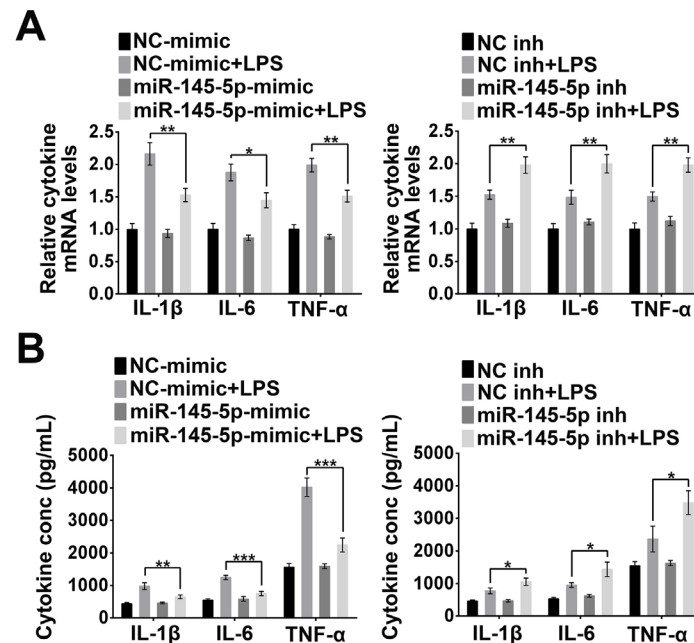


Fig. 3. miR-145-5p inhibited pro-inflammatory cytokine production in LPS-induced AT II cells. **A)** mRNA levels of IL-1 β , IL-6 and TNF- α in LPS-induced AT II cells transfected with miR-145-5p mimic or inhibitor, were determined by qRT-PCR. **B)** Protein levels of IL-1 β , IL-6 and TNF- α in LPS-induced AT II cells transfected with miR-145-5p mimic or inhibitor. (** $p < 0.05$; * $p < 0.01$; *** $p < 0.001$).

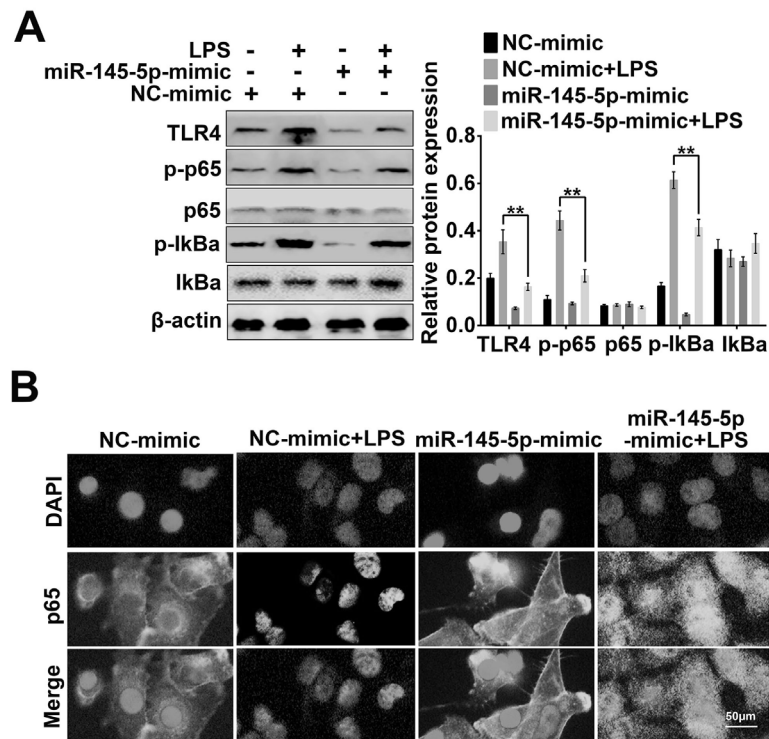


Fig. 4. miR-145-5p inhibited LPS-induced activation of NF- κ B pathway in AT II cells. **A)** Western blotting was used to detect the protein levels of TLR4, p-p65, p65, p-I κ B α and I κ B α . **B)** Immunofluorescence was used to detect the translocation of p65 from cytoplasm to nuclei. (** $p < 0.01$).

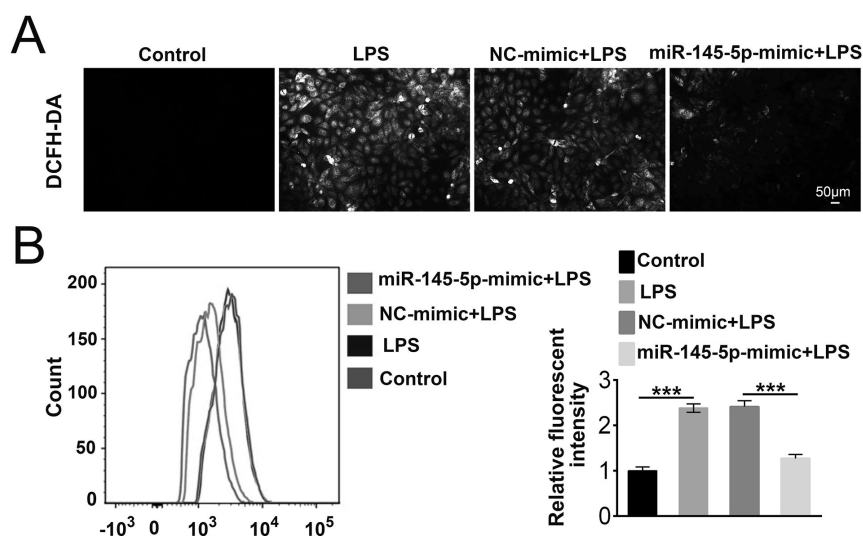


Fig. 5. miR-145-5p decreased LPS-induced ROS accumulation in AT II cells. **A)** ROS level in LPS-induced AT II cells that transfected with miR-145-5p mimic was detected by DCFH-DA kit. **B)** Flow cytometer detected ROS level in LPS-induced AT II cells transfected with miR-145-5p mimic. (***) $p<0.001$.

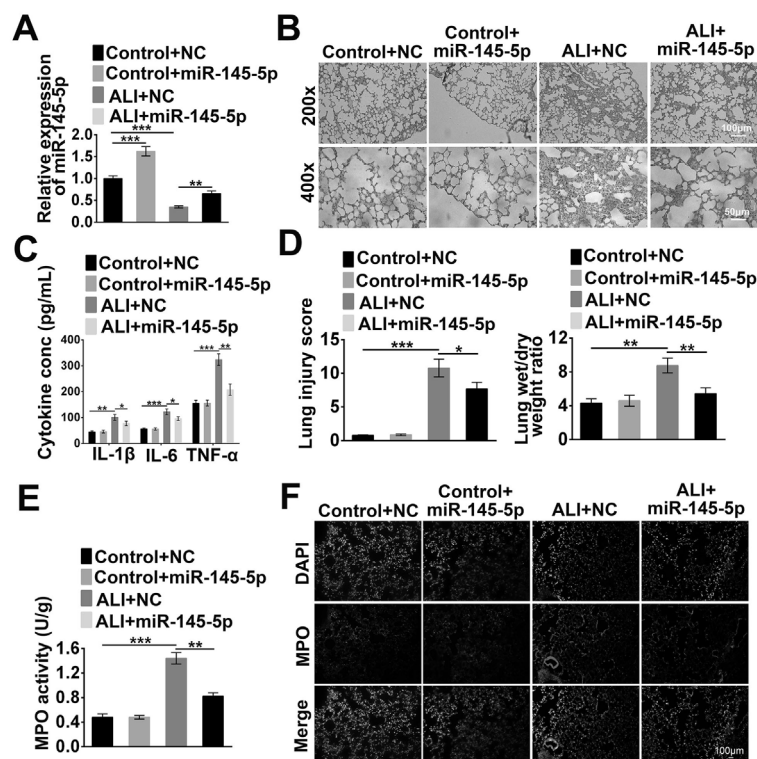


Fig. 6. miR-145-5p alleviated lung injury in mice of acute lung injury. **A)** qRT-PCR detected the expression of miR-145-5p in mice with ALI. **B)** HE staining was performed to assess lung injury in mice with ALI treated by miR-145-5p mimic. **C)** ELISA assay was used to determine the protein levels of IL-1 β , IL-6 and TNF- α in mice with ALI that were treated by miR-145-5p mimic. **D)** The lung injury score and lung wet/dry weight were determined in mice with ALI treated by miR-145-5p mimic. **E)** ELISA assay was used to detect the activity of MPO in mice with ALI treated by miR-145-5p mimic. **F)** Immunohistochemistry was used to detect the expression of MPO in mice with ALI treated by miR-145-5p mimic. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

activation and decreasing ROS accumulation in the progress of ALI.

Traditionally, microRNAs (miRNAs) are generally considered to regulate gene expression post-transcriptionally by inhibiting translation or inducing target mRNA (11). Here, TLR4 was targeted and negatively regulated by miR-145-5p in the luciferase assay. Previous studies showed that TLR4 signaling played a vital role in activating alveolar macrophage in ALI (27, 28). C3H/HeJ mice exhibited resistance to LPS stimulation due to a mutation of TLR4 protein intracellular domain, and TLR4 mutation could regulate the severity of ALI (26, 29). In addition, Lv et al. reported TLR4 was targeted by miR-27a, therefore regulating LPS-induced inflammatory response (30). Taken together, these studies demonstrated that TLR4 exerted vital effects on the progression of ALI. Based on these evidence, we inferred miR-145-5p might regulate lung injury through targeting TLR4. It has been reported that ALI was mediated by TLR4-TRIF-TRAF6-NF- κ B signaling (26), the present study also confirmed that miR-145-5p could suppress NF- κ B pathway activation in LPS-induced AT II cells, raising a possibility that the viral role of miR-145-5p played in ALI might be associated with TLR4-TRIF-TRAF6-NF- κ B signaling.

Accumulating evidence demonstrated miR-145 could induce macrophage-mediated inflammation through targeting Arf6 in LPS-treated THP-1 cells to simulate chronic metabolic inflammation (31). Zhang et al. (32) reported miR-145-5p was significantly upregulated in bone marrow-derived macrophages in the polarized activation processes of M1 and M2, suggesting miR-145-5p also might be related to macrophage polarization. Therefore, we hypothesized miR-145-5p might regulate inflammatory function in macrophages and other immune cells, which finally contribute to ALI. This hypothesis should be further explored in future studies.

Collectively, our research provided the first evidence demonstrating that miR-145-5p participated in the progression and development of ALI by decreasing pro-inflammatory cytokine production, inhibiting NF- κ B pathway and

suppressing ROS accumulation, shedding light on miR-145-5p as a potential therapeutic target for the treatment of ALI.

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BRAIN FUNCTIONS AND UNUSUAL β -AMYLOID ACCUMULATION IN THE HYPERTENSIVE WHITE MATTER LESIONS OF RATS

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This study used Sprague Dawley (SD) rats with stroke-prone renovascular hypertension (RHRSP) to establish an animal model of hypertensive white matter lesions (WML), so as to explore the brain functions and unusual β -amyloid (A β) accumulation in WML. Hypertensive WML and brain dysfunctions were evaluated by measuring the caudal arterial pressure of model rats, and by observing the histomorphological deformations of the prefrontal lobe, temporal lobe, hippocampus and corpus callosum, as well as by counting of the number of neurons using Hematoxylin and Eosin (H&E) staining, and by evaluating the changes in rat brain functions, including memory and the ability of visual space learning, using the Morris Water Maze Test. In addition, the study discussed the correlation between A β accumulation and hypertensive WML cognitive impairment by adopting an enzyme-linked immunosorbent assay (ELISA) to detect the level of A β 1-42, and by detecting the expression of amyloid precursor protein (APP) and Beta-secretase 1 (BACE1) using Western blot. Results of the study showed that at 4 weeks, 8 weeks, 12 weeks and 16 weeks after operation, the blood pressure and brain A β expression in the rats of the model group notably increased ($P < 0.01$), along with deformed and degenerated brain tissues, confirming that the unusual A β accumulation may participate in the occurrence and development of hypertensive WML as well as the induction of cerebral cognitive decreases.

White matter lesions (WML), also known as leukoaraiosis (LA), white matter changes (WMC) and white matter hyperintensities (WMH), are a type of cerebral small vessel diseases (CSVD) (1). WML are mainly manifested as flaky changes in the white matter under the cerebral cortexes and/or around the ventricles, while the CT and MRI scans of WML are featured by low density shadows and high signal intensity of the T2 weighted imaging (WI), respectively (2). WML are most common in

lateral ventricles, followed by corona radiata, basal ganglia and semiovale central areas. Under the cerebral cortex, WML are featured by a reduced number of nerve fibers, partial demyelination and gliocyte proliferation. In addition, the chronic ischemia caused by cerebral atherosclerosis may be important for the occurrence and development of WML (3). Furthermore, WML around the ventricles are often manifested as linear structure damages and gliocyte proliferation in the brain ependyma, along

Key words: hypertensive; WML; A β , HE staining; ELISA

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with nerve demyelination around the lesions. The obstruction of cerebrospinal fluid circulation and damage to the blood-brain barrier may also play important roles in the pathogenesis of WML (4). The prevalence of WML among the elderly is extremely high, and research data indicate that approximately 1/3 of WML cases aged over 60 can be detected by positive MRI results. In addition, the diagnosis rate of WML by MRI increases with age (5).

As a polypeptide of 4KDa coded by chromosome 21, β -amyloid ($A\beta$) is generated by the amyloid precursor protein (APP) after its reaction with proteolytic enzymes (6). $A\beta$ is one of the major pathological signs for type-AD dementia among the elderly, and the formation, accumulation and degradation of $A\beta$ affect the whole pathologic process of dementia. Recent researchers have found that the increase in the plasma levels of $A\beta$ in WML patients is associated with the severity of WML and memory deterioration (7). It has also been widely accepted that hypertension is an important risk factor of WML. In clinical research, a low level of cerebrospinal fluid $A\beta$ 1-42 is usually observed with an increased level of $A\beta$ accumulation inside the brain (8), suggesting the presence of a correlation between an unusual level of $A\beta$ accumulation and the risk of WML. Furthermore, $A\beta$ may also contribute to WML-induced cognitive function impairment. However, due to the limitations of past clinical studies, the early pathological changes of WML and the specific locations of $A\beta$ accumulation in brain tissues remain unknown. Therefore, in this study, an animal model of hypertensive WML was established to clarify the correlation between $A\beta$ accumulation and hypertensive WML-induced cognitive impairment. It was hoped that the results of this study may help the development of preventive treatments for WML-induced cognitive impairment.

MATERIALS AND METHODS

Animals

For this study, 155 healthy male Sprague-Dawley (SD) rats aged 2-3 months were taken as experiment animals (90-120g, purchased from Shanghai Hengdelao Biological Co., Ltd., China). All rats were

given free access to a common granular feed and tap water. Housing environment: specific pathogen free (SPF) grade animal facility, circadian rhythm of 12/12h, environment temperature of 20~25°C, and humidity of 50~60%. After one-week of adaptation, the rats with abnormal behaviors and slow responses were excluded (5 rats in total were excluded, with 150 rats remaining). This study was approved by the Ethics Committee of The Third Affiliated Hospital of Qiqihar Medical University. All animal experiments were conducted in strict accordance with the international laws of animal studies.

Establishment of a stroke-prone renovascular hypertension experimental model

The 150 SD rats were randomly divided into 3 groups: control group (50 rats), sham-operated group (50 rats) and hypertension group (50 rats). Then, each group was divided into 5 subgroups according to the following time points: before operation, 4 weeks (4W) after operation, 8 weeks (8W) after operation, 12 weeks (12W) after operation, and 16 weeks (16W) after operation, with 10 rats in each subgroup of the control group, 10 rats in each subgroup of the sham-operated group and 10 rats in each subgroup of the model group.

Rats in the hypertension group were first anaesthetized with a 10% chloral hydrate solution (Abcam, USA) (0.35mL/per 100 g body weight) through intraperitoneal injection. After routine skin disinfection, the rats were covered by an aseptic drape and their skin was opened along the abdominal mid-line to sequentially cut open the tendon, peritoneum, and the abdominal cavity. After clamping the left renal artery and wrapping the abdominal organs with a piece of gauze soaked in saline, the organs and the renal vein were pushed rightward with a wet cotton swab to expose the left kidney and left renal pedicle before 5 mm of the left renal artery was carefully separated from the vein and horizontally clamped using a U-shaped silver clip with a 0.3 mm inner diameter (Shanghai Alcott Biotech Co. Ltd., China). After injecting 0.075 mL of gentamicin (Henan Runhong Pharmaceutical Co., Ltd., China) into the left abdominal cavity, the abdominal organs were pushed leftward to expose the right kidney and

right renal pedicle, and the right renal artery was also clamped using the procedure described above, followed by injection of 0.075 mL of gentamicin into the right abdominal cavity. After double renal artery stenosis was formed, the abdominal cavity and skin incision were closed and sutured; After the operation, the rats were placed in a supine position under a heater to maintain their body temperature before they woke up naturally. Then, the rats were returned to their cages and given free access to food and water. During the first 3 days after operation, the rats were given gentamicin (0.125mL/100g/per rat/per day) through intraperitoneal injection to prevent infection, and the operation site was disinfected daily with 75% alcohol. The skin incision, mental status, urine, feces and food/water intake of each rat were carefully observed. The rats in the sham-operated group underwent the same operational procedures, including medication and postoperative care, as those of the model rats, except that the sham-operated rats were not subjected to the clipping of bilateral renal arteries. No treatments were carried out in the control group. In the sham-operated group, the other procedures of operation, medication and nursing were identical with those of the model group except for bilateral renal artery clamping.

Criteria for the successful establishment of the stroke-prone renovascular hypertension (RHRSP) model (9) were normal systolic blood pressure (SBP) of about 110 mm Hg in the control group, and SBP of >16.6 k Pa (125mm Hg) in the model rats at 1 week after model establishment. However, if the blood pressure of a model rat showed no significant increase after the operation, the model establishment was considered as a failure.

Blood pressure measurement and evaluation

The rats in each group were divided into 5 subgroups, respectively: before operation, 4 weeks after operation, 8 weeks after operation, 12 weeks after operation and 16 weeks after operation. A Rat Tail Artery Cuff Method Blood Pressure System (Shanghai Alcott Biotech Co., Ltd., China) was used to measure the caudal arterial SBP of conscious rats in each subgroup according to the instruction of the manufacturer. The SBP of each rat was measured

3 times using the same method before the average SBP was taken. Subsequently, the caudal artery blood pressure of rats in the control, model and sham groups was measured before the operation and at 4 weeks, 8 weeks, 12 weeks and 16 weeks after the operation.

Brain function evaluation

In this study, a Morris Water Maze (Beijing Institute of Physiology, Chinese Academy of Sciences, China) was used to evaluate the brain functions of rats. The Morris Water Maze was consisted of a cylindrical pool coated with stainless steel, a small camera, and an image collecting and monitoring system. Fixed room furnishing objects outside the water maze were used as the reference objects throughout the entire test. During the adaptive training, the total distance by which all rats in a group swam freely in 2 min reflected the swimming capacity of the rats in that group.

The Morris Test was carried out for the rats in the sham-operated and hypertension groups as followings: Firstly, two groups of rats were trained adaptively, and the platform was removed. With North-East (NE) as the entry point, the rats were put into the water facing the wall of the pool. The rats were allowed to swim freely in the pool for 2 min. The swimming trajectory of the rats was recorded and the swimming distance was calculated. Positioning navigation experiments were carried out from day 1 to day 5, which lasted for five days. The platform was fixed in the SW (South-West) quadrant center and immersed about 1 cm underwater. Training was performed at the platform four times daily at the same interval. During the training, a water entry point (day 1: N-E-SE-NW; day 2: SE-N-NW-; day 3: NW-SE-E-; day 4: E-NW-SE; day 5: N-SE-NW) was randomly selected, and the rats were put into the water facing the wall of the pool. The time required by each rat to find and climb back up to the platform, i.e. the escape latency, was observed and recorded. All rats had to use 4 different entry points throughout the 4 trainings conducted on each day. If a rat failed to find the platform within 90 s, the rat was led to the platform and stood on it for 15 s, and the latency under such circumstance was recorded as 90 s. The

intervals between trainings were 15~20 min. Once the training was finished, all rats were taken away to have their fur dried. When necessary, the rats were put under a heating lamp for 5 min before they were sent back to their cages. On the 7th day, the platform was removed so that the rats could enter the water facing the wall of the pool opposite of the origin quadrant of the platform, i.e. NE quadrant. The number of trips made by each rat to swim across the original position of the platform (the platform diameter was 12 cm) within 2 min was recorded, which was used to evaluate the spatial memories of the rats regarding the original position of the platform.

Preparation of paraffin sections

After finishing the Morris Water Maze Test, all rats were injected with 10% chloral hydrate solution (0.35mL/per 100 g weight) intraperitoneally for anesthesia. Next, their left ventricles were pierced and perfused with 0.9% saline (Beijing Chemical Works, China) followed by 4% paraformaldehyde (Beijing Chemical Works, China). After successful perfusion, the brain tissues of the rats were completely taken out and put onto a dial scale. A point located at 2 mm from the posterior half of the optic chiasma was used as the datum point, and a 3 mm-thick sample of brain tissues was incised horizontally extending toward the cerebellum direction along the posterior half of the optic chiasma and was put into a 4% paraformaldehyde solution for 24 h of fixation at 4°C. Then, all brain tissue samples were taken out of the fixation liquid, rinsed with 70% alcohol (Beijing Chemical Works, China) to remove residual liquid, and dehydrated until the tissues became transparent. With the anterior frontal plane facing down, all the tissue samples were immersed successively into a wax cup (I) → a wax cup (II) → a wax cup (III) (0.5~1h each) to allow the paraffin wax to infiltrate into the brain tissues. Next, with the anterior frontal plane facing down, the wax-infiltrated tissues in the wax cup (III) were placed horizontally into paraffin wax embedding boxes (Leica, Germany), which were marked and numbered in advance and filled with melted paraffin wax. The embedded tissues were then taken out for consecutive horizontal sectioning after the paraffin wax was cooled and solidified.

The thickness of the sections was 4 μ m. The sliced wax sections were then flattened in lukewarm water, dried at room temperature, and baked 4~6h in a 60°C incubator for future applications.

Hematoxylin-Eosin staining

Tissue sections were stained with Hematoxylin and Eosin (H&E) (Beijing Solarbio Science & Technology Co., Ltd., China) and mounted to observe and compare the histomorphological deformations of rat brain regions, including the hippocampus, corpus callosum, prefrontal lobe and temporal lobe, under an Olympus Optical Microscope (Olympus, Japan; 10×40 magnification), so that the number of neurons in the hippocampus CA1 region, corpus callosum, prefrontal lobe and temporal lobe could be calculated. Meanwhile, 3 adjacent visual fields were selected on each section to analyze the results of staining using Image-Pro Plus 6.0 software.

Detection of A β 1-42 expressions in rat hippocampus by enzyme-linked immunosorbent assay

Rats were injected with a 10% chloral hydrate solution (0.35mL/per 100g weight) intraperitoneally to induce anesthesia. Next, their skull was quickly opened to collect brain tissues. Then, the hippocampus and corpus callosum of the rats were precisely weighed and put into 2 mL centrifuge tubes. In accordance with the volume of 1mL/per 100 g of fresh tissues, a radio immunoprecipitation assay (RIPA) premix (Shanghai Beyotime Biotechnology, China) (1 μ L 100 m M PMSF in 1 mL RIPA) was added into each tube before the tissues were homogenized by sonication on ice thrice (10 s each). Then, the homogenate was put into an ice-box for 30 min and centrifuged at 12000r/min for 5 min at 4°C (Eppendorf, Germany). Afterwards, the supernatant was aspirated into sterile tubes and stored at -80°C until use.

Determination of total protein concentration by bicinchoninic acid assay

The standard solution was prepared according to the instructions of the bicinchoninic acid assay (BCA) protein concentration determination kit (Shanghai Biyuntian Company, China). The BCA

working solution matching the number of samples to be tested was prepared according to the ratio of 50:1 (reagent A: reagent B), and then fully shaken and blended. The standard proteins of 0, 1, 2, 4, 8, 12, 16 and 20 μL were added into 96-well plates and supplemented with PBS until 20 μL . The corresponding concentrations of standard proteins were 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL, respectively. 2 μL volume sample was added to a 96-well plate, and PBS was added until 20 μL . Each well was incubated at 37°C for 30 min with 200 μL BCA working solution. The absorbance of A562 was determined by enzyme labeling. According to the measured absorbance value and the standard curve drawn by the standard solution, the protein concentration of the tested sample was obtained.

Enzyme-linked Immunosorbent Assay

Required Enzyme-linked immunosorbent assay (ELISA) reagents including the standard solution, washing solution and anti-rabbit Ig GHRP (Horseradish Peroxidase) secondary antibody were prepared according to the manufacturer's instructions. Subsequently, 100 μL per well of the standard and sample solutions were added into a 96-well microtiter plate, while an empty well was used as the blank. After incubating the plate for 2 h at room temperature in the dark, the plate was washed and 100 μL of primary antibodies (MS A β 1-42 Detection Antibody) (Shanghai Beyotime Biotechnology, China) were added into each well (except for the blank well). After incubating the plate for 1 h at room temperature in the dark, 100 μL of secondary antibodies (Anti-Rabbit Ig GHRP) (Shanghai Beyotime Biotechnology, China) were added into each well (except for the blank well) and the plate was incubated for 30 min at room temperature in the dark. After plate washing, 100 μL of colorimetric solutions were added into each well (except for the blank well) and the plate was incubated for 30 min at room temperature in the dark. Next, 100 μL of terminating solutions were added into each well to terminate the reaction and the absorbance value of each well was measured at a 450 nm wavelength using a plate reader (Thermo, USA).

Detection of the expression of APP and BACE1 in the hippocampus and corpus callosum by Western blotting

Sample proteins were first separated using gel electrophoresis (10% separation gel, 5% stacking gel). At the same time, a 0.45 μm polyvinylidene fluoride (PVDF) film (Millipore, USA), was activated in a methanol solution for 30 s until it became translucent, and then soaked in a transfer buffer for about 10 min. Then, a preprocessed filter, which had been soaked in the transfer buffer for 24 h, was used to transfer the proteins onto the PVDF film for 30~45 min at a constant current of 0.09A. Thereafter, the PVDF film was washed with a TBST solution for 5 min $\times$ 3 times, soaked in a blocking solution of 5% skimmed milk + TBST (1.65 mL 20% Tween-20 solution + 700 mL TBS solution) for 1 h at room temperature, washed with TBST again, and incubated with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-phosphoesterase (AP) and anti-beta-secretase 1 primary antibodies, respectively, overnight in a 4°C ice-box (Haimen Kylin-Bell Lab Instruments Co., Ltd., China) under shaking. The following day, the PVDF film was washed with TBST and then incubated with fluorescence-labeled secondary antibodies (Goat anti-Rabbit Ig G) for 2 h at 4°C under shaking. After washing, the PVDF film was scanned by an Infrared Imaging System (LI-COR, USA) to collect the images of the protein bands. Image J Analysis Software was adopted to calculate the gray values of protein bands.

Statistical analysis

SPSS 17.0 Statistical Software was applied to analyze the collected data. The measurement data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). Inter-group comparisons (control group vs sham-operated group; hypertension model group vs sham-operated group) were carried out for data collected at the same time points using normality tests and homogeneity tests of variance. The normal distribution was tested with independent sample *t*-test or approximate *t*-test. Inter-subgroup comparisons of the control group, sham-operated group and hypertension model group were carried out using one-way ANOVA. Inter-

group pair comparisons were carried out using the Least Significant Difference (LSD) method. The variance of heterogeneous data was tested using the Welch Approximate F Test. The inter-group pair comparisons were carried out using the Dunnett's T3 method. All tests were bilateral and $P < 0.05$ indicated significant difference.

RESULTS

Comparisons of caudal arterial pressure in rats of each group

The results showed that there was no statistical significance between the control group and the sham-operated group, so the sham-operated group and the model group were compared. Compared with sham-operated group, blood pressure in the model group increased significantly at 4, 8, 12 and 16 weeks after operation ($P < 0.01$). The comparisons of rats in the subgroups of the model group showed that their blood pressure increased obviously ($P < 0.01$) at 4 weeks, 8 weeks, and 12 weeks after the operation, while the blood pressure at 16 weeks and 12 weeks after the operation was not significantly different ($P > 0.05$), indicating that the blood pressure became more stable at 12 weeks after model establishment. The

intra-group and inter-group comparisons between the control and sham-operated groups showed no significant difference ($P > 0.05$, see Fig. 1). After the establishment of the RHRSP model, 1 rat was found dead at 8 weeks in the hypertension model group, while 3 and 5 rats were found dead at 12 weeks and 16 weeks, respectively, in the hypertension model group. Their death rates were 8.3%, 18.8% and 31.3%, respectively. All dead rats suffered sudden death and their autopsy results indicated that the cause was hemorrhagic stroke. The remaining 51 live rats in the hypertension model group showed no signs of serious stroke, including hemiplegia, paraplegia and limb muscular spasm.

Brain function assessment of model rats

The swimming distance of the rats in the sham-operated group gradually increased over age (Fig. 2A). In the hypertension model group, the swimming distance of the rats also increased at 4, 8 and 12 weeks after operation, but decreased at 16 weeks after operation. At 16 weeks after operation, the swimming distance of the rats in the hypertension model group decreased significantly compared to those in the sham-operated group, ($P < 0.05$), indicating that long-term hypertension would reduce

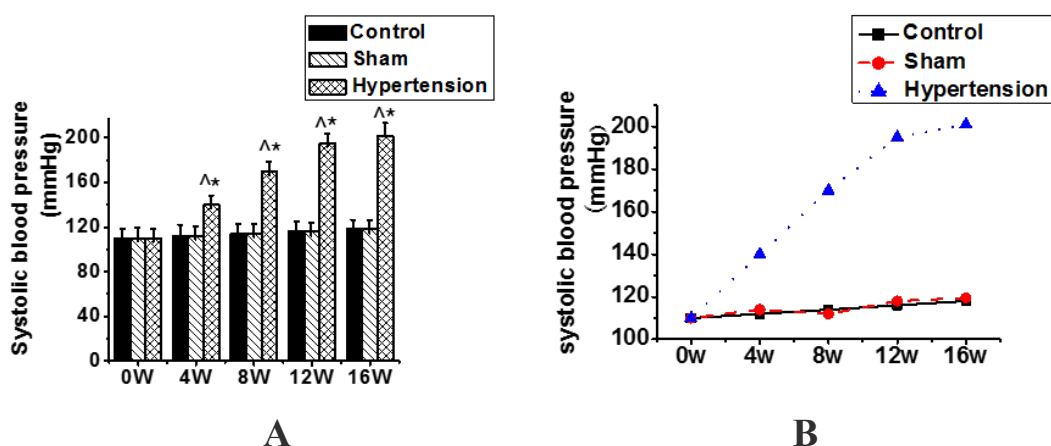


Fig. 1. SBP Comparison of rats in each group. **A)** SBP of rats in each group; **B)** Variation of rats in each group at different time points (* $P < 0.01$)

the motor ability.

As seen in Fig. 2C, compared with those in the sham-operated group, the escape latency of rats in the hypertension model group became longer at 4 weeks, 8 weeks, 12 weeks and 16 weeks after operation, especially at 12 and 16 weeks ($P < 0.01$, Fig. 2B). Therefore, during the 5-day navigation training, the escape latency was gradually shortened as the training proceeded because the memory of rats was strengthened. However, at 12 and 16 weeks after operation, the memory of the rats in the model group deteriorated.

As seen in Fig. 2D, compared with the sham-operated group, the number of trips made by the rats in the hypertension model group when they crossed the platform decreased sharply at 12 and 16 weeks after operation, indicating that hypertension reduced the long-term memory and spatial orientation of rats ($P < 0.01$).

Observation of histomorphological deformations and the number of neurons in the hippocampus, prefrontal lobe, temporal lobe and corpus callosum by H&E staining

Compared to the sham-operated group, the hippocampus CA1 region of rats in the hypertension model group showed a decreased number of granulosal cell layers, loose and disordered cell arrangement and massive deficiency of neurons, especially at 12 and 16 weeks after operation ($P < 0.01$, Fig. 3A), along with neuron pyknosis and necrosis, as well as occasional vesicle formation in the matrix. Among the rats in the hypertension model group, the cortical areas of the prefrontal and temporal lobes showed significant cell dissociation and abnormal distribution at 12 and 16 weeks after operation, along with decreased cell density and a reduced number of neurons ($P < 0.01$, Fig. 3, B and C). Furthermore, the hypertension model group showed the degeneration, karyopyknosis and anachromasis of pyramidal cells, along with occasional microglia activation as well as dispersed arrangement of gliocytes in the corpus callosum region. Finally, at 12 and 16 weeks after operation, the quantity of gliocytes increased sharply ($P < 0.01$, Fig. 3D), along with the clustered aggregation of gliocytes.

Detection of hippocampus A β 1-42 expressions by enzyme-linked immunosorbent assay

As can be seen from Fig. 4, unusual A β accumulation was observed in the cerebral cortex neurons of rats at 4 weeks after operation. As the blood pressure increased during the development of WML, the sign of A β accumulation became more apparent. The content of hippocampus A β 1-42 in the rats of the sham-operated and hypertension model groups showed no significant difference ($P > 0.05$).

Detection of APP and BACE1 expressions in the hippocampus and corpus callosum by Western blotting

As shown in Fig. 5, unusual A β accumulation was observed in the neurons of the cerebral cortex in WML rats at 8 weeks after operation. As the blood pressure increased during the development of WML, the sign of A β accumulation became more apparent. The expression of APP and BACE1 in the hippocampus and corpus callosum of rats in the sham-operated and hypertension groups showed no significant difference ($P > 0.05$).

DISCUSSION

Occupying nearly 50% of the human brain, white matter mediates fiber connections between different gray matter regions in the whole brain (10). WML are considered as comprehensive pathological changes in demyelination, lacunar infarction and axonal loss, which not only lead to ischemic and hemorrhagic stroke, but are closely related to reduced motor, memory and executive ability in dementia (11). An RHRSP model studies the role of “hypertension” as a pathological basis in WML development. However, with the presence of genetic heterogeneity, it is difficult to establish RHRSP, making RHRSP animals inferior to normal control animals of the same species. By utilizing U-shaped silver clips to induce controllable stenosis of bilateral renal arteries, an RHRSP model can be established to study hypertension animals (12). Using H&E staining, this study found that the tissue thickness of corpus callosum splenium of rats significantly

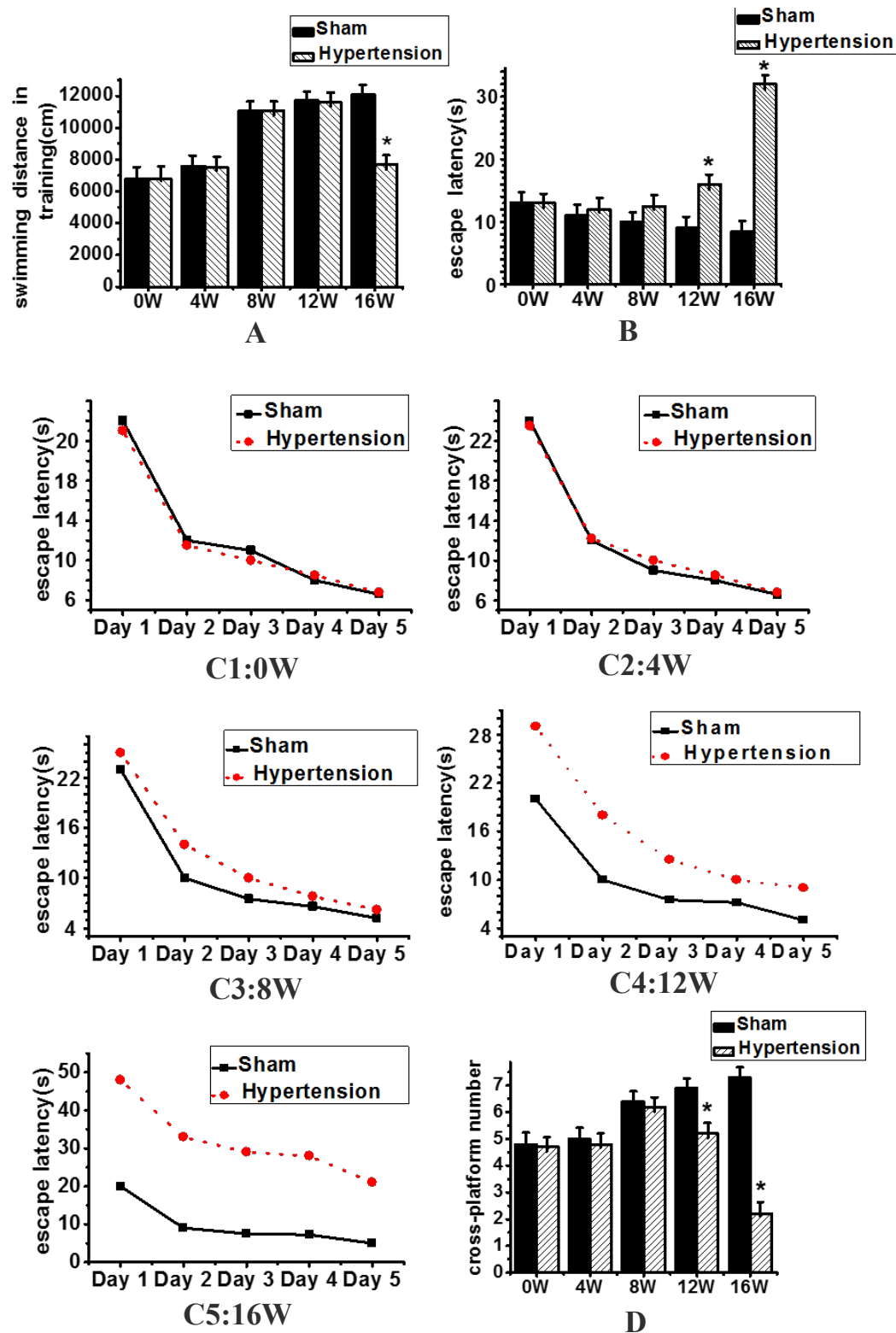


Fig. 2. Cognitive ability of Morris water maze in sham-operated and model rats *A*) Comparison of total swimming distance of rats in two groups in adaptive training; *B*) Comparison of 5d-average escape latency of rats in two groups in navigation training; *C*) Variation of 5d-escape latency of rats in two groups in navigation training; *D*) Comparison of cross-platform trips of rats in two groups in spatial search test (* $P<0.01$).

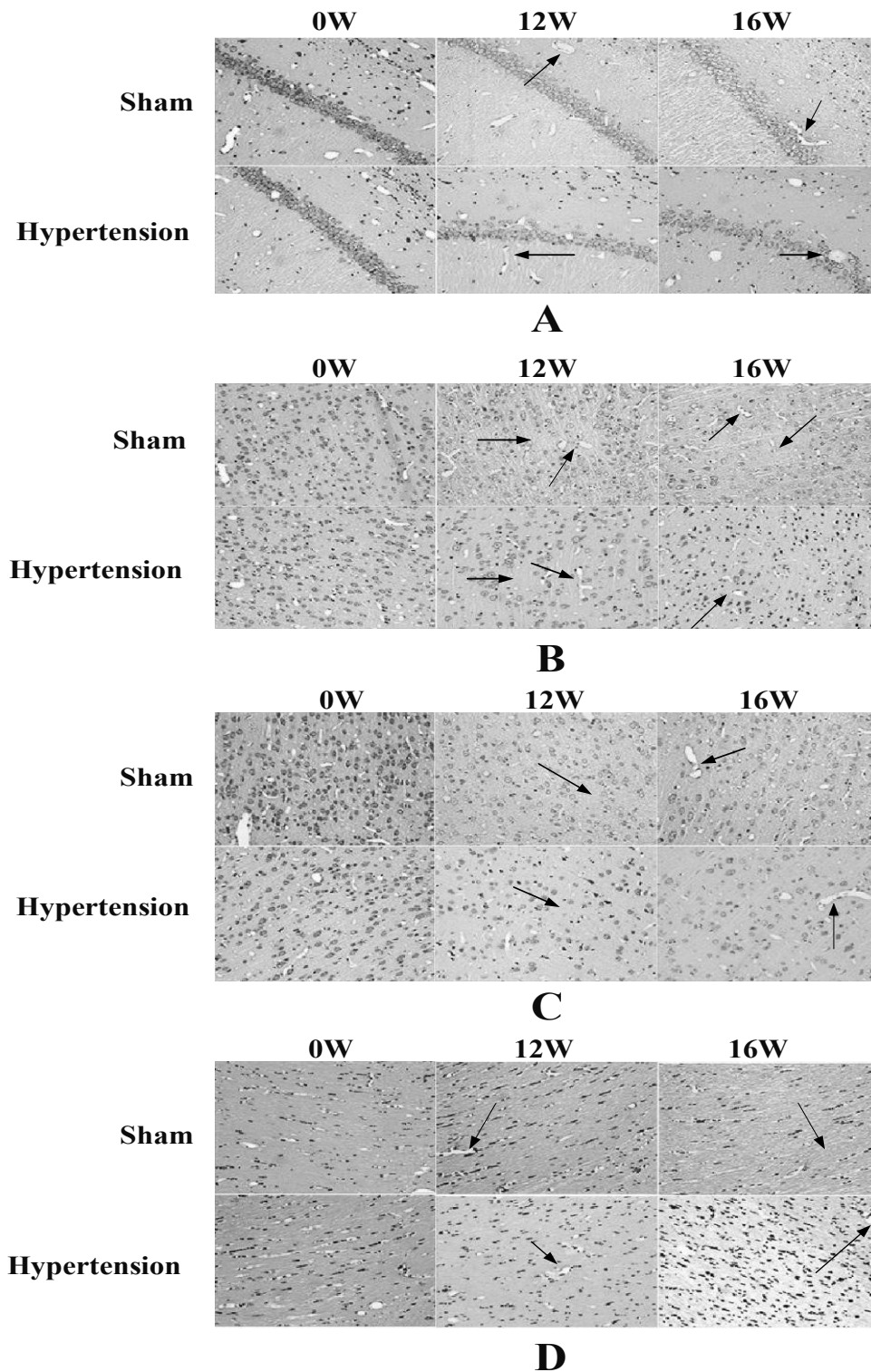


Fig. 3. The number of neurons in the brain of rats in sham-operated group and model group revealed by HE staining (40x). **A)** Neuron quantity in the hippocampus CA1 region of rats in two groups; **B)** Neuron quantity in the prefrontal lobe of rats in two groups; **C)** Neuron quantity in the temporal lobe of rats in two groups; **D)** Quantity of gliocytes in the corpus callosum of rats in two groups.

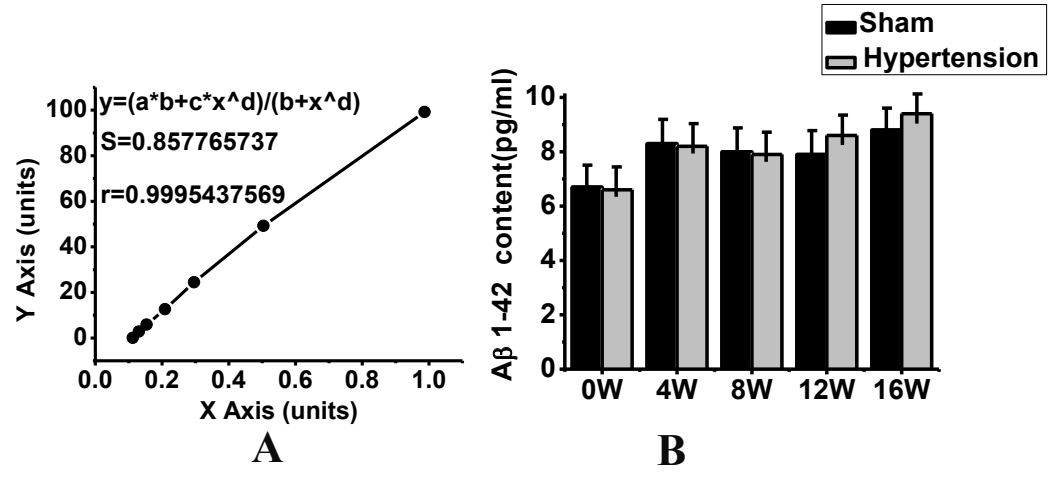


Fig. 4. Relative content of Aβ 1-42 protein in hippocampus of rats in sham-operated group and model group. *A)* Standard curve; *B)* Relevant content of Aβ1-42 $P < 0.05$.

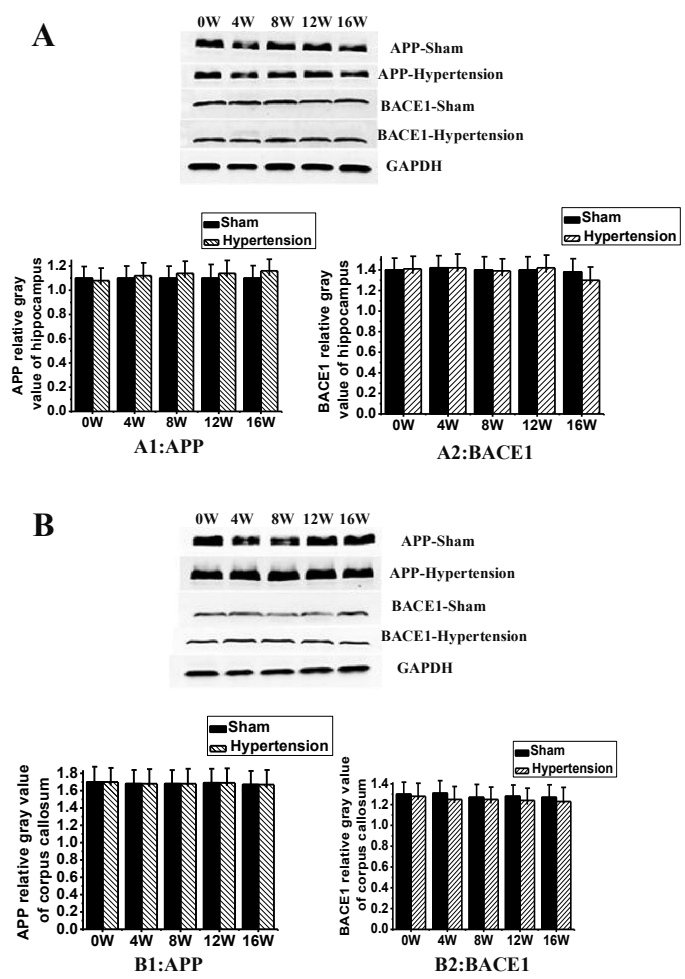


Fig. 5. Expressions of APP and BACE1 in hippocampus and corpus callosum of rats in sham-operated group and model group. *A)* Hippocampus; *B)* Corpus callosum $P < 0.05$

decreased at 12 and 16 weeks after the establishment of a hypertension model, along with vesicle-like deformations, nerve demyelination around the lateral ventricle, loose white matter structure, and proliferative neuroglia cells in the corpus callosum region. As the blood pressure and the duration of hypertensive condition increase, other pathological changes of WML, including white matter nerve demyelination and reactive proliferation of neuroglia cells, become more obvious. This study has further proved the feasibility of establishing a hypertensive WML rat model through RHRSP.

This study utilized a Morris Water Maze (13) to evaluate the changes in cognitive functions, such as learning, memory and spatial discrimination ability, at different time points preoperatively and postoperatively. Results of the study showed that at 16 weeks after the RHRSP operation, the total swimming distance of model rats in adaptive training was significantly shortened compared with that in the sham-operated group. At 12 weeks and 16 weeks after operation, the escape latency of model rats in navigation training was also shortened compared to that of sham-operated rats, along with a reduced number of trips used to cross the original position of the missing platform in the spatial searching test. Consequently, the brain functions including motor ability, learning ability and spatial memory of the rats declined.

A β is derived from APP under the action of β - and γ -secretase (14) by cleaving APP into β -APPs and C99 fragment polypeptide, which is then converted into A β 1-40 and A β 1-42 by γ -secretase. As the major component of A β , A β 1-42 can form fiber-like oligomers more easily to induce stronger neurotoxicity (15). In the study, it was found that at 8 weeks after operation, A β starts to accumulate inside the cerebral cortex neurons of hypertensive WML rats. With the further increase in blood pressure and WML severity, the amount of A β accumulation gradually increased. However, the correlation between the increased A β accumulation in the cerebral cortex of hypertensive WML rats and the occurrence and development of cognitive decreases remains elusive and should be studied by further experiments.

In summary, hypertension, especially sustained hypertensive conditions, causes neuronal injuries in multiple brain regions, including prefrontal lobe, temporal lobe and hippocampus, and damages the white matter in corpus callosum and around the lateral ventricle, which in turn damages brain functions. Moreover, with the increase in blood pressure and the progression of WML, the expression level of A β in the cortical areas of WML rats also increases, indicating that the unusual A β accumulation in the cortex may participate in the occurrence and development of hypertensive WML and WML-induced cerebral cognitive decrease.

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EFFECTS OF AIF-1 INFLAMMATORY FACTORS ON THE REGULATION OF PROLIFERATION OF BREAST CANCER CELLS

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The purpose of this study was to explore the effect of Allograft Inflammatory Factor 1 (AIF-1) on the regulation of proliferation of breast cancer cells. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), cell culture and counting, and mass spectrometry were performed. The biologically active high-purity recombinant protein rhAIF-1 was obtained by optimizing the rhAIF-1 protein purification system, and MDA-MB-231 and MDA-MB-361 breast cancer cell lines were used. After adding to the culture medium, rhAIF-1 was found to promote cell proliferation in dose-dependent and time-dependent manners. The purified protein rhAIF-1 was marked with rhodamine and incubated with the cells. Confocal imaging analysis revealed that the foreign protein was localized in the cytoplasm, and rhAIF-1 was unevenly distributed in the cytoplasm. Although AIF-1 accumulates around the nucleus, it can not enter the nucleus, suggesting that other factors might be involved in the regulation of cell proliferation. In order to find the possible interacting protein of rhAIF-1, protein immunoprecipitation technique and mass spectrometry were employed, and it was indicated that ADAM28m was the possible interacting protein of rhAIF-1. The interaction between rhAIF-1 and ADAM28m was validated by immunoprecipitation along with Western blotting. It was found that rhAIF-1 could precipitate ADAM28m protein by immunoprecipitation. The results indicated that IF-1 participates in the development of breast cancer by interacting with ADAM28m and activating downstream signaling pathways. It was concluded that AIF-1 provides a new idea for the molecular mechanism of breast cancer cell proliferation and acts as a new target for the prevention and treatment of breast cancer in the future.

In China, breast cancer is increasing at a rate of 3-4% yearly. In-depth analysis of the pathogenesis of breast cancer and the search for new targets of breast cancer are of great significance for the diagnosis and treatment of breast cancer (1). Pre-laboratory data indicated that AIF-1 was highly expressed in breast ductal carcinoma. Moreover, it was found that the expression of AIF-1 could significantly promote tumor growth in nude mice (2, 3). *In vitro* experiments also proved that up-regulation

of AIF-1 expressions promoted the proliferation of breast cancer cells (4). After the AIF-1 expression was down-regulated by RNAi interference, the proliferation of breast cancer cells was significantly inhibited (5-7). However, there are few in-depth studies on the molecular mechanisms by which AIF-1 promotes the proliferation of breast cancer.

Based on this, the purpose of this study was to further verify the role of AIF-1 in breast cancer proliferation and its possible molecular mechanism,

Key words: AIF-1 inflammatory factor; mass spectrometry; recombinant protein; breast cancer cell proliferation

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so as to provide new clues and ideas for the treatment of breast cancer.

MATERIALS AND METHODS

Expression and purification of recombinant protein rhAIF-1

The stored BL21 *Escherichia coli* containing pET32-a(+)-AIF-1 were inoculated into LB medium containing Amp (Amp: LB medium = 1:1000 at a ratio of 1:100, and cultured at 37°C for 12-14 h. After the strains were activated, the bacterial suspension was transferred to Amp-containing medium at a ratio of 1:100, and then cultured at 37°C for 2.5 h in a shaker (250 r/min). Thereafter, 0.1 ml IPTG (GILSON, Finland) was added at a ratio of 1:2000, and the mixture was placed on a shaker (BD Company, USA) at 30°C for 2-3 h. The bacterial solution was centrifuged at 8000 rpm and 4°C for 10 min, the supernatant was discarded, and the cells were stored for subsequent use. The collected cells were resuspended in 10 mM imidazole solution (BD Company, USA) (1L of the cells was resuspended in 20-30 ml of imidazole solution) and placed on ice. The ultrasonication (BIO-RAD, USA) was carried out with a power of 400w, an ultrasonic of 5 s and an interval of 5 s. The specific cycle times were selected according to the concentration of the bacteria solution. Finally, the cells were centrifuged at 4°C for 30 min at 8000 rpm, and the supernatant (the target protein) was collected and directly added into a new tube and filtered through a 0.45 µm filter paper. A 15x50 mm chromatographic column was selected, and the lower end was connected with the sample inlet of the nucleic acid protein meter. the chromatographic column was filled with 3 ml Ni-NTA resin, and the 5-10 column bed volumes were rinsed with degassed ultra-pure water, followed by rinsing with the equilibrium solution of 10 mM imidazole, until the reading of the nucleic acid protein meter stabilized at around 2000 and the sensitivity was 0.5. After several column bed volumes were balanced with 10 mM imidazole, the above filtered samples (about 20-30 ml) were added, and the flow rate was controlled at 0.5ml/min. At the end of sampling, impurities were eluted with an equilibrium solution of 10 mM imidazole until the monitor reading returned to 000. The chromatographic column was washed with 50 mM imidazole. The effluent liquid was collected when the reading of the displayer

began to change. The protein of interest was collected by affinity chromatography. In order to obtain the protein with higher purity, the molecular sieve method was used to remove imidazole.

Desalination column with a diameter of 26 × 300 mm was selected as the chromatographic column and the gel medium was Sephadex G-25 medium. Column packing: 20 g of Sephadex G-25 medium gel particles were soaked in ultrapure water overnight to allow the gel particles to fully absorb water and swell; after washing several times, impurities in the upper layer of the gel were removed as much as possible. The outlet end of the gel column was connected with the nucleic acid protein detector (Shanghai Jiapeng Technology Co., Ltd., China), and the sample was taken after the reading of the nucleic acid analyzer was stabilized. After the sample completely penetrated the gel, the eluent was full. As the readings on the meter began to steadily rise, the eluent was collected until approximately one-third of the maximum reading was collected (salt was not collected). The collected peaks were transferred into a conical flask, sealed with filter paper, and placed in a -80°C refrigerator (Shanghai Jiapeng Technology Co., Ltd., China). They were then lyophilized overnight, and the protein dry powder was sealed and stored at -20°C for subsequent use.

SDS-PAGE gel electrophoresis

The plastic glass boards were cleaned with tap water, washed with single steam water, and were blown dry. The glass plates were aligned to prevent leakage. According to the formula of SDS-PAGE separation adhesive solution, the prepared separation adhesive was poured into the glass plate, and isopropanol was added to press the rubber surface. After standing for 30 min until the gel was solidified, the glass plate was inverted to remove isopropyl alcohol. The concentrated gel was prepared according to the 5% SDS-PAGE gel formulation, and the glass plate was filled with the concentrated gel. According to the requirements of the experiment, an appropriate comb was selected and carefully inserted into the concentrated adhesive. The speed was fast to avoid glue solidification and prevent generation of bubbles when inserting the comb. After the gel was solidified, the entire glass plate was removed and placed in a gel electrophoresis tank, into which the prepared 1× electrophoresis buffer was carefully poured. Before sampling, the comb was pulled

out carefully, and the needle was cleaned before each sampling. The power supply was connected. The protein strip was concentrated with 80V voltage in the upper glue layer, and it was changed to 110V voltage when the protein strip reached the lower glue layer. The electrophoresis time was determined by molecular weight of the protein of interest. After electrophoresis, Coomassie brilliant blue R250 solution was dyed for 1 h at room temperature, and the decolorizing solution was eluted overnight at room temperature.

Cell fluorescence staining

After the rhAIF-1 molecule was marked with different fluorescent dyes (BIO-RAD, America), the expression and localization of the marked molecule in cells and tissues were observed under a fluorescence microscope (BIO-RAD, America). In line with the cell passage, Rhodamine marked rhAIF-1 was added after the cell confluency reached 80%. After incubation, the solution was drained and each well was washed six times with 500 μ l PBS to remove redundant rhAIF-1. Each well was fixed with 300 μ l of 4% paraformaldehyde (BIO-RAD, America) solution for 10 min. After the fixation was completed, the stationary liquid was drained and the well was washed 4-6 times with PBS to remove the redundant stationary liquid. 300 μ l of DAPI (4', 6-diamidino-2-phenylindole) staining solution was added to each well and stained for 3 min. Then DAPI staining solution was drained and the well was washed 6-8 times with 500 μ l of PBS. Lens wiping paper was used to remove as many impurities as possible from the surface of the slide, and one end of the glass slide was marked. A small amount of glycerin was applied to the slide so that the cellular side of the cover glass contacted with the glycerol. Nail polish was spread along the edge of the coverslip, the coverslip was fixed, and the sample was placed in the dark. Rhodamine staining results were observed at 552nm excitation light and 575 nm emission light under a Confocal microscope, and DAPI staining results were observed at 405nm excitation light and 445nm emission light.

Immunoprecipitation

The cells were harvested, and an appropriate amount of cell IP lysis buffer (containing protease inhibitor) was added. The cells were lysed on ice or centrifuged at 2000 g and 4°C for 30 min, and the supernatant was

transferred into an EP (eppendorf) tube. A small amount of lysate was taken for Western blot analysis, and 1 μ g of the corresponding antibody and 10-50 μ l of protein A/G-beads were added to the remaining lysate and incubated at 4°C overnight. After immunoprecipitation, the sample was centrifuged at 3000 g and 4 °C for 5 min, and the protein A/G-beads were located at the bottom of the tube. The supernatant was carefully drained and the protein A/G-beads were washed with 1 mL of lysis buffer for 3-4 times. Finally, 15 μ l 2 $\times$ SDS sample buffer was added, boiled for 10 min, and analyzed by SDS-PAGE.

Cell culture and counting

Before the experiment, the complete medium was pre-incubated in a 37°C water bath. The frozen cells were removed from the liquid nitrogen tank and quickly thawed in a water bath. They were then transferred to a petri dish containing complete medium. The old medium in the petri dish was then removed with a pipette and the dish was washed once with warm pre-PBS. The cells were digested with an appropriate amount of cell digestive juice and observed under a microscope. After digestion, an appropriate amount of complete medium was added to terminate the digestion. The cells were carefully pipetted, and centrifuged at a low speed for 5 min. The supernatant was removed with a pipette gun, and an appropriate amount of cell cryopreservation solution was added (prepared medium: FBS: DMSO= 7: 2: 1). Cells were collected, and resuspended with 1 ml complete medium. The 10 ul cell suspension was diluted 10 times in 900 ul of fresh medium, and mixed gently. The cell counting board and cover glass slides were cleaned with 75% alcohol and wiped with a lens wiping paper. The cover glass slides were covered. 10 ul of the diluted cell suspension was added dropwise, and allowed to stand for 3 min. Under a microscope, the number of cells in the counting plate was counted. The calculation formula was as follows: number of cells/ml- total number of cells in the four grids / $4 \times 10^4 \times 10$.

Mass spectrometry

In the sample tube, 200-400 ul decolorizing solution (30% ACN/100ml MNH_4HCO_3) was added and cleaned and decolorized to transparency. The supernatant was absorbed and discarded, and 100 ml MDTT was added after freeze-drying. The sample was placed in a 56 $\square$ incubator for 30

min, and the supernatant was aspirated. 200 mM IAA was added and protected from light for 20 min. The supernatant was aspirated, and 100 ml of NaHCO_3 was added and incubated for 15 min at room temperature. The supernatant was discarded, and 100% ACN (Biyuntian Biological Co., Ltd., China) was added. After incubation for 5 min, the supernatant was lyophilized, and then 2.5–10 ng/ μl Trypsin solution (Biyuntian Biological Co., Ltd., China) was added and reacted at 37°C for 20 h. The enzymatic hydrolysate was transferred to a new EP tube, and 100 μl of the extract (60% ACN 0.1% TFA) was added to the gel and sonicated. Capillary high-performance liquid chromatography: solution A was an aqueous solution of 0.1% formic acid, and solution B was an ethyl acetate solution containing 0.1% formic acid and 84% ethyl ester (Boshide Engineering Co., Ltd., China). After the chromatographic column was balanced with 95% liquid A, the sample was loaded into Trap column by an automatic sampler. Chromatographic gradient: 0–50 min, linear gradient of liquid B from 4% to 50%; 50–54 min, linear gradient of liquid B from 50% to 100%; 54–60 min, liquid B maintained 100%. Acquisition of mass spectral data: 20 fragment maps were acquired after each full sweep. Based on the peptide information, the corresponding database was retrieved to obtain a series of candidate protein information.

Cell proliferation detected by CCK-8 assay

The WST-8 contained in the CCK-8 kit can be reduced by dehydrogenase in mitochondria to produce

orange and yellow products, the color of which indirectly reflected the proliferation of cells. The cells were collected by cell passage, and the supernatant was removed. 1 ml of fresh medium was added to resuspend the cells. After the cells were counted, they were diluted to 1×10^5 cells/ml. Several 96-well plates were taken (the number of plates was determined according to the times of cell proliferation), and 100 μl of the corresponding cell suspension was added into each well at a density of 10^4 cells per well. Four wells were set up for each step to eliminate accidental factors and placed in an incubator for 24 h (37°C , 5% CO_2). After the pre-culture, the complete medium was removed by a pipette, and the culture medium without serum was added to each well and incubated for 12 h. 100 μl of fresh serum-free medium containing different concentrations of rhAIF-1 was added and the plates were incubated in an incubator for 24 h. One plate was taken at each time point, and 10 μl of CCK-8 solution was added to each well. The plate was placed in the incubator and the OD value of each well was measured at a wavelength of 450 nm after 2 h.

Statistical analysis

SPSS 17.0 statistical software was used for statistical analysis, and the data were expressed as mean $\pm$ standard deviation. The data of different groups were compared by one-way analysis of variance (ANOVA). $P < 0.05$ indicated significant difference.

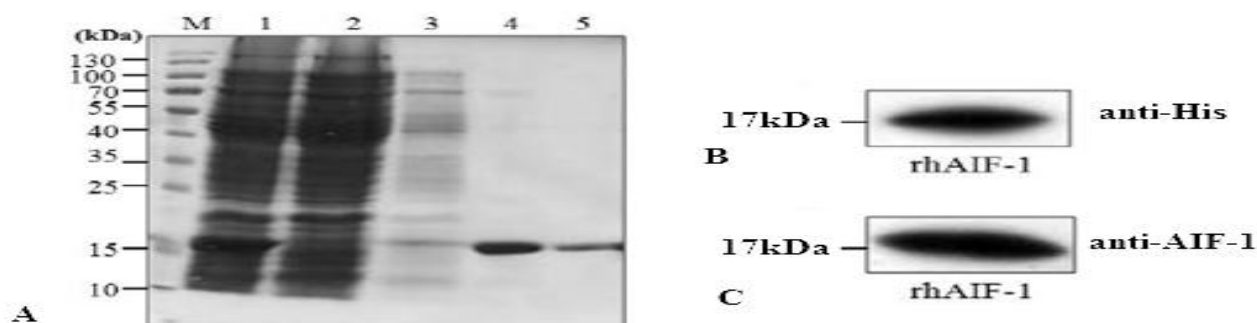


Fig. 1. Optimization and identification of rhAIF-1 recombinant protein purification. **A)** Determination of rhAIF-1 affinity by SDS-PAGE. Lane 1: induced whole liquid BL21 [pET32a(+)-AIF-1]; lane 2: the waste liquid after passing the Ni-NTA resin; lane 3, impure protein eluted with 10 mM imidazole eluate; lane 4, the target protein rhAIF-1 eluted with 50 mM imidazole eluate; lane 5: detection after desalting in lane 4; **B)** rhAIF-1 fusion protein detected by the tag antibody His-tag; **C)** rhAIF-1 fusion protein detected by the AIF-1 antibody.

RESULTS

Optimization and identification of rhAIF-1 protein purification system

The results of optimization and identification of the rhAIF-1 protein purification system are shown in Fig. 1 (A-C). In Fig. 1A, lane 1 represents the expression of the target protein before passing through the Ni-NTA affinity chromatography column. Lane 2 represents the outflow of non-target proteins after passing through the affinity column. It was found that the target protein was well bound to the affinity column. Lane 3 was the impure protein eluted with 10 mM imidazole eluent, and it was found that impure proteins were precipitated under this eluting condition. Abundant pure target proteins were immediately obtained with 20 mM imidazole eluent, with almost no contamination of impure proteins (lane 4). The purified unsalted target protein in lane 4 was harvested after passing through the desalting column (Sephadex G-25 medium).

The new experimental system avoided loss of the target protein in the elution process, shortened the time, avoided degradation of the target protein, and improved the yield. Since the purified protein carried the His-tag tag, the purified protein was further detected by the immunoblotting technique with the His-tag antibody and the AIF-1 antibody, respectively, which was the desired protein rhAIF-1, as shown in Fig1, B and C.

rhAIF-1's promoting effect on proliferation of breast cancer cells in a concentration-dependent manner

As shown in Fig. 2 (A and B), rhAIF-1 with different concentrations of 0 ug/ml, 1 ug/ml, 50 ug/ml, 100 ug/ml, and 200 ug/ml was added to culture for 36 h, respectively. Compared with blank control group, a low concentration of rhAIF-1 (1 ug/ml) failed to promote the proliferation of MDA-MB-231 and MDA-MB-361 cells. However, when the concentration of rhAIF-1 was more than 50 ug/ml, proliferation of the two cell lines was significantly

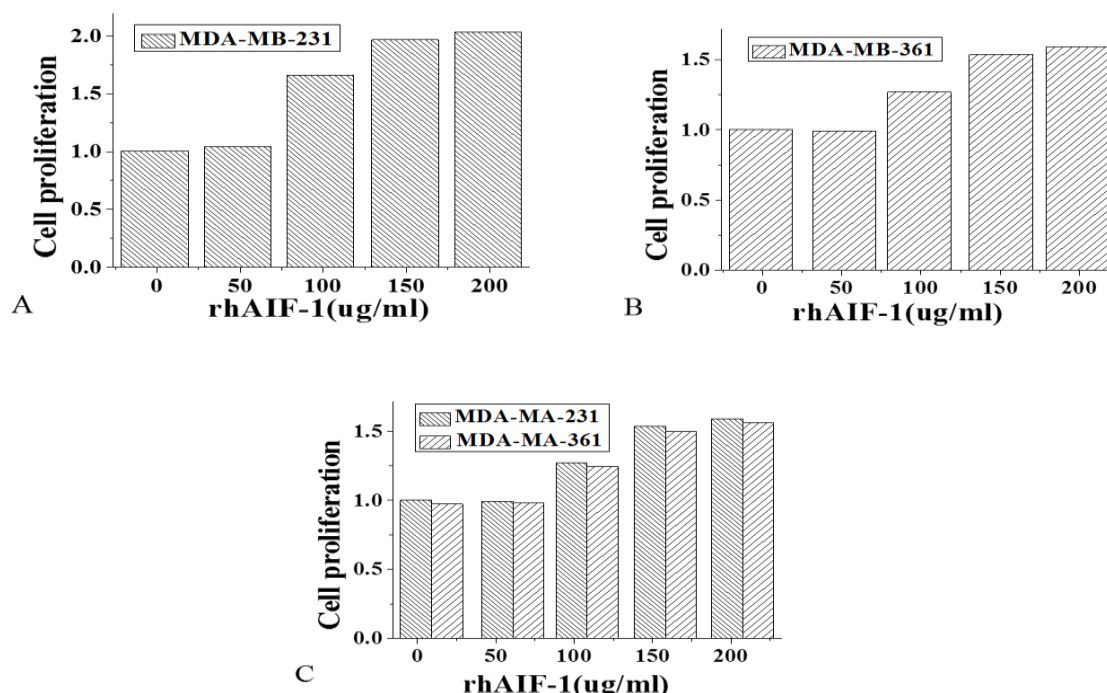


Fig. 2. *rhAIF-1 promoting effect on proliferation of breast cancer cells MDA-MB-231 and MDA-MB-361. A) The proliferation of MDA-MB-231 cells after 36 h incubation with rhAIF-1 at 0 ug/ml, 1 ug/ml, 50 ug/ml, 100 ug/ml and 200 ug/ml concentrations, revealed by CCK-8 method; B) The proliferation of MDA-MB-361 cells after 36h incubation with rhAIF-1 at 0 ug/ml, 1 ug/ml, 50 ug/ml, 100 ug/ml and 200 ug/ml concentrations, revealed by CCK-8 method; C) The proliferation of MDA-MB-231 and MDA-MB-361 cells after incubation with rhAIF-1 at 0 ug/ml, 1 ug/ml, 50 ug/ml, 100 ug/ml and 200 ug/ml.*

promoted. The higher the concentration of rhAIF-1, the stronger the proliferation efficiency of the cells. By comparing proliferation of the two different human breast cancer cell lines, MDA-MB-231 and MDA-MB-361, it was found that MDA-MB-231 cells were more sensitive to rhAIF-1. The proliferation capacity of MDA-MB-361 cells at the same concentration was significantly higher than that of MDA-MB-361 cells (Fig. 2C). It was suggested that rhAIF-1 could promote proliferation of breast cancer cells in a concentration-dependent manner.

The promoting effect of rhAIF-1 on proliferation of breast cancer cells in a time-dependent manner

As shown in Fig. 3, A-B, proliferation of MDA-MB-231 and MDA-MB-361 cells was not different at various time points after the stimulation by rhAIF-1

cells of a low concentration (1 $\mu\text{g/ml}$). When the cells were stimulated by a high concentration (200 $\mu\text{g/ml}$) of rhAIF-1, MDA-MB-231 cells showed a concentration dependence. As the time increased, the cells proliferated significantly. After 12 h of stimulation by rhAIF-1, the proliferation of MDA-MB-361 cells increased to approximately 50%, and increased slightly after 24 h of stimulation, without significant difference. MDA-MB-361 cells showed stronger proliferation capacity than MDA-MB-231 cells when stimulated for less than 24 h (Fig. 3C). MDA-MB-231 cells grew faster than MDA-MB-231 as stimulation time increased.

Properties of rhAIF-1 localized in the cytoplasm

Confocal scanning microscope imaging results are shown in Fig. 4. It was found that rhAIF-1 in

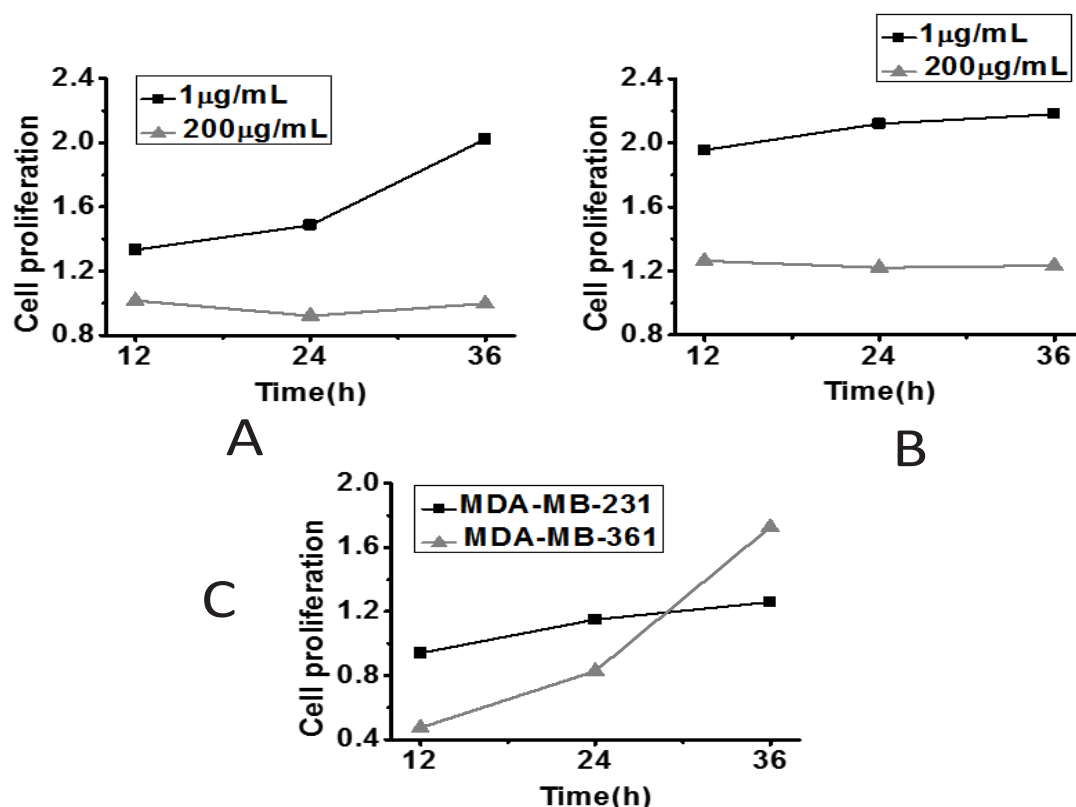


Fig. 3. The effect of rhAIF-1 on the proliferation of breast cancer cells after 12 h, 24 h and 36 h incubation. **A)** Proliferation of cells detected by CCK-8 after MD-MB-231 cells were stimulated by rhAIF-1 for 12h, 24h and 36h, respectively; **B)** Proliferation of cells detected by CCK-8 after MDA-MB-361 cells were stimulated by rhAIF-1 for 12h, 24h and 36h, respectively; **C)** Proliferation of MD-MB-231 cells and MDA-MB-361 cells (cells were stimulated with rhAIF-1 at a high concentration of 200 $\mu\text{g/ml}$) after 12 h, 24 h and 36 h, respectively.

MDA-MB-231 and MDA-MB-361 cells entered the cytoplasm through the cell membrane (as shown by the arrow in Fig. 4) and were distributed unevenly in the cytoplasm. The results showed that AIF-1 could indirectly regulate the development of breast cancer cells, which requires involvement of other factors.

Preliminary analysis of candidate proteins interacting with rhAIF-1

Mass spectrometry was used to analyze proteins that might interact with rhAIF-1, and a series of candidate proteins were obtained by comparing with the database of other proteins. Table I shows

Table I. Preliminary analysis results of possible candidate proteins interacting with rhAIF-1.

NO.	Protein Name	MV(KD)	Location
1	ADAM28m	87	Plasmakmma
2	ACAD11	87	Miochondron
3	ADCY5	102,139	Plasmalkmma
4	BRCA2	384	Cytoplasm. nuckus
5	DSP	332	Cytoplasm
6	FAAH	63	Cytoplasm
7	IRS1	132	Cytoplasm
8	KIAA1033	136	Endosome
9	MMP23	42	ECM
10	MRM1	39	Mitoc hondrion
11	PRDX1	22	Cytoplasm
12	RNF149	43	Plasmalkmma
13	TXN	12	Cytoplasm, nuckus

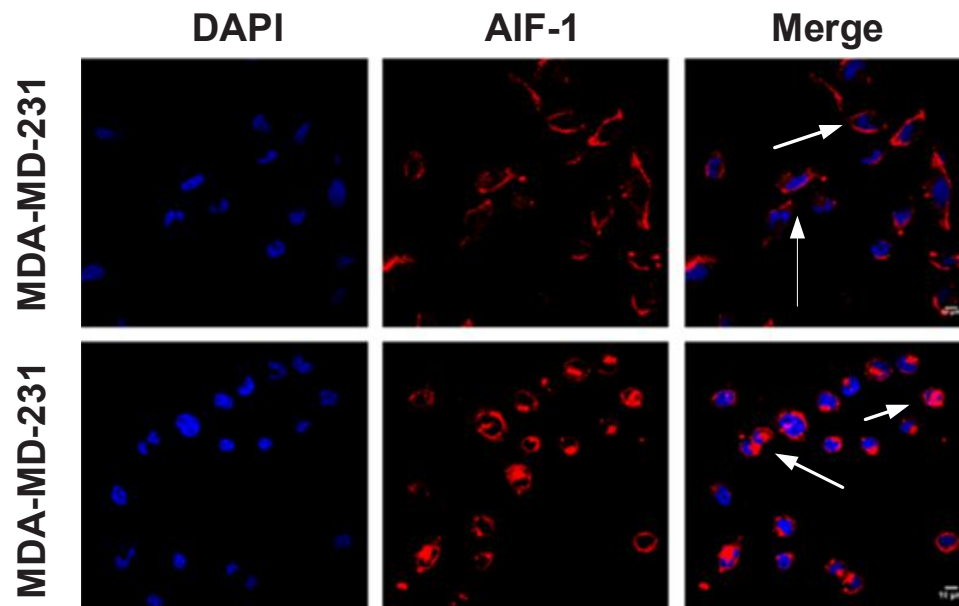


Fig. 4. Properties of rhAIF-1 localized in the cytoplasm. Under the confocal fluorescence microscope, the localization of rhodamine-labeled rhAIF-1 in breast cancer cells MDA-MB-231 and MDA-MB-361 was observed, and the nuclei were labeled with DAPI dye. Ruler: 10 μ m.

the possible interacting proteins of rhAIF-1 after elimination of the non-specific protein of the antibody itself compared with the control group. It was found that although the molecular weight of the diced protein was 70-100 KDa, molecular weight of the protein obtained after mass spectrometry was not in this range, suggesting that further analysis of the interacting protein of rhAIF-1 was required.

As shown in Fig. 5A and Table I, it was found that the largest proportion of candidate proteins was related to cell proliferation, which indirectly proved that rhAIF-1 was indeed involved in the development of breast cancer. Moreover, these candidate proteins were classified according to the subcellular localization. As shown in Fig. 5B, more than 80% of the candidate proteins were localized in the cytoplasmic matrix and subcellular organelles, which indirectly verified the results of previous confocal scans. rhAIF-1 not only enters cells through the cell membrane, but also participates in the deterioration process of breast cancer cells by interacting with candidate proteins in the cytoplasm. Based on the molecular weight of the protein, protein localization and functional analysis, it

was preliminarily determined that the interaction of ADAM28m and rhAIF-1 was involved in the development of breast cancer.

Interaction between rhAIF-1 and ADAM28m revealed by immunoblotting

The results of protein co-precipitation technique and Western blot are shown in Fig. 6. The expression of ADAM28m protein in MDA-MB-231 cell lysate can be used as the positive control. The His-tag antibody and the IgG antibody as negative controls were used to display the candidate protein ADAM28m. Compared to the control, a band was detected at 130 KDa, indicating that rh AIF-1 could interact with ADAM28m *in vitro*.

DISCUSSION

As a novel inflammatory factor, AIF-1 is widely involved in various diseases related to inflammation (8). The correlation between inflammation and tumors is a hotspot, and there have been some studies on AIF-1 and tumors. AIF-1 can promote proliferation and migration of endothelial cells

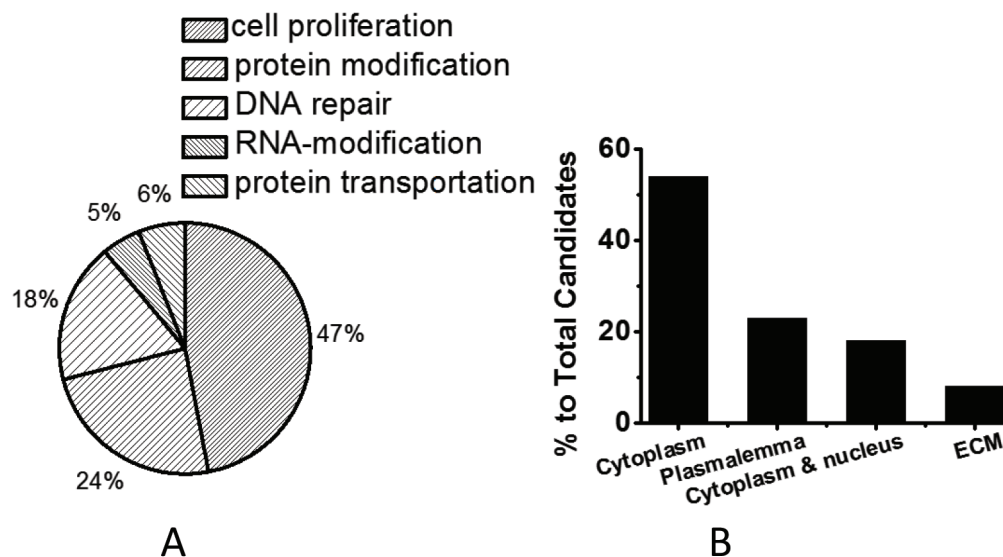


Fig. 5. Preliminary analysis of candidate proteins interacting with rhAIF-1. *A)* Candidate proteins were classified according to function, and a pie chart was used to show the proportion of each protein among the total candidate protein; *B)* Candidate proteins were classified according to the subcellular localization, and the histogram shows the proportion of each protein among the total candidate proteins.

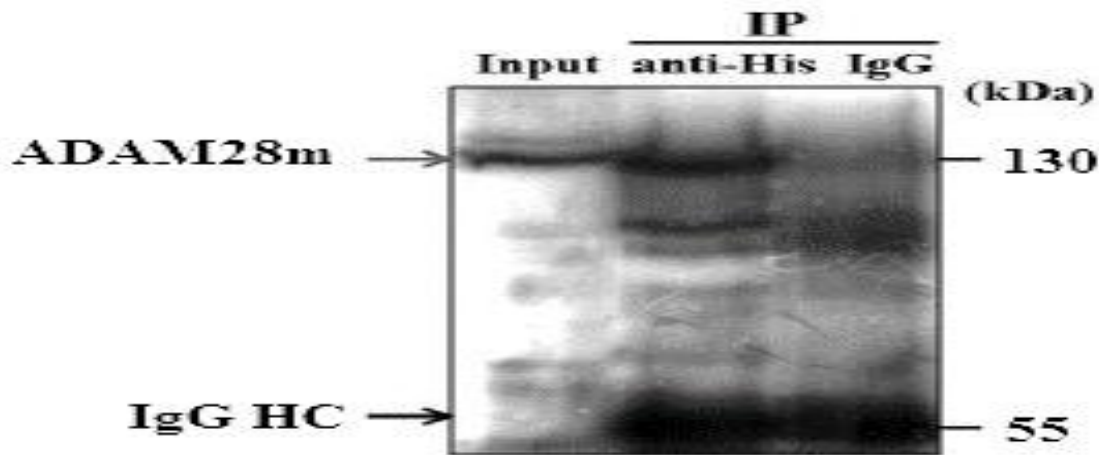


Fig. 6. Interaction between rhAIF-1 and ADAM28m by immunoblotting

involved in the regulation of vascular tumor progression and activate microglia in the generating process of glioma. Clinical studies found that AIF-1 was highly expressed in breast ductal carcinoma tissues and positively correlated with tumor size (9, 10). When the tumor diameter was less than 2 cm, the positive rate was 44.4%, and when the tumor diameter was between 2.1 cm and 4 cm, the positive rate increased to 73.3%. Cell experiments also confirmed that the proliferation was significantly accelerated after AIF-1 was highly expressed by breast cancer cells MDA-MB-231, while the cell proliferation was significantly inhibited after AIF-1 expression was down-regulated (11-15). However, the molecular mechanism by which AIF-1 regulates proliferation remains to be investigated. In this experiment, the high-purity active recombinant protein rhAIF-1 was purified by optimizing the experimental system based on previous studies. At the same time, different molecular subtypes of breast cancer cell lines MDA-MB-231 and MDA-MB-361 were used as experimental models to study the effect of rhAIF-1 on the proliferation of breast cancer cells at the cellular level. It was found that rhAIF-1 promoted the proliferation of these two cell lines, and was concentration-dependent and time-dependent within a certain range. Interestingly, when the proliferation of MDA-MB-231 and MDA-MB-361 cell lines was compared, the proliferation

efficiency of the two cells was found to be different. They represented two different types of breast cancer cell lines, respectively, and MDA-MB-231 was a triple-negative breast cancer cell line with a high degree of deterioration and strong metastatic ability, while MDA-MB-361 was a HER-2 overexpressing breast cancer cell line with relatively weak metastatic potential. This suggested that the ability of AIF-1 in promoting cell proliferation may be related to the expression of cell surface receptor HER-2, which remains to be further explored.

The mechanism of rhAIF-1 entering cells and the study on interaction between proteins and rhAIF-1 are important for the analysis on its cytoplasmic localization and function in the cytoplasm (16-18). Studies have shown that ADAM28m is expressed in a variety of cancers (19, 20). This suggested that ADAM28m, as a proteolytic enzyme, can become active from inactive rhAIF-1 by splitting rhAIF-1 to activate the downstream signal pathway. However, the molecular structure of ADAM28m also contained a signal peptide and a precursor domain, and rhAIF-1 may also be an upstream activating protein of ADAM28m. After ADAM28m was activated by rhAIF-1, ADAM28m could promote the release of TNF- α , or activate other signaling pathways involved in cytokines. The current study has still not given a clear answer.

Besides ADAM28m, a series of other proteins

interacting with rhAIF-1 were screened out. In addition, according to the subcellular localization of candidate proteins interacting with AIF-1, most of the proteins were located not only in the cell membrane and cytoplasm, but also in the mitochondria and lysosome sub-organelles, most of which were enzymes, suggesting the transport mechanism of AIF-1 at the cellular level to some extent. These findings provide a new idea for future research. However, whether these proteins are involved in the occurrence and development of breast cancer and through which signaling pathways they play a role remain to be further studied.

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LETTER TO THE EDITOR

INHIBITION OF MITOCHONDRIAL CYTOCHROME C OXIDASE BY METABOLIZED NOBILETIN IN YEAST

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To the Editor,

Nobiletin (3',4',5,6,7,8-hexamethoxyflavone) is a flavonoid and a major component of citrus fruits, particularly in the peel of sweet oranges (*Citrus sinensis*) and mandarin oranges (*Citrus reticulata*). Nobiletin attracts special attention because of its anti-inflammatory, antitumor, and neuroprotective properties. Without a glycoside moiety, Nobiletin is easily absorbed, due to its high lipophilic nature and high permeability (1). Recent evidence shows that Nobiletin is a multifunctional pharmaceutical agent, and its various pharmacological activities include neuroprotection, cardiovascular protection, antimetabolic disorder, anticancer, anti-inflammation, and antioxidation (2). Even though the beneficial physiological and biological effects of Nobiletin have been widely studied (2), very few studies have addressed the possible modulation of cell energy metabolism by this molecule (3, 4). Here, we investigated the influence of Nobiletin on cellular energetic metabolism in two distinct yeast strains: *Saccharomyces cerevisiae* and *Candida utilis*. The *Saccharomyces cerevisiae* strain exhibits two external Nicotinamide adenine dinucleotide (NADH) dehydrogenases and one internal NADH dehydrogenase that have no proton pumping activity.

The yeast *Candida utilis* mitochondria exhibits a complex I that is proposed to pump protons and an external NADH dehydrogenase that does not pump protons. We have shown that Nobiletin temporarily inhibits mitochondrial respiratory chain on whole cells in a similar fashion to *Saccharomyces cerevisiae* and *Candida utilis*. The observed inhibition of mitochondrial respiratory chain, and more specifically mitochondrial cytochrome c oxidase, requires cellular incubation in the presence of Nobiletin or treatment of isolated mitochondria with metabolites extracted from cells incubated with Nobiletin. We therefore propose that this inhibition is not a direct inhibition by Nobiletin but is more likely due to one of its metabolites.

MATERIALS AND METHODS

Yeast strains, culture medium and growth conditions

The yeast strains *C. utilis* CBS621 and *Saccharomyces cerevisiae* (yeast foam) were used in this study. Cells were grown aerobically at 28°C in the following medium: 0.175% yeast nitrogen base w/o sulphate (Difco), 0.2% casein hydrolysate (Merck), 0.5% (NH₄)₂SO₄, 0.1% KH₂PO₄, 2% lactate (w/v) (Prolabo), pH 5.5, 20mg/L L-tryptophan (Sigma), 40mg/L adenine hydrochloride

Key words: Nobiletin, yeast, mitochondria, respiratory chain, cytochrome oxidase

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(Sigma) and 20mg/L uracil (Sigma). Growth was measured at 600 nm in a Safas spectrophotometer (Monaco). Dry weight determinations were performed on samples of cells harvested throughout the growth period and washed twice in distilled water.

Mitochondria preparation

Yeast cells were harvested in the exponential growth phase and spheroplasts were obtained by enzymatic digestion of the cell wall with Zymolyase 10 mg/g dry weight (ICN iomedicals) as previously described with small modifications (5).

Cells were pelleted at room temperature by centrifugation at 3000g for 10 min and washed three times with distilled water. The obtained yeast pellet was resuspended in prewarmed SH buffer (100 mM Tris, pH 9.3, 0.5 M β -mercaptethanol, 20ml/g DW) and shaken slowly (approx 80 rpm) at 32°C for 10 min. After centrifugation at 3000g for 5 min, the pellet was washed three times in KCl buffer (KCl 0.5M, Tris 10 mM, pH 7) and then resuspended in zymolyase buffer (1.35 M sorbitol, EGTA 1mM; 0.2M sodium phosphate, pH 7.4) 10ml/g DW containing 10 mg zymolyase per gram wet weight and shaken slowly at 30°C for 30–45 min. Cell wall digestion was followed by assessing OD_{600nm} in water. The cells were harvested by centrifugation at 13000g for 10 min and the pellet was washed with zymolyase buffer. The obtained pellet was resuspended in ice-cold homogenization buffer (0.5 M mannitol, 10 mM Tris-Maleate, pH 7.4, 2 mM ethylenediaminetetraacetic acid (EGTA), 10 mM Tris-orthophosphate, 0.2% (w/v) bovine serum albumin pH6.8). Spheroplasts were homogenized with 15 strokes using a glass-Teflon homogenizer, then the sample was diluted twofold with homogenization buffer and centrifuged at 600g for 10 min to pellet cell debris and nuclei. The obtained supernatant was centrifuged at 13000g for 10 min. Crude mitochondrial pellet was washed in ice-cold homogenization buffer and centrifuged again at 600g for 10 min to pellet cell debris and nuclei. Supernatant then was centrifuged at 13000g for 10 min and the obtained mitochondrial pellet was gently resuspended in the following buffer: 0.6 M mannitol, 10 mM Tris-Maleate, pH 7.4, 2 mM EGTA, 10 mM Tris-orthophosphate, pH 6.8 to a final concentration of 60mg/mL protein with ice-cold buffer.

Protein concentration was measured by the biuret

method using bovine serum albumin as standard (6).

Spheroplasts preparation

Spheroplasts were obtained according to Avéret et al. (7) and were resuspended in the following buffer: 1 M sorbitol, 1.7 mM NaCl, 0.5 mM EGTA, 10 mM KCl, 1 mM potassium phosphate, 10 mM Tris-HCl, 4 mM iodoacetate, and 1% bovine serum albumin, pH 6.8 and, permeabilized by nystatin, as described (8). If unspecified, respiratory substrates were 10 mM, Cytochrome c oxidase activity measurement was carried out in the presence of 5 μ M antimycin A, 0.25 mM N,N,N',N'-tetramethyl-1,4-benzenediamine, dihydrochloride (TMPD) and 1 mM ascorbate.

Respiration assay

The oxygen consumption was measured polarographically at 28°C using a Clark oxygen electrode in a 1-ml thermostatically controlled chamber. Respiratory rates (JO₂) were determined from the slope of a plot of O₂ concentration *versus* time. For whole cells, oxygen consumption rate, growing cell suspension (1 ml) was quickly transferred to the respirometer and the “spontaneous cell respiration” was determined. The uncoupled respiratory rate was assessed in the presence of 100 mM ethanol (to avoid a kinetic control upstream the respiratory chain) and 10 μ M carbonyl cyanide m-chlorophenylhydrazone (CCCP). The non-phosphorylating respiratory rate was assessed in the presence of 0.2 mM triethyltin bromide (TET), an inhibitor of mitochondrial ATP synthase (8). For isolated mitochondria respiration measurements, mitochondria (0.33 mg/mL) were incubated in 1 mL mitochondrial buffer (0.65 M mannitol, 0.36 mM EGTA, 10 mM Tris-maleate, 10 mM Tris-phosphate, pH 6.8). Respiratory rates (JO₂) were then determined from the slope of a plot of O₂ concentration *versus* time, and expressed as nanoatoms O/min/OD (cells) or nanoatoms O/min/mg mitochondrial protein (mitochondria).

Metabolites extraction

Cellular extracts were prepared by an ethanol extraction method adapted from the one described by Loret et al. (9). Briefly, cells were harvested by rapid filtration on nitrocellulose filter (1 μ m). The filter was immediately dropped into a glass tube containing 5 ml

ethanol/Hepes 10 mM pH 7.2 (4/1), and the tube was then incubated at 80°C for 3 min. The mixture was cooled down on ice for at least 3 min, and the ethanol/Hepes solution was eliminated by evaporation using a rotavapor apparatus. The residue was resuspended in a final volume of DMSO equivalent to twice the cellular volume). Insoluble particles were eliminated by centrifugation (12 000g, 10 min, 4°C).

RESULTS

Nobiletin influence on cellular energy metabolism was investigated in two yeast strains: *Saccharomyces cerevisiae* and *Candida utilis*. Whereas *Candida utilis* harbours a proton pumping complex I at the level of mitochondrial respiratory chain (10), the *Saccharomyces cerevisiae* strain harbours two external NADH dehydrogenases and one internal NADH dehydrogenase. The cellular respiratory rate in the absence of Nobiletin was shown to be stable all throughout the experiment (Fig. 1, A and B). Upon Nobiletin addition (250 and 500 μ M), spontaneous cell respiration was greatly decreased (about 65% $\pm$ 4%) in both yeast strains (Fig. 1, A and B). After one hour of incubation in the presence of Nobiletin, spontaneous cell respiration tended to recover from the induced decrease and after five hours, only a fraction of the initial inhibition could be assessed in both strains (Fig. 1, A and B).

In order to decipher whether the decrease in spontaneous cellular respiration is due to an inhibition of mitochondrial respiratory chain and/or of the phosphorylating systems, we assessed the uncoupled respiratory rate on both strains in the presence of the well-known uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The uncoupled respiratory rate in the absence of Nobiletin was shown to be stable all throughout the experiment (Fig. 1, C and D). Upon Nobiletin addition (250 and 500 μ M), uncoupled respiratory rate was greatly decreased (60% $\pm$ 4%) in the *Saccharomyces cerevisiae* cells and 50% $\pm$ 3.7% in the *Candida utilis* cells (Fig. 1, C and D). After one hour of incubation in the presence of Nobiletin, uncoupled cell respiration tended to recover from the induced decrease, and after five hours, only a

fraction of the initial inhibition could be assessed in both strains (Fig. 1, A and B).

Under non-phosphorylating respiration condition, *Saccharomyces cerevisiae* and *Candida utilis* cells exhibited an inhibition profile upon Nobiletin treatment similar to that assessed under spontaneous and uncoupled respiration, again pointing to a respiratory chain inhibition by Nobiletin rather than an effect on the phosphorylating systems (data not shown).

To further study the kinetics of respiratory rate inhibition by Nobiletin, we assessed all three respiratory states (spontaneous, uncoupled, non-phosphorylating) every 10 min after Nobiletin addition during the first hour of treatment. Fig. 1E shows that spontaneous and non-phosphorylating respiratory rates reached their maximal inhibition rate after about 10 min in *Saccharomyces cerevisiae* cells whereas uncoupled respiratory rate was fully inhibited after 40 min. This inhibition was quite stable afterward up until 1 h of incubation. The respiratory rate inhibition profile in *Candida utilis* cells was a bit different since spontaneous and uncoupled respiratory rates reach their maximal inhibition after 30 min of incubation with Nobiletin and this inhibition tended to reverse slowly thereafter (Fig. 1F).

In order to identify the respiratory chain target of Nobiletin, we investigated the induced inhibition on mitochondria isolated from either *Saccharomyces cerevisiae* cells or *Candida utilis* cells. Because Nobiletin partition is mostly membranous, the concentration used on isolated mitochondria was decreased compared that used on whole cells. When Nobiletin addition to isolated mitochondria was acute (i.e. injected directly into the oxygraph chamber), no respiration inhibition was observed (data not shown). Three distinct respiratory rates were assessed: non-phosphorylating (ethanol as a respiratory substrate), phosphorylating [in the presence of adenosine diphosphate (ADP)] and uncoupled (in the presence of CCCP). These were assessed either after 5 min preincubation with Nobiletin (Fig. 2A) *Saccharomyces cerevisiae* and *Candida utilis* mitochondria (Fig. 2C) or after 10 min preincubation with Nobiletin *Saccharomyces cerevisiae* (Fig. 2B) and *Candida utilis* mitochondria (Fig. 2D). Fig. 2 shows that, whichever the

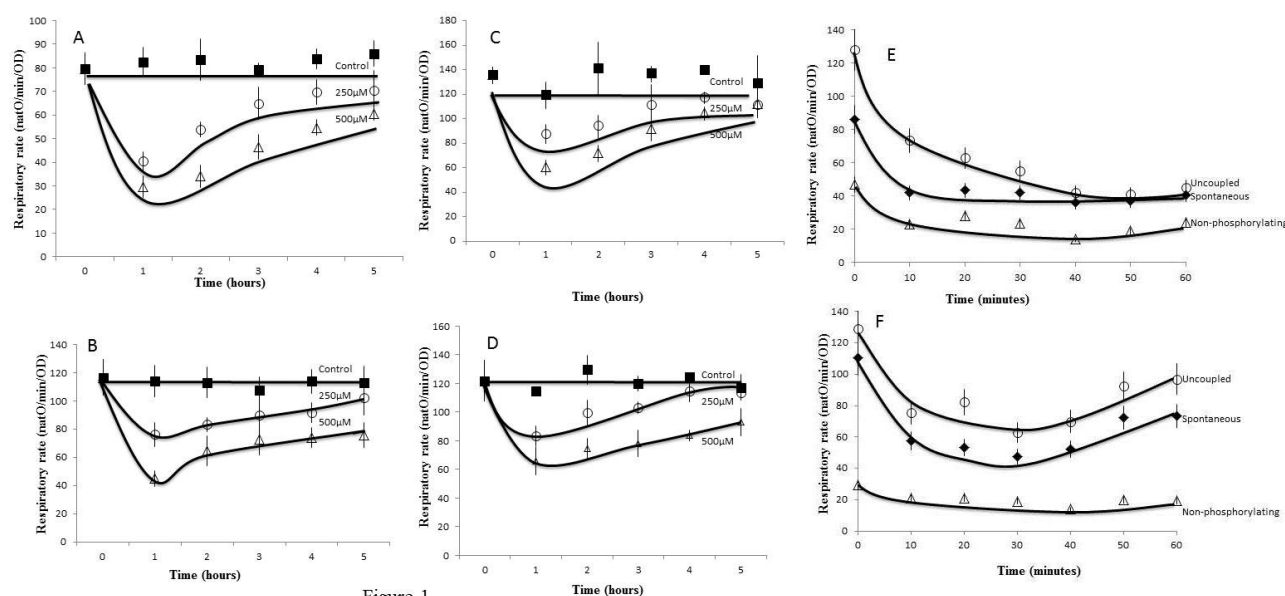


Figure 1

Fig. 1. Influence of Nobiletin on cellular and uncoupled respiration in *Saccharomyces cerevisiae* and *Candida utilis* cells. **(A)** *Saccharomyces cerevisiae*; **(B)** *Candida utilis*. **(C)** *Saccharomyces cerevisiae* uncoupled respiratory rate; **(D)** *Candida utilis* uncoupled respiratory rate. Black squares - control, empty circle 250 μM - Nobiletin, empty triangle - 500 μM Nobiletin. The uncoupled respiratory rate was assessed in the presence of 40 μM carbonyl cyanide *p*-chlorophenylhydrazone (CCCP) as described under "Experimental Procedures." Short term kinetics of the influence of 250 μM Nobiletin on cellular, uncoupled and non-phosphorylating cellular respiration of *Saccharomyces cerevisiae* (**E**); and *Candida utilis* (**F**). The respiratory rate in the absence of phosphorylation (empty triangle), indicated by triethyltin (TET) was assessed in the presence of 0.2 mM triethyltin and the uncoupled respiratory rate (empty circle) was assessed in the presence of 40 μM carbonyl cyanide *p*-chlorophenylhydrazone (CCCP) as described under "Experimental Procedures." Respiratory rates were assessed on cells grown aerobically in minimal medium containing 2% (w/v) DL-lactate. Spontaneous respiratory rate was directly assessed on cells from the growth medium. Results are mean $\pm$ S.D. of at least three measurements performed on three independent cell cultures.

experimental condition, there was no significant inhibition of mitochondrial respiratory chain by Nobiletin on isolated mitochondria.

To decipher the origin of the absence of inhibition of mitochondrial respiratory chain by Nobiletin on isolated mitochondria, we studied the effect of that molecule on spheroplasts isolated from *Candida utilis* cells. When Nobiletin (up to 250 μM) addition to permeabilized spheroplasts was acute (i.e. injected directly in the oxygraph chamber), no respiration inhibition was observed whichever the respiratory substrate used (data not shown). A comparable result was obtained when Nobiletin (up to 250 μM) was added to unpermeabilized spheroplasts with 5 and 10 min incubation (Fig. 3A) or without any incubation

(data not shown). Since Nobiletin-induced maximal respiration inhibition on whole cells required about 30 min, we decided to add Nobiletin on whole cells for 30 min before spheroplast isolation. Then the spheroplasts were isolated and permeabilized according to the experimental procedures section. The respiratory rate was then assessed with various respiratory substrates without any further addition of Nobiletin. Fig. 3B shows that, whichever the respiratory substrate, the respiratory rate was inhibited in spheroplasts isolated from cells treated with 250 μM Nobiletin for 30 min. Furthermore, assessment of cytochrome c oxidase activity in these spheroplasts (Fig. 3B) clearly shows that this enzyme is strongly inhibited in spheroplasts isolated

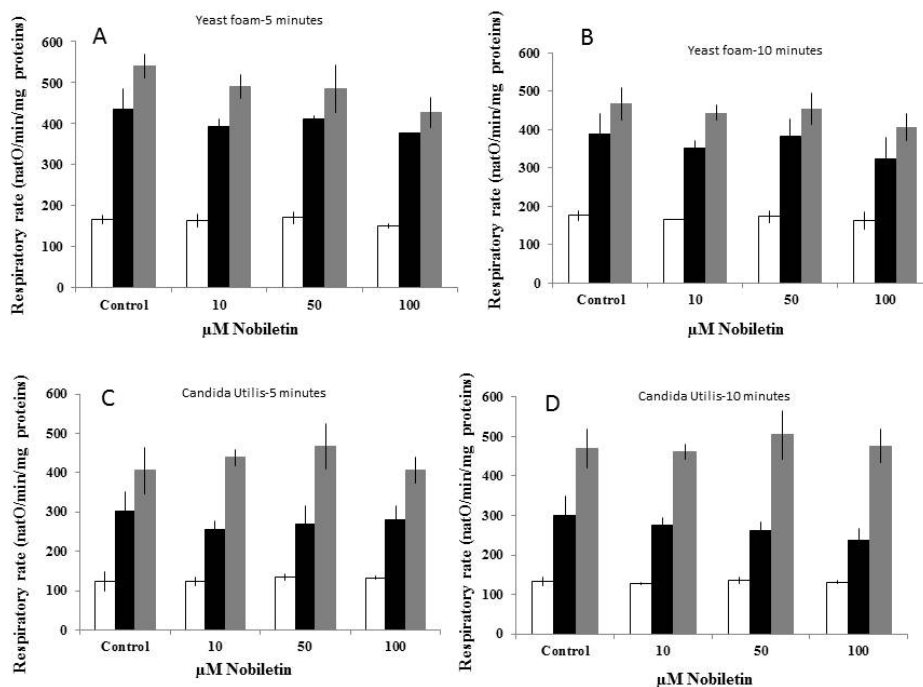


Fig. 2. Influence of Nobiletin on mitochondrial respiration in *Saccharomyces cerevisiae* and *Candida utilis* isolated mitochondria. Mitochondria were isolated from *Saccharomyces cerevisiae* and *Candida utilis* cells as described under “Experimental procedures”. Respiratory rates were assessed using ethanol (100 mM) as a respiratory substrate. White - non-phosphorylating respiratory rate, black- phosphorylating respiratory rate in the presence of 1mM ADP and gray - uncoupled respiratory rate in the presence of 10 μ M CCCP. **A)** *Saccharomyces cerevisiae* mitochondria incubated for 5 min with Nobiletin; **(B)** *Saccharomyces cerevisiae* mitochondria incubated for 10 min with Nobiletin; **(C)** *Candida utilis* mitochondria incubated for 5 min with Nobiletin; **(D)** *Candida utilis* mitochondria incubated for 5 min with Nobiletin. Results are mean $\pm$ S.D. of at least three measurements performed on three independent mitochondria preparation.

from cells treated with 250 μ M Nobiletin for 30 min.

The above-mentioned results led us to hypothesize that, since Nobiletin had no effect on isolated mitochondria but the mitochondrial respiratory chain was inhibited in cells/spheroplasts treated with this flavonoid, the observed inhibition might arise from Nobiletin metabolism within the cell. We thus extracted cellular metabolites from both control cells and cells treated with Nobiletin for 30 minutes. These metabolite extracts were then used on isolated mitochondria while monitoring distinct respiratory states. Fig. 3C shows that all three respiratory states were significantly inhibited by cellular metabolites from Nobiletin-treated cells compared to untreated cells.

DISCUSSION

In this paper we investigated the influence of Nobiletin, a flavonoid abundantly present in citrus peel, on yeast energy metabolism. Studies on yeast cells harboring either a proton pumping complex I or NADH dehydrogenases that do not pump protons showed a comparable inhibition of mitochondrial respiration in both strains, indicating that the observed inhibition did not rely on the presence of a proton pumping complex I. Further studies of cellular respiration in distinct respiratory states (i.e. uncoupled or non-phosphorylating) showed a comparable inhibition of mitochondrial respiration whichever the respiratory state, pointing

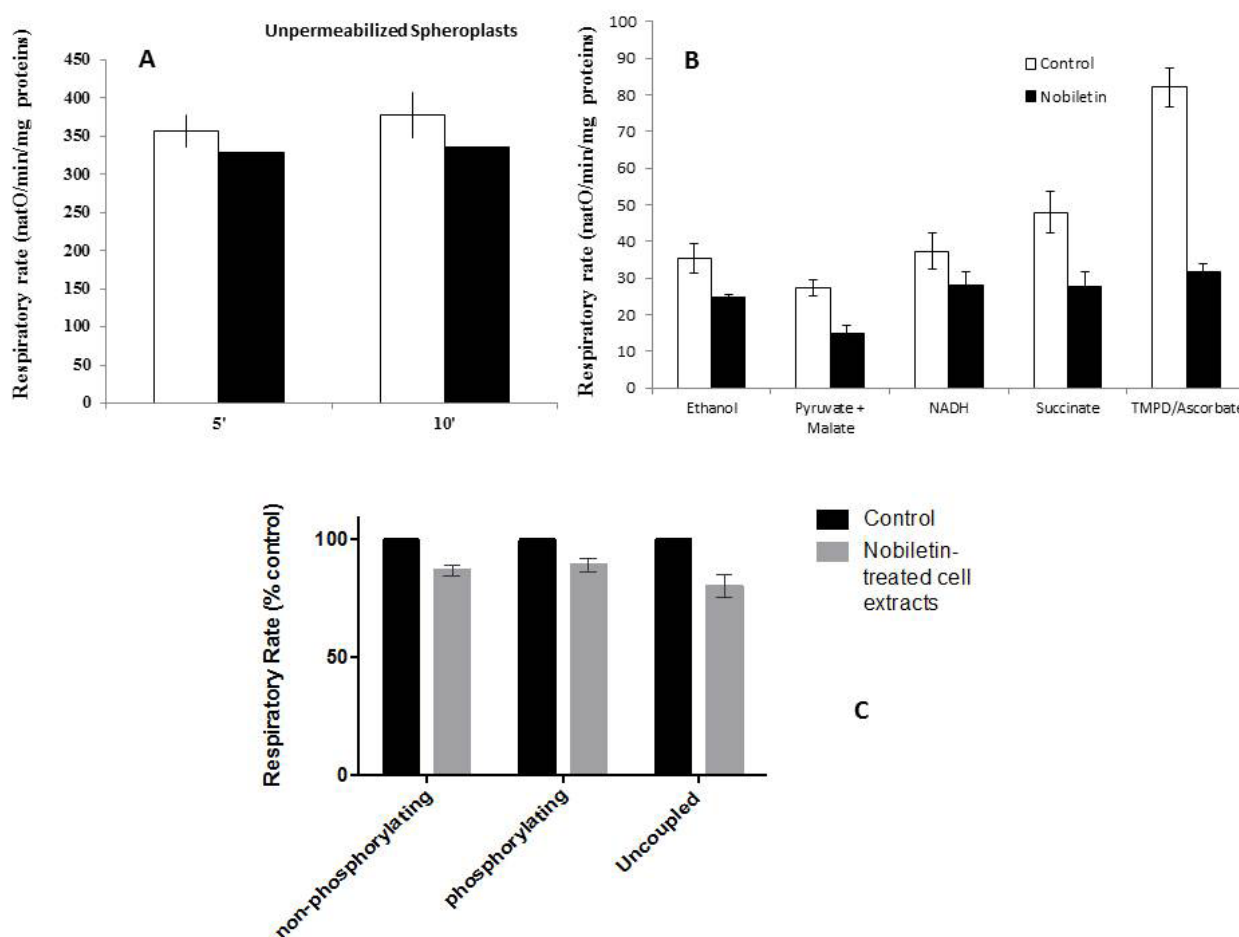


Fig. 3. Influence of Nobiletin on isolated spheroplasts respiration and Nobiletin's metabolites on mitochondrial respiration. Spheroplasts were isolated as described under "Experimental procedures". Respiratory rates were assessed using ethanol as a respiratory substrate (unpermeabilized spheroplasts) (A); or the indicated substrate for spheroplasts permeabilized from cells treated for 30 min with 250 μ M Nobiletin (B). C) Respiratory substrate, non-phosphorylating respiratory rate, phosphorylating respiratory rate in the presence of 1mM ADP, ethanol (100 mM) and uncoupled respiratory rate in the presence of 10 μ M CCCP. *Saccharomyces cerevisiae* mitochondria were incubated with metabolites from cells treated with Nobiletin (gray) or from control cells (black). Results are mean $\pm$ S.D. of at least three measurements performed on three independent mitochondria and spheroplasts preparation.

to a respiratory chain inhibition upon Nobiletin addition to cells. Short term kinetic experiments showed a maximal inhibition of mitochondrial respiratory chain after about 30 minutes of cell incubation with Nobiletin, whichever the yeast strain under consideration. Surprisingly, acute Nobiletin addition on isolated mitochondria or spheroplasts (either permeabilized or not) showed no respiratory chain inhibition. Observed cellular respiratory chain inhibition in spheroplasts isolated from cells

preincubated with Nobiletin for 30 minutes clearly points to a required cellular metabolism of Nobiletin in order to obtain a respiration inhibition. Moreover, the cellular metabolites from Nobiletin-treated cells inhibit mitochondrial respiratory chain. We have shown that inhibition of respiration in yeast cells by Nobiletin requires its intracellular metabolism and is thus due to an intermediate of Nobiletin metabolism.

Nobiletin's and its metabolites' effect on the mitochondria could be also explained by the

involvement of yeast's certain regulatory systems. One of the possible candidates is cAMP-dependent protein kinase that is known as one of the main target of Nobiletin (11). This type of protein kinase involves in the regulation of mitochondrial cytochrome c content as well as cytochrome-c-oxidase activity (12). Further studies will be required to assess the consequences of this respiration inhibition on cell physiology.

ACKNOWLEDGEMENTS

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LETTER TO THE EDITOR

ABNORMAL BONE ARCHITECTURE IN MICE EXPRESSING MyD88 IN CELLS OF THE OSTEOCLAST LINEAGE

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To the Editor,

Myeloid differentiation primary response gene 88 is a 33 kilo Dalton cytosolic protein first reported in 1990 (1). The 296 amino acid polypeptide consists of an N-terminal death domain, followed by a short intermediate domain, and a C-terminal Toll/interleukin 1 receptor homology (TIR) domain (2). The latter facilitates association with the cytosolic TIR domain of IL1R, IL18R, or TLRs, and upon ligand binding mediates IRAK signaling which then leads to the downstream activation of NF-κB-mediated transcription. The transcriptional activation via MyD88 is well established in inflammation research and its targets includes pro-inflammatory cytokines that mediate the innate and adoptive immune response (3, 4). NF-κB also plays a role in other signaling pathways. For example, it has a pivotal role in osteoclastogenesis (5), thus providing a strong rationale to begin to assess the function of MyD88 in bone.

Data on the function of MyD88 in bone is still

limited. Adachi and co-workers described global MyD88 deficient mice (MyD88^{-/-}) (6), and Sato et al. subsequently reported, as part of a larger study, a histomorphometric analysis of spinal cancellous bone of MyD88^{-/-} mice and reported a pronounced reduction in the bone volume fraction BV/TV, and hence characterized these mice as osteopenic (7). Because both osteoblastic and osteoclastic cancellous bone surfaces were reduced about equally in MyD88^{-/-} mice, the data implied a reduced osteoblast function and/or hyperactivity of osteoclasts. Based on these findings, we decided to study mice in which we maintained the MyD88-deficient background but selectively re-express MyD88 in all cells of the myeloid lineage, which in bone restores MyD88 expression in osteoclasts. Our underlying hypothesis was that in these mice the osteopenic phenotype would either remain or be counteracted dependent on the significance of MyD88 expression in the osteoclast lineage.

Key words: bone; bone architecture; osteoclast; osteoblast; MyD88 (myeloid differentiation primary response 88); TLR (toll-like receptor) signaling

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MATERIALS AND METHODS

Animals and bone preparation

This research was approved by the IRB (No.4-009-17) of the authors' affiliated institutions. Use of the lysozyme M (LysM) promoter to drive Cre expression in osteoclasts has been described by the Teitelbaum lab (8). We previously validated that LyM-Cre mice (9) crossed into MyD88^{LSL/LSL} mice, which harbor a knock-in/knock-out allele with a floxed transcriptional termination element inserted between exons 1 and 2 of the MyD88 gene, results in MyD88-deficient hosts exclusively expressing MyD88 under control of the LysM promoter (10). These mice were created on a C57BL/6 genetic background and termed MyD88^{OC}. MyD88^{OC} (n=8) and wt (n=9) female mice from our colony were sacrificed at 12-14 weeks of age by cervical dislocation, and the tibias and femurs immediately dissected free of soft tissues. Tibias and femurs for microCT and mechanical testing, respectively, were fixed in 4% paraformaldehyde for 48 h at room temperature, washed three times in phosphate buffered saline (PBS), and stored in PBS at 4°C. Femurs for gene expression analysis were split in half, flushed carefully with ice cold PBS to remove the bone marrow, and immediately snap frozen in liquid nitrogen.

Micro computed tomography (microCT)

Bone microCT was carried out on a Scanco microCT 35 (Scanco Medical, Brüttisellen, Switzerland) system, guided by recommendations made by Bouxsein et al. (11). Acquisition parameters included 6 μm voxel size, 55 KVp, 0.36 degrees rotation step (180 degrees angular range), and a 400 ms exposure per view. Samples were scanned in PBS at RT. Fields-of-view for cortical and trabecular bone were a 1.35 mm of the distal part of femurs starting at 100 μm from the growth plate, and a 1.4 mm part of the mid-diaphysis, respectively. The Scanco μCT software (HP, DECwindows Motif 1.6) was used for 3D reconstruction and viewing of images. After 3D reconstruction, volumes were segmented using a global threshold of 0.4g/cc. For trabecular bone, the tissue mineral density (TMD in $\text{mg HA}/\text{cm}^3$) and apparent density (in $\text{mg HA}/\text{cm}^3$) were measured and calculated, while morphometric parameters included bone volume fraction (BV/TV in %), specific bone surface (BS/BV in mm^2/mm^3), trabecular number (Tb.N

in $1/\text{mm}$), trabecular thickness (Tb.Th in mm), trabecular separation (Tb.Sp in mm), degree of anisotropy (DA), and connectivity density (Conn.D in mm^{-3}). For cortical bone, the tissue mineral density and apparent density were also measured and calculated, while morphometric parameters included bone volume fraction (BV/TV), bone area (BArea in mm^2), total area (TArea in mm^2), cortical thickness (Ct.Th in mm), maximum moment of inertia (Imax in mm^4), minimum moment of inertia (Imin in mm^4), polar moment of inertia (J in mm^4), and cortical porosity (Ct.Po in %).

Mechanical testing

Femurs were subjected to three-point bending experiments using a Bose Test Bench LM 1 ElectroForce (Bose, Eden Prairie, MN). A 50-lb (225 N) load cell was used. Test conditions included a temperature and humidity of 24°C and 26.8%, respectively. Femura were mounted anterior-posterior for bending tests at a 8.5 mm span width between the end supports. A deflection rate of 0.1 mm/s was applied. The bending stiffness, maximum load, load to failure and deflection at failure were calculated using a routine written in MATLAB (The Mathworks, Inc. Natick, MA, USA).

Gene expression analysis

Bone tissue was pulverized mechanically by grinding in liquid nitrogen. Upon evaporation of the liquid nitrogen, Trizol Reagent (Ambion, Carlsbad, CA, USA) was added and the bone powder resuspended. RNA was extracted using the RNeasy Plus Mini Kit (Cat. no.: 74134, Qiagen, Hilden, Germany). Subsequent reverse transcription was performed using the Quantitect Reverse Transcription Kit (Cat. no.: 205313, Qiagen) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (QRT-PCR) was carried out on a StepOnePlus Real-Time PCR System (Applied Biosystems by LifeTechnologies, Carlsbad, CA) employing the QuantiTect SYBR Green PCR Kit (Qiagen). Briefly, each target was amplified in a total reaction volume of 20 μl , containing 10 μl QuantiTect SYBR Green PCR Master Mix, 2 μl QuantiTect Primer, 2 μl cDNA and 6 μl RNase-free water. The following QuantiTect Primer Assays (Qiagen) were used: MyD88 (Cat. no.: QT02589475), TRAP (Cat. no.: QT01757840), CTSK (Cat. no.: QT02589489), RANK (Cat. no.: QT02589503), COL1A1 Cat. no.: QT02589482), OC

(Cat. no.: QT01057049), RANKL (Cat. no.: QT02589496), OPG (Cat. no.: QT02589510), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cat. no.: QT00079247) and β -actin (ACTB; Cat. no.: QT00095431). Target amplification was started with an initial incubation step of 15 min at 95°C, followed by 40 cycles of a 15 s denaturation step at 95°C, a 30 s annealing step at 55°C and a 30 s extension step at 72°C. For data analysis, the comparative $\Delta\Delta$ CT method was used. Relative gene expression was measured, normalized to the corresponding values of the housekeeping genes (GAPDH and ACTB).

Statistical analysis

Statistical analyses were conducted using GraphPad software. An unpaired *t*-test was used and statistical significance was defined for $p < 0.05$.

RESULTS

To determine potential differences in morphometric parameters and bone mineral density

between wt and MyD88^{OC} mice, we carried out microCT analysis on tibia bone. Fig. 1 shows representative images from the analysis. Differences were observed for both trabecular and cortical bone. Compared to wt bone, trabeculae in MyD88^{OC} had fewer connections, appeared thicker and seemed reduced in numbers. Images acquired from cortical bone showed that the MyD88^{OC} bone geometry had a stocky appearance when compared to wt bone. Thus, particularly the geometric organization of MyD88^{OC} bone was abnormal when compared to wt bone. These findings prompted a quantitative analysis of the microCT data.

Table I summarizes the quantitative analysis of microCT data obtained from trabecular bone. Of the 9 parameters analyzed, 7 were significantly altered. The four most pronounced differences in MyD88^{OC} trabecular bone were decreases in connectivity density (-44%, $p < 0.0001$), trabecular thickness (14%, $p < 0.0001$), and in trabecular number (-15%, $p < 0.0001$), with increases in trabecular spacing

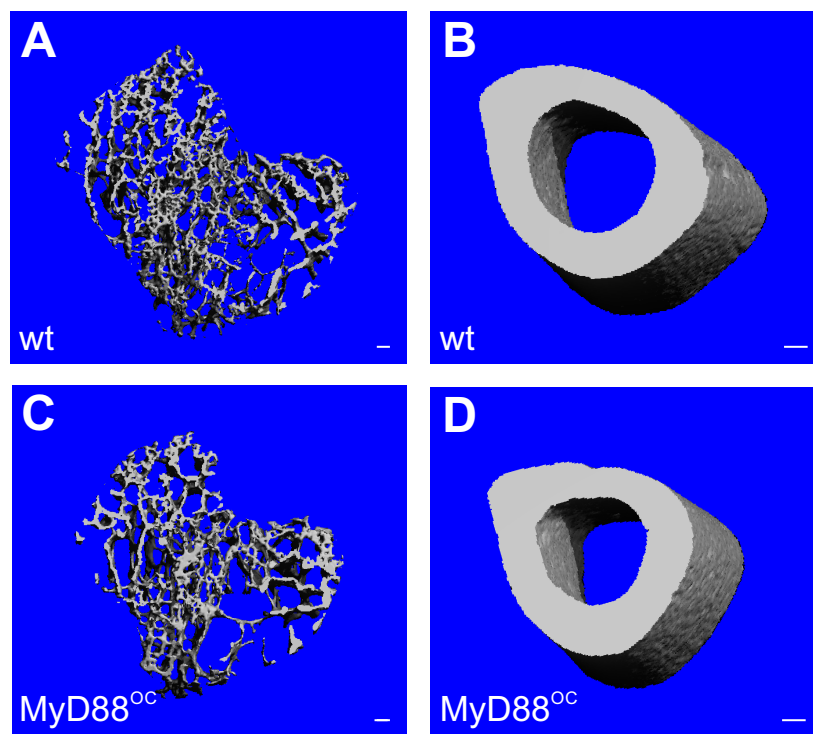


Fig. 1. MicroCT images of bone from wt and MyD88^{OC} mice. Representative images of (A) cancellous and (B) cortical tibia of wt (A and B) and MyD88^{OC} (C and D) mice. The scale bar corresponds to 100 μ m.

Table I. Quantitative microCT.

Parameter	Cancellous bone				Cortical bone			
	wt	MyD88 ^{OC}			wt	MyD88 ^{OC}		
	Mean ($\pm$ SD)	Mean ($\pm$ SD)	Δ	p	Mean ($\pm$ SD)	Mean ($\pm$ SD)	Δ	p
BV/TV	0.11 $\pm$ 0.01	0.11 $\pm$ 0.01	0%	0.97	0.59 $\pm$ 0.01	0.62 $\pm$ 0.02	5%	<0.01
Apparent Density	111.60 $\pm$ 11.26	109.10 $\pm$ 8.97	-2%	0.62	659.78 $\pm$ 14.49	711.17 $\pm$ 34.30	8%	<0.01
TMD	845.60 $\pm$ 10.88	876.39 $\pm$ 14.61	4%	<0.001	N/A	N/A	N/A	N/A
Tb.N	4.90 $\pm$ 0.34	4.19 $\pm$ 0.50	-15%	<0.01	N/A	N/A	N/A	N/A
Tb.Th	0.03 $\pm$ 0.001	0.04 $\pm$ 0.002	14%	<0.0001	N/A	N/A	N/A	N/A
Tb.Sp	0.20 $\pm$ 0.02	0.24 $\pm$ 0.03	18%	<0.05	N/A	N/A	N/A	N/A
BS/BV	81.87 $\pm$ 2.35	71.28 $\pm$ 3.54	-13%	<0.0001	N/A	N/A	N/A	N/A
DA	2.21 $\pm$ 0.06	2.36 $\pm$ 0.09	7%	<0.01	N/A	N/A	N/A	N/A
Conn-Dens.	421.30 $\pm$ 79.93	237.76 $\pm$ 30.95	-44%	<0.0001	N/A	N/A	N/A	N/A
TMD	N/A	N/A	N/A	N/A	1078.65 $\pm$ 5.07	1098.71 $\pm$ 18.59	2%	<0.01
Ct.Th	N/A	N/A	N/A	N/A	0.18 $\pm$ 0.004	0.18 $\pm$ 0.01	-1%	0.76
Porosity	N/A	N/A	N/A	N/A	5.53 $\pm$ 0.58	0.18 $\pm$ 0.004	12%	<0.05
J	N/A	N/A	N/A	N/A	0.11 $\pm$ 0.01	0.08 $\pm$ 0.01	-27%	<0.0001
Imax	N/A	N/A	N/A	N/A	0.04 $\pm$ 0.01	0.06 $\pm$ 0.01	-31%	<0.0001
Imin	N/A	N/A	N/A	N/A	0.04 $\pm$ 0.003	0.03 $\pm$ 0.01	-20%	<0.001
BArea	N/A	N/A	N/A	N/A	0.53 $\pm$ 0.01	0.47 $\pm$ 0.05	-11%	<0.01
TArea	N/A	N/A	N/A	N/A	0.90 $\pm$ 0.03	0.76 $\pm$ 0.06	-15%	<0.0001
BA/TA	N/A	N/A	N/A	N/A	0.59 $\pm$ 0.01	0.62 $\pm$ 0.02	5%	<0.01

Abbreviations: BArea: bone area; BA/TA: bone area index; BS/BV: specific bone surface; BV/TV: bone volume fraction; Conn-Dens.: connectivity density; Ct.Th: cortical thickness; DA: degree of anisotropy; J (pMOI): polar moment of inertia; Imax: maximum 2nd moment of area; Imin: minimum 2nd moment of area; N/A: not applicable; TArea: tissue area; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation; TMD: tissue mineral density; Δ : difference (wt = 100%); p: p-value.

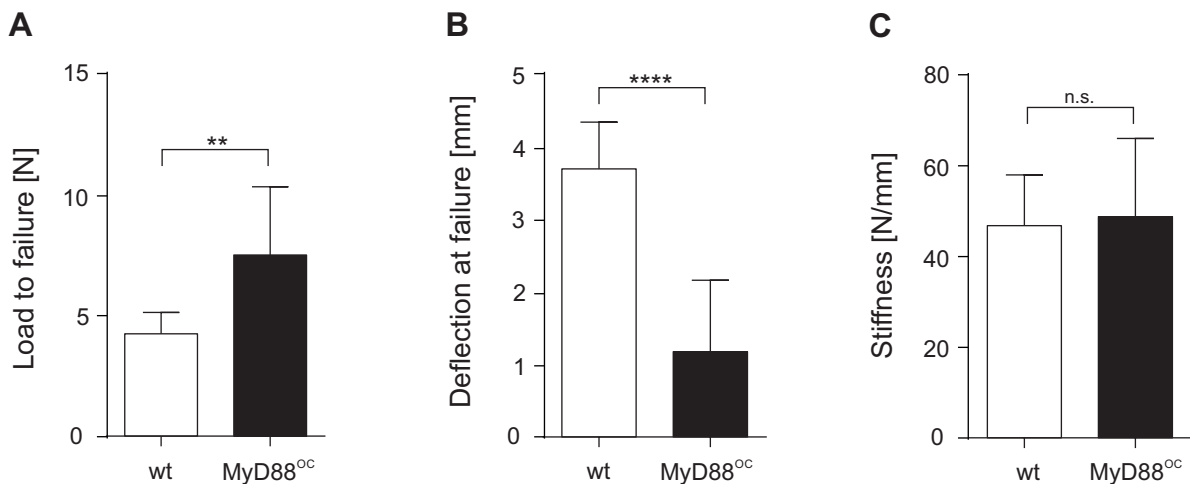


Fig. 2. Mechanical testing of wt and MyD88^{OC} femurs. In three-point bending experiments, (A) load to failure, (B) deflection at failure, and (C) stiffness were measured.

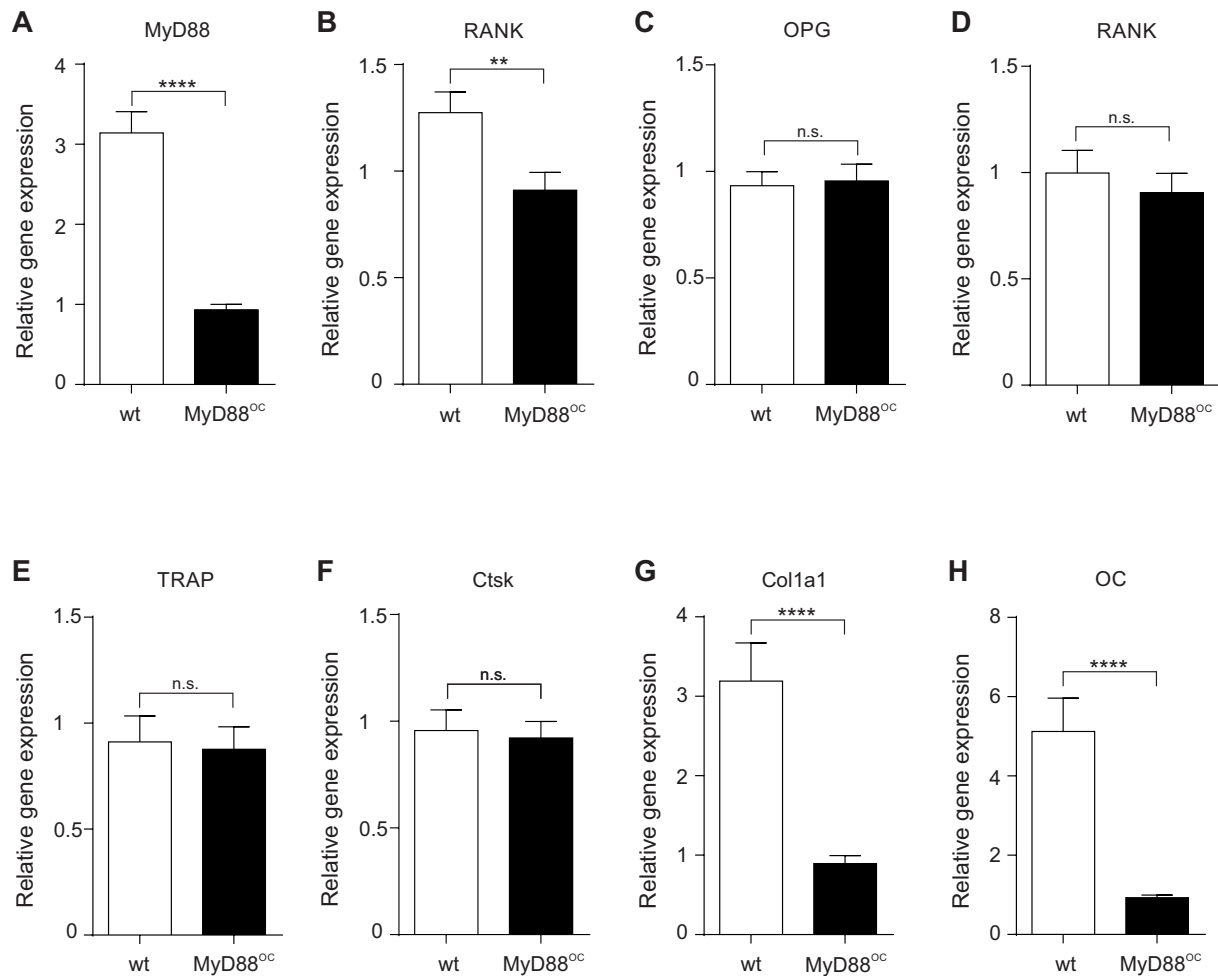


Fig. 3. Expression of bone coupling, osteoclast and osteoblast markers in bone from wt and MyD88^{OC} mice. RNA was extracted from pooled femurs, stripped of bone marrow, of either MyD88^{OC} or wt mice and analyzed by QRT-PCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

(18%, $p < 0.05$). With respect to BV/TV, no change was detected, while TMD was increased (4%, $p < 0.001$) in MyD88^{OC} trabecular bone. Table I presents the quantitative analysis of the microCT data obtained from cortical bone. Of the 11 parameters analyzed, 10 were significantly altered. The three most pronounced differences were recorded for I_{max} (-31%, $p < 0.0001$), I_{min} (-20%, $p < 0.001$), J (-26%, $p < 0.0001$). Regarding BV/TV, an increase in both BV/TV (5%, $p < 0.001$) and TMD (2%, $p < 0.001$) was observed in MyD88^{OC} cortical bone.

Three-point bending experiments were performed to validate the predicted changes in mechanical

properties. Comparing femura obtained from wt and MyD88^{OC} mice, we recorded a 77% increase ($p < 0.01$) in load to failure (Fig. 2 A). This was accompanied by a 68% ($p < 0.01$) decrease in deflection at failure (Fig. 2 B). The bone stiffness was increased by approximately 2%, but this increased did not reach significance (Fig. 2 C).

To explore cellular functions of MyD88 in bone, we measured the expression of markers for bone coupling, bone resorption, and bone formation. To validate our gene expression analysis, we also measured MyD88 expression, which was 70% ($p < 0.0001$) reduced in MyD88^{OC} mice compared

to wt mice (Fig. 3 A). The comparison between the primary bone coupling markers RANKL, OPG and RANK demonstrated a significant difference between wt and MyD88^{OC} only for RANKL (Fig. 3 B-D). A 29% ($p < 0.01$) decrease was measured for this marker. Assessment of osteoclast markers TRAP and CTSK showed no significant change (Fig. 3, E and F). In contrast, osteoblast markers COL1A1 and OC were both significantly reduced in MyD88^{OC} compared to wt bone. The measured reductions in COL1A1 and OC expression were 72% ($p < 0.0001$) and 82% ($p < 0.0001$), respectively.

DISCUSSION

We demonstrated here that the re-expression of MyD88 in the osteoclast compartment in MyD88^{OC} mice counteracted the profound osteopenia (about -25% BV/TV) measured by Sato et al. in trabecular bone of MyD88^{-/-} mice by histomorphometry (7), thus confirming our initial hypothesis. Furthermore, we discovered profound changes in bone geometry in cancellous and cortical bone of MyD88^{OC} mice, suggesting a previously unnoted and osteoclast-independent role of MyD88 in maintaining bone architecture. Functionally, these changes resulted in altered biomechanical properties of the bones. On the molecular level, our data suggested that MyD88 expression in cells of the osteoclast lineage contributes to roughly one-third of the overall MyD88 expression in the skeletal compartment. Despite a reduction in RANKL expression, we observed almost normal expression of the osteoclast markers TRAP and CTSK, indicating that *in vivo* sufficient RANKL was produced to drive a normal osteoclastogenesis, however, follow-up studies need to directly measure bone resorption rates. In addition, data on bone formation rate will be desirable to better understand the observed reduction in bone formation parameters, which may result from the change in bone architecture and thus cellular response to load; however, a major reduction in bone mass was not produced in the young adult female mice we studied. In future studies, we aim to determine whether changes in bone geometry occur in both sexes at the developmental, modeling or remodeling stage

of the skeleton, and to further elucidate underlying pathways. Lastly, recent data demonstrated a function of MyD88 in bone repair post skeletal defect (12). Lack of MyD88 resulted in accelerated bone healing as it compensated the IL-1 β -driven inhibition of growth factor-mediated proliferation and differentiation of osteoblast precursors (12). Thus, MyD88 is essential for the suppressor function of IL-1 β in bone healing. Together with our study, those findings support the notion of possibly important skeletal functions of MyD88 and warrant further investigations.

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LETTER TO THE EDITOR

ESTABLISHMENT OF A MODEL FOR PREDICTING GLEASON SCORE ≥ 7 BEFORE PROSTATE BIOPSY

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To the Editor,

Prostate cancer (PC) is one of the most common malignant tumors in the male genitourinary system (1). Worldwide, more than 660,000 new cases of PC are diagnosed every year, accounting for 10% of all new cases of male cancers (2). In recent years, the incidence of PC in China has increased significantly due to population aging, changes in dietary structure and environmental carcinogenesis and ranks third, after bladder and kidney cancer, among urogenital tumors (3, 4). Gleason score (GS) can be used for evaluating the invasiveness of PC (5), and is an effective prognostic indicator (6). GS has important guiding significance for diagnosis and treatment strategy before treatment (7). At present, the main method of clinical diagnosis of PC and GS score is transrectal ultrasonography (TRUS)-guided prostate biopsy (8). However, transrectal ultrasound guided systemic puncture still has a high false negative rate, so that many patients need repeated puncture or even multiple punctures before clear diagnosis. Some studies have established relevant predictive models to reduce unnecessary biopsies. Karakiewicz et al. (9) found that age, prostate-specific antigen (PSA) and digital rectal examination (DRE) were important factors to evaluate of the risk of PC. Nam et al. (10)

pointed out that age, race, family history, prostate volume (PV), DRE and PSA had a great impact on PC. They also developed a prostate biopsy system which could be accepted by patients. This study established a risk prediction model for predicting GS ≥ 7 before surgery to eliminate unnecessary puncture and improve the detection rate of GS ≥ 7 .

MATERIALS AND METHODS

Research subjects

In the first stage, the data of 756 patients who had undergone TRUS-guided 12+1-core prostate biopsy from August 2014 to December 2016 were retrospectively analyzed. The average age of the patients was (70.41 ± 6.72) years. Clinical data included age, serum PSA, free PSA (f PSA), free/total PSA (f/t), prostate volume (PV), PSA density (PSAD), prostatic texture in DRE, with or without nodules and with or without hypoechoic nodules or microcalcification in B ultrasound. PSAD is defined as the ratio of PSA to PV. The second stage, from December 2017 to August 2018, included 270 cases with an average age of (71.43 ± 5.89) years. In this stage, GS ≥ 7 risk evaluation model was used to assist the judgment of whether puncture was necessary, and 214 patients finally received puncture, according to the evaluation results.

Key words: prostatic cancer; Gleason; PSA

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All the selected patients signed an informed consent, and the study was approved by the ethics committee of our hospital.

Methods

TRUS-guided 12+1-core prostate biopsy was used. The first 12 cores followed the 12-core distribution sites put forward by Gore et al. (11). Based on the early 6-core biopsy, one core was added at the bottom, in the middle and at the top of the bilateral peripheral zone of the prostate. The 1st, 2nd, 3rd, 7th, 8th and 9th cores were at the central zone of the prostate, and the 4th, 5th, 6th, 10th, 11th and 12th cores were in the peripheral zone of the prostate. The 13th core was in the low-echo area under B ultrasound or at the zone with abnormal signal displayed in Magnetic Resonance Imaging (MRI). If B ultrasound and MRI had no significant abnormalities, then the 13th core was at the prostatic apex. The positive biopsy point was recorded after surgery. Moreover, the patients underwent TRUS-guided B ultrasound before surgery.

Statistical analysis

SPSS 21.0 was used for statistical analysis. All data

were expressed by mean $\pm$ standard deviation. Mean of continuous variables between groups was compared by *t*-test or Wilcoxon rank-sum test, and classified variables or rates were compared by χ^2 test. In the first stage, multivariate logistic regression analysis with backward elimination was used for screening out independent predictors before establishment of the model. The formula of risk assessment model was established according to the results of Logistic regression analysis. Only the indicators with statistical significance ($P < 0.05$) were included in the model formula. Receiver operating characteristic curve was used for evaluating the prediction efficiency of the new model and other indicators. $P < 0.05$ meant difference had statistical significance.

RESULTS

The establishment of model for predicting risk of GS ≥ 7

The percentage of patients with GS ≥ 7 in the first stage among the 756 patients who received TRUS-guided prostate biopsy was 71.43% (540/756). The patients were divided into a group of GS < 7 and a group of GS ≥ 7 according to GS.

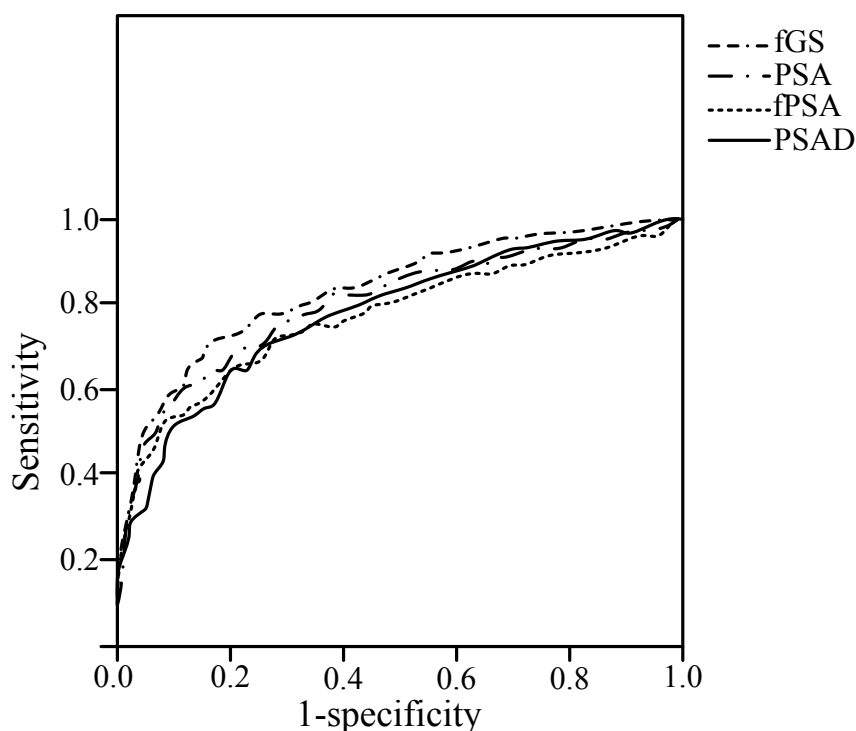


Fig. 1. ROC curve.

The comparison of PSA, fPSA, PSAD, prostatic texture in DRE, nodules in DRE and low-echo area in B ultrasound between the two groups showed statistically significant differences ($P < 0.05$), but age, PV, f/t and microcalcification of the two groups had no statistically significant differences ($P > 0.05$, Table I).

Then the factors with statistically significant differences were introduced to multivariate logistic regression analysis. Backward elimination was used. PSA, prostatic texture in DRE, with or without nodules in DRE and with or without low-echo nodules in B ultrasound were screened as independent predictive factors, as shown in Table II. Finally the formula of the model of GS predictive value (fGS) was obtained according to the regression analysis results:

$$fGS = e^{0.893 + 0.042 \times PSA + 0.632 \times DRE \text{ texture} + 0.815 \times DRE \text{ nodules} + 0.381 \times Hypoechoic/1 + e^{0.893 + 0.042 \times PSA + 0.632 \times DRE \text{ texture} + 0.815 \times DRE \text{ nodules} + 0.381 \times Hypoechoic}}$$

Then the receiver operating characteristic (ROC) curve was drawn according to the model,

and the newly established model and the prediction efficiency of the other indicators were evaluated. The area under ROC curve (AUC) of the model was 0.832, which was significantly higher than that of the traditional independent predictive indicator (Fig. 1), such as PSA (0.802), fPSA (0.769) and PSAD (0.781).

In addition, considering that the prediction model needs high sensitivity, the cut-off point selected on the ROC curve was 0.87, the corresponding sensitivity was 90%, and the corresponding specificity was 45.8%, which was significantly higher than that of other independent predictive indicators under that sensitivity, such as PSA (37.6%), fPSA (24.5%) and PSAD (35.3%). According to the cut-off point of the model, patients with predictive value of $fGS \geq 0.87$ were set as group of high risk of $GS \geq 7$, while the others with predictive value of $fGS < 0.87$ were set as group of low risk. In the first stage, the percentage of patients with $GS \geq 7$ was 80.23% (483/602) in the high risk group, which was significantly higher than 37.01% (57/154) in the group of low risk ($\chi^2 = 7.894$, $P < 0.05$).

Table I. The baseline data of the patients in the first stage of study

Variable		Group of GS score < 7 (n=216)	Group of GS score $\geq$ 7 (n=540)	P
Age (year)		70.35 $\pm$ 6.57	71.05 $\pm$ 7.39	>0.05*
PSA (ng/mL)		17.82 $\pm$ 19.71	123.39 $\pm$ 447.58	<0.05*
PV (cm ³)		52.56 $\pm$ 29.82	1.76 $\pm$ 49.08	>0.05*
f PSA (ng/mL)		2.06 $\pm$ 2.25	13.42 $\pm$ 0.78	<0.05*
PSAD [ng/(mL·cm ³)]		0.15 $\pm$ 0.09	0.11 $\pm$ 0.08	<0.05*
f/t		0.13 $\pm$ 0.05	0.14 $\pm$ 0.07	>0.05*
Prostatic texture in DRE [n(%)]	Soft or tough	145(67.13%)	226(41.85%)	<0.05#
	Hard	71(32.87%)	314(58.15%)	
Nodules in DRE [n(%)]	Without	179(82.87%)	337(62.41%)	<0.05#
	With	37(17.13%)	203(37.59%)	
Low echo in B ultrasound [n(%)]	Without	86(39.81%)	129(23.89%)	<0.05#
	With	130(60.19%)	411(76.11%)	
Microcalcification [n(%)]	Without	129(59.72%)	291(53.89%)	>0.05#
	With	87(40.28%)	249(46.11%)	

*indicates that t-test was used; # indicates that bilateral χ^2 test or Fisher's exact test was used.

Verifying the clinical significance of the prediction model

In the second stage, a newly established risk assessment model was used for predicting the risk of $GS \geq 7$ of 270 PC patients to consider whether prostate biopsy was necessary. The baseline data of patients in the two stages showed no significant difference in basic data and GS distribution (Table III). Only patients who were predicted having high risk of $GS \geq 7$ by the risk assessment model in the first stage needed prostate biopsy; otherwise close observation continued. According to the results of

comprehensive evaluation, 214 patients underwent prostate biopsy. The detection rate of $GS \geq 7$ increased significantly from 71.43% (540/756) in the first stage to 79.26% (214/270) in the second stage, and the difference had statistical significance ($\chi^2 = 3.628$, $P < 0.05$).

DISCUSSION

Gleason scoring system is the most widely used grading system for histological grading of

Table II. The results of multivariate Logistic regression analysis.

Variable	B value	P value	OR (95% CI)
Intercept	0.893	<0.05	
PSA	0.042	<0.05	1.042(1.032~1.051)
Prostatic texture in DRE	-0.632	<0.05	0.531(0.383~0.742)
Nodules in DRE	-0.815	<0.05	0.443(0.300~0.649)
Low echo in B ultrasound	-0.381	<0.05	0.683(0.479~0.973)

Table III. The comparison of baseline data between the two stages.

Variable		Patients in the first stage(n=756)	Patients in the second stage(n=270)	P
Age (year)		70.41±6.72	71.43±5.89	>0.05*
PSA (ng/mL)		92.85±380.37	76.73±208.39	>0.05*
PV (cm ³)		55.34±35.17	56.61±44.97	>0.05*
f PSA (ng/mL)		10.12±38.51	8.73±25.09	>0.05*
PSAD[ng/(mL·cm ³)]		0.14±0.07	0.13±0.06	>0.05*
f/t		0.13±0.05	0.14±0.07	>0.05*
Prostatic texture in DRE [n(%)]	Soft or tough	371(49.07%)	128(47.41%)	>0.05 [#]
	Hard	385(50.93%)	142(52.59%)	
Nodules in DRE [n(%)]	Without	516(68.25%)	171(63.33%)	>0.05 [#]
	With	240(31.75%)	99(36.67%)	
Low echo in B ultrasound [n(%)]	Without	215(28.44%)	84(31.11%)	>0.05 [#]
	With	541(71.56%)	186(68.89%)	
Microcalcification [n(%)]	Without	420(55.56%)	164(60.74%)	>0.05 [#]
	With	336(44.44%)	106(39.26%)	
Gleason score [n(%)]	<7	216(28.57%)	56(20.74%)	>0.05 [#]
	≥7	540(71.43%)	214(79.26%)	

*indicates that t-test was used; [#] indicates that bilateral χ^2 test or Fish's exact test was used.

PC and has a close correlation with prognosis. One study (12) showed that treatment measures were significantly different for $GS \geq 7$ or $GS < 7$. When $GS < 7$, conservative treatment or close follow-up observation were undertaken, while $GS \geq 7$ required active treatment, such as surgery and radiotherapy. This study established and applied a model for predicting the risk of $GS \geq 7$ based on PSA, PSA-related indicators and other examination results. The prediction efficiency of the model was improved. The area under ROC was 0.832, which was higher than previous independent factors, PSA (0.802), fPSA (0.769) and PSAD (0.781). According to the cut-off value, the patients with predictive value of $GS \geq 0.87$ were set as group at high risk of $GS \geq 7$, and the others were set as a group at low risk. In the first stage, the percentage of patients with $GS \geq 7$ in the high risk group was significantly higher than in the low risk group. The detection rate of $GS \geq 7$ in the second stage when the model was used was much higher than that in the first stage.

To sum up, the model mentioned above can be used to distinguish patients with $GS < 7$, and follow-up monitoring can be carried out without delaying the diagnosis and treatment of patients, therefore reducing unnecessary puncture biopsy. Although the application of the new model is satisfactory, there are some problems. Some factors are not included in the model, such as body mass index, biochemical indicators, etc., which is because that some clinical data of patients were missing. The model had a good result in the second stage of our center, but it has not yet been applied and verified in other centers; hence multi-center study with larger sample size is needed.

ACKNOWLEDGEMENTS

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LETTER TO THE EDITOR

DISTRIBUTION OF SINGLE NUCLEOTIDE POLYMORPHISM WITH A LOSS OF HETEROZYGOSITY STATUS AND ITS RELEVANCE ON GENE EXPRESSION IN CHINESE HAN

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To the Editor,

Loss of heterozygosity (LOH) is a gross chromosomal event that results in loss of the entire gene and the surrounding chromosomal region. The classical definition of LOH was originated from Knudson's two-hit hypothesis (1). Recently, the advent of large-scale genome-wide single nucleotide polymorphism (SNP) array offers a high-resolution genomic approach to infer the SNP with an LOH status (LOH-SNP) (2). LOH affects the development of various cancers that can also be observed in healthy population, probably because of early mitotic errors and autozygosity.

Genetic variants (e.g. SNP, deletion, insertion) at the DNA level may have biological functions and impact gene expression regulation. LOH is regarded as a type of structure variation (SV). As SV, LOH may have potential capacity in regulating gene expression. However, such regulatory functions have not yet been testified. Expression quantitative trait locus (eQTL) analysis is the most common approach used to dissect the effects of genetic variation on gene expression. The eQTL analysis enables us to study the genetic basis of gene expression variation

across individuals (3). By using eQTL analysis, previous studies have identified a long list of regions that contain DNA sequence variants that influence expression level of one or more genes (4, 5). However, to date, no eQTL analysis has been performed for LOH.

In this study, we conducted a genome-wide eQTL analysis in peripheral blood mononuclear cells (PBMCs) of 41 Chinese Han female population to identify the distribution characteristics of LOH-SNP and explore how LOH influences gene expression.

MATERIALS AND METHODS

Study samples

A total of 43 Chinese Han female subjects were recruited from Suzhou city of China. Each participant signed an informed consent document before entering the study. The study was approved by the ethics committee of Soochow University.

Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from 15 mL whole blood by density gradient

Key words: loss of heterozygosity ; eQTL; gene expression; distribution

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centrifugation immediately after blood withdrawal. PBMCs per subject were equally divided into two parts, one for DNA extraction, and the other for RNA extraction which was stored at -80°C with TRIzol reagent treatment (Invitrogen, Carlsbad, California, USA) to avoid RNA degradation.

DNA extraction and SNP microarray analysis

DNA extraction from PBMC was performed in the laboratory of CapitalBio Corporation (Beijing, China) using phenol-chloroform extraction and ultrapurification method. Affymetrix Genome-Wide Human SNP Array 6.0 chips were used for SNP genotyping by following the protocol recommended by the manufacturer. Affymetrix software tool Genotyping Console 4.2 (GTC4.2) was used to analyze the microarray data. We adopted contrast quality control (QC) >0.4 and a QC call rate $>95\%$ as sample QC thresholds. Finally, 41 samples passed the thresholds (Mean contrast QC 1.64 and QC call rate 98.38%). SNPs with a minor allele frequency less than 5% or a call-rate less than 95% were excluded. SNPs on sex chromosomes were also excluded in view of the samples were all female. Finally 841,932 SNPs were included in our study for further analysis.

RNA extraction and mRNA expression microarray analysis

Total RNA was extracted according to the instructions recommended by the manufacture and then quantified by using NanoDrop ND-1000 (Thermo Scientific, Wilmington, Delaware, USA) spectrophotometer. The RNA sample with the purity ($\text{OD}_{260}/\text{OD}_{280} \geq 1.8$) and the integrity rRNA ratio ($28\text{S}/18\text{S}$) ≥ 1.5 was used in a further microarray experiment. Genome-wide mRNA expression was profiled using lncRNA and mRNA Human Gene Expression Microarray v. 4.0 (CapitalBio Corp, Beijing, China) according to the manufacturer's instructions. The data was extracted by Agilent Feature Extraction (V10.7) and was summarized, normalized and controlled for quality using GeneSpring GX program (V12.0). After filtering out probes with detection rate less than 80% and/or incomplete annotation information, a total of 17,566 unique mRNA probes were used for further analysis.

Identification of LOH-SNP

LOH analysis was processed using the GTC4.2 software. This analysis first created an CN/LOH Reference

Model file using the HapMap270 file supplied by Affymetrix. Then each sample CEL file we selected was re-analyzed against the new Reference Model file. From this comparison, the sample's copy number and LOH data were generated. The LOH analysis is carried out with the genotype calls made on the fly by the BRLMM-P+ algorithm using the Reference Model data. LOH status at each SNP locus of each subject was inferred based on the genotype calls at that locus. 1 means that these SNPs are LOH status, 0 means these SNPs have an undefined LOH status, and null refer to no call (Affymetrix Genotyping Console 4.2 User Manual). Finally 141,816 SNPs with LOH = 1 were selected for further analysis.

Statistical analysis

In order to assess the correlation between LOH-SNP and gene expression, eQTL analysis was performed with the R package MatrixeQTL using linear model. SNPs were included in the cis-eQTL analysis if they were located within 1 megabase (Mb) distant from the transcription start site (TSS) or transcription end site (TES) of mRNAs. In contrast, if SNPs located distal from the gene, either far away from the gene ($>1\text{Mb}$) on the same chromosome, or where an SNP and gene were on different chromosomes, were determined as trans-eQTL. Age was treated as covariate in the regression analyses to adjust the impact on mRNA expressions. Benjamini-Hochberg false-discovery rate (FDR) was used to correct for multiple testing. For the significant pairs, we defined the corresponding SNPs with a LOH status and target mRNA as effect SNPs and effect mRNA (emRNA), respectively.

To determine whether the significant effect SNPs were enriched within certain functional domains on the genome, the online tool Table Browser (<http://genome.ucsc.edu/cgi-bin/hgTables>), which can provide a powerful and flexible graphical interface for querying and manipulating the Genome Browser annotation tables, was used to annotate the effect SNPs. The total SNP data were used as control group, Fisher's exact test was conducted to evaluate whether cis- or trans-effect SNPs enriched in certain functional regions.

To systematically extract biological functions for the genes detected from eQTL analysis, the online tool DAVID (the database for annotation, visualization and integrated discovery, freely available at <http://david.abcc.ncifcrf.gov/>) bioinformatics resources were used.

DAVID provided a comprehensive set of functional annotation tools for investigators to understand biological meaning behind large list of genes. GO (Gene Ontology) function and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analyses were conducted for emRNAs.

RESULTS

The distribution LOH-SNP

The total SNPs with an LOH status in 41 samples were 141,816 and were observed in all chromosomes, the counts of LOH-SNP were associated with the physical length of chromosome, the longer the chromosome, the more the counts of LOH-SNP. The largest occurred on chromosome 2 (18,256) and the least occurred on chromosome 21 (940)

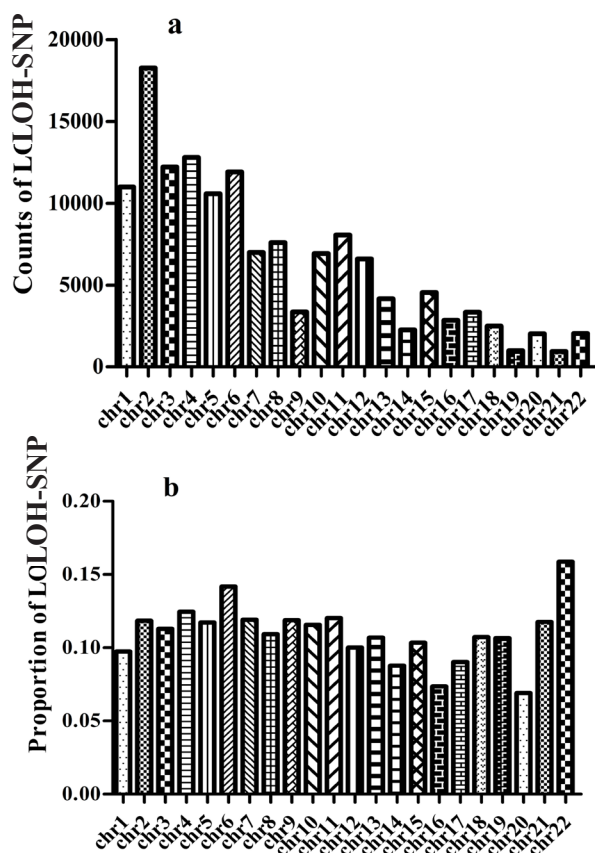


Fig. 1. Distribution characteristics of LOH-SNP. *a)* Counts of SNPs with a LOH status on each chromosome; *b)* Density of LOH-SNP on each chromosome.

(Fig. 1a). However, the mean density of SNP with an LOH status (number of SNP with an LOH status/physical length of chromosome) in 22 chromosomes showed various patterns, the largest density occurred on chromosome 2 and the least density occurred on chromosome 19 (Fig. 1b).

Genome-wide identification of eQTLs

To assess how SNPs with an LOH status influence gene expression, a genome-wide eQTL analysis was conducted. A total of 20,825 cis-SNP-probe combinations ($P < 0.01$) and 7,659 trans-SNP-probe combinations ($P < 10^{-6}$) were identified. In addition, there was no obvious peak around the starting transcription region, suggesting that the physical distance seemed to have little effect on the expression levels of mRNAs.

After eQTL analysis, each eQTL pair had their own beta value which represented effect degree of SNP with an LOH status on gene expression. Fig. 1 shows the distribution of beta value. We found that the beta value formed two peaks that mainly concentrated at ± 0.5 for cis-effect eQTLs, and concentrated at ± 1.0 for trans-effect eQTLs.

Functional annotation and enrichment analysis

We also examined the functional properties of the cis- and trans-effect SNPs and found that cis-effect SNPs were significantly enriched in coding-synonymous, missense, ncRNA, near-gene-3 and untranslated-5 (5-UTR) regions, while trans-effect SNPs were significantly enriched in coding-synonymous and ncRNA regions (Fig. 3) (Fisher's exact test $P < 0.05$).

After eQTLs analysis, 1168 (709 cis-effect and 459 trans-effect) genes were identified. In order to explore the biological functions of these genes, DAVID informatics resources 6.8 was used for functional annotation and gene enrichment analysis. A total of 201 GO terms were generated and 113 terms had $P < 0.05$. After controlling the FDR at 0.05, five GO terms (protein binding, membrane, cytosol, extracellular exosome and poly(A) RNA binding) were identified (Table I).

Twenty-one KEGG pathways were generated and 5 pathways (Insulin resistance, Neurotrophin signaling pathway, Systemic lupus erythematosus,

Table I. The enriched GO terms from 1168 mRNAs.

GO ID	Term	P-Value	FDR	Count
GO:0005515	protein binding	7.95E-12	1.26E-08	624
GO:0016020	membrane	6.12E-08	9.14E-05	189
GO:0005829	cytosol	7.70E-08	1.15E-04	264
GO:0070062	extracellular exosome	3.38E-06	5.06E-03	221
GO:0044822	poly(A) RNA binding	1.71E-05	2.71E-02	102

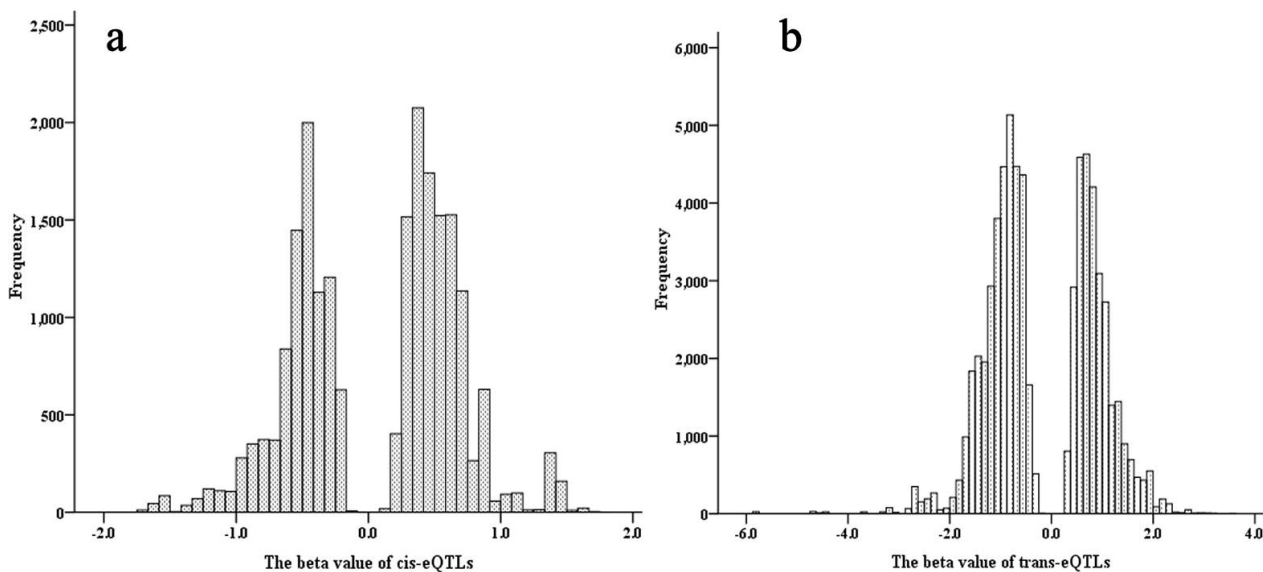


Fig. 2. Frequency distribution of beta value from eQTL analysis. *a)* cis-eQTL, *(b)* trans-eQTL.

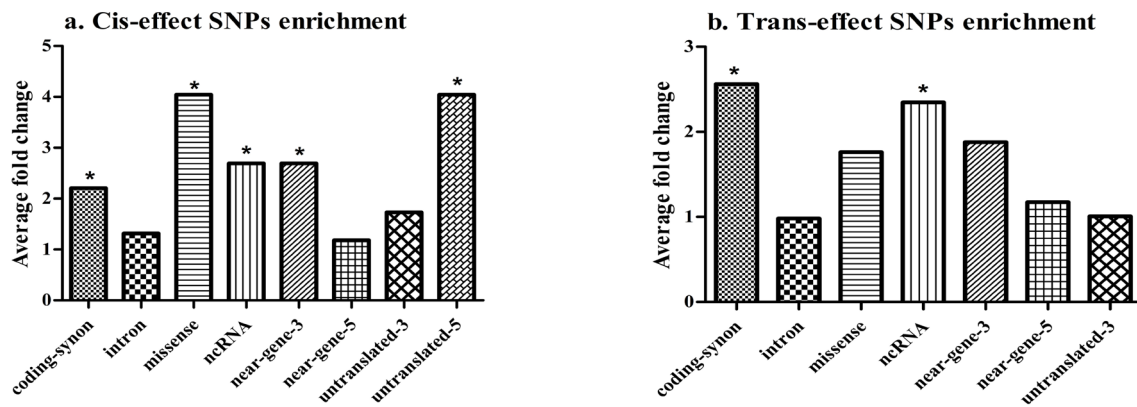


Fig. 3. Annotation and enrichment analysis for cis- and trans-effect SNPs. Note: * $P < 0.05$ (Fisher's exact test); coding-synon: coding-synonymous

Table II. *The enriched KEGG pathways from 1168 mRNAs.*

KEGG ID	Term	P-Value	Genes
hsa04931	Insulin resistance	1.50E-02	PTPRF, PIK3CB, RPS6KA2, GSK3B, CREB3L2, SLC27A6, RPS6KB2, CREB3L4, PRKAA1, IKBKB, PRKCD, STAT3, PTPN11, PYGB
hsa04722	Neurotrophin signaling pathway	1.53E-02	PIK3CB, CAMK2G, KIDINS220, PRKCD, PTPN11, MAP3K3, RPS6KA2, BAX, SOS1, GSK3B, GAB1, RAP1A, SHC1, SH2B2, IKBKB
hsa05322	Systemic lupus erythematosus	1.75E-02	C7, HIST1H2BD, SNRPD1, ACTN3, H2AFJ, C8G, CD86, HIST1H2BK, H2AFV, HIST1H4A, H2AFZ, HIST1H4F, HIST1H4D, HIST1H3F, HIST1H2AL, CD28
hsa04070	Phosphatidylinositol signaling system	3.80E-02	MTMR2, INPP1, DGKQ, PIK3CB, DGKZ, PIP5K1, INPP4B, PIP5K1A, INPP4A, ITPR3, PIP4K2C, ITPR1
hsa05211	Renal cell carcinoma	4.51E-02	PAK2, PIK3CB, SOS1, GAB1, TGFA, RAP1A, ARNT, RBX1, PTPN11

Phosphatidylinositol signaling system and Renal cell carcinoma) pass the threshold of $P < 0.05$ (Table II).

DISCUSSION

LOH was a common genetic alteration in genome, which derived from heterozygous deletion of one of the two alleles, or duplication of a maternal or paternal chromosome or chromosomal region and concurrent loss of the other allele. LOH is generally considered to be the major molecular feature of tumor suppressor gene variants and the important marker of certain normal cells change. Study found that individual genetic background strongly affected the frequency of LOH and the loss of which parental allele on different chromosomes by genome-wide LOH analyses in radiation-induced thymic lymphomas (6). Our study found that SNP-LOH occurred on all chromosomes, probably suggested that LOHs are frequently observed variants. However, this study only represents the preliminary inference of LOH status for SNP based on the SNP Arrays, and further studies are needed to confirm the inferred LOH status.

Current accumulating evidence has shown that genetic variation appeared to have important regulation effect on mRNA in normal PBMCs, for example,

miRNA, lncRNA, and DNA methylation (7-9). Our study firstly confirmed that important association existed between LOH-SNP and gene expression in normal PBMC. However, the underlying regulatory mechanisms by which LOH impact gene expression remained unknown. A possible mechanism was that LOH was polymorphism in nature that changed the binding strength of transcription factor binding sites, thereby affecting the rate of mRNA transcription (10). These results provided useful insights into molecular biological function of LOH in PBMC, and might shed light on PBMC-relevant human physiology and pathology.

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LETTER TO THE EDITOR

VALSARTAN/HYDROCHLOROTHIAZIDE INDUCED PROSTATE CARCINOMA IN A PATIENT WHO SUBSEQUENTLY DEVELOPED MELANOMAG. TCHERNEV^{1,2} and I. TEMELKOVA^{1,2}

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To the Editor,

Angiotensin receptor blockers (ARBs) are drugs that are thought to have potentially carcinogenic effects (1). In 2018 from the pharmaceutical market, products containing valsartan and irbesartan were withdrawn due to their content of N-Nitrosodimethylamine/N-nitrosodiethylamine (NDMA/NDEA) substances that are identified as potential carcinogens (2, 3). There are no official data on the incidence of tumors and the type of cancer that may occur after taking sartans, although probably such should be available (4).

The common view is that ARBs have also a potential pro-cancerogenic effect, which is confirmed in part by *in vitro* studies, according to which this group of drugs affect melanogenesis (5, 6).

We inform about a 72-year-old man in good general health. The patient suffers from arterial hypertension for which he was initially on a 4-month treatment with a combined product valsartan/hydrochlorothiazide 160/12.5mg (1-0-0), followed by monotherapy with valsartan 160 mg (1-0-0) for a period of 13 months. Subsequently, the angiotensin receptor blocker was replaced with a combined drug containing again valsartan/hydrochlorothiazide 150/12.5 mg (1-0-0), but from another pharmaceutical company for a period of 7

months. In the same year, due to the rise in prostate specific antigen (PSA) (7.79) a prostate gland biopsy was performed in a urological compartment and prostate adenocarcinoma was diagnosed. Radical prostatovesiculectomy was performed and the subsequent postoperative histological examination revealed the presence of prostate adenocarcinoma, Gleason score 4 + 3 = 7, accounting for about 10% of one half and about 90% of the test tissue volume in the other half of the prostate gland with a small focus (1%) of ductal carcinoma; presence of peri- and intra-neural invasion, seminal vesicles and extra-prostate soft tissues - without tumor infiltration. Three months after surgical intervention, definitive radiotherapy was performed in the area of the tumor bed. Postoperative control computer tomography established a normal finding in a small pelvis without enlarged abdominal and pelvic lymph nodes, with no focal changes in the examined bone structures. Since the beginning of 2016, after a successful surgery for prostate cancer, the patient was again given sartan therapy with irbesartan 300 mg (1-0-0), which he continued until 2018. In 2018, the same patient underwent a melanoma surgery by the one-step melanoma surgery (OSMS) method, which has already been described by our team and published.

It is precisely the moment to note that there is a

Key words: valsartan, irbesartan, hydrochlorothiazide, prostate carcinoma, drug related cancer

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description of another case in the literature of long-term intake of sartans and development of three types of carcinomas over a different time intervals, but in parallel with sartan intake (17-year intake), namely laryngeal squamous cell carcinoma, prostatic carcinoma and cutaneous melanoma (7). This case is indicative of: i) the possibility that sartans trigger not only melanomas but also other types of carcinomas; ii) in some cases simultaneous triggering of several carcinomas may occur in the same patient; and iii) in the worst cases of drug-induced cancer they may be associated with a more aggressive course of development, such as metastatic type melanomas (7).

In our previous work, we described three different drugs from the group of ARB blockers produced and distributed on the European market that induce cutaneous melanomas (4, 8, 9).

According to data from 2018, the second active substance in the combined drugs (which our patient has been taking) - hydrochlorothiazide, is also associated with an increased risk of melanoma, especially the nodular type and lentigo maligna melanoma (10).

In the present case, the patient underwent treatment for arterial hypertension with three different medications (sartans/hydrochlorothiazide) over a period of 5 years (in total for both types of cancer), during which he developed, independently of each other, two types of cancer [prostate cancer - described in the current publication, as well as melanoma – described in our previous publication (11)].

The fact that a third possible carcinogen in sartans was recently discovered, neuromuscular blocking agent (NMBA) (in addition to the previously described NDEA and NDMA), adds to the claim that multifactorial determined carcinogenesis is a completely possible phenomenon, as well as the emergence of three types of cancer after the use of a single drug (7,12).

Since the group of angiotensin receptor blockers generally has a risk of new cancer occurrence (1), we believe that it is entirely possible to develop two different types of cancer in the same patient as a result of sartan/hydrochlorothiazide therapy, as

in our case. Importantly, in this patient, it is worth noting that: i) the development of both types of cancer was shortly after the administration of ARBs/hydrochlorothiazide, i.e. a short-term potentiation of carcinogenesis within approximately 2 years (as in previously described cases) (4, 8, 9); and ii) medicines produced by different companies but with one active ingredient, valsartan/irbesartan and/or hydrochlorothiazide. This suggests that the carcinogenic effect could be related to the action of the parent substance or the contamination by different carcinogens (4). However, literature data suggest that carcinogenesis may also be triggered in cases of long-term intake of sartans (7).

It is also relevant the fact that a number of European companies import their substances from countries outside Europe, including India and China, and, according to the current legislation for a producer country, it is considered to be the place where the medicine is packaged, regardless of the area or region where the original substance was produced. This would further complicate/make it impossible for data to be accurately interpreted, as well as the possibility of defining guilt within eventual legal proceedings.

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LETTER TO THE EDITOR

CHANGES OF HEPCIDIN LEVELS AND THEIR CORRELATION WITH INTERLEUKIN-6 AND C-REACTIVE PROTEIN IN LIVER CANCER PATIENTS WITH CHRONIC INFLAMMATORY ANEMIA

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To the Editor,

Hepatic bactericidal protein (Hepcidin) is a polypeptide hormone which is mainly expressed in the liver (1), and Hepcidin-25 is the most common. It is also prevalent in blood and other body fluids as well as spinal cord, heart and lungs, but not in other organs (2, 3). Hepcidin travels with blood circulation, and acts on monocytes, macrophages and intestinal tract, which can regulate iron absorption, utilization and balance of storage, and achieve iron homeostasis (4-6).

Inflammatory anemia, also known as anemia of chronic disease (ACD) (7), is positive cytochrome anemia, which may develop into small cell hypochromic anemia, usually secondary to infection, malignant tumors, arthritis, trauma and other chronic diseases. It is accompanied by decreased serum iron (SI) concentration and increased serum ferritin (SF) concentration, but total iron binding capacity (TIBC) is normal or slightly reduced (8, 9). Inflammation is often present in patients with hepatocellular carcinoma as the disease progression, which stimulates changes in the Hepcidin expression in the liver (10) and belongs to ACD. In this study, changes in the serum levels of Hepcidin, erythropoietin (EPO), interleukin-6 (IL-6), C-reactive protein (CRP) and

iron parameters in liver cancer patients with chronic inflammatory anemia (CIA) were observed. The expression level of Hepcidin in liver cancer patients with CIA and its correlation with CRP, IL-6 and iron parameters were further explored to provide theoretical basis for clarifying the mechanism of CIA in liver cancer patients, and provide new ideas for exploring effective treatment modalities.

MATERIALS AND METHODS

Subjects

Thirty-five liver cancer patients with CIA who visited Dezhou People's Hospital from April 2016 to July 2018 were enrolled in the study. The diagnosis was made based on histopathological examination combined with laboratory examination, imaging examination and endoscopy. The patients were enrolled in the experimental group, and all of them suffered normocytic normochromic anemia (which can be divided into anemia with normal bone marrow reaction, myelodysplastic anemia, bone marrow infiltration anemia, anemia caused by decreased production of erythropoietin, etc.) with an average hemoglobin of 81.32g/L. The specimens of the healthy control group

Key words: CIA; Hepcidin; IL-6; CRP; EPO

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(35 subjects) were derived from healthy people who visited the hospital for physical examination, with an average hemoglobin of 137.26g/L. The healthy controls were free from anemia, and antigens of hepatitis B and hepatitis C were negative, with normal liver function and no abnormal results of physical examination. Informed consent was signed by all participants and the protocol was approved by the Hospital Ethics Committee of Dezhou People's Hospital.

The diagnostic criteria of CIA in liver cancer: chronic infection, rheumatic immune disease and malignant tumor disease existed for more than 1-2 months, and hemorrhage, renal insufficiency, and bone marrow invasion or suppression caused by these diseases were excluded; normal cytochrome anemia or small cell hypochromic anemia was usually manifested, and erythropoiesis reduction led to a decrease in reticulocytes; SI, TIBC and TRF saturations were reduced, SF was increased, and serum soluble TRF receptors were normal; mildly compensatory proliferation of the erythroid (due to anemia, the body's erythroid (erythropoiesis system) produces compensatory proliferation that returns the body to normal levels), increased extracellular iron of bone marrow iron staining and stored iron in monocytes and macrophages, but decreased intracellular iron [sideroblasts (1 to 5 small blue iron particles were visible around the nucleus of normal young erythrocytes. Young erythrocytes containing iron particles are called sideroblasts)].

Exclusion criteria of the experiment group: hypoferric anemia; diluted anemia in advanced stage of the tumor; bone marrow metastasis of the tumor; drug-induced bone marrow suppression; blood loss caused by chronic disease itself; severe liver and kidney dysfunction.

Collection, processing and storage of samples

All the patients fasted overnight, and 5-8 mL of venous blood samples were taken from the cubital fossa at 8a.m. the next day. 2 mL of blood was added into an EDTA-K2 anticoagulant vacuum tube and mixed with sufficient anticoagulant (the ratio of anticoagulant to blood was 1:9) for routine blood test. In addition, the remaining whole blood was injected into a procoagulant tube without heat source and endotoxin for determination of other

indicators. The blood was subjected to centrifugation at 3000 rpm for 10 min, and the supernatant was collected and frozen in the refrigerator at -20°C (Beijing Fuyi Electrical Appliances Co., Ltd., China).

Determination of Hepcidin by ELISA

Hepcidin kit (Shanghai Jianglaibio Co., Ltd., China) was used for detection. The microporous plates were pre-coated with Hepcidin antibodies, and standard samples, test samples and horseradish peroxidase (HRP) labeled antibodies were added, incubated and then washed thoroughly. Substrate tetramethylbenzidine (TMB) was used for coloration, and the depth of color was positively correlated with Hepcidin in the sample. The absorbance (OD) value was measured at a wavelength of 450 nm under a microplate reader (Shenzhen Highcreation Co., Ltd., China), and the concentration of samples was calculated. 100 µl test samples were added to each sample well, and three parallel wells were set for each sample; two negative control wells were set up, and 100 µl cell lysis buffer of the untreated group was added into each well; a blank control well was set up, and 100 µl of pure cell lysis buffer was added. The ELISA plate was placed at 4°C and coated overnight. Washing of the ELISA plate: the reaction solution in the well was drained and washed once with the washing solution (after the washing liquid was filled into the plate well, it was removed). Then the washing liquid was filled into the plate well, soaked for 1-2 minutes, and intermittently shaken. After the liquid in the well was removed, it was dried on the absorbent paper. Washing was repeated 3-4 times. 50 µl PBS was added to each negative control well, and 50 µl of AIF antibody solution diluted at a ratio of 1:500 was added to each sample well and each blank well. The ELISA plate was placed in a humid chamber and incubated at 37°C for 60 min. After the plate was washed, 100 µl of a HRP-labeled goat anti-rabbit antibody working solution diluted at a ratio of 1:5000 was added to each well. Then the ELISA plate was placed in a humid chamber and incubated at 37°C for 60 min. After the plate was washed, 100 µl of TMB coloring solution was added to each well, gently mixed for 10 s, and allowed to react in the dark at 37°C for 15-20 min. The reaction was terminated by adding 100 µl of 2 mol/l H₂SO₄ to each well. The light absorption value W1 at a wavelength of 450 nm and light absorption value W2 at a wavelength of 630 nm were measured,

respectively, and the OD value finally measured was the difference between them (W1-W2), so as to reduce the light interference caused by scratch or finger mark on the container.

Determination of IL-6, EPO, SF and TRF in serum by ELISA

IL-6 kit, SF kit, EPO kit and TRF kit (Wuhan Elabscience Biotechnology Co., Ltd., China) were used for detection, respectively: the microporous plates were pre-coated with IL-6 (or EPO, SF, TRF) antibodies, and standard samples, test samples and HRP (Horseradish peroxidase) labeled antibodies were added. They were incubated and then washed thoroughly. The substrate TMB was colored, which turned blue after being catalyzed by peroxidase and then yellow under the action of acid. The depth of color was positively correlated with IL-6 (or EPO, SF, TRF) in samples. The OD value was measured at a wavelength of 450 nm, and the concentration of samples was calculated (the unit of IL-6, EPO, SF and TRF was pg/mL, IU/L, ng/mL and g/L, respectively).

Detection of SI by spectrophotometry

Under the reductant and acidic solution, the protein in TRF was separated from iron so that the high valent

iron in serum was reduced to ferrous, and ferrous was combined with dipyrindine to form a pink complex. In a certain range, the content of iron ion was proportional to the color. Serum iron kit (Nanjing Senbeijia Biotechnology Co., Ltd., China) was used for detection. A blank tube, a standard tube and a test tube were set up for the experiment. 0.5 mL double distilled water was added to the blank tube, 0.5 mL 2mg/L standard iron solution (provided in the kit) was added to the standard tube, and 0.5 mL sample serum was added to the test tube. 1.5 mL iron chromogenic agents were added to the blank tube, standard tube and test tube, respectively. After mixing, it was subjected to a boiling water bath for 5 min, followed by centrifugation at 3500 rpm for 10 min after cooling. 1.0 mL supernatant was taken, and the absorbance OD value of each tube was measured under a light path of 0.5 cm, a wavelength of 520 nm and zero adjustment for double distilled water. $SI (mg/L) = (OD \text{ value measured} - \text{Blank OD value}) / (\text{standard OD value} - \text{Blank OD value}) \times \text{standard concentration}$ (note: 2mg/L standard iron solution = 2000μg/L ÷ Fe atomic weight [55.847] = 35.81μmol/L).

Determination of TIBC by spectrophotometry

TIBC kit (Shanghai Yu Bo Biological Technology

Table I. *The basic characteristics of patients in the experimental group.*

Category	Experimental group	Control group
Age (years)	53.81±13.68	52.11±16.43
Hb (g/L)	81.32±15.33**	137.26±11.16
WBC (×10 ⁹ /L)	9.56±4.11**	6.06±1.58
Hepcidin (ng/mL)	17.03±6.14	14.236±5.15
IL-6 (pg/L)	16.22±7.16**	1.83±0.15
CRP (mg/L)	26.53±19.26**	1.18±0.76
EPO (IU/L)	14.62±7.25**	5.12±2.38

*Note: Hb, hemoglobin; WBC, white blood cell; Hepcidin, hepatic bactericidal protein; IL-6: interleukin-6; CRP: C-reactive protein; EPO: erythropoietin. *P<0.01 and **P<0.001, compared with the control group.*

Co., Ltd., China) was used for detection: excessive iron (when the added iron ions were excessive, the excess iron ions can not be combined with the transferrin, so that the solution would be yellow, which can be judged according to the color) was added to the serum so that TRF was all bound by iron, and then iron adsorbent was added to absorb excess iron. The iron content was determined by SI method, which was also TIBC. According to the formula, the serum TIBC ($\mu\text{mol/L}$) = (measured OD value - Blank OD value) / (OD value - Blank OD value) $\times$ standard sample concentration $\times$ dilution times before test (note: standard product concentration, $17.91\mu\text{mol/L}$; dilution times, twice).

Detection of Hb and CRP

LH750 automatic blood analyzer (Beckman Coulter, Inc, America) was used for routine blood test, and IMMAGE 800 specific protein analysis system (Beckman Coulter, Inc, America) was employed for determination of CRP.

Statistical analysis

Statistical software SPSS19.0 was used to analyze the experimental data. The experimental results were tested by homogeneity of variance test and expressed in the form of mean $\pm$ standard deviation (there were 35 cases of liver cancer anemia and 35 healthy controls, and the mean $\pm$ standard deviation was calculated). Data of the two groups were compared by *t*-test (*t*-test for homogeneous variance and corrected *t*-test for uneven variance). Pearson correlation analysis was employed to

analyze correlation between variables. $P < 0.05$ indicated significant difference.

RESULTS

Expressions of Hepcidin, IL-6, CRP and EPO

Compared with the control group, expression levels of Hepcidin were increased in the experimental group ($P > 0.05$). Compared with the control group, serum levels of IL-6, CRP and EPO in the experimental group were increased significantly ($P < 0.001$) (Table I).

Changes of indexes of iron parameters in serum

As shown in Table II, levels of SF and TRF in the experimental group were significantly higher than those in the control group ($P < 0.001$), and SI was significantly decreased ($P < 0.001$).

Correlation analysis between Hepcidin and various parameters

There was no obvious correlation between Hepcidin and EPO, CRP, IL-6, SF, TRF, TIBC, and SI in liver cancer patients with CIA ($P > 0.05$) (Fig. 1).

DISCUSSION

Hepcidin of the experimental group was higher than that of the control group ($P > 0.05$), but not positively correlated with IL-6 ($P > 0.05$).

EPO in the experimental group was increased

Table II. *Changes of indexes of iron parameters in serum.*

Category	Experimental group	Control group
TIBC ($\mu\text{mol/L}$)	55.25 $\pm$ 12.13	55.18 $\pm$ 11.58
SF (ng/L)	69.581 $\pm$ 7.59**	21.36 $\pm$ 12.11
TRF (g/L)	1737.24 $\pm$ 332.18*	1291.26 $\pm$ 458.37
SI (mg/L)	10.23 $\pm$ 3.16**	25.56 $\pm$ 5.73

Note: TIBC, total iron binding capacity; SF, serum ferritin; TRF, transferrin; SI, serum iron. * $P < 0.01$ and ** $P < 0.001$, compared with the control group.

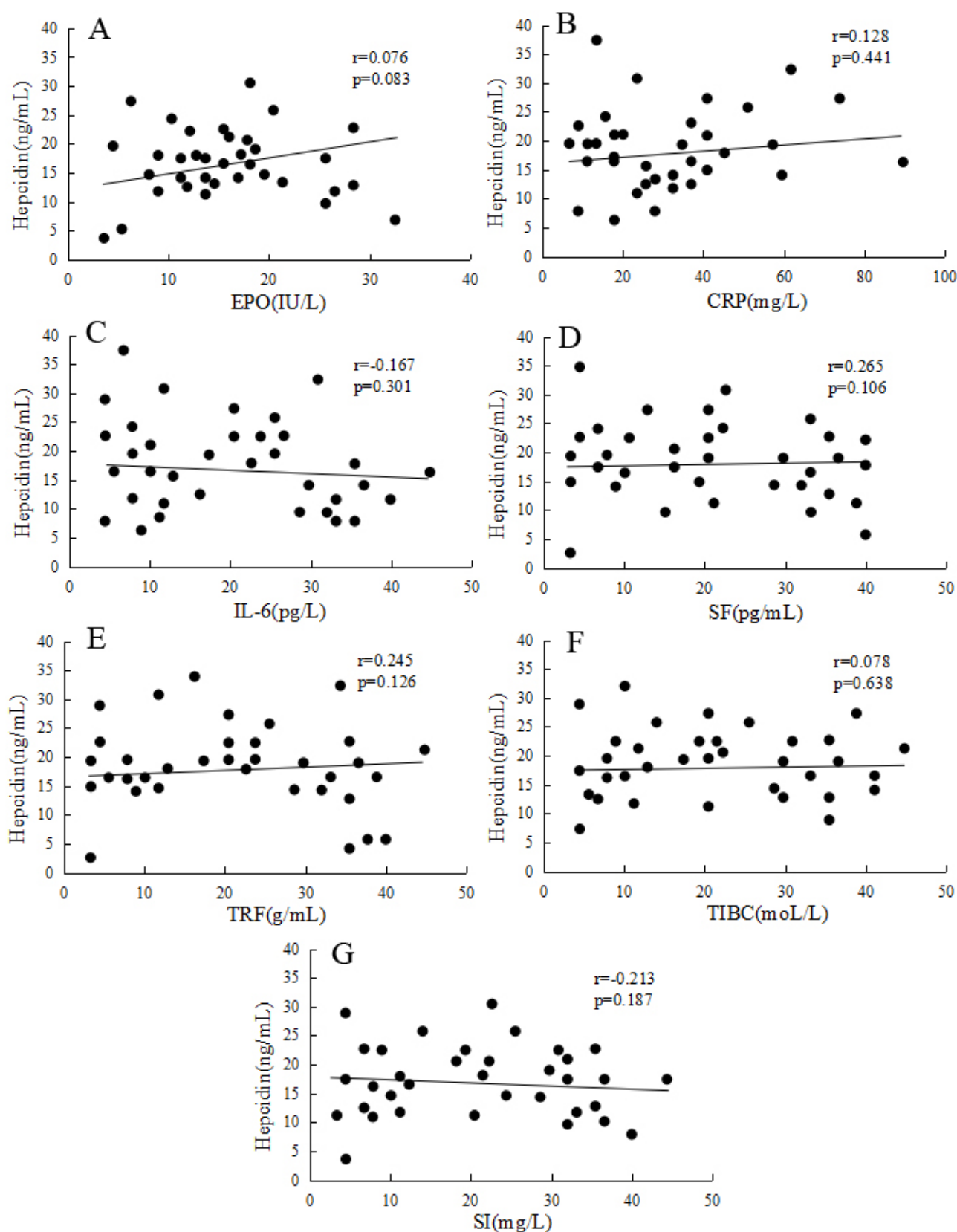


Fig. 1. The correlation between Hepcidin and various parameters: A) EPO; B) CRP; C) IL-6; D) SF; E) TRF; F) TIBC; G) SI.

significantly ($P < 0.01$), and Hepcidin was also increased and positively correlated with EPO ($P > 0.05$). Nevertheless, Hepcidin was not obviously increased as expected. The possible reasons are as follows: iron metabolism is affected by EPO during the process of hematopoiesis, and iron absorption is increased by duodenum, thereby affecting Hepcidin; due to the increased EPO, the expression of Hepcidin is inhibited by reducing expressions of inflammatory factors through its own bacteriostatic effect. Whether the expression of Hepcidin is affected by cancer tissues when liver cancer occurs remains to be further studied.

It was found that the levels of SF and TRF in liver cancer patients with CIA were increased. It is hypothesised that there is a certain correlation between the degree of increase and the degree of lesion development. The level of SF is increased with the increase of lesion degree (11). The concentration of TRF is regulated by iron supply. Under iron deficiency, TRF is increased. In this experiment, SI was decreased ($P < 0.001$) and TRF was increased ($P < 0.01$). When iron deficiency is improved, TRF levels are decreased and return to normal.

To sum up, serum expressions of IL-6 and CRP in liver cancer patients with CIA were increased obviously, while the expression of Hepcidin was not changed apparently, which may be related to the inhibition of EPO.

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LETTER TO THE EDITOR

INCREASED HEPCIDIN EXPRESSION IN ADIPOSE TISSUE AS A PRIMARY CAUSE OF OBESITY-RELATED INHIBITION OF IRON ABSORPTION

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To the Editor,

Obesity is a growing global health problem. Accumulating evidence shows that obese people have higher rates of iron deficiency (ID) than do their normal weight peers (1), and the iron-regulatory hormone, hepcidin, may play a key role in the occurrence of obesity-related ID since plasma hepcidin is elevated in obese patients (2, 3). Hepcidin is synthesized primarily in the liver and adipose tissue and acts at nanomolar serum concentrations by inducing degradation of its receptor, the cellular iron exporter ferroportin (Fpn), to restrict dietary iron absorption by enterocytes and the release of stored iron from hepatocytes (4, 5). Hence, studies should determine whether ID in obesity is a nutritional deficiency (reduced absorption) or functional deficiency (increased sequestration) of iron. Research is also needed to clarify whether the elevated hepcidin in obesity originates from liver or adipose tissues.

In addition to the hepcidin-Fpn axis, which controls the release of iron from enterocytes and hepatocytes into the circulation, various iron transporters regulate the cellular iron uptake process. For example, the divalent metal transporter 1 (DMT1) transports dietary iron across the apical membrane into enterocytes (6), while transferrin receptors (TfRs) import iron into liver cells, which are the main sites of iron storage

(7). Their involvement in the pathogenesis of obesity-related ID remains unclear.

We hypothesized that both reduced intestinal iron absorption and impaired mobilization of hepatic storage iron are involved in the occurrence of obesity-driven ID, due to upregulation of systemic hepcidin sourced from the liver or/and adipose tissues. We further hypothesized that iron transporters play a role in this process.

MATERIALS AND METHODS

Animals and treatment

Six-week-old female C57BL/6J mice were obtained from the Experimental Animal Center of Capital Medical University (Beijing, China) and were housed under specific pathogen-free (SPF) conditions in individually ventilated cages of 5 mice per cage. After acclimating for one week, the mice were randomly allocated to either a high fat diet with 60% energy from fat (lard) and 20% from protein or a standard diet with 10% kcal from fat and 20% from protein for 16 weeks to be used as either diet-induced obese model (DIO) mice or normal control (NC) mice, respectively. All animals had free access to food and water and were weighed weekly. The Animal Ethics Committee of Capital Medical University approved all experiments.

Key words: intestinal iron absorption; obesity; iron transporter; iron deficiency; hepcidin

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Duodenal iron absorption and hepatic iron storage assessment

The stable iron isotope ^{57}Fe (96% enrichment, Frontier Scientific Inc., Logan, Utah, USA) was used to assess the *in vivo* absorption of iron as per a previously reported procedure (8).

Briefly, a 0.4 mol/L solution of $^{57}\text{FeSO}_4$ was prepared by overnight dissolution of 22.85 g ^{57}Fe in 0.4 mol/L H_2SO_4 (Sigma Aldrich, Milano, Italy). The obtained $^{57}\text{FeSO}_4$ solution was stored at 4°C. Before its use, 87.7 mg sucrose and 0.83 mg ascorbic acid per mg iron were added to the $^{57}\text{FeSO}_4$ solution to yield final concentrations of ^{57}Fe of 20 mmol/L, ascorbic acid of 5.38 mmol/L, and sucrose of 10%. An analogous solution without tracer was prepared as the negative control. Both the $^{57}\text{FeSO}_4$ -labeled and the control solution were adjusted to pH 7 by adding the required volume of 1 mol/L NaOH. Next, 100 μL of $^{57}\text{FeSO}_4$ -labelled solution (22.8 μg ^{57}Fe) was orally administered to five DIO mice and five NC mice, all of whom had been fasted overnight. Tissue samples were collected 90 min after administration. Another five DIO and NC mice were given the vehicle solution, representing the “0” time point of the treatment. Blood samples were drawn via direct cardiac puncture under chloral hydrate anesthesia [0.4 mg/g, intraperitoneal (i.p.)], and the sera were immediately separated by centrifugation.

The duodenum and liver tissue samples were excised after transcardially perfusing the mice with 0.1 mol/L phosphate-buffered saline (PBS), washed in PBS, blotted dry, snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis. ^{57}Fe isotopes and total Fe in the samples were then determined using inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 7700 $\times$, Agilent Technologies, Santa Clara, CA, USA). Two hundred microliters of serum or 200 mg of the liver or duodenal specimen were lysed with 2 mL of concentrated HNO_3 (70%, GR, Sinopharm Chemical Reagent Co. Ltd., Beijing, China) under microwave heating at 60°C for 20 min. After lysis, each sample volume was brought to 3 mL with ultrapure water, then analyzed via ICP-MS. A natural abundance iron standard solution was analyzed during the sample runs to check for changes in the systematic bias. The calibration curve was obtained using four standard solutions for iron absorption (Sigma-Aldrich) ranging from 0.2–0.05 $\mu\text{g}/\text{mL}$. Results were expressed as μg of ^{57}Fe per g of wet tissue or per mL of serum, considering

the amount of naturally occurring ^{57}Fe that was checked before the assessment.

Real-time PCR detection of hepcidin gene expression in the liver and adipose tissue

The liver, abdominal, and perirenal adipose tissues from five DIO and five NC mice which received the vehicle solution gavage were used for this detection. Total RNA was extracted from ~100 mg of tissue with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Next, 1 μg of total RNA was reverse-transcribed into first-strand cDNA using the Reverse Transcription System (Promega, Madison, WI, USA). PCR was then performed in triplicate using SYBR Green protocol on an iCycler IQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The hepcidin-specific primer sequences were CTGAGCAGCACCACCTATCTC (forward primer) and TGGCTCTAGGCTATGTTTTGC (reverse primer). The reaction conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. Hepcidin mRNA content was calculated relative to the β -actin mRNA content using the $\Delta\Delta\text{Ct}$ method, assuming exact doubling of the amplified DNA in each PCR cycle.

Western blot analysis for duodenal and hepatic iron transporter proteins

The protein expression levels of DMT1 and Fpn1 in the duodenum and of DMT1, Fpn1, TfR1 and TfR2 in the liver were determined via Western blot. For total protein extraction, tissue samples were sonicated and homogenized in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] containing protease inhibitors (100 $\mu\text{g}/\text{mL}$ phenylmethylsulfonyl fluoride (PMSF), 45 $\mu\text{g}/\text{mL}$ aprotinin, and 100 mM sodium orthovanadate) on ice. Protein samples were electrophoresed on 12% SDS-polyacrylamide gel (PAGE), transferred to polyvinylidene difluoride membranes (Bio-Rad), blocked for 1 h, then incubated with the following primary antibodies: anti-DMT1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ferroportin (Alpha Diagnostic, San Antonio, TX, USA), anti-TfR1 (Invitrogen, Mulgrave, Australia), anti-TfR2 (Invitrogen, Mulgrave, Australia), and anti-GAPDH (Millipore, North Ryde, Australia) overnight at 4°C in blocking buffer. After washing and incubating

with HRP-conjugated secondary goat anti-mouse IgG antibody (Santa Cruz Biotechnology) for 1 h at room temperature, immunoreactive proteins were visualized using SuperSignal Pico electrochemiluminescence (ECL) reagent (Pierce, Rockford, IL, USA) and exposed to X-ray film. Band volume and density were quantified using SynGene GeneTools, version 4.0 (Syngene Booth 916, Synoptics, Cambridge, UK).

Serum hepcidin measurement

Blood samples from mice that were not orally administered iron were used to measure the serum hepcidin concentration via enzyme-linked immunosorbent assay (ELISA) using a mouse hepcidin ELISA kit (EMD Millipore, St. Charles, MO, USA). All procedures were performed as per the manufacturer's instructions. Absorbance was measured at 450 nm with an ELISA microplate reader (Biochrom Anthos 2010, Biochrom Ltd., Cambridge, UK). All samples and standards were run in duplicate.

Statistical analysis

Results were expressed as the mean $\pm$ SD. Statistical analyses were performed in SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA), using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for between-group comparisons. Bivariate correlations between plasma hepcidin and tissue hepcidin mRNA levels (Ct values from real-time PCR) or intestinal Fpn1 protein levels (OD values from the Western blot) were assessed using Spearman correlation coefficient analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Identification of the DIO mouse model

Weekly body weight monitoring revealed that 2 weeks after beginning the experiment, the DIO model mice had gained more weight per week than did the NC mice. At the end of experiment, the mean body weight of the DIO mice was 35.34 ± 2.18 g, while the NC mice weighed 23.67 ± 1.29 g ($P < 0.05$). Body fat was calculated for each mouse in the DIO group using the formula: obesity degree (%) = [(actual body weight of the DIO mouse - mean body weight of the NC mice)/mean body weight of

the NC mice] $\times 100$. Although all mice in the DIO group were heavier than their NC counterparts, only those mice with an obesity degree greater than 30% were identified as obese animal models and used for further testing.

Effect of obesity on duodenal iron absorption and hepatic iron storage

After orally administering the ^{57}Fe -containing solution, a considerably high amount of ^{57}Fe was detected in the duodenal tissues in both the DIO and NC mice compared with the "0" time-point control treatment with vehicle solution (Table I). Comparing the DIO and NC groups showed that DIO mice retained significantly more duodenal ^{57}Fe than did NC mice 90 min after ^{57}Fe gavage (Table I, Fig. 1), while no difference in the duodenal ^{57}Fe content existed at the "0" time-point between the two groups (Table I).

Iron within enterocytes can be released into the bloodstream through the basolateral membrane for distribution to other tissues and organs. Increased ^{57}Fe levels in the liver were detected 90 min after its oral administration to both DIO and NC mice (Table I). Comparing the hepatic ^{57}Fe concentrations between both groups showed that the DIO mice had accumulated significantly less iron in their livers than did the NC mice in response to oral iron supplementation (Fig. 1).

The serologic test results also showed that the DIO and NC groups differed significantly in serum ^{57}Fe concentrations. Specifically, the DIO mice had less circulating ^{57}Fe than did the NC mice 90 min after iron administration (Fig. 1), whereas the "0" time-point serum ^{57}Fe levels were comparable between the groups (Table I).

In short, these results support the existence of obesity-related malabsorption of iron because, after oral iron supplementation, the DIO mice had lower serum iron and liver iron levels than did the NC mice, while more iron was retained in the duodenal cells.

Expression of iron transporters DMT1 and Fpn1 in the duodenum

The body's iron status strongly affects iron homeostasis by regulating the expression of

Table I. Measurement of *in vivo* ^{57}Fe concentration by ICP-MS pre- and post- oral ^{57}Fe administration to mouse (ug/g or ug/mL, $X \pm \text{SD}$, $n=5$)

	“0” time-point		90 min post iron gavage	
	DIO	NC	DIO	NC
Duodenum	1.47 $\pm$ 0.33	1.68 $\pm$ 0.18	52.15 $\pm$ 3.72*	32.38 $\pm$ 4.10*
Liver	1.02 $\pm$ 0.02	1.06 $\pm$ 0.12	4.73 $\pm$ 0.67*	10.06 $\pm$ 1.89*
Blood	3.57 $\pm$ 1.22	3.67 $\pm$ 1.19	13.88 $\pm$ 1.80*	24.56 $\pm$ 1.24*

* $P < 0.01$ compared to the corresponding group at the “0” time-point. DIO: diet induced obese mouse; NC: normal control mouse

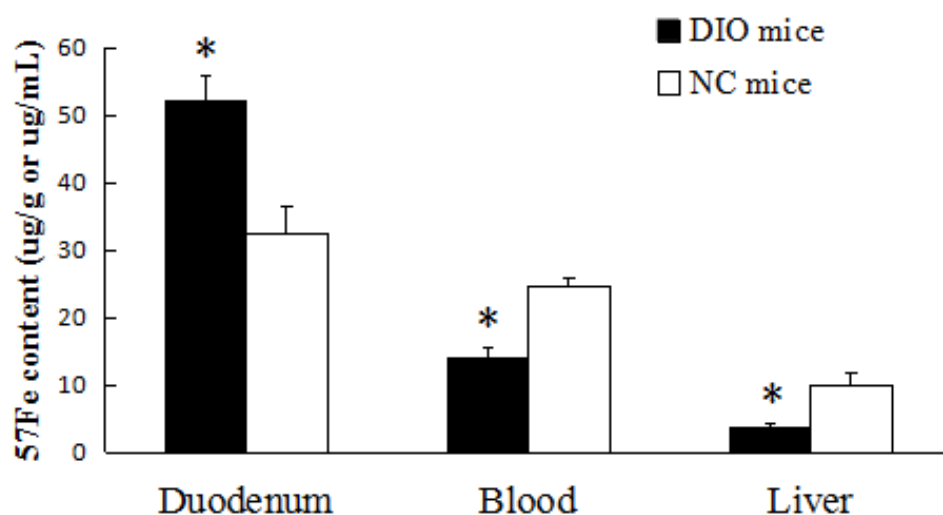


Fig. 1. Assessment of iron absorption in mouse by ICP-MS 90 min after oral administration of a 20 mmol/L ^{57}Fe solution. Values are expressed as ug ^{57}Fe /g tissue or ug ^{57}Fe /mL serum. Data represent mean $\pm$ SD. Abbreviations: DIO, diet induced obesity; NC, normal control. $n=5$ for each group. * $P < 0.05$ compared with the corresponding NC group.

iron transporters. Therefore, all tissue samples used to detect iron transporter gene expression were collected from mice that were not orally administered iron. Western blot analysis of DMT1 and Fpn1 in the duodenum showed that the relative protein expression levels of DMT1 were similar in the DIO and NC mice; the two groups did not statistically significantly differ ($P > 0.05$). However, the Fpn1 protein abundance in the DIO mice was evidently lower than that in the NC mice ($P < 0.05$). This may explain why DIO mice exhibited higher duodenal ^{57}Fe and lower serum ^{57}Fe concentrations than did their NC counterparts: decreased duodenal

Fpn1 hinders iron efflux from enterocytes into the blood circulation. Moreover, correlation analysis demonstrated a significantly negative relationship between plasma hepcidin and duodenal Fpn1 protein levels ($r = -0.517$, $P < 0.05$), manifesting the negative regulatory effect of systemic hepcidin on the protein abundance of intestinal Fpn1 (9).

Expression of the DMT1, TfR1/2, and Fpn1 iron transporters in the liver

Fig. 2 shows the Western blot results for the hepatic iron transporters. Most iron transporter expression levels in the liver, including those of

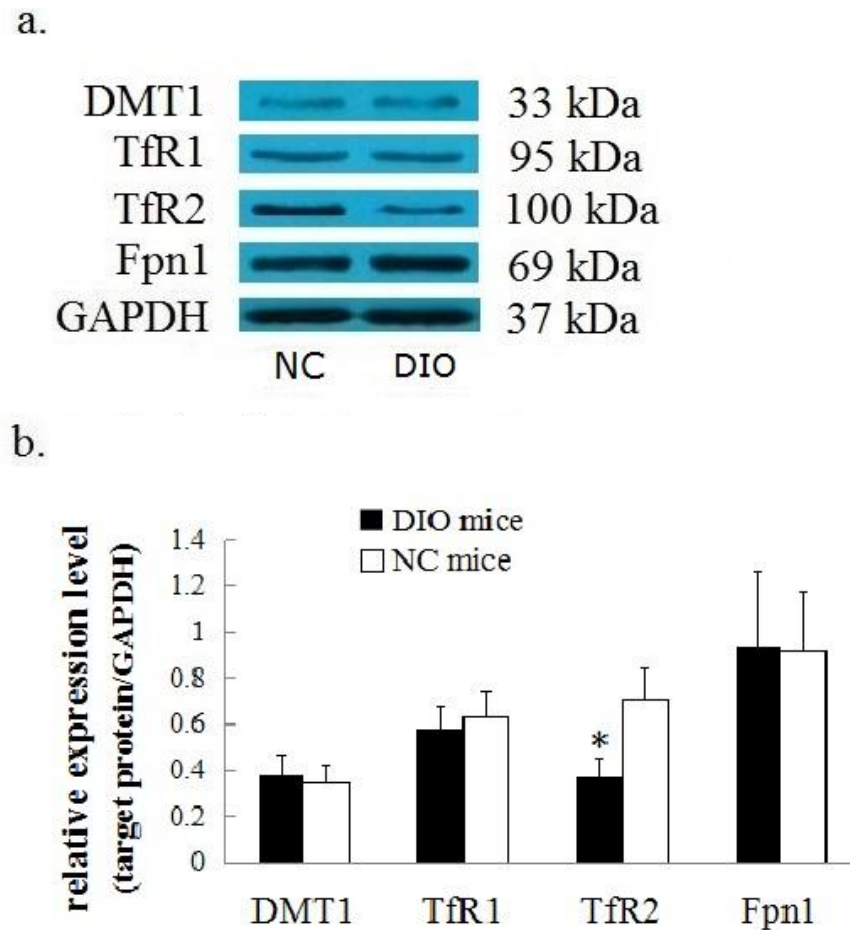


Fig. 2. Western blot analysis of protein expression levels of hepatic iron transporters in different group of mice ($n=5$). **a)** Representative blots of the investigated proteins, the GAPDH protein was simultaneously detected as internal control. **b)** Quantitative analysis of signal intensities. The relative expression level is shown as the ratio of the band intensity of the target protein to that of GAPDH protein. Bars represent mean and SD of the 5 mice in each group. The data are analyzed by one-way ANOVA followed by Tukey post-hoc test. * $P<0.05$ compared with the corresponding NC group. Abbreviations: DIO, diet induced obesity; NC, normal control.

DMT1, TfR1, and Fpn1, were uninfluenced in the DIO mice compared with those of the NC mice, that is, no intergroup differences in the protein contents of the iron uptake and release transporters achieved statistical significance ($P>0.05$). This indicated that the hepatic iron uptake in the DIO mice and the mobilization and release of liver storage iron were normal. One notable exception was that TfR2 protein abundance was markedly decreased in the DIO group than in the NC group ($P<0.05$), demonstrating that TfR2 was the molecule in the liver that was potentially involved in regulating the obesity-related iron metabolism. The reduced TfR2 may help explain

why hepatic hepcidin expression in the DIO mice was not increased as predicted — its role in stimulating hepcidin expression (10) was reversed.

Changes in the iron regulatory hormone hepcidin

To quantitatively assess hepcidin expression (a small 25-amino acid peptide that is difficult to detect by Western blotting), we measured its mRNA levels in hepatic and adipose tissues via real-time PCR. The nonrelative expression values were used for the calculation, and no statistically significant differences were detected in hepcidin mRNA between the DIO and NC mice ($P>0.05$) either in

the liver or the abdominal fat tissue. Nevertheless, in the perinephric adipose tissue, hepcidin mRNA expression levels in the DIO mice were higher than those in the NC mice ($P < 0.05$). In addition, the ELISA results indicated that the DIO mice had strikingly higher plasma concentrations of hepcidin than did the NC mice (22.51 ± 7.53 vs 14.03 ± 1.08 $\mu\text{g/L}$, respectively, $P < 0.05$). Furthermore, the plasma hepcidin and hepcidin expression in the perirenal fat tissue were significantly correlated ($r = 0.582$, $P < 0.05$), while the plasma and hepatic hepcidin levels were not ($r = 0.344$, $P > 0.05$). This indicates that the elevated systemic hepcidin concentration in obesity is likely derived from the exaggerated fat mass, particularly visceral adipose tissue, rather than from the liver.

DISCUSSION

Iron uptake and release through the duodenal epithelial cells are two distinct and modulated processes for iron absorption (11). Our present study demonstrated that duodenal iron absorption was reduced in obesity, characterized by decreased iron efflux from enterocytes into the bloodstream due to a decreased basolateral Fpn1 content, while the DMT1-mediated mucosal iron uptake from the enteric canal remained unchanged. The elevated concentration of circulating hepcidin in obesity is likely the initial cause of iron malabsorption, and the adipose-derived hepcidin (particularly from the expansive visceral adipose tissues) rather than hepatic hepcidin may be the main source contributing to the increased systemic hepcidin in obesity. In other words, the adipose hepcidin-duodenal Fpn1 axis may play a vital role in the occurrence of obesity-driven ID. No iron accumulation in the liver was observed in the DIO mice. Therefore, we deduced that obesity-driven ID is more likely a true deficiency due to hindered intestinal iron absorption rather than a functional deficiency due to impeded mobilization of hepatic storage iron. The unaltered hepatic hepcidin expression in obesity may be partially attributed to Tfr2, which was decreased in the DIO mouse livers. Tfr2 can upregulate hepcidin expression by activating the bone morphogenetic protein signaling

pathway under normal conditions (10). The liver hepcidin expression levels during obesity may result from the strong competition between this gene's positive and negative regulators (12). Our findings suggest that obesity can lead to impaired intestinal iron absorption and an absolute iron deficiency due to increased production of adipose-derived hepcidin instead of inflammation-mediated production of hepatic hepcidin. Thus, adjusting the hepcidin-Fpn1 axis by reducing body fat to improve intestinal iron absorption may be a necessary adjuvant measure to treat obesity-related refractory ID.

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LETTER TO THE EDITOR

EFFECT OF DEXMEDETOMIDINE ON POSTOPERATIVE COGNITIVE FUNCTION IN ELDERLY PATIENTS UNDERGOING ANESTHESIA WITH CONTROLLED HYPOTENSION FOR ENDOSCOPIC SINUS SURGERY

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To the Editor,

MATERIALS AND METHODS

Postoperative cognitive dysfunction (POCD) is a central nervous system complication characterized by confusion, anxiety, and personality changes, as well as deterioration of cognitive functions including reduction of memory and attention (1). POCD often occurs one week after general anesthesia, and in most patients who are over 65 it often occurs 1-3 days after surgery (2). Patients undergoing endoscopic sinus surgery generally need controlled hypotension. However, elderly patients are extremely sensitive to hypotension and hypoperfusion, and intraoperative hypotension is one of the risk factors for postoperative cognitive dysfunction in these patients. The protective role of dexmedetomidine in postoperative cognitive function in patients given general anesthesia has been widely reported (3, 4), but its effects on postoperative cognitive function in elderly patients with controlled-hypotension have not been studied. In this study, elderly patients who underwent general anesthesia with controlled-hypotension for endoscopic sinus surgery were treated with dexmedetomidine or saline and the changes in vital signs, VAS Score and Ramsay Score were recorded at different time points. Mini-Mental State Examination (MMSE) Score was used to evaluate the cognitive function in elderly patients.

From March 2017 to May 2017, a total of 115 patients aged 60-80 years, American Society of Anesthesiologists (ASA) class I-II, underwent anesthesia with controlled-hypotension for endoscopic sinus surgery. Two patients had a history of mental illness, 8 patients had a history of hypertension, 1 patient had a history of using long-term sedative medication, and 4 patients had abnormal liver function. One hundred patients were finally included in the study, including 51 cases of nasal polyps, 30 cases of sinusitis, and 19 cases of nasal septum deviation. The patients were divided into two groups: a dexmedetomidine group (Group A, 50 cases) and a control group (Group B, 50 cases). The inclusion process of the study is shown in Fig. 1. The patients signed informed consent to participate in this research.

The exclusion criteria were: abnormalities in heart; lung; liver; kidney function and coagulation; anemia (preoperative hemoglobin concentration Hb <100 g/L); illiteracy; visual and auditory disorders; history of hypertension; history of cerebrovascular accidents; history of traumatic brain injury; history of mental illness; long-term use of sedatives and senile dementia. The Mini-Mental State Examination (MMSE) (4) was also used to exclude patients with cognitive dysfunction.

One day prior to surgery, the MMSE score was

Key words: anesthesia-controlled hypotension, dexmedetomidine, elderly patients postoperative cognitive function, endoscopic sinus surgery

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evaluated by an investigator who was blinded to the treatment group. On the day of surgery general anesthesia was used to induce intubation. The experimental group was given a maintenance dose of dexmedetomidine at the beginning of the anesthesia induction. Both groups were given nitroglycerin 3 min after intubation to induce controlled-reduction of blood pressure. Hemodynamic values were recorded at the same time point.

Electrocardiogram (ECG), blood pressure (BP), and partial pressure of oxygen in the arterial blood (PaO₂) were routinely monitored after admission to the operating room. Under local anesthesia, the right internal jugular vein was retrogradely punctured and the catheter was inserted in the radial artery so that the tip of the catheter was at the level of the internal jugular vein, and the heparin was sealed for blood collection. Intravenous infusion of lactated Ringer's solution (8 ml/kg) was used as supplement. An anesthesia monitor was connected to monitor ECG, pulse levels of hemoglobin saturation (SpO₂), non-invasive blood pressure. An arterial transducer was connected to monitor mean arterial pressure (MAP), systolic arterial pressure (SAP), and diastolic arterial pressure (DAP). The forehead skin was scrubbed with 75% alcohol, and after drying, the bispectral index (BIS) electrode was attached and connected to a BIS monitor to monitor the bispectral BIS value of the intraoperative patient. Anesthesia induction mask was used for inhalation of pure oxygen 6L/min, followed by target-controlled infusion (TCI) of propofol (from 3µg/ml), remifentanyl (from 3ng/ml), and benzenesulfasuccinamide (0.2mg/kg intravenous bolus). Three to five minutes after insertion of the endotracheal tube, an anesthesia machine was connected to control breathing (total gas flow 2L/min, FiO₂ 60%, tidal volume 8~12 ml/kg, respiratory rate was 10-12 times/min at a I:E ratio of 1:2). Anesthesia was maintained with a TCI infusion of propofol and remifentanyl. During the operation, the anesthetic dosage and ventilator parameters were adjusted in time to maintain the end-expiratory carbon dioxide pressure (PETCO₂) between 30-35 mmHg and BIS 40-60. When cotton swab was inserted into the nasal cavity, the anesthetic was gradually reduced to withdrawal, and intravenous fentanyl (0.15µg/kg) was given. After the patient was naturally awakened (eye blinking) and achieved full recovery of spontaneous breathing, tidal volume and swallowing, and cough reflex, the tracheal tube was removed.

Group A was injected with dexmedetomidine via a micropump at the beginning of anesthesia induction. Dex solution 200 µg was diluted to 50 ml with 0.9% saline to prepare a concentration of 4 µg/ml to maintain a dose of 0.5 µg/kg/h. The Group B was given the same dose of physiological saline at the beginning of anesthesia induction. The two groups of patients received controlled-hypotension treatment 3 min after endotracheal intubation. The target of blood pressure reduction was 20-30% of the baseline blood pressure, but the MAP was not lower than 50 mmHg. If blood pressure exceeded 30% of this range during the blood pressure reduction process, it was recorded as a rise in blood pressure. If the blood pressure was lower than 30% of this range, it was recorded as a drop in blood pressure. The blood pressure and HR values measured 5 min after the patient entered the operating room were used as the baseline. The hypotension procedure started with nitroglycerin delivered via a micropump; 10 mg of nitroglycerin stock solution was diluted with 0.9% normal saline to 50 ml at a final concentration of 200pg/ml. This allowed for the starting dose of 0.5µg/kg/min, and the nitroglycerin was adjusted to about 0.3 µg/kg/min every minute until the hypotension was satisfactory. During the operation, the rate of nitroglycerin infusion was adjusted according to the patient's arterial blood pressure and the amount of bleeding, and appropriately supplemented the crystal colloid to ensure sufficient blood volume. When the patient's HR was less than 50times/min or more than 100times/min, intravenous atropine 0.25 mg/time or esmolol 2mg/time was given, when appropriate. When the blood pressure was lower than 30% of the baseline for more than 5 min, ephedrine was intravenously administered 5mg/time, after the adjustment of nitroglycerin infusion failed. Infusion of dexmedetomidine and nitroglycerin were stopped at the end of surgery.

Patient basic characteristics were recorded including age, body mass index (BMI), gender, ASA grade, hypertension, heart condition, anesthesia and surgical procedure (Table I), as well as mean arterial pressure, mean heart rate, and mean oxygen saturation at different time points: 5 min after patient admission to the operating room (T0), before induction of anesthesia (T1), after tracheal intubation (T2), 5 min (T3), 10 min (T4), and 15 min (T5) after controlled hypotension (Table II). We also recorded VAS score (0-10 points, the higher the score, the

more obvious the pain) and Ramsay score. The Ramsay score used the points representing dysphoria: 2 points represented calm and cooperative, 3 points represented sleepiness with active reaction to instruction and slurred speech, 4 points represented the condition of sleep and could be awoken, 5 points represented slow response to calling, 6 points represented the condition of deep sleeping or anesthesia and no response to calling. We reported the occurrence of adverse events 1 day after extubation. The Mini-Mental State Examination (MMSE) score is a 30-point questionnaire that is used extensively in clinical and research settings to measure cognitive impairment (no cognitive impairment=24-30; mild cognitive impairment=18-23; severe cognitive impairment = 0-17). An investigator who was blinded to the two treatment group conditions evaluated MMSE score at 1 day, 2 days, or 4 days after surgery. A decrease of 2 points before and after surgery was considered to be POCD.

In addition, the time required for the controlled-hypotension to reach the target value was recorded, as well as the time required for the blood pressure to rise after the controlled-hypotension, the number of times the blood pressure exceeded or fell below the target range during controlled-hypotension, and the occurrences of adverse reactions during surgery (hypotension, bradycardia). Finally, the operation time, anesthesia time, the patient's spontaneous breathing recovery time and extubation after stopping the infusion of anesthetic drugs (not shown in results) were recorded.

Statistical analysis

SPSS 22.0 (IBM Corporation, NY, USA) and Excel 2016 (Microsoft, Redmond, MA, USA) were used for the data analysis. Quantitative variables are presented as mean $\pm$ standard deviation and paired *t*-test was used to compare means between groups. Categorical variables are presented as cases and percentages, and Pearson *chi*-squared test was used for data analysis in Group B comparison. $P < 0.05$ was considered as a statistically significant difference.

RESULTS

We found that there was no significant difference in the basic characteristics of the two groups, including age, BMI, gender, ASA class, hypertension, heart

disease, anesthesia and surgical procedures ($P > 0.05$) (Table I).

There was no significant difference in mean arterial pressure and mean oxygen saturation at 5 minutes after patient admission to operating room (T0), before induction of anesthesia (T1), after tracheal intubation (T2), or at 5 minutes (T3), 10 minutes (T4), and 15 minutes (T5) after controlled hypotension ($P > 0.05$). The average heart rate of patients in Group A after tracheal intubation (T2) and at 5 minutes (T3) after controlled hypotension was significantly lower than that of Group B ($P < 0.05$) (Table II).

There were no significant differences in VAS scores and Ramsay scores between Group A and Group B ($P > 0.05$).

There was no significant difference in the incidence of adverse events at 1 day after extubation between the two groups ($P > 0.05$).

The MMSE score of Group B was significantly lower than that of Group A 1 day after surgery (Table III).

DISCUSSION

Endoscopic sinus surgery has multiple challenges because the sinonasal surgical field is narrow and the sinus is rich in blood vessels and adjacent to important structures. It requires less intraoperative bleeding and clear surgical field. Hence, it is often necessary to perform controlled-hypotension during surgery (5-7).

Elderly patients with intraoperative hypotension are prone to postoperative cognitive dysfunction, however, in order to have successful surgery, they need anesthesia with controlled-hypotension for endoscopic sinus surgery. Nitroglycerin is characterized by a rapid increase in heart rate when blood pressure reaches the target value of hypotension. Excessive heart rate can lead to a short diastolic phase in the cardiac cycle, which affects coronary perfusion. Dexmedetomidine is a potent α_2 -adrenergic receptor agonist that acts as an adjunct medication to general anesthesia which can slow the heart rate, reduce blood pressure, and help hemodynamic stability (8). The pharmacological properties of dexmedetomidine lead it to cause a drop

Table I. Comparison of characteristics of the patients at baseline.

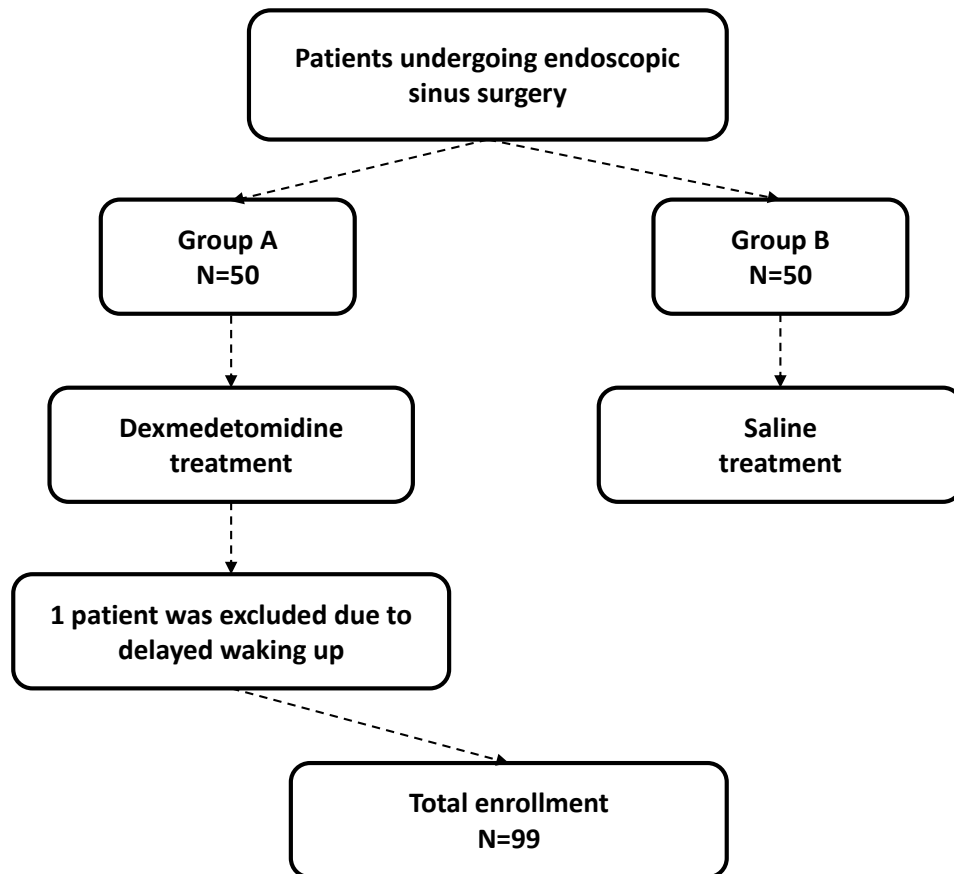
Variable	Control Group(n=50)	Dexmedetomidine Group(n=49)	t value	P value
Age (years)	73.5±3.1	73.8±3.5	0.454	0.651
BMI	28.1±4.0	28.1±3.8	0.128	0.898
Male, NO.(%)	23(46%)	25(50%)		0.160
ASA status, NO.(%)				
I-II	35(70%)	37(74%)		0.198
III	15(30%)	13(26%)		0.198
Hypertension, NO.(%)	21(42%)	19(38%)		0.167
Heart disease, NO.(%)	5(10%)	6(12%)		0.102
Anesthesia duration, min	140±32.1	141±33.5	0.152	0.879
Surgical duration, min	118.6±32.0	120.5±28.6	0.089	0.930

Table II. Comparison of mean arterial blood pressure, average heart rate, and average oxygen saturation (SaO₂) at different time points.

Variable		Control Group(n=50)	Dexmedetomidine Group(n=49)	t value	P value
Mean arterial blood pressure (mmHg)	T0	115.2±16.8	118.1±14.5	0.274	0.783
	T1	122.5±17.5	123±15.5	0.045	0.965
	T2	116.5±16.3	115.8±15.8	0.218	0.828
	T3	82.6±10.5	83.6±9.9	0.490	0.625
	T4	97.6±11.2	100.2±10.6	1.116	0.702
	T5	107.2±13.2	110.2±12.2	1.180	0.241
Heart-rate(time/min)	T0	78.2±8.2	80.3±7.9	1.304	0.195
	T1	82.1±9.0	83.2±8.7	0.621	0.536
	T2	88.5±8.6	84.5±7.1	2.536	0.013
	T3	93.1±8.9	83.3±7.0	6.12	0.000
	T4	86.5±8.0	84.5±7.1	1.322	0.189
	T5	82.6±8.5	81.5±7.5	0.686	0.494
SaO ₂ (%)	T0	98.2±2.3	98.3±2.1	0.227	0.821
	T1	99.1±1.6	98.9±2.0	0.552	0.582
	T2	99.2±1.5	99.3±1.9	0.292	0.771
	T3	98.8±1.3	99.1±1.5	1.069	0.288
	T4	98.7±1.4	98.9±1.5	0.689	0.492
	T5	98.6±1.3	99.1±1.3	1.923	0.057

Table III. Comparison of POCD scale at different time points.

Variable		Control Group(n=50)	Dexmedetomidine Group(n=49)	t value	P value
MMSE scale	T ₁ 0	29.3±0.4	29.2±0.5	1.104	0.272
	T ₁ 1	26.5±0.5	28.2±0.4	18.773	0.000
POCD NO. (%)	T ₁ 2	28.6±0.4	28.7±0.5	1.104	0.272
	T ₁ 3	29.0±0.4	29.1±0.4	1.250	0.214
	T ₁ 4	29.2±0.5	29.1±0.4	1.104	0.272

**Fig. 1.** Inclusion process of the study.

in blood pressure after infusion, which will synergize with nitroglycerin. In addition, the effect of slowing the heart rate by dexmedetomidine can reduce the increased heart rate caused by nitroglycerin. Hence, these synergistic effects are extremely beneficial for elderly patients. Notably, in this study, when the two drugs were combined, it was found that the average heart rate of Group A at T2 and T3 was lower than Group B, while the hemodynamics were more stable (Table II), indicating that dexmedetomidine can reduce cardiovascular stress and synergize with nitroglycerin to make controlled hypotension more stable (9).

Dexmedetomidine acts as a sedative and analgesic and reduces the anesthetic dose by regulating sympathetic nerve activity. In this study, the MMSE scores at days 1, 2, and 4 after surgery were lower than those before surgery. The MMSE score in Group B at 1 day after surgery was significantly lower than in Group A. These results showed that dexmedetomidine is safe and effective, can be used in elderly patients with controlled-hypotension and has a protective effect on postoperative cognitive function.

ACKNOWLEDGEMENTS

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LETTER TO THE EDITOR

**PURIFICATION, CRYSTALLIZATION AND PRELIMINARY X-RAY ANALYSIS OF
NicX, A KEY ENZYME IN NICOTINIC ACID DEGRADATION FROM *PSEUDOMONAS*
PUTIDA KT2440**

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To the Editor,

Enzymes capable of degrading aromatic rings are of increasing interest because of lack of efficient and clean methods for their removal. Dioxygenases play key roles in the degradation of aromatic compounds. Nicotinic acid (NA) is a carboxylic derivative of pyridine that is widely distributed in nature as part of pyridine cofactors and alkaloids (1). The biochemical pathways involved in the degradation of this *N*-heterocyclic aromatic compound have been used as a source of novel and unusual enzyme activities as well as metabolic intermediates, such as 6-hydroxynicotinic acid (6HNA) and 2,5 dihydroxypyridine (2,5 DHP), that can funnel the degradation of toxic compounds of major environmental concern, such as nicotine and hydroxypyridines (2). The aerobic catabolism of NA is considered to be a model system for degradation of *N*-heterocyclic aromatic compounds. 2,5 Dihydroxypyridine dioxygenase converts 2,5-dihydroxypyridine to *N*-formyl maleamic acid and were expressed in *E. coli* BL21(DE3) (Fig. 1). *Pseudomonas putida* metabolizes nicotinic acid to fumarate in six steps, however, little is known from this route about structural bases and biochemical function for extradiol ring-cleavage dioxygenase.

Determination of the three-dimensional crystal structure of this enzyme would be valuable to understand the biochemical function. The elaborate information obtained from the three-dimensional structure of NicX underlies our understanding of the biochemical role and its enzymatic mechanisms in nicotinic acid degradation. In this study, we expressed purified and crystallized native and selenomethionine-substituted NicX proteins. Diffraction data were collected from native and labeled crystals and processed to 1.77 Å and 2.08 Å resolution, respectively. These results will provide a basis for the ultimate structure determination of NicX which can be explain the biochemical role of this essential protein in the aerobic nicotinic acid degradation.

MATERIALS AND METHODS

Cloning and protein expression

The gene of 2,5-DHP dioxygenase from *Pseudomonas putida* KT2440 was PCR amplified using the following forward primers: 5'GCCATATGCCGGTGAGCAATGCACAAC3' and the reverse primer 5'GCGAATTCTGGGTGGAAAGAGGCTGGCC3'.

The 1.1-kb PCR fragment containing *nicX* gene

Key words: NicX; 2,5Dihydroxypyridine; selenomethionine; crystallization

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was double digested with NdeI/HindIII and ligated into NdeI-HindIII sites of pET28a (Novagen). Successfully cloned plasmids were verified by sequencing and transformed into *E. coli* BL21(DE3). The recombinant *E. coli* cells were cultured in Luria Broth (LB) with 50 µgml⁻¹ kanamycin, grown to mid-log phase (OD_{600nm} of 0.6-0.8) at 310 K. The culture was induced with 0.2 mM isopropyl-d-1thiogalactopyranoside and grown for another 16-18 h at 289 K.

Protein purification

Cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris-HCl) pH 8.0, lysed by sonication. Cell debris was removed by centrifugation at 10500 g for 40 min at 277 K. The filtered supernatant was applied onto a Ni-NTA agarose resin equilibrated with 20 mM imidazole, 50 mM Tris-HCl pH 8.0 buffers. The target protein was finally eluted using 200 mM imidazole pH 8.0 buffer followed by a washing steps of 20 mM imidazole pH 8.0, 50 mM Tris-HCl pH 8.0 buffers. The eluted protein was further purified by gel-filtration chromatography using a superdex 200

10/300 G column (GE Healthcare) equilibrated with 100 mM NaCl, 2 mM DTT (10 mM DTT) and 10 mM Tris-HCl pH 8.0 buffer. The purity of the protein was verified by SDS-PAGE and Coomassie blue staining. Protein concentration was determined at 595 nm UV-Vis maximum absorbance. The purified protein was concentrated to 10 mg ml⁻¹, frozen in liquid nitrogen and stored at -80°C until use. In order to purify selenomethionine, pET28a-nicX plasmid was transformed into BL21-derived auxotrophic strain *E. coli* B834(DE3), cultured in modified SeMet minimal medium. The protein was purified as described above.

Crystallization

Purified native and selenomethionine-containing NicX proteins were adjusted to 10 mg/ml in 25 mM Tris-HCl pH 8.0, 200 mM NaCl and 2 mM DTT (10 mM DTT) for selenomethionine-SeMet. NicX has screened for crystallization by the sparse-matrix method with commercial screens from Hampton Research (Riverside, California, USA) in crystallization trials using the hanging-drop vapour-diffusion method in 48-well plates. Equal volumes

Table I. Data collection and processing statistics.

Data information	Native NicX	SeMet NicX
Beam line	BL-19U1	BL-17U1
Wavelength (Å)	0.9997	0.99917
Space group	P222	PP222 ₁
Resolution:	1.77Å	2.08Å
CC1/2	0.721(>0.5)	0.526
Cell volume:	2125169	217379
Completeness (%)	98.4. %(96.9%)	100 %(100%)
I:= error	2.7:1.4(>1)	
Monomers per ASU	6	6
R-factors	0.134(<0.15)	
Redundancy:	11.1(>3)	
Unity cell parameters	<i>a</i> =118.51Å, <i>b</i> =125.18Å, <i>c</i> =143, 25Å	<i>a</i> =119.32Å, <i>b</i> =126.63Å, <i>c</i> =145,33Å
Solvent content	45.86 %	47.60%
Resolution range (Å)	50.0-1.77	50.0-2.08
Unique Reflections	203,334	130,065
Temperature (K)	100	100
Exposure time per image	0.5s	0.5s
I/σ _I	17.5(1.93)	17,8(1.54)
R-merge	13.4 %(127.9%)	31.3 %(279.7%)

Values in parentheses are for the outermost resolution shell.

$$R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I(hkl)_i - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I(hkl)_i}, \text{ where } I(hkl) \text{ is the intensity of observation of reflection } hkl$$

of protein solution (1 μ l) of 10 mg/ml and precipitant solution (1 μ l) were set up at 287 K equilibrated against (80 μ l) reservoir solution of different screens. High-quality crystals were grown to full size ($\sim$ 0.2 mm) within two weeks. Among the appeared crystals well diffraction quality crystals were obtained from a hanging drop containing 1 μ l protein solution (10 mg ml⁻¹ in 100 mM NaCl, 2 mM DTT (10 mM DTT) and 20 mM Tris-HCl pH 8.0, mixed with the same volume (1 μ l) of reservoir solution consisting of 20% w/v PEG 3-350, 0.2 M sodium formate pH 7.0. SeMet crystals were obtained from the same screening solution.

Data collection and processing

Crystals for X-ray analysis were mounted in nylon

cryoloops with 25% glycerol in the cryoprotectant buffer and were flash-cooled at 100 K in a cold liquid nitrogen using nylon loops on copper pins (Hampton Research). The crystal diffraction data sets of native and SeMet-NicX were collected at the BL19U1 and BL17U1 beam lines, respectively, at Shanghai Synchrotron Radiation Facility (SSRF) by using an ADSC Quantum 315 r CCD area detector in 0.5 s exposure time. Intensity data were processed and scaled by using the program package of HKL2000 (3). The statistics of the data collection are summarized in Table I.

Enzyme assay

The 2,5-DHP dioxygenase activity of NicX was measured, using UV-2550 Spectrophotometer

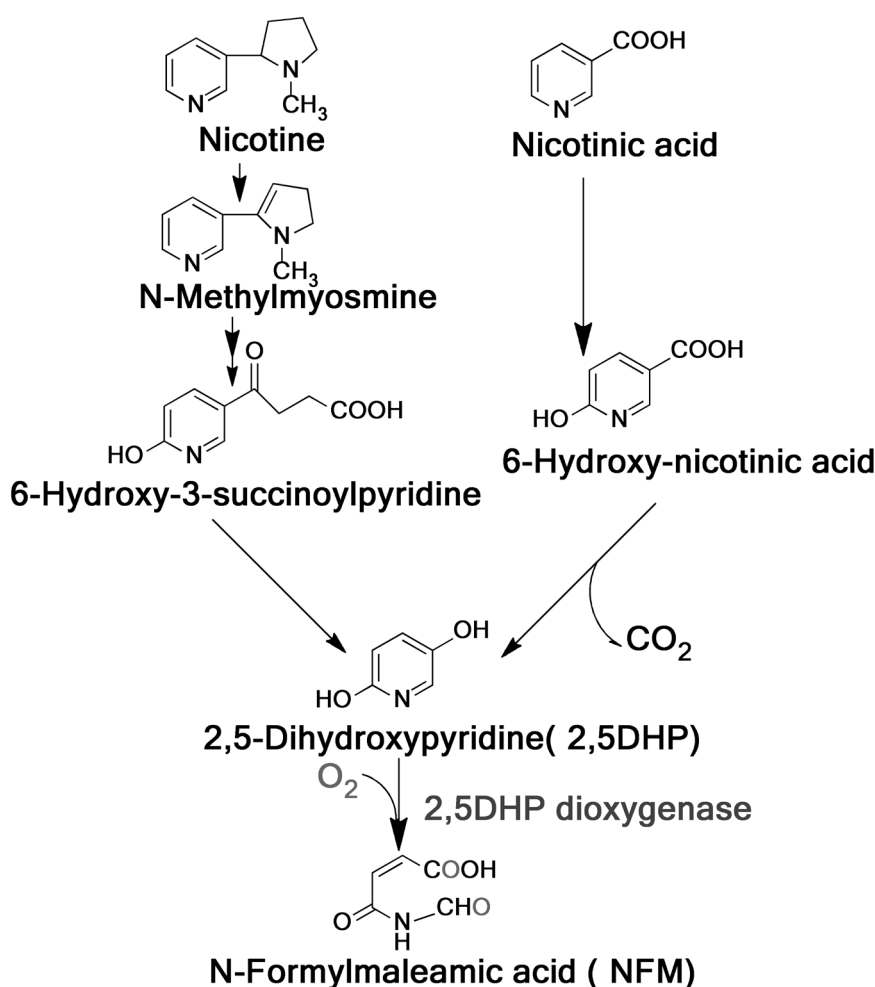


Fig. 1. Degradation pathways of pyridine derivatives through 2, 5-DHP and catalyzed conversion of 2, 5-DHP and NFM in the aerobic nicotinic acid degradation pathway.

(Shimadzu, Kyoto, Japan) at absorbance change of 320 nm ($\epsilon=5200 \text{ M}^{-1} \text{ cm}^{-1}$) as described previously (4). Activity was determined in the mixture containing 25 mM of 2, 5-DHP (100 mM final concentration in the reaction mixture), 0.025 M ferrous sulfate and 1 μl of 10 mg/ml enzymes. The reaction mixture was then diluted with 0.1M $\text{Na}_2 \text{HPO}_4\text{-NaH}_2 \text{PO}_4$, pH 7.4 (1 ml total volume) NicX solutions and the absorbance of 2,5-DHP was continuously monitored at 298 K.

RESULTS

2,5-Dihydroxypyridine is a vital metabolic intermediate in catabolism of many pyridine derivatives and commonly transformed to N-formylmaleamic acid. Characterizing three dimensional structure of NicX is crucial to understand its molecular mechanism in nicotinic acid degradation. The *nicX* gene was expressed in *E. coli* BL21(DE3) from cells carrying the recombinant plasmid pET-nicX (5). The expression of 39 kDa protein was supposed in cells harboring pET-nicX upon induction with 0.2 mM IPTG at an $\text{OD}_{600 \text{ nm}}$ of 0.6-8. The expression levels were apparent during 16-18 h after induction at 289 K. The final yield of soluble NicX was estimated to be roughly

16 mg per 1000 ml of liquid culture with a purity of greater than 95% quality, as determined by SDS-PAGE analysis. The SDS-PAGE result also exactly showed that a band whose apparent molecular mass (39 kDa) corresponded with that predicted for the NicX gene product (Fig. 2). Native and SeMet crystals were obtained from the same screening solution (Fig. 3A and 3B). An initial structural model was obtained using molecular replacement and anomalous scattering method and revealed the position of Se atoms and derived initial phases for electron-density calculation. All data were indexed, integrated and scaled with the XDS package (6). The number of molecules in the crystallographic asymmetric unit was estimated using the Matthews probability calculator (7). There were six molecules in each asymmetric unit. In this case, the Matthews coefficient was $2.27 \text{ \AA}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 45.86% in native and 47.6% in SeMet (8). The collected data sets were 98.4% (96.9%) integrity to 1.77 $\text{\AA}$ resolution and R-merge 13.4% (127.9%) for the native and 100% (100%) integrity to 2.08 $\text{\AA}$ resolution and R-merge 31.3% (279.7%) for SeMet (Fig. 3C). The crystals most probably belong to the orthorhombic space group P222 or P222₁ with unit-cell parameters $a = 118.51 \text{ \AA}$

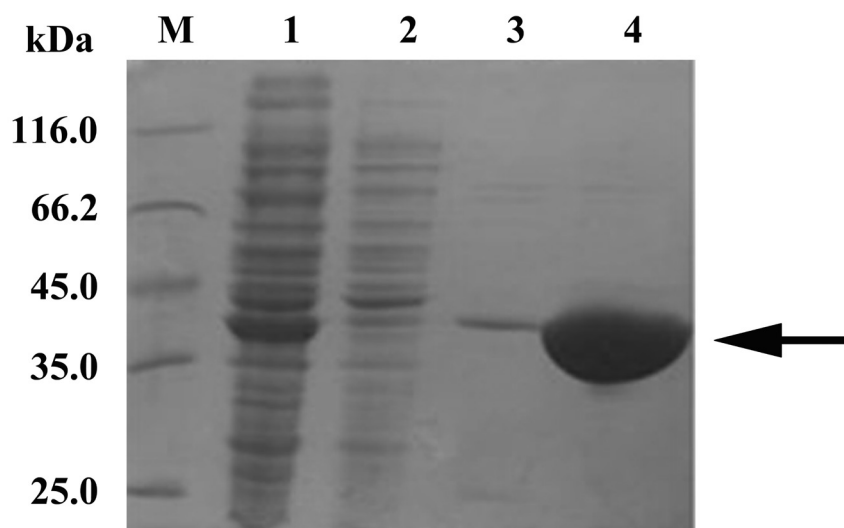


Fig. 2. SDS-PAGE analysis of purified recombinant NicX. Purification of NicX by size exclusion, Column showing a large peak with a small shoulder. Lane M, protein molecular weight marker (labeled in kDa). Lanes 1, supernatant, lanes 2 and 3, the washing fractions with 20 and 50, mM imidazole, respectively. Lane 4 was further purified using Size exclusion chromatography. Fractions were pooled, concentrated to 10mg/ml and used for crystallization trials. The protein fractions were resolved on a gradient SDS-PAGE gel (15%) and stained using Coomassie blue for visualization.

$b = 125.18 \text{ \AA}$ $c = 143.25 \text{ \AA}$ $a = 119.32 \text{ \AA}$ $b = 126.63 \text{ \AA}$ $c = 145.33 \text{ \AA}$ and crystal class is $\alpha = \beta = \gamma = 90^\circ$ for native and SeMet, respectively. The data completeness and average $I/\sigma(I)$ values of the collected data set were 98.4% and 17.5, and (96.6 % and 1.93 for the highest resolution shell, respectively). Se-NicX analysis using PHENIX revealed that there are 12 methionine in a molecule, 6 molecules in an asymmetric unit, but the Autosol found only four selenium atoms. As a result, to ensure data reliability, the X-triage data evaluation was conducted to rule out the possibility of twins. The entered data resulted in a value of 1.905. Similarly, we assessed selenium as with wild type whether selenium generation was twinning; and a value of 1.149, less than 3.5, was also obtained. It was generally considered that if the value is less than 3.5, the crystal is not considered as a twin. Therefore, the quality of the data set from all aspects of indicators revealed that the set of data was highly reliable and can be used. However, the minor numbers of selenium atoms in the selenite data analysis precluded to solve the final structure of this enzyme. Currently, to obtain the phase information for robust structure determination, a multiple anomalous dispersion (MAD) study of selenomethionine-substituted (SeMet) is under way. The biochemical activity of 2,5-DHP dioxygenase was measured at absorbance change of 320 nm and

continuously monitored at 298 K. The enzyme was most active at pH 7.4 $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ and 25°C . The K_m for 2,5-DHP and V_{max} values were $69 \mu\text{M}$ and $2.3 \mu\text{M min}^{-1} \text{mg}^{-1}$, respectively.

In conclusion, this study was conducted to determine the molecular mechanisms and structural analysis of NicX, a key enzyme in nicotinic acid degradation. We successfully expressed and purified recombinant native NicX and seleno-methionine-substituted (SeMet) and obtained crystals of high resolutions. Diffraction data were collected to a resolution of $1.77 \text{ \AA}$ native and $2.08 \text{ \AA}$ selenomethionine. An initial structural model was obtained using the molecular replacement method and revealed the position of Se atoms and derived initial phases for electron-density calculation. The estimated Matthews coefficient is $2.27 \text{ \AA}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 45.86% in native and 47.60% in selenomethionine-containing NicX, which is consistent with the presence of six protein molecules in each asymmetric unit. Crystal structure analysis of NicX would be essential in revealing the nature of ligand interaction, conformation of the active site and the structural basis for substrate specificity. These results may provide a basis for the elucidation of robust structure, which should shed light on the function of this essential enzyme in the aerobic nicotinic acid degradation.

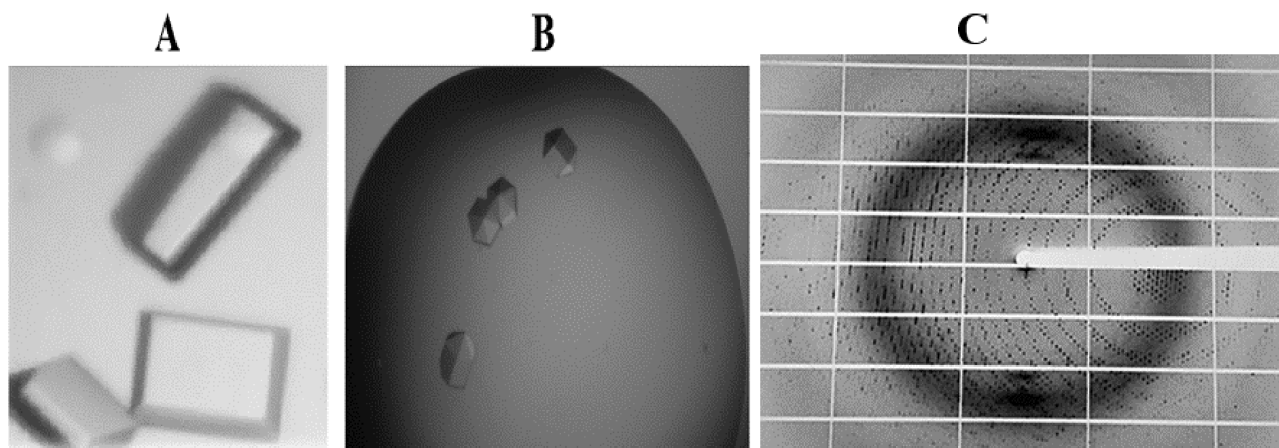


Fig. 3. Typical crystals of recombinant NicX (A). Typical crystals of seleno-methionine-substituted (SeMet) NicX (B). Typical X-ray diffraction pattern of the native NicX protein crystal (C). The crystal diffraction dataset was processed to $1.77 \text{ \AA}$ resolution.

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LETTER TO THE EDITOR

INHIBITORY EFFECTS OF APOLIPOPROTEIN M ON EXPRESSIONS OF NF- κ B, IL-6, IL-1 β AND OTHER RELATED FACTORS IN MICE WITH MYOCARDITIS

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To the Editor,

The American Heart Association and the American College of Cardiology have listed myocarditis as the third leading cause of sudden cardiac death in competitive athletes (1, 2). At present, the pathogenesis of myocarditis is not completely clear, and the treatment still relies on pathogen suppression, immunomodulation and heart failure treatment, with no specific therapeutic methods available (3, 4).

Apo M is a member of the human lipoprotein superfamily. Past studies found that the Apo M expression is regulated at multiple levels (5, 6). For example, Xu et al. (7) found that the platelet activating factor can significantly enhance the expression level of Apo M in human hepatoma Hep G2 cells. In addition, current clinical studies on Apo M found that the Apo M expression is closely related to the onset of some inflammatory diseases (8). By studying patients with severe lower limb ischemia, Ahnstrom et al. (9) found that serum levels of Apo M were significantly lower than those in healthy people and negatively correlated with the levels of inflammation-related factors such as IL-6, TNF- α and C-reactive protein. A previous study found that the NF- κ B pathway is important for inducing inflammatory responses in mouse myocardium, and the blockage of NF- κ B p65 protein transfer can significantly reduce ischemia-reperfusion-induced myocardial inflammation

(10). In this study, by detecting and comparing the changes in the expression of factors related to myocardial inflammation, such as NF- κ B, IL-1 β , IL-6 and ICAM-1, in mice with myocarditis before and after the LPS treatment, the protective effect of Apo M was investigated to provide a new direction for future studies.

MATERIALS AND METHODS

Animals

Forty C57BL/6 mice aged 6-8 weeks (20 males and 20 females) were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd., China. An Apo M knockout mouse model was constructed with the assistance of the Animal Research Institute of Tangshan Worker's Hospital. The mice were housed under room temperature at 20~23°C, humidity 50~60%, and light 12 hours per day. Feed and water were supplied by specially trained animal caretakers. The first mating age of the mice was 12 weeks. The newborn mice were weaned at the age of 3 weeks and were fed with the feed. At the same time, ear and tail markers were used for identification of different genotypes. Gene identification was performed by a base quenching technique. The growth of mice was closely observed throughout the feeding process. Twenty-four healthy

*Key words: apolipoprotein M; myocarditis; NF- κ B; IL-1 β ; IL-6**Corresponding Author:*

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male Apo M^{+/+} and 24 healthy male Apo M^{-/-} mice were used for experimental studies. The protocol of this study was approved by the ethics committee of Tangshan Worker's Hospital, and the experiment was carried out following the regulations of the State Science and Technology Commission on the management of laboratory animals.

Grouping

Twenty-four Apo M^{+/+} mice were randomly divided into three groups (eight mice in each group): 0h LPS treatment group, 1h LPS treatment group, and 3h LPS treatment group.

i) ApoM^{+/+} mice in the 0 h LPS treatment group: After 40-50 mg/kg of 2% pentobarbital sodium was used to anesthetize the mice, the mice were sacrificed by spine dislocation and their myocardial tissue samples were removed and used as the blank control group;

ii) ApoM^{+/+} mice in the 1h LPS treatment group: Each mouse was intraperitoneally injected with 0.1 ml of 2.0 mg/ml LPS solution (dissolved in saline). At 1 h after intraperitoneal injection of the LPS solution, 40-50 mg/kg of 2% sodium pentobarbital was used to anesthetize the mice. Then, the mice were sacrificed by dislocation of the spine to collect their myocardial tissue samples;

iii) ApoM^{+/+} mice in the 3h LPS treatment group: Each mouse was intraperitoneally injected with 0.1 ml of 2.0 mg/ml LPS solution (dissolved in saline). At 3 h after intraperitoneal injection of the LPS solution, 40-50 mg/kg of 2% sodium pentobarbital was used to anesthetize the mice. Then, the mice were sacrificed by dislocation of the spine to collect their myocardial tissue samples;

Twenty-four ApoM^{-/-} mice were randomly divided into three groups (eight mice in each group): 0h LPS treatment group, 1h LPS treatment group, and 3h LPS treatment group.

i) ApoM^{-/-} mice in the 0h LPS treatment group: After 40-50 mg/kg of 2% pentobarbital sodium was used to anesthetize the mice, the mice were sacrificed by spine dislocation and their myocardial tissue samples were removed and used as the blank control group;

ii) ApoM^{-/-} mice in the 1h LPS treatment group:

Each mouse was intraperitoneally injected with 0.1 ml of 2.0 mg/ml LPS solution (dissolved in saline). At 1 h and 3 h after intraperitoneal injection of the LPS solution, 40-50 mg/kg of 2% sodium pentobarbital was used to anesthetize the mice. Then, the mice were sacrificed by dislocation of the spine to collect their myocardial tissue samples;

iii) ApoM^{-/-} mice in the 3h LPS treatment group: Each mouse was intraperitoneally injected with 0.1 ml of 2.0 mg/ml LPS solution (dissolved in saline). At 1 h and 3 h after intraperitoneal injection of the LPS solution, 40-50 mg/kg of 2% sodium pentobarbital was used to anesthetize the mice. Then, the mice were sacrificed by dislocation of the spine to collect their myocardial tissue samples.

At 0 h, 1 h, and 3 h after sacrifice the cardiac tissues of all mice were extracted. The tissues were frozen in liquid nitrogen (Beijing Dingguo Changsheng Biotechnology Co., Ltd., China), while repeated freeze-thaw cycles were avoided when the samples were used in subsequent experiments.

Detection of NF- κ B, IL-1 β , IL-6 and ICAM-1 mRNA expressions in myocardial tissues

Total RNA was extracted using a K352 total RNA extraction kit (Shanghai Shenneng Gaming, China) according to the manufacturer's instructions and stored in a cryo container at -70°C (Thermo Fisher Scientific Co., Ltd., China). The absorbance of the samples was measured at 260 nm and 280 nm by a nucleic acid protein analyzer (Eppendorf, Germany) to calculate their RNA concentration and OD260/280 ratio. The cDNA was synthesized using a cDNA first strand synthesis kit (Termo scientific, USA) in a 12- μ l reaction system containing 2 μ g of total RNA, 1 μ l of oligo (d T) 18, and DEPC (diethyl pyrocarbonate) water. After centrifugation at 65°C for 5 min, the mixture was placed in a -20°C cryo container for 3 min before 4 μ l of 5 $\times$ reaction buffer, 2 μ l of 10 Mm d NTP suspension, 1 μ l of M-Mu LV reverse transcriptase (200 U/ μ l) and 1 μ l of Ribolock RNA enzyme inhibitor (20 U/ μ l) were sequentially added to the mixture to establish a 20- μ l reaction system for reverse transcription. The mixture was then incubated at 42°C for 60 min before incubation at 70°C for 5 min to terminate the

reaction. The solution was placed on ice for cooling and then centrifuged at room temperature for 5 s. The resulting solution was collected from the bottom of the tube and the obtained cDNA was stored in a -20°C container for later use. Primers and probes for mouse NF- κ B, IL-1 β , IL-6, ICAM-1 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were designed by Primer Premier 5.0 (Primer, Canada) and synthesized by Human Bioengineering (Shanghai) Co., Ltd. (China). Justice primers 1 and 2 were used to identify wild-type and knockout Apo M alleles, respectively. Probes 1 and 2 were used to detect Apo M wild type and Apo M knockout type genes, respectively. Details are listed in Table I.

Statistical analysis

Data were statistically analyzed and plotted using Graph Pad Prism 6.0 statistical software (GraphPad Software, USA). The trend of experimental data was expressed in median. Comparisons between the two groups were performed using the Mann-Whitney U rank sum test. The interaction between two factors was analyzed by two-way analysis of variance. The difference was considered statistically significant at $P < 0.05$.

RESULTS

Changes in the mRNA expression of nuclear transcription factor NF- κ B

In Apo M^{+/+} and Apo M^{-/-} mice (Fig. 1), the mRNA expression of NF- κ B in the 1h group and 3h group was significantly higher than that in the 0h group ($P < 0.05$). Compared with that in the 0h group, the

mRNA expression of NF- κ B in Apo M^{+/+} mice of the 1h group and 3h group increased 2.19- and 2.65-fold, respectively. Similarly, compared with that in the 0h group, the mRNA expression of NF- κ B in Apo M^{-/-} mice of the 1h group and 3h group increased 9.41-fold and 12.10-fold, respectively.

Between Apo M^{-/-} and Apo M^{+/+} mice, the difference in the mRNA expression of NF- κ B in the 1h group and 3h group was statistically significant ($P < 0.05$), while no significant difference was observed in the 0h group (Fig. 1).

Two-way analysis of variance was used to analyze the effect of Apo M and LPS on the expression of NF- κ B mRNA in myocardial tissues of the 1h and 3h mice. The results showed that the effects of both factors were statistically significant ($P < 0.05$).

Changes in the mRNA expression of inflammatory cytokine IL-1 β

In the Apo M^{+/+} and Apo M^{-/-} mice, the mRNA expression of IL-1 β in the 1h group and 3h group was significantly higher than that in the 0h group ($P < 0.05$). Compared with the 0h group, the IL-1 β expression in Apo M^{+/+} mice of the 1h group and 3h group increased 58.54-fold and 15.67-fold, respectively. Similarly, compared with the 0h group, the IL-1 β expression in Apo M^{-/-} mice of the 1h group and 3h group increased 259.95-fold and 98.81-fold, respectively. Between Apo M^{-/-} and Apo M^{+/+} mice, the mRNA expression of IL-1 β was significantly different in the 1h group and 3h group, while no significant difference was observed in the 0h group (Fig. 1).

Two-way analysis of variance was used to analyze

Table I. Primers and probes for identification of mice genotypes by base quenching probe technology.

Primer probe	Sequence
Justice primer 1	GCCCAGACATGAAAACAGACCT
Justice primer 2	AGCGTTGGCTACCCGTGATA
Probe 1	CTGTGCAATGTCAGCCA-FAM
Probe 2	GGATCCACTAGTTCT-FAM

the effect of Apo M and LPS on the expression of IL-1 β mRNA in myocardial tissues of mice in the 1h group and 3h group. The results showed that the effects of both factors were statistically significant ($P < 0.05$).

Changes in the mRNA expression of inflammatory cytokine IL-6

In Apo M^{+/+} and Apo M^{-/-} mice, the myocardial expression of IL-6 in the 1h group and 3h group was significantly higher than that in the 0h group ($P < 0.05$) (Fig. 2). Compared with the 0h group, the IL-6 levels in the myocardial tissues of Apo M^{+/+} mice in the 1h group and 3h group increased 171.47- and 861.90-

fold, respectively. Similarly, compared with the 0h group, the IL-6 levels in the myocardial tissues of Apo M^{-/-} mice in the 1h group and 3h group increased 745.89-fold and 2733.57-fold, respectively. Between Apo M^{-/-} and Apo M^{+/+} mice, the mRNA expression of IL-6 was significantly different in the 1h group and 3h group, while no significant difference was observed in the 0h group (Fig. 2).

Two-way analysis of variance was used to analyze the effect of Apo M and LPS on the expression of IL-6 mRNA in myocardial tissues of mice in the 1h group and 3h group. The results showed that the effects of both factors were statistically significant ($P < 0.05$).

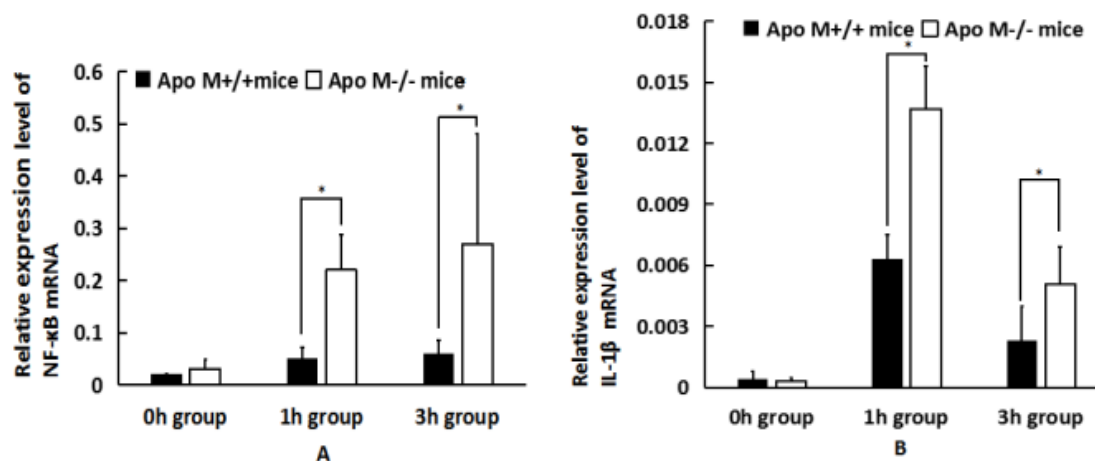


Fig. 1. Effects of LPS and Apo M on the expression of NF- κ B and IL-1 β mRNA in the myocardium of Apo M^{+/+} and Apo M^{-/-} mice. **A)** Effects of Apo M on the expression of NF- κ B mRNA in myocardium of mice; **B)** Effects of Apo M on the expression of IL-1 β mRNA in myocardium of mice. * $P < 0.05$

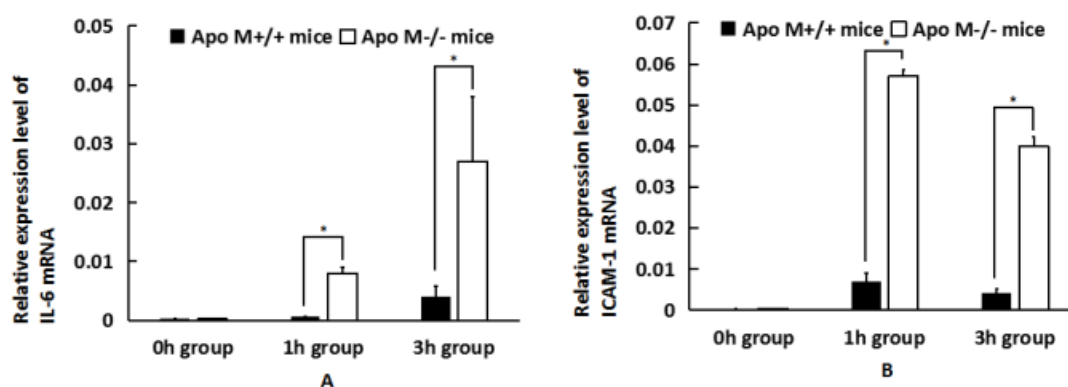


Fig. 2. Effects of LPS and Apo M on the expression of myocardial IL-6 and ICAM-1 mRNA in Apo M^{+/+} and Apo M^{-/-} mice. **A)** Effects of Apo M on the expression of myocardial IL-6 mRNA in mice; **B)** Effects of Apo M on the expression of ICAM-1 mRNA in myocardium of mice. * $P < 0.05$

Changes in the mRNA expression of inflammatory factor ICAM-1

The mRNA expression of ICAM-1 in the myocardial tissue of Apo M^{+/+} and Apo M^{-/-} mice in the 1h group and 3h group was significantly higher than that in the 0h group ($P < 0.05$) (Fig. 2). Compared with the 0h group, the ICAM-1 levels in the myocardial tissues of Apo M^{+/+} mice in the 1h group and 3h group increased 177.65-fold and 124.24-fold, respectively. Similarly, compared with the 0h group, the ICAM-1 levels in the myocardial tissues of Apo M^{-/-} mice in the 1h group and 3h group increased 177.98-fold and 124.16-fold, respectively. Between Apo M^{-/-} and Apo M^{+/+} mice, the mRNA expression of ICAM-1 was significantly different in the 1h group and 3h group, while no significant difference was observed in the 0h group (Fig.2).

Two-way analysis of variance was used to analyze the effect of Apo M and LPS on the expression of ICAM-1 mRNA in myocardial tissues of mice in the 1h group and 3h group. The results showed that the effects of both factors were statistically significant ($P < 0.05$).

DISCUSSION

In this paper, changes in the mRNA expression of NF- κ B, IL-1 β , IL-6 and ICAM-1 were detected in mouse myocardial tissues at different time points before and after intraperitoneal injection of the LPS solution. It was found that the expression of the factors above was significantly higher after injection of the LPS solution, suggesting that the inflammation models in the two genotypes were successfully established.

At the same time, the results of this experiment also showed that after LPS treatment, the expression levels of inflammatory factors IL-1 β , IL-6 and ICAM-1 in Apo M^{-/-} mice were significantly higher than those in Apo M^{+/+} mice. The interaction analysis of Apo M and LPS also showed statistically significant difference, indicating that Apo M also inhibits LPS-mediated expressions of myocardial IL-1 β , IL-6 and ICAM-1. Therefore, it is hypothesised that Apo M can inhibit the expression of various myocardial inflammatory factors and reduce the damage to

cardiomyocytes during the onset of a myocardial inflammatory response. Some scholars have studied the over-expression of Apo M in hepatocellular carcinoma Hep G2 cells (11), where Apo M was found to inhibit the expression of ICAM-1 via the NF- κ B pathway. Consistent with this experiment, the above results showed that the expression of NF- κ B and its downstream inflammatory factors, such as IL-1 β , IL-6 and ICAM-1, was significantly higher in Apo M^{-/-} mice than that in Apo M^{+/+} mice, suggesting that the anti-inflammatory effect of Apo M in the myocardium may be regulated by the NF- κ B pathway.

In summary, Apo M inhibits the expression of NF- κ B, IL-1 β , IL-6 and ICAM-1 in LPS-induced myocardial inflammation in mice.

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LETTER TO THE EDITOR

PD-1 AND PD-L1 EXPRESSION ON THE PROGNOSIS OF OVARIAN CANCER

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To the Editor,

According to the statistics of American Cancer Society (ACS), ovarian cancer has the highest mortality among gynecological tumors (1). Although chemotherapy and surgical treatment have significantly improved the prognosis of ovarian cancer patients in the past two decades, the efficacy of these methods in patients with advanced ovarian cancer remains poor (2). As a member of the CD28/CTLA-4 family, programmed death receptor (PD-1) is mainly expressed on the surface of activated T/B cells and macrophages (3). The ligands of PD-1 include PD-L1 and PD-L2. In particular, PD-L1 is widely expressed in ovarian cancer, malignant melanoma and other malignant tumors (4). The binding of PD-L1 to PD-1 is believed to promote immune escape. When PD-L1-positive tumor cells are bound to PD-1-positive T cells, the proliferation and differentiation of T cells can be inhibited, while the apoptosis of T cells can be induced. Several clinical trials of anti-PD-L1 and anti-PD-1 antibodies have shown that these antibodies exerted significant anti-tumor effects on some malignant tumors, including malignant melanoma (5). Similarly, anti-PD-L1 and anti-PD-1 antibodies have shown efficacy in the adjuvant treatment of ovarian cancer (6).

As a new method of cancer treatment, immunotherapy can control and kill tumor cells by activating the immune system to enhance the anti-

tumor immunity of the tumor microenvironment (7). At present, it remains unclear whether PD-L1 can be used as a biomarker in tumor treatment. Therefore, the analysis of the correlation between PD-L1 expression and the prognosis, outcomes and survival of ovarian cancer patients will deepen our understanding of the PD-L1 and PD-1 signaling pathway, to provide a new direction for treating ovarian cancer with immunotherapy.

MATERIALS AND METHODS

Subjects

Paraffin-embedded ovarian cancer tissues were collected from 79 ovarian cancer patients [aged 54.1±9.9 years (range, 26 to 73 years)] undergoing surgery in Lanzhou University Second Hospital from 2012 to 2018. Of these patients, 55 were cases of serous cystadenocarcinoma and 24 were of non-serous cystadenocarcinoma. The age, tumor size, CA125 levels, Federation of Gynecology and Obstetrics (FIGO) stage, tumor metastasis and other basic clinical data were collected for statistical analysis. The tumor size was 1124.6±1326 cm³ (range, 2-5400 cm³). Sixteen patients were of FIGO stage I+II and 63 patients of stage III+IV; 54 had poorly differentiated tumor while 25 had moderately differentiated tumor. The serum level of CA125 was elevated in 75 patients, and tumor metastasis was found in 72. None of the patients were treated by chemotherapy or radiotherapy before surgery.

Key words: PD-1; PD-L1; ovarian cancer; prognosis

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During surgery, primary tumor tissues were collected as specimens. In addition, this study enrolled 8 cases of ovarian cysts undergoing ovariectomy during the same period as the negative control group.

All 79 cases of ovarian cancer had complete follow-up data. Survival was defined as the interval from the date of surgery to the date of death or the last follow-up. Thirty-three patients survived and 46 patients had died from ovarian cancer at the last follow-up. The protocol was approved by the Medical Ethics Committee and informed consent was signed by all patients.

Elivision™ immunohistochemical staining

Paraffin-embedded slices were dried for 2 h at 90°C, and dewaxed. They were then treated by xylene - dimethylbenzene - 100% alcohol - 100% alcohol - 95% alcohol - 90% alcohol - 80% alcohol - 70% alcohol, 5 min each. Subsequently, the slices were rinsed three times with Phosphate Buffered Saline (PBS), pH 7.4, microwaved to 100°C in an ethylene diamine tetraacetic acid (EDTA) buffer (Shanghai Beino Biotechnology Co., Ltd., China), dewaxed and hydrated for 10 min in a 100°C buffer solution. They were cooled naturally, rinsed twice with distilled water and PBS separately, and then incubated with 0.5 mL 3% H_2O_2 for about 20 min at 25°C to block the activity of endogenous peroxidase. After being rinsed three times with PBS, each slice was incubated overnight at 4°C with 0.5 mL of monoclonal mouse anti-human PD-L1 antibody (1:800 dilution) (Novus Biotechnology Company, USA) or monoclonal mouse anti-human PD-1 antibody (Beijing Zhongshanjinqiao Biotechnology Co., Ltd., China). After being rinsed three times with PBS, each slice was incubated at room temperature for 20 min with 0.5 mL of polymer reinforcing agent (Fuzhou Maixin Biotechnology Development Co., Ltd., China). After being rinsed three times with PBS, each slice was incubated at room temperature for 30 min with 0.5 mL of enzyme-labeled anti-mouse/rabbit polymer (Fuzhou Maixin Biotechnology Development Co., Ltd., China). After being rinsed three times with PBS, each slice was developed using 0.5 mL of fresh DAB (Diaminobenzidine) solution (prepared by adding 0.5 mL of developer A) to 1 mL of deionized water, followed by the addition of 0.5 mL of developer B and a drop of developer C, and then observed under a microscope. Subsequently, the slices were counterstained with hematoxylin (Fuzhou Meixin

Biotechnology Development Co., Ltd., China), and differentiated in 0.1% HCl. After rinsed with distilled water, they were blued, dried by gradient alcohol, and cleared in xylene. Finally, the slices were incubated with 70% alcohol - 80% alcohol - 90% alcohol - 95% alcohol - 100% alcohol - 100% alcohol - xylene - xylene, 2 min each, and sealed in neutral gum.

Determination of immunohistochemical staining results

The criteria of Al-Shibli (8) were used to determine the positive results of PD-L1 on stained slices. The presence of yellow to brown granules in cytoplasm or cell membrane indicated positive. In addition, 5 tumor regions were randomly selected under a 40× high power lens. The staining intensity of PD-L1 positive cells in tumor tissues was graded as follows: 0, negative staining; 1, light yellow staining; 2, brown yellow staining; and 3, chocolate-brown staining. The average staining intensity of the 5 tumors was taken as the staining intensity of the tumor case. Immunohistochemical scores were determined as follows: 0, -; 1, +; 2, ++; 3, +++, among which - and + were defined as low expression, while ++ and +++ were defined as high expression.

Cells with positive PD-1 expression were mainly interstitial lymphocytes, while the positive staining referred to yellow to brown granules in the cytoplasm or on the membrane of interstitial cells. PD-1 positive cells were counted as follows: First, the whole section was observed under a low power lens to randomly select 5 high power microscopic fields (×200) to count the number of positive cells. The average number of positive cells in the 5 high power microscopic fields was used as the number of PD-1 positive cells in this tumor case. The average number of PD-1 positive cells in all 79 tumor cases was used as the threshold value. Subsequently, the 79 cases were divided into high PD-1 expression group and low PD-1 expression group.

Statistical processing

SPSS 23.0 and Graphpad prism6 were used for statistical analysis. Measurement data were expressed as mean ± standard deviation. Expansion test and Fisher's exact probability method were used for the comparison among different groups. Survival analysis was performed using the Kaplan-Meier survival curve method and Log-rank test. PD-L1 and PD-1 expression, FIGO stage, age,

cell differentiation, metastasis, histological type and tumor volume were analyzed by the COX model.

RESULTS

Immunohistochemical results of PD-L1 and PD-1 protein expression

The contrast map of PD-L1 expression is shown in Fig. 1, A-D. PD-L1 was expressed in the cytoplasm and on the membrane of tumor cells as yellow to brown granules or masses (Fig. 1E). In addition, the expression of PD-L1 was low in 8 cases of ovarian cysts and high in 79 cases of ovarian cancer (43.04% (34/79) (Fig. 1F).

The high expression rate of PD-1 protein was 41.77% (33/79) in 79 cases of ovarian cancer. In addition, PD-1 protein was mainly localized in the cytoplasm and/or on the membrane of tumor infiltrating lymphocytes (TILs) as brown-yellow to chocolate-brown granules (Fig. 1G).

Correlation between the expression of PD-L1 and the infiltration of PD-1 positive lymphocytes

Among the 79 specimens of ovarian cancer, the counted number of PD-1 positive cells was 0-80, with an average of (14.80 ± 12.15) . The mean number of PD-1 positive lymphocytes was 14.80 and was used as the threshold. There were 46 cases of low infiltration of PD-1 positive lymphocytes, including 27 cases of low expression of PD-L1 and 19 cases of high expression. And there were 33 cases of high infiltration, among which 18 cases of low expression of PD-L1 and 15 cases of high expression. χ^2 test showed no significant correlation between the expression of PD-L1 and the infiltration of PD-1 positive lymphocytes in ovarian tissues ($P > 0.05$, Table I).

Correlation between the expression of PD-L1/PD-1 protein and the clinicopathological parameters of ovarian cancer

The expression of PD-L1 in ovarian cancer cells was closely related to the clinical stage ($P < 0.05$) and age ($P < 0.05$) of the patients, however, was not related to other clinicopathological parameters, such as histological type, cell grade, tumor size,

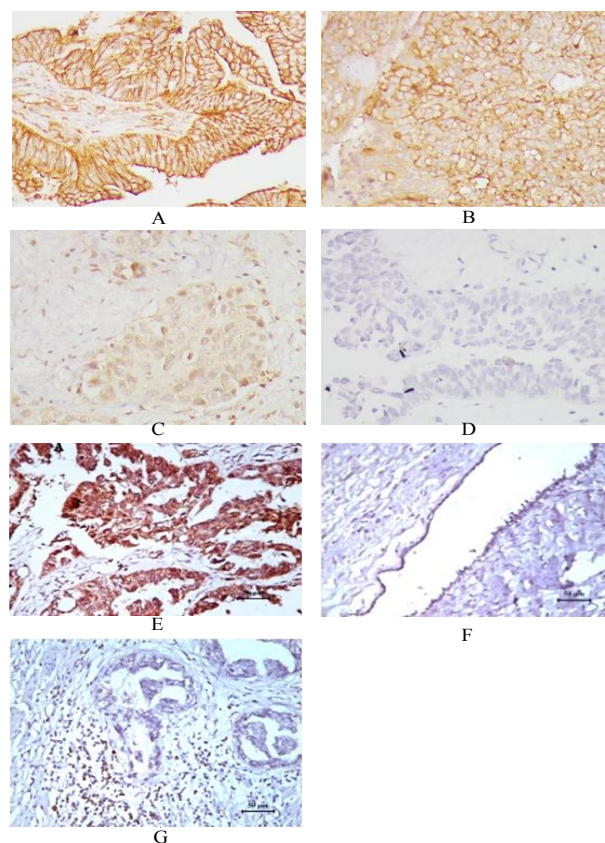


Fig. 1. Comparison of the expression of PD-L1 and the expression of PD-L1 molecule in ovarian tissue and ovarian cancer interstitial lymphocytes (Positive color development was observed with yellow to brown particles on the cytoplasm or cell membrane. **A)** the control immunohistochemical image (x40) with strong positive expression of PD-L1, the stained part in the figure was brown, and the positive expression of PD-L1 was strong. **B)** The control immunohistochemical image (x40) with positive expression of PD-L1, the stained part in the figure was yellow, and the expression of PD-L1 was positive. **C)** The control immunohistochemical images (x40) with weak positive expression of PD-L1, in which the stained part was light yellow and the positive expression of PD-L1 was weak. **D)** the control immunohistochemical images (x40) with negative expression of PD-L1, there was no yellow staining part in the figure, and PD-L1 was not expressed. **E)** PD-L1 was positively expressed in ovarian cancer cells (x200), the stained part in the figure was brown, and the positive expression of PD-L1 was strong. **F)** PD-1 was negatively expressed in ovarian cyst tissue (x200), the stained part in the figure was light yellow, and the expression of PD-L1 was weak. **G)** PD-1 was positively expressed in ovarian cancer interstitial lymphocytes (x200), and brown granules appeared on the cytoplasm or cell membrane of the cytoplasmic cells, which was positive development for PD-1.

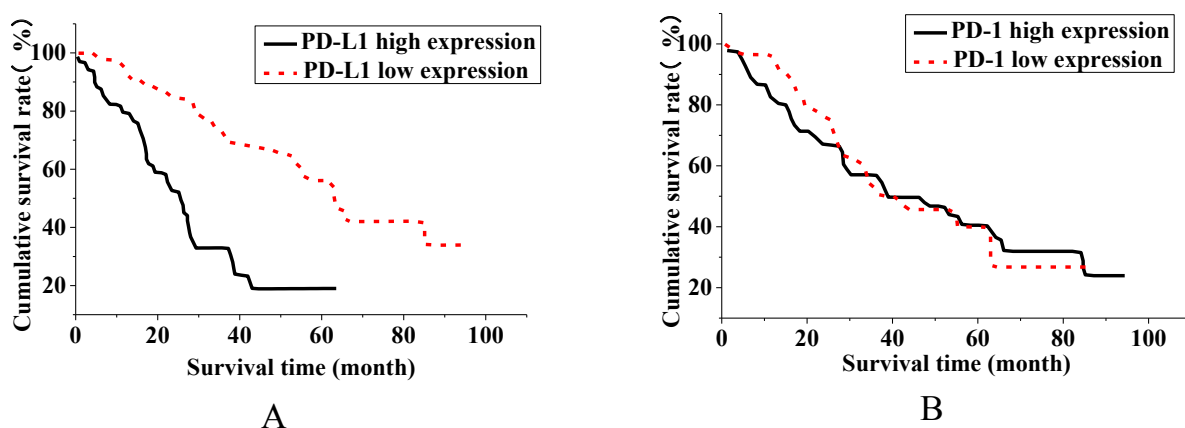


Fig. 2. OS curves of patients with different expression levels of PD-L1 and PD-1. **A)** OS curves of patients with different expression levels of PD-L1; **B)** OS curves of patients with different expression levels of PD-1)

Table I. Relationship between the expression of PD-L1 and PD-1 protein and the clinicopathological parameters of ovarian cancer.

Gene	Variable	Case	High expression	Low expression	χ^2	P
PD-L1 expression	Age / year	≤ 54	35	10	5.36	0.021
		> 54	44	24		
	Histological type	Serous	55	25	0.43	0.511
		Non-slurry	24	9		
	Cell grade	Moderately differentiated	25	11	0.01	0.906
		Poorly differentiated	54	23		
	FIGO stage	I+ II stage	16	3	5.05	0.025
		III+ IV stage	63	31		
	Tumor size / cm^3	< 120	13	8	2.17	0.14
		≥ 120	66	26		
PD-1 expression	CA125	Normal	4	2	0	1
		Increased	75	32		
	Metastases	Yes	72	32	0.16	0.682
		No	7	2		
	Age/years	≤ 54	35	11	2.76	0.096
		> 54	44	22		
	Histological type	Serous	55	25	1	0.315
		Non-slurry	24	8		
	Cell grade	Moderately differentiated	25	9	0.5	0.479
		Poorly differentiated	54	24		
	FIGO stage	I+II stage	16	7	0.03	0.857
		III+IV stage	63	26		
	Tumor size/ cm^3	< 120	13	7	0.93	0.334
		≥ 120	66	26		
	CA125	Normal	4	1	0.032	0.859
		Increased	75	32		
	Metastasis	Yes	72	32	1.3	0.253
		No	7	1		

Table II. COX regression multivariate analysis on the relationship between PD-L1 expression and OS.

Variables		Univariate analysis			Multivariate analysis		
		HR	95%CI	P	HR	95%CI	P
Age/years	≤54/>54	0.498	0.272-0.911	0.024	0.616	0.314-1.207	0.158
Histological types	Serous/non-serous	0.955	0.511-1.786	0.886	1.001	0.510-1.966	0.998
Cell grading	Low/medium and high differentiation	1.708	0.867-3.367	0.122	1.603	0.792-3.246	0.190
FIGO stage	I-II/III-IV stage	0.155	0.047-0.505	0.002	0.112	0.015-0.835	0.033
Tumor size/cm ³	<120/≥120	1.512	0.725-3.152	0.271	0.863	0.395-1.883	0.711
Metastasis	Yes/no	0.295	0.071-1.232	0.094	3.547	0.302-41.719	0.314
PD-L1 expression	Low/high	0.288	0.154-0.539	<0.001	0.355	0.181-0.694	0.002

CA125 expression and metastasis ($P > 0.05$, Table I). In addition, there was no significant correlation between the expression of PD-1 protein in tumor infiltrating lymphocytes and the clinicopathological parameters of ovarian cancer ($P > 0.05$, Table I). The age at which ovarian cancer is highly prone to occur is about 54 years, therefore this was used as a boundary.

Relationship between the expression of PD-L1/PD-1 in ovarian cancer tissues and the post-operative overall survival

The overall survival (OS) of patients with different levels of PD-L1 and PD-1 expression was measured by the Kaplan-Meier method. The results showed that the OS, (23.8±14.8) months *vs* (48.6±23.6) months, respectively, of patients with high and low PD-L1 expression was significantly different ($\chi^2=16.8$, $P<0.001$) (Fig. 2A). In addition, the OS, (38.8±26.2) months *vs* (36.7±19.8) months, respectively, of patients with high and low PD-L expression showed no significant difference ($\chi^2 = 0.02$, $P > 0.05$) (Fig. 2B).

Results of COX regression multivariate analysis

Univariate analysis and COX regression multivariate analysis were performed for age/year,

histological type, cell grading, FIGO stage, tumor size/cm³, and metastases. It was found that among the multiple factors, the single factor analysis of PD-L1 expression was $P<0.001$, the multifactor analysis was $P=0.002$, the single factor analysis of FIGO stage was $P=0.002$, and the multifactor analysis was $P=0.033$, which were independent prognostic factors affecting the survival of ovarian cancer patients (Table II).

DISCUSSION

The results of this study are consistent with those reported by studies on PD-L1 in other tumors (9). Ohigashi et al. studied patients with esophageal cancer and found that those with positive PD-L1 expression had shorter survival, worse prognosis and a higher risk of recurrence and metastases than those with negative PD-L1 expression (10). Wu et al. analyzed the expression of PD-L1 in 102 cases of gastric cancer, and it was found that the expression of PD-L1 was negative in normal gastric tissues and high in gastric cancer tissues. The expression of PD-L1 was correlated with the degree of tumor invasion and lymph node metastasis as well as the size of tumor. The survival of patients with high PD-L1 expression was significantly shorter than those

with low PD-L1 expression, and the expression of PD-L1 was an independent prognostic indicator for gastric cancer patients (11). All these studies indicated that the negative immune regulation of PD-L1 plays an important role in the immune escape of tumors. Interestingly, PD-L1/PD-1 is also expressed in normal tissues and may be related to the establishment of autoimmune tolerance and tolerance for excessive inflammation.

According to the literature, two hypotheses have been proposed regarding the role of increased expression of PD-L1 in tumor tissues: one is that ovarian cancer cells can induce PD-L1 expression by direct contact with effector cells or by promoting the action of INF- γ paracrine from T cells. The other is that the expression of PD-L1 is significantly up-regulated due to the local hypoxic microenvironment of tumor cells, thus significantly enhancing the tolerance of tumor cells for CTL-mediated lysis. However, it remains unclear whether the expression of PD-L1 in ovarian cancer is affected by the above mechanisms.

In conclusion, the increased expression of PD-L1 may lead to poor prognosis and shorter survival of ovarian cancer patients. Nevertheless, more studies on PD-L1 are required to provide a new direction for developing novel immunotherapy for the treatment of ovarian cancer.

ACKNOWLEDGEMENTS

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LETTER TO THE EDITOR

PREVALENCE AND ECHOCARDIOGRAPHIC CHARACTERISTICS OF ARTERIAL HYPERTENSION IN PATIENTS WITH ATRIAL FIBRILLATION: A SINGLE CENTER STUDY

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To the Editor,

With prevalence of approximately 3%, atrial fibrillation (AF) is the most common sustained arrhythmia on discharge from hospital (1). It is estimated that hospital admission for AF has increased 2/3-fold during the last 20 years (2). AF is more common in older people and patients who suffer from diabetes mellitus, chronic kidney disease, coronary heart disease, valvular heart disease, heart failure or arterial hypertension. This arrhythmia is associated with left ventricular dysfunction and heart failure, impaired quality of life, increased stroke risk, and increased risk of all-cause mortality. While stroke risk can be significantly reduced by using anticoagulation, cardiovascular deaths in patients with AF remain common even in treated patients (3).

Arterial hypertension is an independent risk factor for AF development (4). It causes left ventricular hypertrophy which leads to impaired ventricular filling that causes structural changes and enlargement of the left atria which then leads to AF. There are parameters that can help us predict AF development in patients with hypertension. Hypertension is one of the powerful risk factors for stroke in AF and, since it is modifiable, its regulation is very important to reduce this risk (5). The best medications for this may be

renin- angiotensin-aldosterone (RAAS) blockers (4).

The aim of this study was to determine the prevalence of hypertension and assess the echocardiographic parameters in patients suffering from atrial fibrillation (AF), with or without hypertension, and to assess the current status of the use of anticoagulation for the treatment of AF.

MATERIALS AND METHODS

The present study included 167 patients with AF, [116 males (69.46%) and 51 females (30.54%)] who were all hospitalized at the University Hospital, Institute for Treatment and Rehabilitation Niska Banja, Nis, Serbia between 2014 and 2016. Data regarding AF and comorbidities were taken from medical history and by medical examination. For 140 patients an echocardiogram was taken and the size of the left atria, ejection fraction and pressure in pulmonary artery were assessed. All patients signed an informed consent before being enrolled in the study.

RESULTS

The average age of the patients was 68.3 years (males 67.7, females 69.8). Fifteen patients were diagnosed with newly manifested AF, 61 patients

Key words: atrial fibrillation, arterial hypertension, echocardiography, prevalence

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suffered from paroxysmal, 21 patients from persistent, 1 from “long standing” persistent, and 64 patients (38.2 %) from permanent AF (Fig. 1).

Out of the 167 patients with AF, 133 (79.6%) were treated for hypertension; 59.3% of the patients had had hypertension for more than 10 years, 12% between 5 and 10 years, and 23.4% for less than 5 years, while 5.4% did not answer this question. The most common type of AF in patients with hypertension was paroxysmal AF (37.5%), while in the group without hypertension it was permanent AF (40.2%). There were no statistically significant differences in the distribution of AF type in the examined groups ($p = 0.402$).

A total of 112 patients (67.1%) suffered from coronary heart disease diagnosed by coronarography, while 14 of them (8.4%) were diagnosed with renal failure. Six patients (3.6%) suffered from chronic obstructive pulmonary disease, and 7 (4.2%) from anemia. A total of 91 patients (54.5%) stated to be non-smokers, 40 (24.0) to be active smokers, 32 (19.2%) to be former smokers, and 4 patients (2.4%) made no comment on the subject. Thirty-three patients (19.7%) out of 167 who participated in the study were treated for diabetes, of whom 22 (66.7%) were treated medically, and 11 (33.3%) received insulin therapy. Most patients (61.7%) had

elevated levels of lipids in the blood, or were treated by statins.

The average size of the left atrium in patients with first diagnosed AF was 43.93 mm, while it was 36.53 mm with paroxysmal, 50.22 mm with persistent and 51.15 mm with permanent AF.

The average size of the left atrium in patients with hypertension was 48.3 mm, and was 47.4 mm in the subjects without hypertension. Patients with and without hypertension did not differ significantly according to the size of the left atrium in different types of atrial fibrillation (Table I).

The average ejection fraction (EF) value in first diagnosed AF was 49%, 55.4% in paroxysmal, 48.7% in persistent, 45% in long standing persistent, and 43.98% in the permanent AF. The ejection fraction in patients with hypertension was 49.5%, while it was 48.8% in patients without these co-morbidities. Statistical analysis showed that patients with newly diagnosed atrial fibrillation and hypertension had statistically higher values of EF than patients with first diagnosed atrial fibrillation without hypertension ($Z=2.062$; $p=0.042$) (Table II).

The average value of the pressure in the pulmonary artery in patients with first diagnosed AF was 32.3 mmHg, 28.5 mmHg with paroxysmal, 35.5 mmHg with persistent, 38.7 mmHg with long standing persistent

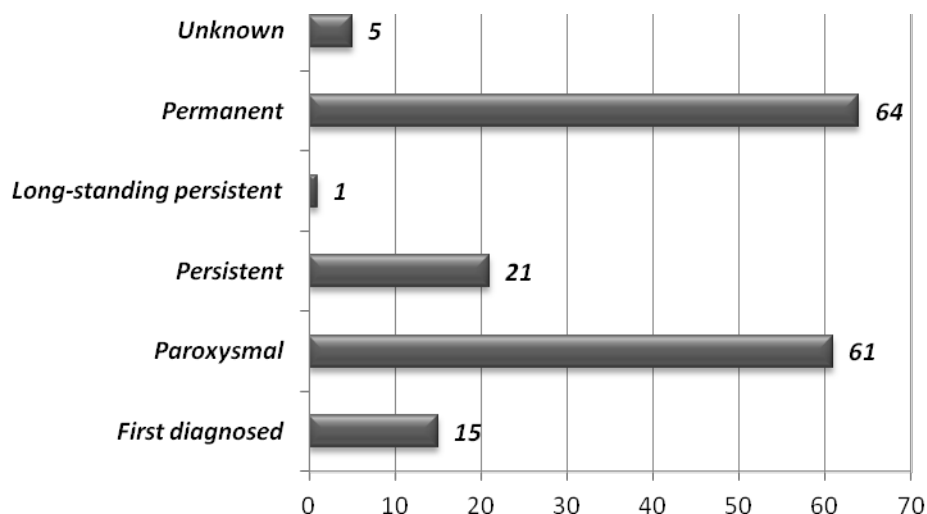


Fig. 1. Different types of atrial fibrillation and their distribution in the study population.

Table I. Left atrium in patients with or without hypertension in different types of atrial fibrillation

	Hypertension		Z	p
	no	yes		
First diagnosed	44.34±11.69	45.70±3.94	0.102	0.118
Paroxysmal	46.27±7.05	44.79±4.52	0.496	0.621
Persistent	50.50±14.85	49.77±3.14	1.004	1.000
Permanent	51.67±9.67	51.48±7.37	0.016	0.987

Z- Man-Whitney U test

Table II. Ejection fraction with or without hypertension in different types of atrial fibrillation.

	Hypertension		Z	p
	no	yes		
First diagnosed	39.17±10.55	53±14.59	2.062*	0.042
Paroxysmal	56.54±12.4	54.77±9.89	0.852	0.394
Persistent	42.5±3.53	51.38±15.98	1.111	0.267
Permanent	45±17.32	43.25±14.75	0.148	0.883

Z- Man-Whitney U test. * statistically significant for $p < 0.05$ **Table III.** Pressure in the pulmonary artery in patients with or without hypertension in different types of atrial fibrillation.

	Hypertension		Z	p
	no	yes		
First diagnosed	39.17±10.55	53±14.59	2.062*	0.042
Paroxysmal	56.54±12.4	54.77±9.89	0.852	0.394
Persistent	42.5±3.53	51.38±15.98	1.111	0.267
Permanent	45±17.32	43.25±14.75	0.148	0.883

Z- Man-Whitney U test

and 41.8 mmHg with permanent AF. The mean value of the pressure in the pulmonary artery in the group with hypertension was 35.9 mm Hg, while in the group without hypertension it was 34.4 mm Hg. The groups did not differ according to the level of pressure in the pulmonary artery ($p = 0.639$) (Table III).

On admission to the Institute 98 (58.7%) patients had used oral anticoagulant therapy (OAT), of whom 95 had used vitamin K antagonists. On discharge 127 (76.1%) used oral anticoagulation therapy (OAT). None of the patients had a stroke in the personal history.

The most commonly used drugs in patients with

AF were beta blockers (93.4%), followed by ACE inhibitors (79.6%) and diuretics (55.7%).

DISCUSSION

Patients with hypertension have a significantly greater risk for development of AF compared to those who do not suffer from increased blood pressure (6). AF and hypertension are often in coexistence, which has a great significance in determining the risk for stroke, as both AF and hypertension are independent risk factors for cerebrovascular insult (7). The

probability for the occurrence of hypertension and AF increases with the age. Also, the incidence of AF is significantly higher in men than in women (8).

Left ventricular dysfunction and heart failure increase the risk for AF, which was confirmed in our study (9, 10). Therapeutic approach in patients with atrial fibrillation and arterial hypertension involves keeping blood pressure under control and introducing oral anticoagulant therapy (OAT) in order to prevent stroke (11). The introduction of OAT in patients with AF extensively reduces the risk for the occurrence of thromboembolic complications, but not for other complications such as heart failure or sudden cardiac death (3). The control of blood pressure in patients with AF is very important because hypertension is a variable factor in determining the risk of bleeding and the regulation of blood pressure significantly reduces the risk for both stroke and bleeding (3). Drugs that have proven to have great importance in the treatment of hypertension, as well as in the prevention of AF, are blockers of the RAAS system. In our study, the most commonly used drugs were beta blockers and angiotensin converting enzyme inhibitors (ACEI).

The present study has once again stressed the importance of educating both patients and physicians about the importance of using OAT in patients with AF. At a reception at the Institute, less than two-thirds of the patients used OAT. The use of vitamin K antagonists or non-vitamin K antagonists significantly reduces the risk of thromboembolic complications (12). When introducing OAT, both the risk for thromboembolic complications and the risk of bleeding should be taken into account.

This study showed high prevalence of arterial hypertension in patients with atrial fibrillation, especially in the group of patients with permanent atrial fibrillation. Patients with hypertension had higher pulmonary arterial pressure and increased size of left atria, as well as greater ejection fraction compared to the subjects without hypertension. In spite of the high prevalence of atrial fibrillation, the use of oral anticoagulation therapy is still low.

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LETTER TO THE EDITOR

NEUTROPHIL GELATINASE-ASSOCIATED APOLIPOPROTEIN IN PATIENTS WITH IODINE-CONTRAST NEPHROPATHY

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To the Editor,

Neutrophil gelatinase-associated lipocalin (NGAL) is a glycoprotein extracted from peroxidase-negative granules in neutrophils (1, 2). In recent years, with the in-depth study of NGAL in many fields, it has been gradually recognized that NGAL can maintain the stable expression of RNAs in many human tissues and organs. For example, the NGAL expression is closely related to kidney injuries and diabetes mellitus (3). With the progression of diabetic nephropathy, the symptoms of glomerular dysfunction are aggravated progressively, and the integrity of renal tubular matrix membrane is further damaged. The activity and content of NGAL are positively correlated with the increased severity of proteinuria, indicating that the presence of NGAL in the serum may indirectly reflect the severity of diabetic nephropathy (4). Acute kidney injury (AKI) refers to a clinical syndrome that occurs when renal functions suddenly deteriorate in a short period (hours or days) due to various reasons (5, 6). In recent years, biological markers, such as NGAL, CysC, KIM-1, and IL-18, have been studied in the timely and accurate diagnosis of AKI. Kidney injury molecule-1 (KIM-1) is an epithelial cell adhesion

molecule present in CD4T lymphocytes and renal proximal convoluted tubular epithelial cells (7). In this study, the sensitivity and specificity of serum cystatin C (sCysC), plasma NGAL (pNGAL), urinary NGAL (uNGAL), urinary KIM-1 (uKIM-1) and urinary IL-18 (uIL-18) in the diagnosis of contrast-induced nephropathy (CIN), as well as the correlation between the above indicators and the serum level of creatinine (sCr), were evaluated (8, 9).

MATERIALS AND METHODS*Patients*

Forty-five CIN patients receiving an iodine contrast medium for computed tomography angiography (CTA) or computed tomography perfusion imaging (CTP) at Affiliated Yancheng Hospital, School of Medicine, Southeast University were selected from May 2016 to October 2018 as an experimental group, while 45 non-CIN patients also receiving the same dose of the iodine contrast medium during the same period were randomly enrolled into a control group. The study was approved by the Ethics Committee of Affiliated Yancheng Hospital, School of Medicine, Southeast University and all patients signed the

Key words: CIN; NGAL; Iodine contrast agent; early renal injury

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informed consent to participate in the study.

Diagnostic criteria: The age of the subjects ranged from 36 to 80 years, with an average age of (62.7 ± 11.4) years. There were 240 males with an average age of (62.6 ± 11.7) years, and 127 females with an average age of (62.8 ± 10.7) years. By signing the informed consent, all subjects agreed to undergo cerebral angiography and/or cerebral perfusion imaging.

Exclusion criteria: Subjects who were allergic to iodine compounds; subjects who had undergone radiography or interventional therapy in the previous week; subjects who had used nephrotoxic drugs in the previous week; pregnant and lactating women; elderly subjects (≥ 80 years old); subjects whose eGFR expression was less than $60 \text{ mL/min/1.73 m}^2$ as evaluated by a preoperative DRD formula; subjects with tumors, severe liver dysfunction, coagulation dysfunction, and severe heart failure (with an ejection fraction of less than 40%).

Sample collection

Ten mL of venous blood samples and 10 mL of urine samples were collected from each subject before angiography and at 8 h, 24 h and 72 h after angiography. The 10 mL venous blood and 10 mL urine samples were collected from the subjects in the control group upon admission. After the blood samples had naturally coagulated at room temperature and the urine samples became stationary, the samples were centrifuged at 3000 rpm for 10 min to collect 3 mL of serum and 3 mL of urine supernatant, respectively, which were then stored at -80°C . Subsequently, the levels of sCr, sCysC, NGAL, uNGAL, uKIM-1 and uIL-18 were detected. The sensitivity and specificity of sCr, sCysC, pNGAL, uNGAL, uKIM-1 and uIL-18 in the diagnosis of CIN, as well as the correlation between the levels of the above indicators and the changes in sCr levels, were analyzed by comparing the changes of the above biological indicators before and after use of the contrast agent and plotting of ROC curves.

Detection index

NGAI, KIM-1 and IL-18 were detected by enzyme-linked immunosorbent assay (ELISA) (Shanghai Steady Biotechnology Co., Ltd., China). ELISA was used to detect IL-18 in serum and urine. sCr and sCysC were detected by a spot CBLATCOULIIBAU5800 automatic biochemical analyzer (Beckman Coulter, Inc., USA). Levels of hemoglobin, serum albumin, total cholesterol

and urine proteins were also detected routinely. NGAI, KIM-1 and IL-18 were detected by ELISA kit (Shanghai Hengtai Biotechnology Co., Ltd., China). IL-18 in serum and urine was detected by ELISA; sCr and sCysC were detected by an AU5800 automatic biochemical analyzer (Beckman Coulter, USA); hemoglobin, serum albumin, total cholesterol and urinary protein levels were measured by an XN-1000 blood analyzer (Sysmex, Japan) and VITROS5600 automatic biochemical immunoanalyzer (Johnson, USA); using a b221 blood gas analyzer (Cobas, Japan), femoral artery blood or radial artery blood was collected when breathing natural air at rest to detect arterial blood gas analysis.

Observation index

The difference of sCr and other biological indicators was compared between the contrast group and the control group; the changes of biological indicators before and after the use of contrast medium were compared between the CIN group and the non-CIN group. The ROC curve was drawn and the area under the curve (AUC) was calculated to evaluate the sensitivity and specificity of biological indicators. The correlation between the indicators and the changes of sCr was analyzed.

Statistical analysis

SPSS22.0 software was used for statistical analysis. Measurement data were expressed by mean $\pm$ standard deviation, while categorized data were expressed by percentages. *T*-test was used to compare the values with a normal distribution, while a variance analysis was used to compare multiple groups. Furthermore, *chi*-squared test was used to classify the data. $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

Changes of biological indexes in the CIN and non-CIN groups before and after angiography

In the CIN group, the level of sCr increased significantly at 24 h and 72 h after angiography ($P < 0.05$). In the non-CM group, there was no significant change in the level of sCr before and after angiography ($P > 0.05$). The sCr level in the CIN group was significantly higher than that in the non-CIN group at 24 h and 72 h after angiography ($P <$

0.01) (Fig. 1A). In the CIN group, sCysC increased significantly 24 and 72 hours after angiography compared with before angiography, with significant difference ($P < 0.05$). In the non-CIN group, there was no significant change in sCysC levels before and after angiography ($P > 0.05$). The level of sCysC in the CIN group was significantly higher than that in the non-CIN group since 8 hours after angiography ($P < 0.01$) (Fig. 1B).

In the CIN group, the level of pNGAL started to increase at 8 h after angiography and the difference became significant at 24 h and 72 h after angiography ($P < 0.05$). However, the level of pNGAL at 72 h after angiography was lower than that at 24 h after angiography. In the non-CIN group, the level of pNGAL was not changed significantly before and after angiography ($P > 0.05$). The level of pNGAL in the CIN group was significantly higher than that in the

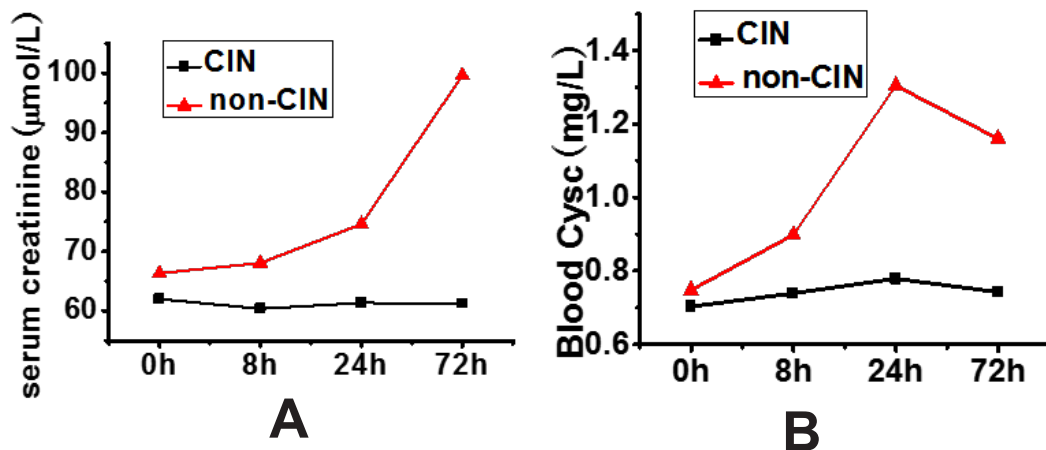


Fig. 1. Changes before and after angiography in the CINN group and non-CINN group. *A)* Changes of serum creatinine. *B)* Changes in the level of CysC.

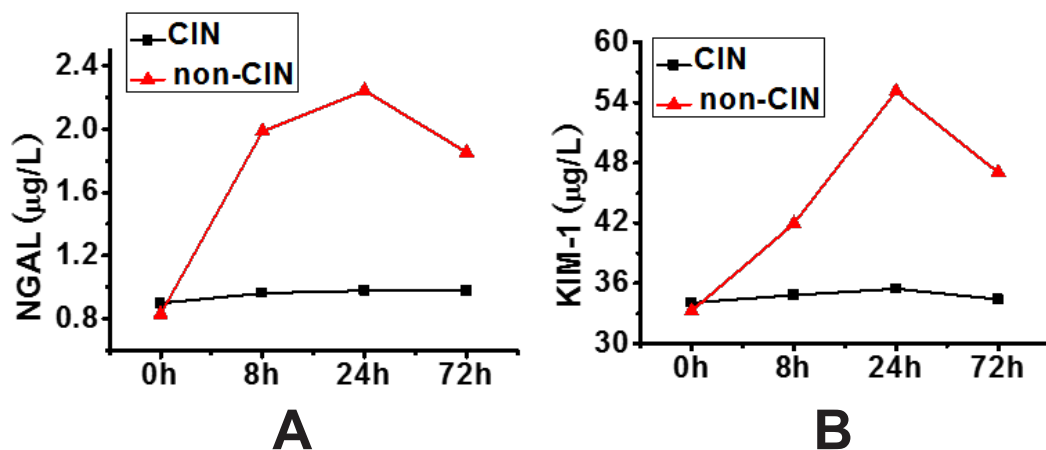


Fig. 2. Changes before and after angiography in the CINN group and non-CINN group. *A)* Changes of urinary NGAL. *B)* Changes of blood KIM-1.

non-CIN group at 8 h after angiography ($P < 0.01$).

In the CIN group, the level of uNGAL started to increase at 8 h after angiography, and the difference became significant at 24 h and 72 h after angiography ($P < 0.05$). However, the level of uNGAL at 72 h after angiography was lower than that at 24 h after angiography. In the non-CIN group, the level of uNGAL at 24 h after angiography was significantly different from that before angiography ($P < 0.05$), but the levels of uNGAL at 8 h, 24 h and 72 h after angiography showed no significant difference ($P > 0.05$). The level was significantly higher than that in the non-CIN group starting from 8 h after angiography ($P < 0.01$) (Fig. 2A).

In the CIN group, the level of uKIM-1 started to increase at 8 h after angiography, and the difference became significant at 24 h and 72 h after angiography ($P < 0.05$). In addition, the level of uKIM-1 in the CIN group reached its peak at 24 h after angiography. In the non-CIN group, the level of uKIM-1 was not changed significantly before and after angiography ($P > 0.05$). Moreover, the level of uKIM-1 in the CIN group was significantly higher than that in the non-CIN group at all time points after angiography ($P < 0.01$) (Fig. 2B).

In the CIN group, the level of uIL-18 started to increase at 8 h after angiography, and the difference became significant at 24 h and 72 h after angiography ($P < 0.05$). In addition, in the CIN group, the level of uIL-18 also reached its peak at 2 h after angiography.

In the non-CIN group, there was no significant change in the level of uIL-18 before and after angiography ($P > 0.05$). The level of uIL-18 in the CIN group was significantly higher than that in the non-CIN group at all time points after angiography ($P < 0.01$).

ROC curves of uNGAL, sCysC, pNGAL, uKIM-1 and uIL-18 in the CIN group

At 8 h after angiography, the AUC of the ROC curve of each biological index was ordered as uNGAL (0.983) > pNGAL (0.975) > uIL-18 (0.856) > uKIM-1 (0.809) > sCysC (0.742) > sCr (0.718). The sensitivity of these indicators was 96.8%, 90.3%, 87.1%, 83.9%, 83.9%, and 80.6%, respectively, while their specificity was 100%, 100%, 90%, 76.7%, 56.7%, and 63.3%, respectively. The value of pNGAL and NGAL in the early diagnosis of CIN became higher at 8 h after angiography, while the diagnostic value of uIL-18, KIM-1 and sCysC was higher than that of sCr.

At 24 h after angiography, the AUC of ROC curve of each biological index was ordered as uNGAL (0.988) > pNGAL (0.984) > uIL-18 (0.954) > uKIM-1 (0.918) > sCysC (0.896) > sCr (0.759). The sensitivity of these indicators was 96.8%, 93.5%, 93.5%, 96.8%, 87.1%, and 87.1%, respectively, while their specificity was 100%, 100%, 100%, 96.7%, 90% and 63.3%, respectively. At 24 h after angiography, the early diagnostic values of various biological indicators became significantly higher

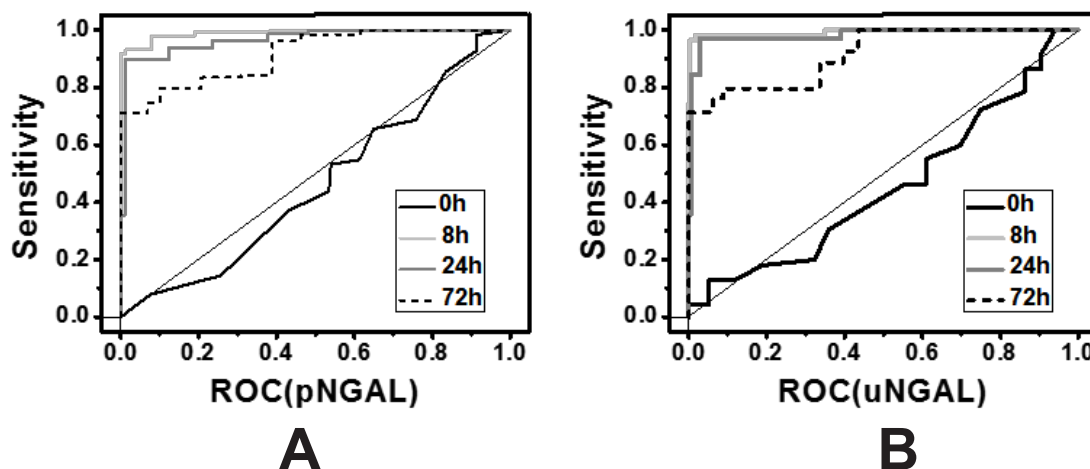


Fig. 3. A) ROC curve of pNGAL. B) ROC curve of uNGAL.

than their corresponding values obtained at 8 h after angiography. In addition, at 24 h after angiography, the diagnostic value of NGAL, pNGAL, uIL-18 and uKIM-1 became higher along with increased sensitivity and specificity, but the diagnostic value of uNGAL was still the highest.

At 72 h after angiography, the AUC of ROC curve of each biological index was ordered as sCysC (0.946) > uKIM-1 (0.92) > uNGAL (0.919) > pNGAL (0.917) > uIL-18 (0.891) > sCr (0.865). The sensitivity of these indicators was 90.3%, 87.1%, 77.4%, 71%, 87.1%, and 83.9%, respectively, while their specificity was 86.7%, 90%, 96.7%, 100%, 96.7%, and 83.3%, respectively. The diagnostic value of sCysC increased to the highest level at 72 h after angiography. In addition, although the diagnostic value of other biological indicators remained high, the diagnostic value of NGAL was slightly reduced compared to its corresponding value obtained at 2 h after angiography.

In the CIN group, the early diagnostic value of combined blood indexes (sCysC and pNGAL) or urine indexes (uNGAL, uKIM-1, and uIL-18) was determined. In respect to the combined blood indexes of sCysC and pNGAL, the AUC values of their ROC curves at 8h, 24h and 72h after angiography were 0.977, 0.984 and 0.962, respectively; their sensitivity was 90.3%, 93.5% and 80.6%, respectively; and their specificity was 100%, 100% and 96.7%, respectively, which were slightly better than the diagnostic value of biological markers, such as NGAL, at 72 h after angiography.

Regarding the combined urine indexes of uNGAL, uKIM-1, and uIL-18, their AUC, sensitivity and specificity were 0.999%, 96.8% and 100%,

respectively. The AUC of the ROC curve was up to 1.0 at 24 h and 72 h after angiography, and the sensitivity and specificity of these indicators were both 100%, which were better than those of biological markers such as NGAL and the combination of sCysC and pNGAL.

Analysis of correlation between biological indexes and sCr

The correlation between the level of sCr and that of sCysC, pNGAL, uNGAL, uKIM-1, and uIL-18 at 8, 24 and 72 h after angiography was ordered as follows: at 8 h after angiography, the correlation coefficient of uKIM-1 (0.778) > sCysC (0.755) > uIL-18 (0.702) > uNGAL (0.674) > pNGAL (0.662); at 24 h after angiography, the correlation coefficient of uNGAL (0.773) > pNGAL (0.723) > sCysC (0.676) > uIL-18 (0.672) > uKIM-1 (0.646); at 72 h after angiography, the correlation coefficient of uKIM-1 (0.872) > uNGAL (0.871) > pNGAL (0.86) > sCysC (0.774) > uIL-18 (0.726). These indexes were significantly and positively correlated with the level of sCr ($P < 0.01$) (Table I).

DISCUSSION

The criteria for diagnosing CIN by IGAL have not yet been unified (11). In this study, the level or extent of creatinine increase could not meet the criteria for the diagnosis of CIN. At this time, the levels of pNGAL and uNGAL increased significantly, which had significant diagnostic value. The level of uKIM-1 in the CIN group also increased, signifying that uKIM-1 could also reflect the early renal injury of CIN. IL-18 is an inactive

Table I. *r* and *P* values of sCysC, pNGAL, uNGAL, uKIM-1, uIL-18 and sCr at different time points after angiography.

Item	8 h after angiography		24 h after angiography		72 h after angiography	
	r	P	r	P	r	P
sCysC	0.758	<0.01	0.673	<0.01	0.768	<0.01
pNGAL	0.665	<0.01	0.725	<0.01	0.876	<0.01
uNGAL	0.678	<0.01	0.769	<0.01	0.872	<0.01
uKIM-1	0.774	<0.01	0.635	<0.01	0.869	<0.01
uIL-18	0.709	<0.01	0.668	<0.01	0.719	<0.01

precursor molecule of 23kDa mainly synthesized by monocytes, macrophages and renal tubular epithelial cells. After inducing the secretive activity of proximal renal tubules, IL-18 is lysed into urine (12). The value of sCysC and pNGAL in the early diagnosis of CIN was only slightly higher than that of NGAL and other biomarkers at 72 h after angiography. The AUC of the combination of urine NGAL, KIM-1 and IL-18 at 24 and 72 h after angiography was up to 1.0, while the diagnostic sensitivity and specificity of these factors were both 100%, significantly higher than those of sCysC, pNGAL, uNGAL, uKIM-1 and uIL-18 alone or of the combination of sCysC and pNGAL. The detection of the combination of these three indicators in urine has the advantages of simple sample collection and non-invasive examination. In summary, compared with the control group, the levels of pNGAL, uNGAL, uKIM-1 and uIL-18 in the experimental group increased significantly at 8 h after angiography ($P < 0.05$), while the level of sCysC increased significantly at 24 h after angiography. The combination of uNGAL, uKIM-1 and uIL-18 showed a better value in the early diagnosis of CIN than NGAL and other biological markers or the combination of sCysC and pNGAL.

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LETTER TO THE EDITOR

CONCURRENT APATINIB AND LOCAL RADIATION THERAPY FOR ADVANCED ESOPHAGEAL ADENOCARCINOMA

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To the Editor,

Esophageal cancer is one of the most fatal diseases worldwide, mainly because of its rapid progression and poor prognosis (1). The incidence of esophageal adenocarcinoma (EAC) has markedly increased in North America and Europe in the last decades. However, esophageal squamous cell carcinoma (ESCC) is still the predominant subtype of esophageal cancer, especially in China. EAC usually presents at a late stage, with most patients presenting with T3 or T4 disease. Radiotherapy and chemotherapy are the main treatments for patients with advanced esophageal cancer or postoperative recurrence and metastasis. However, no effective treatment is available for patients with poor physical conditions who cannot tolerate chemotherapy.

Angiogenesis is one of the most important mechanisms for the emergence and development of malignant tumors (2), and frequently becomes overexpressed in esophageal cancers (3). Studies have shown that high VEGF expression in tumor tissues is significantly associated with radiotherapy resistance. Apatinib is a novel oral antiangiogenic agent that shows efficacy in the inhibition of tumor angiogenesis (4, 5). However, the efficacy of treating esophageal cancer with apatinib is not clear, and no clinical studies have examined the efficacy and safety of concurrent apatinib and local radiation therapy for advanced esophageal adenocarcinoma in China.

Here, we report the case of a 68-year-old male patient with advanced esophageal adenocarcinoma who received concurrent apatinib and local radiation therapy in our hospital.

Case presentation

On February 3, 2017, a 68-year-old man presented with idiopathic swallowing difficulty and obstruction in the previous month. The patient's symptoms gradually worsened and were accompanied by pain after eating. During that period, there had been no fever, no chest pain, no cough, and no hematemesis or melena, and his weight was significantly reduced. Esophagoscopy showed that from the incisors, 37 cm from the esophagus, irregular tumors were blocking the lumen, and the surface of the tumor was uneven, with erosion and necrosis. Esophageal biopsy revealed adenocarcinoma. (Fig. 1). The PET-CT diagnosis was esophageal cancer with multiple peritoneal metastases (peritoneal and pelvic floor peritoneal local thickening) and abdominal and pelvic fluid (Fig. 2).

The patient was administered 2 cycles of chemotherapy with docetaxel and cisplatin from February 3, 2017 to March 13, 2017. After two cycles of chemotherapy, the patient's swallowing pharyngeal sensation improved. However, the treatment was terminated because

Key words: esophageal adenocarcinoma; apatinib; molecular targeted therapy; radiation therapy

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the patient could not tolerate the associated gastrointestinal disturbances. On March 31, 2017, adjuvant radiotherapy was administered. Before radiotherapy, computed tomography (CT) showed abdominal segment esophageal wall thickening, esophageal stricture, peritoneal thickening and a thickened stomach wall. Oral apatinib (500 mg once a day) combined with and following radiotherapy was prescribed. The patient agreed to the treatment plan and signed therapeutic communication and informed consent. In an attempt to improve tolerance to treatment, palliative intensity-modulated radiation therapy (IMRT) was used. The patient received a dose of 45 Gy in 25 fractions to the esophageal lesion and abdominal lymph node. According to the Response Evaluation Criteria in Solid Tumors (RECIST), the clinical effect was partial response (Figs. 3 and 4 A-C). Therefore, the patient received further radiotherapy (50 Gy in 28 fractions) to the esophageal lesion.

Two months after therapy, chest and abdominal CT scans indicated stable disease, and the swallowing obstruction had improved. During the administration of apatinib, the patient experienced some toxicity. In the initial stage of taking this medicine, he experienced a bout of stomach upset and loss of appetite; after administering oral omeprazole, the symptoms were relieved. Hematologic toxicity, hypertension, renal dysfunction, proteinuria, and hand-foot syndrome were not observed during apatinib therapy.

In August 2017, the patient suddenly experienced abdominal pain, and abdominal CT and ultrasound showed that there were large amounts of ascites (Fig. 4 D-F). Therefore, the ascites were drained. and after 2 weeks of therapy, the symptoms were relieved. Tumor markers were evaluated during the treatment (Fig. 5). The patient's condition is presently stable. Currently, the patient is taking apatinib as maintenance treatment; we will continue to follow-up regularly with this patient.

The patient signed informed consent for receiving apatinib, which was approved by the Medical Ethics Committee of Harbin Medical University Tumor Hospital. Written informed consent was obtained from the patient for

publication of this case report and accompanying images.

DISCUSSION

Initial treatment approaches for EAC depend on several factors, including the stage and grade of the tumor, the location of the tumor, the comorbidities and the age of the patient. In the U.S.A., the most common approach involves neoadjuvant chemotherapy and external beam radiotherapy (CRT), but their 5-y rate of survival is less than 15%.

Molecular targeted therapies have the potential to improve oncological outcomes. A growing body of data suggest a correlation between VEGF-VEGFR expression and prognosis in esophageal cancer (6). Apatinib targets the intracellular ATP-binding site of VEGFR-2 (7). The high expression of VEGF in tumor tissues is significantly associated with radiotherapy resistance (8). Apatinib was the first oral VEGFR-2 inhibitor (9). There have been several preclinical and clinical trials proving the effect and safety of apatinib (10). In patients with advanced or metastatic adenocarcinoma of the stomach or gastroesophageal junction, it was also verified that the median overall survival (OS) was significantly improved in the apatinib group compared with the placebo group (6.5 months *vs* 4.7 months, respectively, $P = 0.0149$); the same was true for PFS (2.6 months *vs* 1.8 months, respectively, $P < 0.01$) (11). Recently, many studies have shown that apatinib can directly act on tumor cells and promote their apoptosis.

Although several trials have shown that the addition of conventionally fractionated radiation therapy to antiangiogenic agents is well tolerated, some reports suggest that it could increase luminal gastrointestinal toxicity in combination therapy. In the current study, a recommended dose of 500 mg was used (12).

Advanced esophageal adenocarcinoma may benefit from concurrent apatinib and local radiation therapy when chemotherapy is not tolerated or successful. Further studies are needed to investigate the clinical outcomes and toxicities

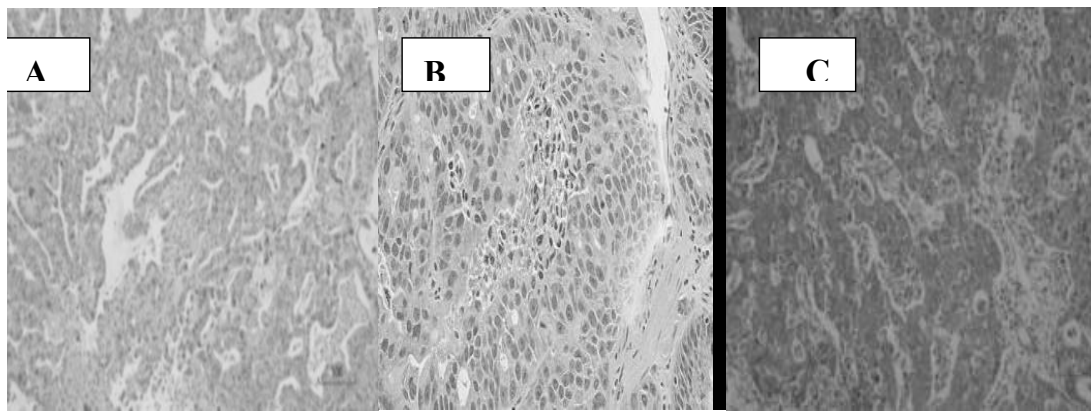


Fig. 1. *A, B)* Hematoxylin and eosin staining indicating differentiated esophageal adenocarcinoma (100×). *C)* Immunohistochemistry showing the expression of VEGF (+) (100×).

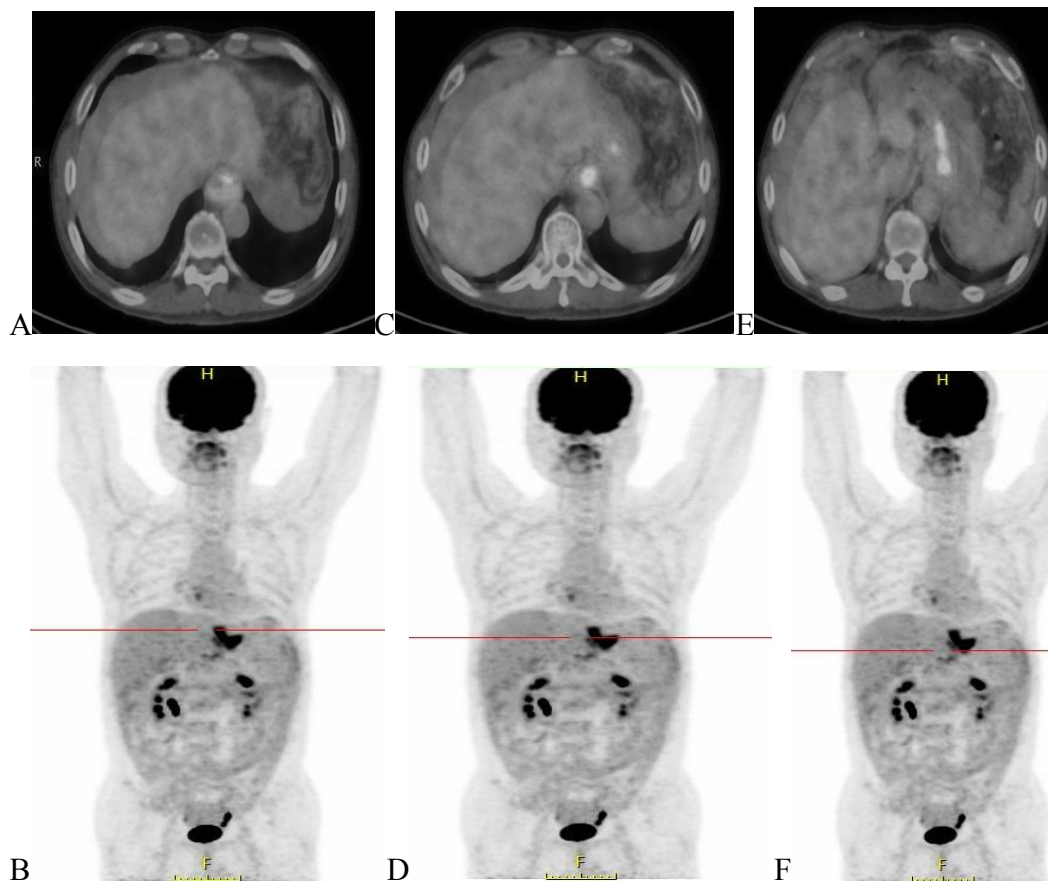


Fig. 2. *PET-CT scans. A-B)* Initial esophageal lesion. *C-D)* Initial esophageal lesion and abdominal lymph node. *E-F)* Initial abdominal lymph node.

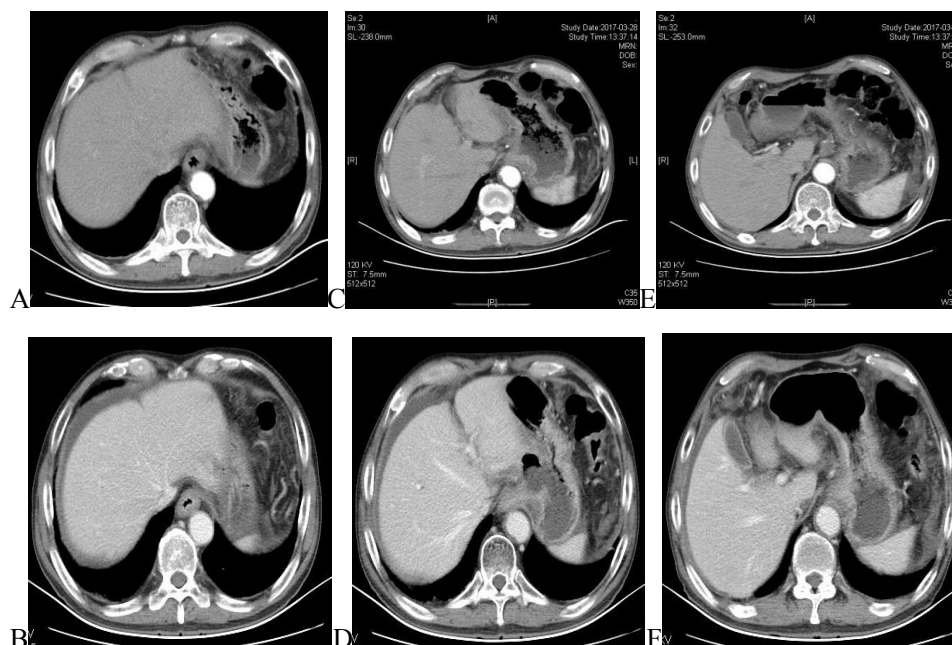


Fig. 3. CT scans. *A)* Esophageal lesion before apatinib treatment. *B)* Esophageal lesion after apatinib treatment. *C)* Esophageal lesion and abdominal lymph node before concurrent apatinib and radiotherapy. *D)* After concurrent apatinib and radiotherapy. *E)* Abdominal lymph node before concurrent apatinib and radiotherapy. *F)* Abdominal lymph node after concurrent apatinib and radiotherapy.

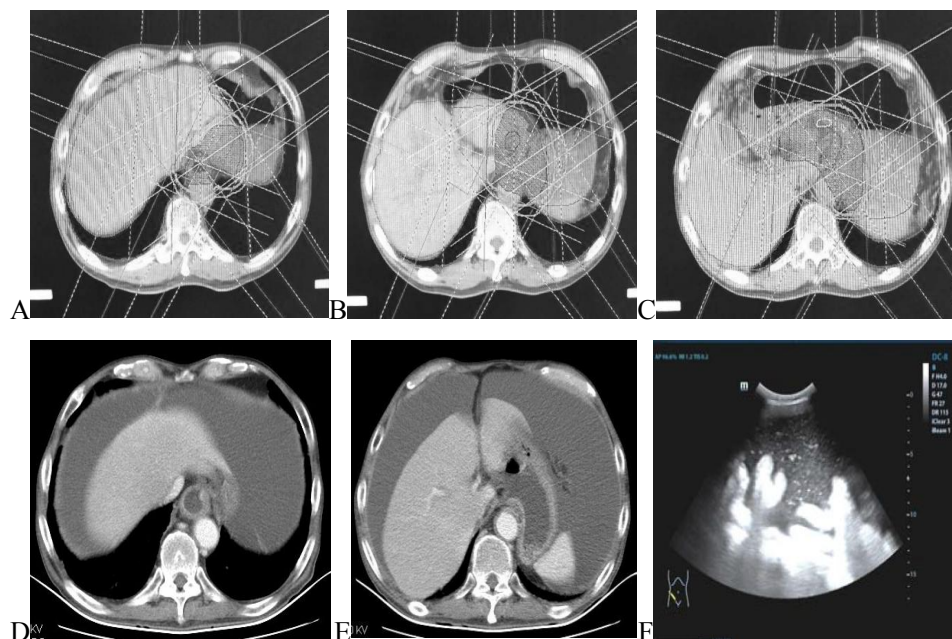


Fig. 4. Pre-radiotherapy and abdominal effusion after radiotherapy. *A)* Planning Target Volume of the esophageal lesion. *B)* Planning Target Volume of the esophageal lesion and abdominal lymph node. *C)* Planning Target Volume of the abdominal lymph node. *D)* CT showing large amount of ascites at the esophagus level. *E)* CT showing large amount of ascites at the abdominal lymph node level. *F)* Abdominal ultrasound showing large amount of ascites.

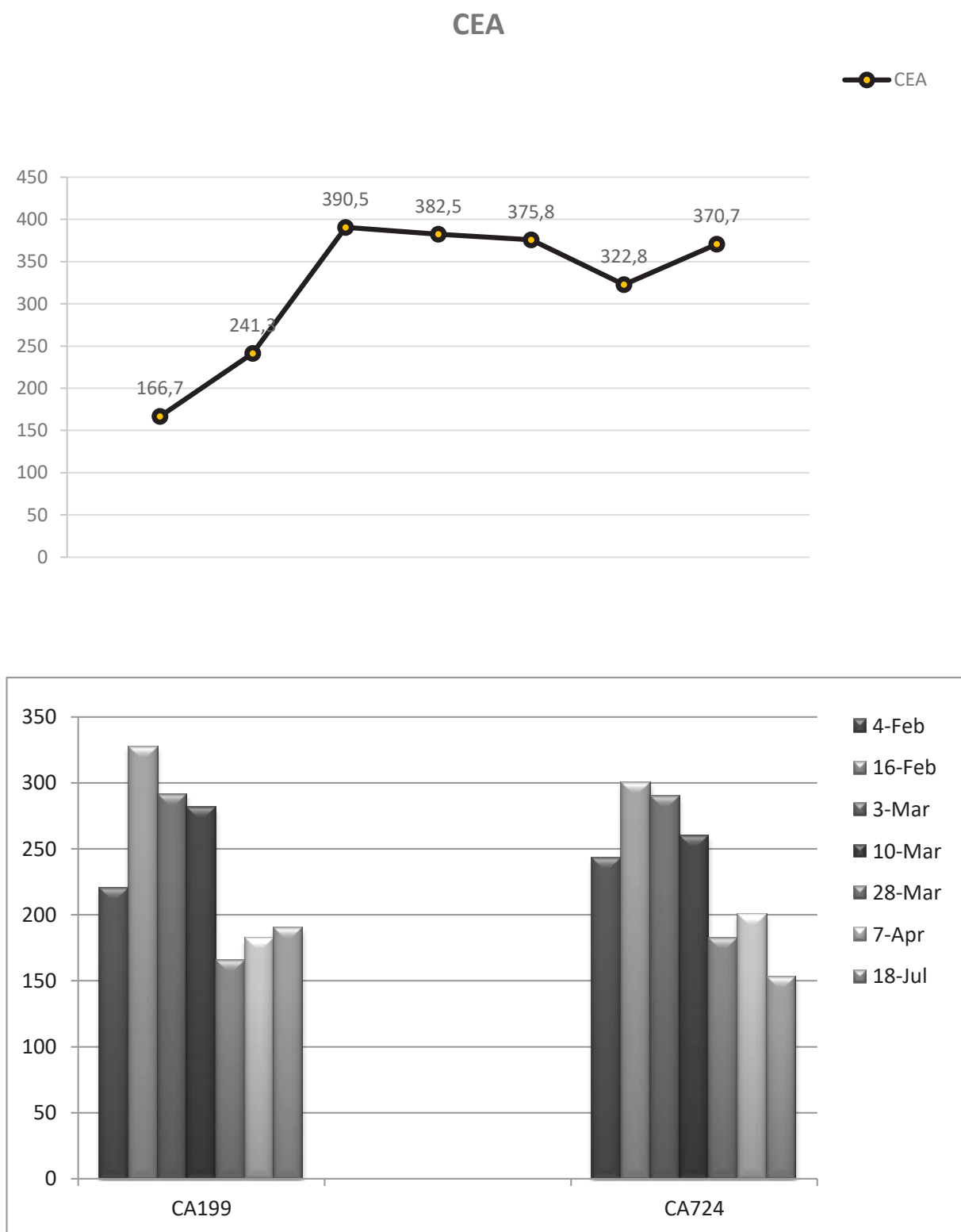


Fig. 5. The time course of the tumor marker concentrations measured in the patient.

associated with concurrent apatinib and radiation therapy in esophageal adenocarcinoma.

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LETTER TO THE EDITOR

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS CAUSED DUODENAL STENOSIS

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To the Editor,

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used in fever, pain and inflammation because they possess antipyretic, analgesic and anti-inflammatory properties (1). The leading causes of benign gastric outlet obstruction are peptic ulcer disease and corrosive ingestion (2). It is well known that the use of NSAIDs is associated with many types of gastrointestinal injuries such as protein losing enteropathy, peptic ulcer disease, and diaphragm disease of the bowel (3). NSAIDs are not only commonly prescribed but are often used by patients chronically. While it is often rare to find gastric outlet obstruction due to chronic NSAID use (4), duodenal web-like strictures due to long-term NSAID use have been reported in several cases (5).

Gastric outlet obstruction is an obstruction in the antropyloric area or in the bulbar or postbulbar duodenal segments (2). Endoscopic balloon dilatation (EBD) plays a critical role in the management of benign gastric outlet obstruction. EBD can also be performed under the guidance of endoscopy (6), and the safety and efficacy of this procedure have been reported in many studies (7). The initial experience of EBD was under fluoroscopic guidance; subsequently it has been shown that EBD can be performed under

endoscopic guidance only (8). Here, we report a case of gastric outlet obstruction caused by NSAID use. The aim of this study was also to examine the nature, range, and causation of the gastrointestinal side effects associated with the use of NSAIDs, and to consider how these may be reduced or modified.

Case presentation

A 45-year-old female patient was admitted to The Second Hospital of Jilin University, Jilin, China, on April 18, 2018, due to nausea and vomiting which had lasted for one month, and was aggravated with fatigue for 2 days. The vomitus contained the stomach's content, no fresh food, no blood and no coffee. The patient had mild abdominal distension, occasional acid reflux, no heartburn, no abdominal pain, or black stool. In the past history, she had rheumatoid arthritis for more than 16 years. She reported pains in hands and feet as well as in small joints. She received "pain relief pills" treatment all year round. Eight months previously, she had undergone a gastroscopy at a local hospital and a pyloric ulcer was found. Post-hospital examination showed that she had chronic disease, malnutrition, no abnormal heart, lungs, soft abdomen and no tenderness. Touch examination showed no enlargement of liver or spleen. The joint

Key words: nonsteroidal anti-inflammatory drugs; endoscopic balloon dilatation; pylorus; duodenal stenosis

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deformities showed in both hands.

The patient referred to the hospital for a gastroscopy, and an ulcer with a diameter of about 0.8 cm at the pylorus was discovered. The ulcer base was covered with white moss and the surrounding mucosa was congested and edematous. The duodenum was apparently narrow in the lumen of the intestine at the junction of the bulb and had an opening diameter of about 0.2 cm. White ulcer scars could be seen in the stenosis. *Helicobacter pylori* were negative. After routine examination, there was no abnormality and the endoscopic

incision was performed. The stenosis was found to be membrane-like with a thickness of about 1.5 mm. After the radial incision, the conventional endoscope passed smoothly and then the dilatation balloon was expanded up to 13 mm. The operation was carried out without problems (Fig. 1). After acid-suppressive treatment, the patient was kept on a semi-liquid diet 12 hours after surgery and she had no obvious abdominal distension and no nausea or vomiting. The patient was then successfully discharged.

The patient was informed gave written informed consent for this study.

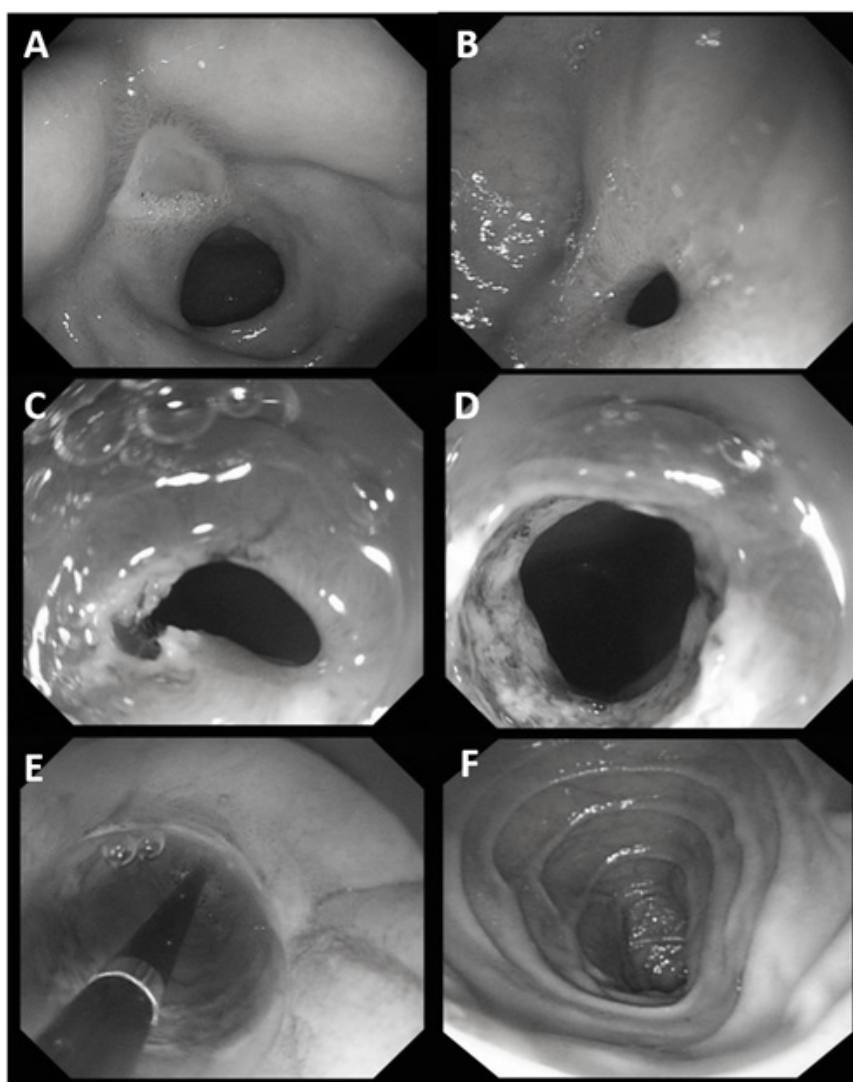


Fig. 1. Gastroscopy examination. *A)* Pyloric ulcer. *B)* Membrane-like stenosis at the junction of the duodenal bulb. *C)* Needle knife starts radial incision. *D)* Stenosis completely cut. *E)* After full incision, the balloon was expanded to a diameter of 13 cm. *F)* Conventional gastroscopy successfully passed through the stenosis into the duodenal descending segment.

DISCUSSION

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, indomethacin, diclofenac, and compound aminopyrine phenacetin, are the most commonly used drugs for inflammation, arthritis and cardiovascular protection. NSAIDs are one of the commonly used drugs for the prevention and treatment of cardiovascular and rheumatic diseases. The adverse reactions are mainly manifested in the gastrointestinal tract, liver, nervous system, blood system, allergy and cardiovascular system (9). Ramakrishnan et al. (10) made a further clinical comprehensive analysis of the clinical manifestations of acute and chronic damage caused by NSAIDs. Endoscopic findings on chronic injury often appear as gastrointestinal mucosal deposition/erosion, erythema, submucosal hemorrhage, small and shallow ulcer and scars. Chronic damage to the gastrointestinal tract has more typical ulcer symptoms and is commonly associated with bleeding and perforation leading to stenosis (11), but the cause is unclear. The hypothesis is that fibrous tissue proliferation may be induced by chronic inflammation. The stenoses are rare and occur in both the small and large intestines.

At present studies, there are three main types of drugs used for preventing gastric and duodenal injury related to NSAIDs, such as misoprostol for proton pump inhibitor and H₂ receptor blocker, but the biggest problem is gastrointestinal adverse reactions, such as abdominal pain, and diarrhea. The occurrence of adverse reactions of misoprostol is positively related to the prescribed dose, which many patients cannot tolerate. However, the membranous stenosis caused by NSAIDs is short, and to achieve the best effectiveness, incision with balloon expansion is needed. This method is simple, convenient, economical and effective and suitable for implementation in hospitals at any level.

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LETTER TO THE EDITOR

EVALUATION OF PLANT GROWTH-PROMOTING ACTIVITY
OF STRAIN HBCD-sjtu

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To the Editor,

Plant growth-promoting bacteria (PGPB) are termed as free-living soil, rhizoplane, rhizosphere and phyllosphere bacteria useful for plants in some certain circumstances (1). They can help plants by promoting their growth, health and development. Bacteria may bring about progress in plant growth in two ways: firstly, indirect influences of soil-created infections via competition for nutrients such as the siderophore-facilitated struggle for iron or the stimulation of systemic resistance in host plants (2); secondly, the direct influences, like the generation of phytohormones and enzymes participating in growth regulator metabolism such as indole acetic acid (IAA), 2,3-butanedial, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, cytokinins (3), permanent nitrogen supply to host plants or the solubilization of soil phosphorus. Several essential physiological functions comprising tissue differentiation, cell growth or division and responses to gravity or light are regulated by IAA, which is the principal auxin in plants (4). An important function of IAA has also been observed in the stimulation of the plant defense system along with its role in plant development (5). In addition, the role of IAA as a signaling molecule has also been shown by some studies on bacterial communications (6). There is a

correlation of PGPB with some plant species existing in various environments (7). The purpose of the present study was to explore the biological properties of the Strain HBCD-sjtu purified from a landfill in Shanghai. Properties of Hexabromocyclododecane (HBCD-sjtu) were investigated for the formation of plant growth stimulating materials.

MATERIALS AND METHODS

Seed germination experiment

The clean seeds were germinated in 20 cm diameter plastic pots, each containing 600 g of soil. The pots were watered with 400 ml Hoagland's solution. Each pot was chosen to germinate three identical seeds. For the control, seeds were treated with 1 mL LB medium while for inoculation treatment strain HBCD-sjtu suspension (1.0×10^9 CFU/mL) one mL per plant was used.

Physiological measurements

Physiological and growth measurements were calculated when the plant was reaped after 60 days. Calculations of the growth parameters like leaf, shoot and root lengths (cm) were made with a ruler after separating the plants from the pots. Leaf area/plant was divided by some leaves/plant for determination of leaf area average. Shoots were directly weighed for fresh weight (g),

*Key words: HBCD-sjtu; plant growth promotion; biofertilizers; indole acetic acid (IAA)**Corresponding Author:*

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however for study of dry weights (g), the plant parts were oven-dried at 75°C for 2 days.

Determination of chlorophyll contents

Chlorophyll contents were calculated by a previously reported method (8). Fresh leaf samples were macerated with 80% acetone and centrifuged at 10,000 rpm at 4°C for 10 min. The optical density (OD) of the supernatants for chlorophyll content were checked at 645 and 663 nm.

Screening of Strain HBCD-sjtu for production of plant growth-promoting substances

The train HBCD-sjtu was isolated from a landfill in Shanghai, People's Republic of China (9). The computational and *in vitro* analysis of an HBCD degrading gene (*dehHZI*) from Strain HBCD-sjtu was performed (10). In the present study, different enzymatic activities, e.g., lipase, amylase, esterase and protease of the HBCD-sjtu strain, were analyzed via plate technique utilizing tween-20, starch, tween-80 and skim milk as substrates, respectively. Certain plates were used to pour bacterial suspensions for individual enzymes, and incubation was performed at 30°C for 48 h. Determination of clear zone was carried out by measuring the diameter. Likewise,

spot inoculation of Strain HBCD-sjtu was performed on Pikovskaya's agar medium followed by incubation at 30°C for 48 h. After incubation, the diameter was measured to calculate the clear zone surrounding the growth which revealed phosphate solubilization (11). Ammonia production was assessed by inoculation of the Strain HBCD-sjtu into 5 ml of sterile peptone broth followed by incubation for 48 h at 30°C. Following incubation, the tubes were supplemented with 0.5 ml of Nessler's reagent. A yellow color occurred specifying ammonia production. In order to screen the production of acetoin, the Voges-Proskauer test was applied as a qualitative technique. MR-VP broth in the amount of 2 ml was used for inoculation of the strain separately and incubated for 48 h at 30°C. After incubation, the tubes were supplemented with 1 ml of bacterial culture, 3 ml of newly made α -naphthol (5%) in absolute ethanol and 1 ml of 40% potassium hydroxide (KOH). Acetoin existence was detected by the appearance of a red color.

Determination of Strain HBCD-sjtu for tolerance in stress conditions

Five ml of nutrient broth was used for inoculation of all plant-related bacteria for 48 h at 30°C. Sodium chloride

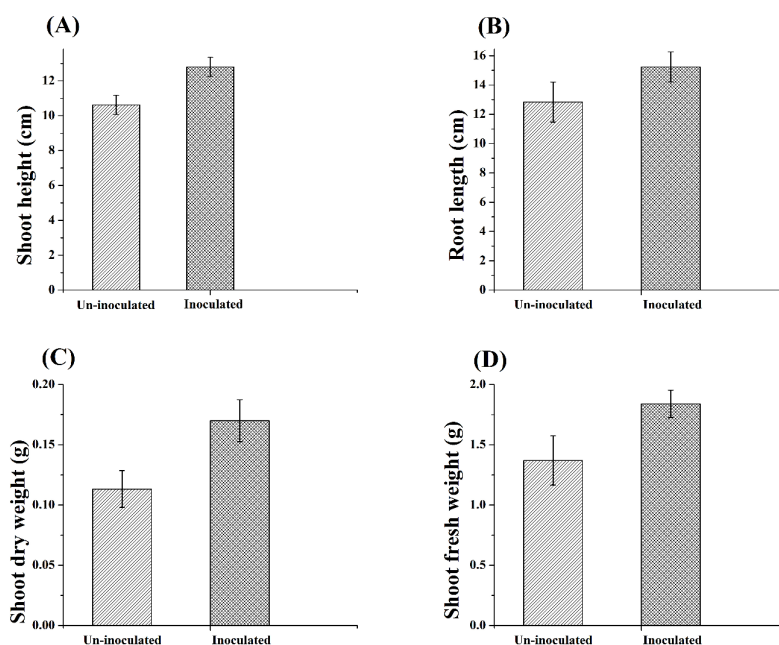


Fig. 1. A) Effect of Strain HBCD-sjtu inoculation on shoot height. B) Root length. C) Shoot dry weight. D) Shoot fresh weight.

(NaCl) 3% and hydrogen peroxide (H_2O_2) 3% were mixed with cell cultures and treated for 2 h. Subsequently, 10 μl of the treated cells were put down on NaCl and allowed to incubate for 24 h at 30°C (1). To determine the stress of metal ion, 10 μl of cell culture were put down on NA augmented with 1 mM of mercury chloride (HgCl_2), ferric chloride (FeCl_3), zinc sulfate (ZnSO_4), manganese sulfate (MnSO_4), nickel sulfate (NiSO_4), copper sulfate (CuSO_4), silver sulfate (AgSO_4), aluminium sulfate (AlSO_4), cadmium sulfate (CdSO_4), lead nitrate ($\text{Pb}(\text{NO}_3)_2$) and cobalt sulfate (CoSO_4). Incubation of the plates was allowed for 48 h at 30°C .

Growth and IAA production of HBCD-sjtu strain

Sixty ml of nutrient broth augmented with 3 mg/ml of L-tryptophan or without L-tryptophan were used for inoculation of Strain HBCD-sjtu and kept for incubation at 30°C . The cultures were assembled at certain incubation phases of 0, 12, 24, 36, 48, 60 and 72 h. Spectrophotometric analysis was carried out to examine bacterial growth by calculating the optical density at 600 nm. Next, centrifugation of cultures was carried out for 10 min at 10,000 rpm. One ml of supernatant was isolated in a fresh tube and an equivalent volume of Salkowski's reagent

was mixed, then kept in the dark at room temperature for 30 min. For screening of IAA production, optical density was evaluated at 530 nm.

RESULTS

Growth, biomass, leaf no and chlorophyll improvement

The Strain HBCD-sjtu showed improvement on the growth of wheat cultivation. Shoot height and root length were increased by 60.23% and 51.21%, respectively, in inoculated plants compared with un-inoculated plants. Biomass of wheat was also noticed to be increased in the plants inoculated with the HBCD-sjtu strain. Shoot fresh weight, shoot dry weight and root dry weight were increased 1-fold 1.6-fold and 1.9-fold in inoculated plants (Fig. 1). The improvement in leaf number, leaf area and chlorophyll contents were 41.2%, 38.2% and 43.5%, respectively, increase in inoculated plants compared to non-inoculated plants (Fig. 2).

Properties of Strain HBCD-sjtu as plant growth-promoting activity

Enzymatic activities of Strain HBCD-sjtu were

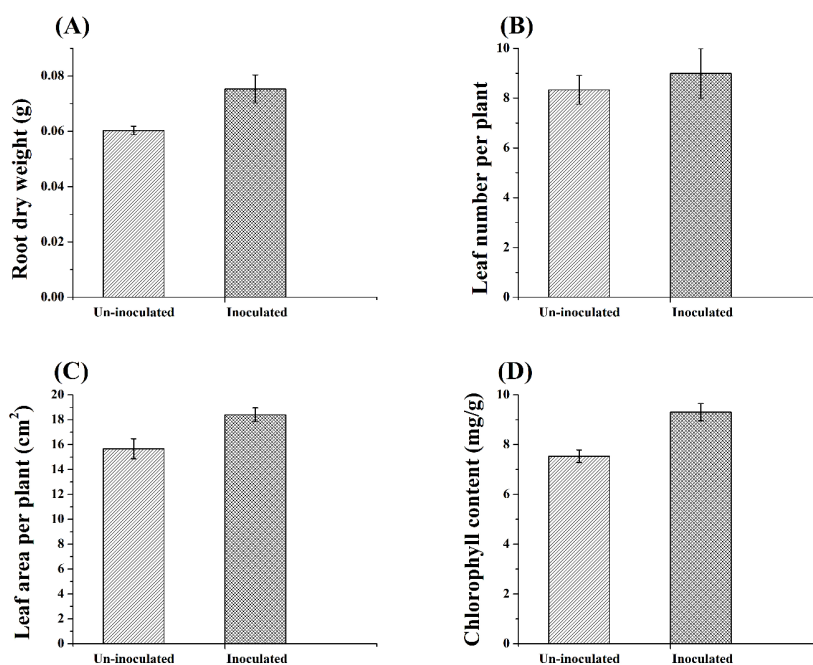


Fig. 2. A) Effect of Strain HBCD-sjtu inoculation on root dry weight. B) Leaf number per plant. C) Leaf area per plant. D) Chlorophyll content.

revealed. Hydrolytic enzymes, such as protease, lipase and esterase, are capable of inhibiting the growth of pathogens and consequently decreasing the pathogenic effects on plants (1). An effective enzymatic activity of the Strain HBCD-sjtu was clear (Table I). Four hydrolytic enzymes were generated by the HBCD-sjtu strain. The strain could produce substances responsible for plant growth-promoting activity. Amylase enzyme helps to improve initial germination by providing starch to plants, and Strain HBCD-sjtu was noticed to increase the activity of this enzyme. Acetoin improves growth of plants and ammonia acts as a signaling molecule in the communication of plants and bacteria (3). It is obvious from phosphate solubilization that phosphate insoluble phosphate might be changed to plant accessible kinds by the HBCD-sjtu strain. It was shown that plant growth could be improved by HBCD-sjtu strain. The characteristics of bacteria to tolerate stress environments were also verified. Making association between plants and bacteria, these characteristics may benefit from host plants existing in stress environments. 3% NaCl and 1 mM of entire assessed metal ions, excluding nickel sulfate, were tolerated by HBCD-sjtu strain. In the case of 1 mM nickel sulfate, the Strain HBCD-sjtu exhibited growth; similarly it showed tolerance to 3% hydrogen peroxide (data not shown). Being an IAA-generating bacteria, the Strain HBCD-sjtu was tolerant with stress. It has been studied that the metabolism of IAA-producing strains was more effective, and produced tolerance in plants during stress conditions.

Growth and IAA production of HBCD-sjtu strain

The Strain HBCD-sjtu was a great producer for IAA production. IAA, being a plant hormone, was involved in strengthening the interaction between bacterium and plant. Throughout the growth of Strain HBCD-sjtu on nutrient broth, both in the presence and absence of L-tryptophan, an observation of the quantitative evaluation of IAA production was carried out (1). There was a link between bacterial growth and IAA production (Fig. 3a) which indicated a correspondence of the enhanced bacterial growth with higher production of IAA.

Salt Tolerance of Strain HBCD-sjtu

The salt tolerance activity of Strain HBCD-sjtu was checked at different concentrations (0.5, 2, 4, 6, 10, 12 and 15%) of NaCl. The effect of different salt concentrations on Strain HBCD-sjtu were measured (Fig. 3b). Maximum growth rate of Strain HBCD-sjtu was noticed at 0.5% NaCl. Increase in salt concentration greatly reduced the growth rate.

DISCUSSION

PGPB reduce stress and stimulate plant growth and development. It is not evidently identified which particular method is used by PGPB for plant growth promotion, however it is hypothesized that plant growth may be promoted by processes such as phytohormones production, inhibition of lethal organisms, stimulation of phosphate solubilization and stimulation of the uptake of mineral nutrient (2). The current study was carried out to assess

Table I. Characteristics of Strain HBCD-sjtu in plant growth-promoting activity.

Enzymatic activity ^a				Plant growth-promoting substances			
Esterase	Lipase	Protease	Amylase	Phosphate solubilize ^b	Acetoin production ^c	Ammonia production ^c	Indole acetic acid (IAA) ^c
+++	++	+	++++	++	++	+	++

^a Zones of enzymatic activity: + 0.1 – 1.0 cm, ++ 1.1 – 2.0 cm, +++ 2.1 – 3.0 cm, ++++ 3.1 – 4.0 cm. ^b Zones of phosphate solubilization: ++ 1.1 – 2.0 cm. ^c Plant growth-promoting substances synthesis : + represents synthesis, ++ represents great synthesis.

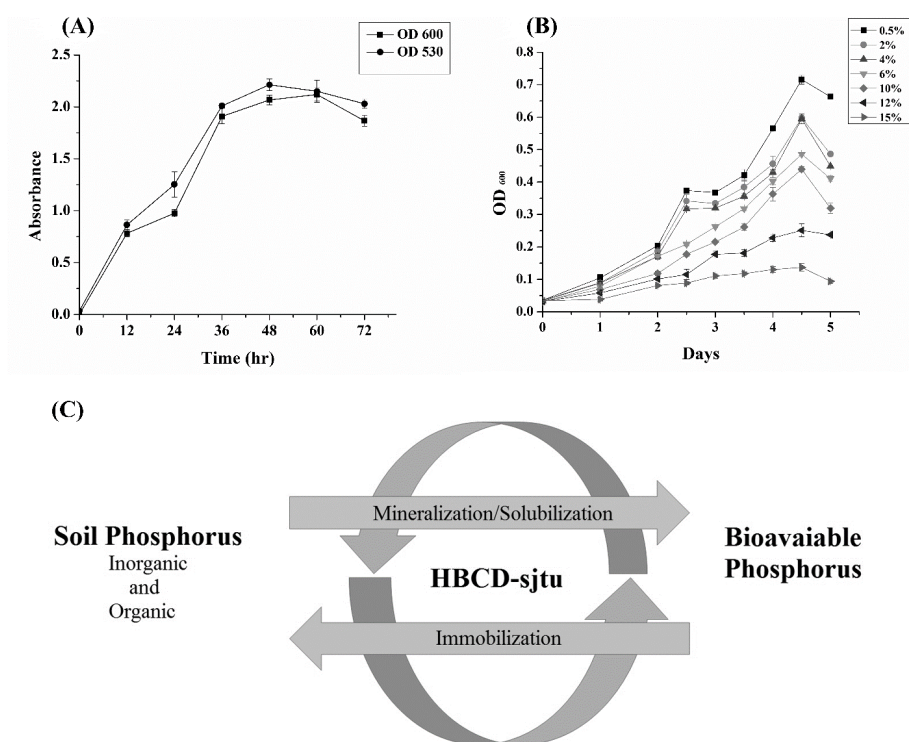


Fig. 3. *A)* Growth and indole acetic acid (IAA) production of HBCD-sjtu strain. *B)* Cell growth of strain *Bacillus sp.* HBCD-sjtu in different salt concentrations. *C)* Schematic diagram of soil phosphorus mobilization and immobilization by HBCD-sjtu strain.

various activities of the Strain HBCD-sjtu related to plant growth improvement and categorized through biochemical analyses. Being one of the highly essential phytohormones, IAA serves as key signal molecule to control plant development (1). Ammonia production is an additional significant property of PGPB, which has an indirect effect on plant growth (8). The Strain HBCD-sjtu was also capable of catalase and ammonia production. Due to exhibiting catalase activity, the Strain HBCD-sjtu should have great resistance to chemical, environmental and mechanical stress. The current study revealed maximum proficiency of the Strain HBCD-sjtu as a plant growth-promoting bacteria, which promoted the production of IAA, catalase, ammonia, acetoin and solubilized insoluble phosphorus (Fig. 3c). This study is essential to support the use of PGPB as biofertilizers or inoculants which is an effective method to substitute chemical fertilizers. Various

properties of plant growth-promoting activities were revealed by HBCD-sjtu strain. Induced systemic resistance (ISR) might be caused by the HBCD-sjtu strain. Additionally, plant growth inducing substances such as acetoin, amylase, phosphate solubilization and ammonia can be generated by the HBCD-sjtu strain. Moreover, the strain showed tolerance to sodium chloride, hydrogen peroxide and metal ions. The characterization of the effective strain will further facilitate the valuation of properties favorable for agriculture.

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LETTER TO THE EDITOR

A SURVEY ON DEMOGRAPHIC FACTORS AND SEROPREVALENCE OF HIV AND OTHER SEXUALLY TRANSMITTED INFECTIONS AMONG FEMALE SEX WORKERSYF. WANG^{1*}, YF. XUN^{1*}, PP. JIANG¹, T. WANG¹, XF. LI² and H. YANG^{1*}¹*Department of Dermatology, 970 Hospital of the PLA Joint Logistics Support Force, Weihai, China;*²*Department of Dermatology, The Affiliated Hospital of Qingdao University, Qingdao, China**Received January 3, 2019 – Accepted May 14, 2019*

*These authors contributed equally the work

To the Editor,

Sexually transmitted infections (STIs) have become a major public health and social problem in many countries around the world (1). STIs are synergistic factors for human immunodeficiency virus (HIV) infection, and high levels of STIs can be used as early warning signs for the detection of HIV and acquired immunodeficiency syndrome (AIDS) (2). The prevalence of AIDS in China is on the rise; the proportion of HIV/AIDS/STI transmission by sexual contact is increasing every year. Female sex workers (FSWs) have become a susceptible population and a bridge population for the spread of these diseases in China (3, 4). However, due to the large number of FSWs, the discreet nature of their activities, and the influence of traditional culture and ignorance of and discrimination by communities, the intervention in this group has been greatly restricted (5).

MATERIALS AND METHODS

The subjects were women who provide sexual services to male customers in the entertainment venues in Qingdao.

Over the years, the project team has conducted outreach work through ‘going out’ to entertainment venues and providing reproductive health services to FSWs, as well

as establishing a trust-based relationship with managers and some target groups in recreational facilities. Some of the individual issues and circumstances included in the questionnaire were not linked to their true identity and specific workplace. They could refuse to answer certain questions. With informed consent, 3–5 ml of venous blood was collected followed by the anonymous questionnaire survey, using codes to strictly match the survey and the blood sample.

Test methods

After blood serum separation, the syphilis test was carried out first using reagents manufactured by Shanghai Rongsheng Biotechnology Co., Ltd. (China) for syphilis TRUST detection. Positive specimens were confirmed by TPPA using the reagent manufactured by Fuji (Japan). The remaining serum was preserved below -20°C and detected by enzyme-linked immunosorbent assay (ELISA) for anti-HIV1/2 and anti-HSV-2-1gG using reagents manufactured by Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.

Statistical analysis

The survey data included age, ethnicity, marital status, residence, education, and other demographic data as well as reproductive health and serological test results of FSWs. The data were analysed using SPSS15.0 software.

*Key words: female sex workers, young, HIV, AIDS, STD, survey**Corresponding Author:*

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RESULTS

Demographic data

A total of 379 valid questionnaires and 338 blood samples were collected. The subjects investigated were 16–46 years old, the average age was 22.75 ± 0.275 years. Sixty-eight participants were <18 years old (18.8%), 246 (68.0%) were 19–29 years old, and 48 (13.3%) were ≥ 30 years old. Three hundred and fifty (96.42%) participants were of Han nationality and 13 were of minority nationalities (3.58%); 68 participants had under primary school education (18.23%), 57 (15.28%) had finished junior high school/secondary school, 243 had finished senior high school (65.15%), and the remaining five (1.34%) had finished college education or further education; 191 (51.07%) participants were unmarried, 94 were married (25.13%), 89 were living with partners (23.80%), and the remaining 89 were living with children (24.05%); 177 (48.23%) were local and 190 (51.77%) came from other provinces.

Social behaviour status

Two hundred and twenty-nine (60.4%) participants had fixed male partners (a husband or a boyfriend), while 136 (35.9%) had no fixed male partner; 91 (38.08%) participants thought that their fixed partner had other female partners; 82 (34.31%) thought that their fixed partner did not have other female partners, and 66 (27.62%) were not sure. Other social behaviour status are shown in Table I.

HIV/AIDS/STIs incidence status in the previous six months

The infection rate was 51.3% (148 participants); 106 (67.95%) had genital warts, 59 (37.82%) had non-gonococcal urethritis, 39 (25%) had gonorrhoea, 15 (9.62%) had syphilis, and 14 (8.97%) had genital herpes. For HIV/AIDS/STIs patients, in the previous six months, the average number of illnesses was 1.36 ± 0.06 (range: 1–7 times). Among these patients, 2.66% were syphilis

Table I. FSWs social behavior status.

Variable	Number	Ratio(%)
The main purpose of engaging in sexual work:	373	
Feed oneself	225	60.32
Parents	145	38.87
Children	72	19.30
Husband / boyfriend	34	9.11
Feed other people	8	2.14
Satisfaction with sexual work:	352	
Satisfied	68	19.32
Dissatisfied	284	80.68
Future plans:	343	
Change or do business	139	40.52
Go home / get married / have children	80	23.32
Other:	74	21.57
Procuress	37	10.79
Be a mistress	13	3.79
Would you be willing to work if you set up a special "entertainment service area" that could ensure safety and health?	347	
Willing	109	31.41
Unwilling	238	68.59

Table II. *Patients in different age groups who had HIV/AIDS/STD in the past 6 months.*

Age status	HIV/AIDS/STD Group		Non HIV/AIDS/STD group	
	n	Percentage(%)	n	Percentage(%)
≤18	32	54.2(32/59)	27	45.8 (27/59)
19~29	105	51.7(105/203)	98	48.3(98/203)
≥30	11	39.3(11/28)	17	60.7(17/28)

TRUST positive, 26 (7.69%) were syphilis TPPA positive, and 105 (76%) underwent an HIV/AIDS/STIs test after nearly three months. Serological test results showed that 35% were anti-HSV-2-IgG positive and HIV antibodies were negative.

The different age groups of patients based on incidence status in the previous six months are shown in Table II.

DISCUSSION

FSWs are an important bridge population and victims of the HIV/AIDS/STI pathways (6). In the interventions for HIV/AIDS/STIs through sexual contact, consideration should be given to the social natural conditions of the FSWs (7).

Most previous studies have focused on the average age of this population but not so much on their age distribution (8). The survey revealed that approximately one-fifth of FSWs were legally minors and 13.3% were 30 years old. Furthermore, the age distribution of FSWs revealed ‘polarisation’ or ‘two age’ (9). The ‘bipolar’ distribution trend increases the difficulty of reproductive health and HIV/AIDS/STIs intervention for FSWs. Other studies revealed that age was a factor for FSWs infected with HIV/AIDS/STIs (10). Similarly, the present study revealed that HIV/AIDS/STIs were most prevalent (54.2%) in the <18 years age group in the surveyed population. In addition, a survey revealed that older FSWs usually had higher rates of HIV/AIDS/STIs (11). Although this survey did not find a higher prevalence of HIV/AIDS/STIs in the 30 years age group, the prevalence rate in this age group was quite high at 39.3%.

This survey revealed that nearly half of the selective work of FSWs was mainly due to poverty or lack of work. Most FSWs came from low-income and low-cultural groups and lacked general professional skills. The survey revealed that FSWs engage in sexual work voluntarily because they think it is a relatively easy way to earn money; they even envy the income of others engaged in sex work. Changes in attitudes of some FSWs towards careers and sex work indicates that the anonymity of FSWs is weakening to a certain extent.

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LETTER TO THE EDITOR

A CLINICAL STUDY OF QINGYI DECOCTION IN THE TREATMENT OF ACUTE PANCREATITIS

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To the Editor,

Acute pancreatitis (AP) is an inflammatory response induced by a variety of etiologies, where trypsin is activated in the pancreatic gland, causing digestion of the pancreatic tissue itself, edema, bleeding, and even necrosis. AP patients with mild conditions only present with epigastric discomfort, and severe patients can develop multiple organ failure, which can even lead to death (1). Therefore, the early treatment of AP can significantly reduce the complications and mortality of patients with severe AP (2). There are two high-risk periods for the disease: one is the early stage of inflammatory response, where inflammatory response is activated, excessive inflammatory factors impair the organ functions of patients, and insufficient blood volume usually concurrently occurs, therefore is the main period of organ function damage (3-4); another is the period of infection in the late stage, where the pancreas may be locally infected, which may induce sepsis, organ failure and even death if not being controlled timely (5). Therefore, severe AP is a high risk factor for mortality (6), especially for those complicated with multiple organ failure.

With the development of traditional Chinese medicine (TCM), TCM treatment has been widely used in AP. Clinical research proved that Qingyi

decoction (QD) administration not only reduces AP-induced acute lung injury (ALI) by reducing alveolar type II epithelial cell (AEC II) apoptosis, inhibiting the overexpression of secretory type II phospholipase A2 (sPLA2), but also improves AP-complicated liver and renal injuries by decreasing high mobility group box protein 1 (HMGB1) expression. In addition, the actions of modified QD involved in the biological processes include directly neutralizing endotoxins, reducing intestinal endotoxin generation and absorption, inhibiting excessive neutrophil activation and NF- κ B expression, and minimizing the release of inflammatory cytokines (7). In the present study, AP patients were treated with Chinese medicine QD to investigate its clinical efficacy.

MATERIALS AND METHODS

A total of 97 AP patients without surgical indications, who were admitted to the Gastroenterology Department of Second Affiliated Hospital of Kunming Medical University, Kunming, China, from May 2014 to May 2016, were included in this study. These patients were randomly divided into a treatment group and a control group. The treatment group ($n=49$) comprised of 30 males and 19 females, aged 17-76 years (average: 47.2 ± 7.8 years). The interval from onset to medical visit was 0.5-6 days,

Key words: acute pancreatitis; qingyi decoction; laboratory index; clinical study

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with an average interval of 2.8 ± 0.3 days. Among these patients, 21 had edematous AP and 28 had hemorrhagic necrotizing AP. The control group ($n=48$) comprised of 29 male and 19 female patients, aged 18-75 years (average: 46.8 ± 7.2 years). The interval from onset to medical visit was 0.5-5.5 days, with an average interval of 2.7 ± 0.5 days. Among these patients, 20 had edematous AP and 28 had hemorrhagic necrotizing AP. Differences in age, gender and course of disease between the two groups were not statistically significant ($P > 0.05$), therefore the two groups were comparable. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Second Affiliated Hospital of Kunming Medical University. All subjects gave informed consent to participate in the study.

All patients met the criteria for the diagnosis of AP in the Guidelines for the Diagnosis and Treatment of Acute Pancreatitis (2014 edition) (8), which was established by the Pancreatic Surgery Group of the Surgery Branch of the Chinese Medical Association, where a clinical diagnosis can be drawn when at least two of the following three criteria are met: (i) abdominal pain consistent with AP, (ii) activity of serum amylase and/or lipase at least three times higher than the normal upper limit, and (iii) imaging changes consistent with AP when revealed by abdominal imaging examination.

Patients who did not meet the diagnostic criteria, pregnant and lactating women with AP, and patients allergic to TCM were excluded from the study.

Therapeutic methods

Control group. Patients received the conventional comprehensive treatment of Western medicine, including: water fasting and gastrointestinal decompression; spasmolysis and pain relief; inhibition of the secretion of trypsin and gastric acid by somatostatin and proton pump inhibitors; application of antibiotics; improvement of the microcirculation of the pancreas; enteral nutrition; intravenous rehydration, and maintenance of acid-base balance and electrolyte balance; the supplement of plasma and albumin if necessary.

Treatment group. On the basis of the treatment in the control group, QD was used. Prescription composition: 10 g of rhubarb (added in the later stage of the decocting time), 10 g of *Magnolia ficalis*, 10 g of fructus *aurantii immaturus*, 10 g of *Scutellaria baicalensis*, 10

g of bupleurum, 10 g of *radices paeoniae*, and 10 g of compound of glauher-salt and liquorice (added when the decoction was going to be taken). According to the principle of “depriving food but not drugs”, under gastrointestinal decompression, one decoction was used daily, which was boiled to 300 ml, and restored to normal atmospheric temperature. This was divided into three portions, and each portion was infused through a gastric tube in the morning, at noon and in the evening. After completion of the infusion, the gastric tube was closed for 60-120 min.

For these two groups, every seven days was set as a course of treatment.

Observation indexes. The observation indexes included the following: relief time of abdominal pain, recovery time of gastrointestinal function, recovery time of blood amylase and white blood cells, recovery time of abdominal positive computed tomography (CT) signs (recover to grade A according to Balthazar classification criteria for grading), length of hospital stay, the number of complications, and the number of deaths.

Evaluation criteria for the curative effect

The criteria were established based on a former study in the literature (9): i) Cured: the symptoms and signs of patients were relieved within three days, and completely disappeared within seven days, and laboratory indicators returned to normal levels; ii) Markedly effective: the symptoms and signs of patients were basically improved within seven days, and the laboratory indicators returned to normal levels. iii) Effective: the symptoms and signs of patients were relieved to a certain extent within seven days, and the laboratory indicators were lowered. iv) Ineffective: the symptoms and signs of patients did not improve within seven days, and the laboratory indicators were not lowered. The endpoint of the study was the total effective rate at seven days. The total effective rate of treatment = (Cured + Markedly effective + Effective) / total number of cases $\times 100\%$.

Statistical analysis

Data were statistically analyzed using statistical software SPSS 17.0. Measurement data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm SD$). Count data were evaluated using the χ^2 -test. Independent samples were compared between these two groups using *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Compared with the control group, the relief time of abdominal pain, recovery time of gastrointestinal function, blood amylase, white blood cell and abdominal positive CT signs, and length of hospital stay were significantly shorter in the treatment group ($P<0.05$, Table I).

In the treatment group, the effective rate was 95.91%. In the control group, the effective rate was 77.08%. The differences were statistically significant ($P<0.05$, Table II).

Two patients died in the treatment group, and four patients died in the control group, all from multiple organ failure (Table III).

DISCUSSION

With the improvement of the living standard and the increase in bad habits, such as alcoholism and overeating, the incidence of AP has steadily increased. AP is one of the most common acute abdominal diseases, and its characteristics are rapid onset, rapid progression and complexity.

Table I. Two groups of clinical symptoms and laboratory index recovery time were compared ($\bar{x}\pm SD$, d).

Type	Group	cases	abdominal pain	gastrointestinal function	blood amylase	white blood cell	abdominal positive CT signs	length of hospital stay
Edematous	treatment group	21	0.9 $\pm$ 0.6	1.9 $\pm$ 0.8	2.9 $\pm$ 0.6	3.8 $\pm$ 0.5	7.2 $\pm$ 1.9	10.2 $\pm$ 2.3
	control group	20	2.8 $\pm$ 0.4	3.9 $\pm$ 0.5	4.3 $\pm$ 0.5	5.5 $\pm$ 0.7	9.3 $\pm$ 1.8	14.3 $\pm$ 2.9
Necrotizing	treatment group	28	2.3 $\pm$ 1.5	4.3 $\pm$ 1.4	3.9 $\pm$ 0.7	6.9 $\pm$ 1.6	14.9 $\pm$ 2.7	26.3 $\pm$ 2.8
	control group	28	4.2 $\pm$ 1.6	6.1 $\pm$ 1.5	5.3 $\pm$ 0.6	8.5 $\pm$ 1.7	17.1 $\pm$ 2.6	31.5 $\pm$ 2.9

Table II. Comparison of clinical efficacy between the two groups of patients(%).

Group	Cases	Cure rate	Markedly effective rate	Effective rate	Invalid rate	Total effective rate
Treatment group	49	38(77.55)	6(12.24)	3(6.12)	2(4.08)	47(95.51)
Control group	48	28(58.33)	7(14.58)	2(4.17)	11(22.92)	38(77.08)

Compared with the control group, $P<0.05$

Table III. Comparison of complications and death cases between the two groups (%).

Group	cases	pancreatic pseudocyst	Pancreatic abscess	upper gastrointestinal hemorrhage	Pancreatogenic encephalopathy	Respiratory distress syndrome	multiple organ failure
Treatment group	49	10(20.41)	5(10.20)	1(2.04)	1(2.04)	3(6.12)	2(4.08) death
Control group	48	15(31.25)	7(14.58)	2(4.17)	1(2.08)	5(10.42)	4(8.33) death

Many organs can be affected and the fatality rate is high. It has been reported that the mortality rate of severe AP complicated with persistent multiple organ failure is up to 80%, while the mortality rate in patients without persistent multiple organ failure is only 14.29% (10). Therefore, early control of inflammatory response and the reduction of inflammation level in patients is the key to the protection of important organ functions (11).

Traditional Chinese herbal prescription QD has effects of clearing fever, detoxifying, soothing the liver, regulating the flow of vital energy and removing obstruction, invigorating the circulation of blood, dissipating blood stasis, and dredging urination and defecation (12). Hence, it is the most common prescription for the treatment of AP. It can enhance gastrointestinal peristalsis, rule out a large amount of enteral bacteria and toxic substances, protect the gastrointestinal mucosal barrier, inhibit intestinal bacterial propagation and flora translocation, reduce inflammation-inducing factors, avoid damage to the pancreas in the ischemic status, improve pancreatic microcirculation, and relieve the symptoms of abdominal pain and abdominal distension. In the present study, after being infused with the decoction through a gastric tube, the patients in the treatment group achieved effects superior to the control group in terms of improvement of abdominal symptoms, recovery of gastrointestinal function, recovery time of the laboratory indexes and length of hospital stay, as well as significantly fewer complications and lower mortality.

Thus, QD is significantly effective, safe and reliable for the treatment of AP, and as it is also economical, it is worthy of clinical popularization and application.

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LETTER TO THE EDITOR

**CARDIAC TROPONIN I, MYOGLOBIN, AND CREATINE KINASE-MB AS
NEW BIOMARKERS FOR DIAGNOSIS OF NEONATAL HYPOXIC ISCHEMIC
ENCEPHALOPATHY**

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To the Editor,

Hypoxic ischemic encephalopathy (HIE) is a neurodegenerative disorder that occurs in fetuses and neonates, and is characterized by hypoxia and decrease or suspension of cerebral blood flow caused by various perinatal factors (1). HIE is one of the major complications of neonatal asphyxia and a common cause of intellectual disability in children (2). The incidence of HIE is between 1.3% and 1.7%, and in moderate and severe forms the incidence of death or severe disability can reach 53-61%. The children that survive the moderate/severe form of HIE have different degrees of neurological sequelae, such as cerebral palsy, epilepsy, mental retardation, representing a heavy economic and living burden to society and families (3-5). Currently, it is generally accepted that the best time window for the treatment of HIE is in the first 6 h after birth (6). Early diagnosis and treatment of HIE can effectively reduce the disability rate of newborns.

Asphyxia is one of the main causes leading to the development of HIE determined by the decrease of neonatal blood oxygen concentration due to the impairment in blood circulation and gas exchange between the mother and the fetus (7). It is implicated in around 50% of the cases of HIE that appear to

newborns (8). HIE is also associated with impairment in systems or organs other than the central nervous system, that influence the long-term prognosis and quality of life of HIE children. Cardiac biomarkers, including serum Cardiac Troponin I (cTnI), Creatine Kinase-MB (CK-MB) and myoglobin (Mb), are correlated with different pathologies and diagnosis in neonatology (9). This study aimed to investigate the correlation between the levels of cTnI, Mb and CK-MB in umbilical cord blood serum, the severity of HIE and the presence of HIE complicated with myocardial injury in order to elucidate their predictive value in the diagnosis of HIE or HIE-related myocardial injury.

MATERIALS AND METHODS*Patients*

Eighty full-term neonates born in the Obstetrics Department of Affiliated Hospital 2 of Nantong University, China between November 2015 and November 2018 were included in the study. Of the neonates included in the study, 60 presented risk factors for developing HIE and 20 were without any risk factors. All the legal guardians of the neonates signed a written informed consent form and the study was approved by the Ethics Committee of

Key words: hypoxic ischemic encephalopathy (HIE); cardiac troponin I (cTnI); myoglobin (Mb); creatine kinase-MB (CK-MB)

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Affiliated Hospital 2 of Nantong University, China.

Clinical data of the selected neonates were collected, including mode of birth, gestational age, gender, birth weight, 1-minute and 5-minute Apgar score (10), placenta, umbilical cord and amniotic fluid, cardiovascular performance, electrocardiogram, cardiac ultrasound.

The neonates were divided into an observational group that included 60 newborns with HIE risk factors and a control group that included 20 newborns without any risk factor for developing HIE. In the follow-up, the observational group was subdivided into a mild HIE group of 20 cases, a moderate HIE group of 26 cases, a severe HIE group of 8 cases and a non-HIE group with 6 cases.

Diagnostic criteria: newborns free of abnormal obstetric conditions that can lead to intrauterine distress or with severe intrauterine distress (fetal heart ≤ 100 beats/min for more than 5 min); III degree amniotic fluid pollution; obvious asphyxia occurred during delivery; severe asphyxia occurred at birth, Apgar score was less than or equal to 3 points after 1 min and remained less than or equal to 5 points after 5 min, or umbilical artery blood gas pH was less than or equal to 7.0 at birth; there were neurological symptoms at birth and the symptoms persisted for more than 24 h; convulsions caused by intracranial hemorrhage, birth injury and electrolyte disturbance other than brain injuries caused by intrauterine infection and genetic metabolic diseases.

Clinical classification of hypoxic ischemic encephalopathy (HIE):

- mild: easy to irritate, excited, in an excessively awakened state, highly irritated such as tremor, shaking, etc., normal sucking reflex and muscle tension, stable breathing, no convulsions, but the hug reflex is more active;
- moderate: in a state of lethargy or shallow coma, weakened muscle tension, sucking reflex, and hug reflex, convulsions or apnea occurred in some patients;
- severe: coma and frequent convulsions, extreme reduction of muscle tension (flaccidity), disappearance of tendon reflex and hug reflex, anisocoria with poor light response, anterior fontanel elevation, apnea or irregular, respiratory failure may occur in some patients.

Exclusion criteria: during pregnancy, the mother had various serious infections such as respiratory tract or vagina, as well as the application of immune preparations,

hormones, etc.; the newborn suffers from congenital, inherited metabolic disease or congenital malformations of respiratory, circulatory and central nervous systems, as well as neuromuscular diseases; the fetus who suffered from respiratory and digestive system inflammation and sepsis after birth; premature rupture of membranes was more than 24 hours; the fetus had edema and hemorrhagic shock; during parturient labor, fetal passive drug poisoning occurred due to the use of high doses of analgesics and magnesium sulfate.

Diagnostic criteria of neonatal asphyxia complicated with myocardial injury: newborns with history of perinatal asphyxia or hypoxia; neonates had the following clinical manifestations: pale complexion, thick fingers, refilling time of capillary >3 s, bradycardia, low and blunt heart sounds, and even heart failure, severe arrhythmia, and cardiac arrest; the electrocardiogram showed changes in ST-T segment and did not recover for more than 2-3 days; abnormal rise in serum CK-MB or cTnI.

For each case included in the study the following parameters were noted and assessed: pregnancy complications, history of intrauterine distress, mode of delivery, gestational age, gender, birth weight, body length, Apgar score, cTnI, Mb, CK-MB, cardiovascular performance, electrocardiogram and cardiac ultrasound.

Serum collection

After the newborn was delivered, after the routine cutting of the umbilical cord, it was disinfected and punctured within 1-2 min, and 4 mL of blood was collected. The blood naturally coagulated for 10-20 min at room temperature, was centrifuged at 3000 r/min for 10 min, and the supernatant was carefully collected. After collecting the supernatant, the samples were kept at -20°C until the moment of testing.

Determination of troponin I and myoglobin

Troponin I and myoglobin were determined by chemiluminescence (double site immunoassay) based on the manufacturer's specifications using an Access 2 Immunoassay System analyzer (Beckman Coulter Co., Ltd, USA). The reagents, quality control products, and calibration liquids were provided by Beckman Coulter Co., Ltd, USA. The batch number for cTnI kit was 624362, with a normal range of 0-0.034 ng/mL and the batch number for the Mb kit was 624434, with a normal range of 0-121 ng/mL.

Detection of CK-MB

CK-MB was determined by an immunosuppression method using a Hitachi Automatic Biochemical Analyzer 7600-210E+ID (Hitachi, Ltd., Japan) according to the manufacturer's instructions. The CK-MB kit was provided by Zhejiang Aodete Biotechnology Co., Ltd, China. Ten μL of the sample was mixed with 1200 μL of reagent and incubated at 37°C for 5 min. Then 50 μL of reagent 2 was added and the incubation was continued at 37°C for another 2 min, followed by detecting the absorbance changes continuously for 1 to 3 min in order to calculate $\Delta A/\text{min}$. The calculation formula used was: $\text{CK-MB(U/L)} = \Delta A/\text{min} \times F = (\text{Total reaction volume (mL)} \times 1000/\text{mmol extinction coefficient} \times \text{sample volume (mL)} \times 1.0) \times 2$, where, A represents the rate, $\Delta A/\text{min}$ represents the rate of change of absorbance per minute, and F is the theoretical factor, $F = 9938$ (when the wavelength is 340 nm).

Statistical analysis

Pearson linear correlation was used to analyze the correlation between cTnI, Mb and CK-MB and the severity of the disease, and Spearman rank correlation was used to analyze the correlation between cTnI, Mb and CK-MB. Logistic regression analysis was used to establish a diagnostic model combining three indicators (cTnI, Mb and CK-MB), and a new variable (PRE) was generated. The three original indicators and the new variable (PRE) were used as the test variables, and the incidence of HIE (1 for patients with disease and 0 for those without disease) was used as the classified variables for ROC

curve analysis, so as to explore the predictive diagnostic value of each indicator for HIE. The ROC curve was generated by MedCalc 13.0 (Beijing Huanzhongruichi Technology Co., Ltd, China) and other statistical analyses were carried out using SPSS 22.0 (IBM, USA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

The levels of cTnI, Mb and CK-MB in umbilical cord serum of neonates

Serum cTnI and Mb levels in the moderate and severe HIE group were significantly higher than those in the mild HIE group, non-HIE group, and control group ($P < 0.05$). There was no significant difference in the umbilical cord serum cTnI and Mb levels between the mild group, the non-HIE group and the control group ($P > 0.05$). Umbilical cord serum CK-MB levels in the moderate and severe groups were significantly increased compared to those in the mild HIE group, non-HIE group, and control group ($P < 0.05$). The umbilical cord serum CK-MB levels in the non-HIE group were significantly increased compared to the control group ($P < 0.05$), as shown in Table I.

Correlation analysis of serum cTnI, Mb, CK-MB and disease

Serum cTnI was positively correlated with CK-MB level in the HIE group ($r = 0.450$, $P < 0.05$), cTnI was positively correlated with Mb level ($r = 0.631$,

Table I. Umbilical cord serum cTnI, Mb and CK-MB levels in the observational and control groups.

Group	cTnI (ng/ml)	Mb (ng/ml)	CK—MB (U/L)
Control group	0.010 $\pm$ 0.002	63.1 $\pm$ 21.1	11.3 $\pm$ 3.3
Non-HIE group	0.011 $\pm$ 0.002	97.4 $\pm$ 21.2	30.0 $\pm$ 11.3
Mild HIE group	0.012 $\pm$ 0.001	116.4 $\pm$ 19.1	47.2 $\pm$ 22.0 ^{*#}
Moderate HIE group	0.022 $\pm$ 0.006 ^{*#&}	185.3 $\pm$ 72.7 ^{*#&}	157.0 $\pm$ 73.0 ^{*#&}
Severe HIE group	0.038 $\pm$ 0.009 ^{*#&}	373.2 $\pm$ 168.1 ^{*#&}	172.6 $\pm$ 125.2 ^{*#&}
F value	39.557	28.688	22.068

Note: * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the non-HIE group; & $P < 0.05$ compared with the mild HIE group.

$P < 0.05$), and CK-MB was positively correlated with Mb level ($r = 0.493$, $P < 0.05$). The levels of serum cTnI, Mb and CK-MB were positively correlated with HIE severity ($r = 0.721$, $P < 0.05$; $r = 0.560$, $P < 0.05$ and $r = 0.437$, $P < 0.05$).

Diagnostic value of serum cTnI, Mb and CK-MB for HIE

Three indicators, cTnI, Mb and CK-MB, were used as independent variables and HIE incidence (1 for patients with disease and 0 for patients without

disease) was used as dependent variables. Logistic regression analysis was used to establish a diagnostic model combining the three indicators and a new variable (PRE) was generated (Table II). The three original indicators, cTnI, Mb and CK-MB, and the new variable (PRE) were used as the test variables, and the HIE incidence (1 for the disease and 0 for disease-free) was used as the classified variables for ROC curve analysis. The area under the ROC curve (AUC) for the diagnosis of HIE by serum cTnI was

Table II. Diagnostic model for HIE established by combining three original indicators (cTnI, Mb, and CK-MB.)

Indicator	B	S.E.	Wald	df	P	OR
CK-MB	0.035	0.016	4.401	1	0.035	1.036
cTnI	2.634	1.981	1.767	1	0.183	13.948
Mb	0.055	0.025	4.735	1	0.030	1.056
Constant	-10.704	3.997	7.168	1	0.006	0.000

PRE: $1/(1+\exp(-Z))$; Z: $-10.704 + 0.055 \times Mb + 2.634 \times cTnI + 0.035 \times CK-MB$.

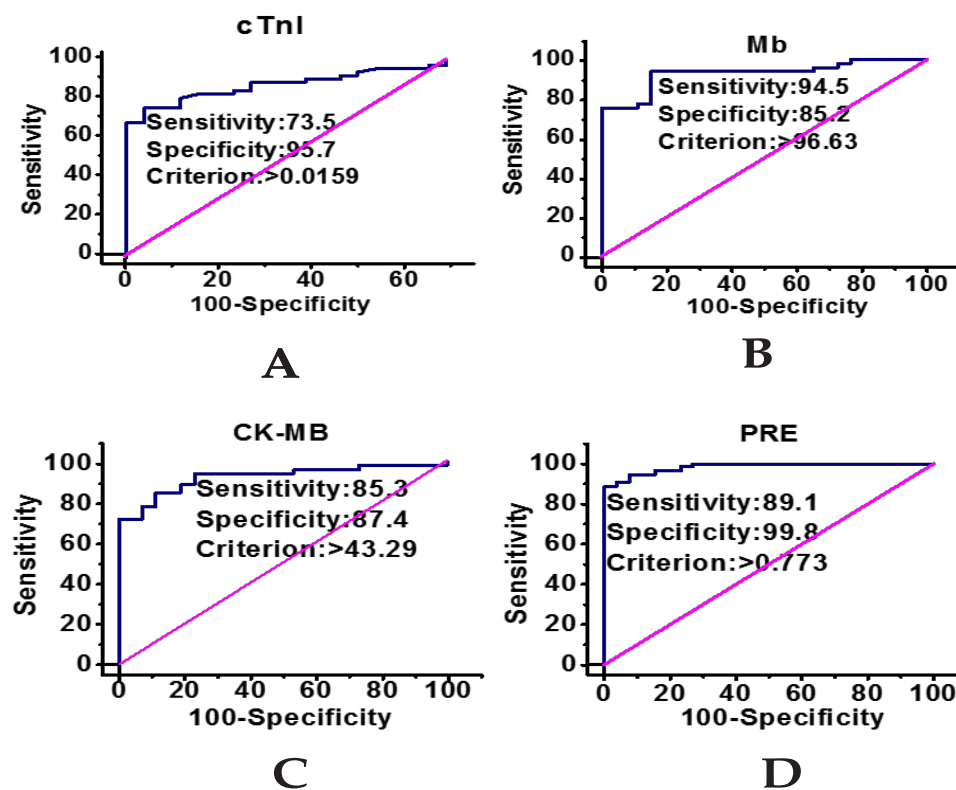


Fig. 1. ROC curve analysis of the diagnostic value of four indicators (cTnI, Mb, CK-MB, and PRE) for HIE. **A)** cTnI; **B)** Mb; **C)** K-MB; **D)** Joint diagnostic model (PRE).

0.896 (95% confidence interval: 0.828 - 0.962), and the best diagnostic boundary was >0.0157 ng/mL. The AUC of serum CK-MB in the diagnosis of HIE was 0.925 (95% confidence interval: 0.844 - 0.972), and the best diagnostic boundary was >43.0 U/L. The AUC of serum Mb for the diagnosis of HIE was 0.932 (95% confidence interval: 0.877 - 0.986), and the best diagnostic boundary was >96.53 ng/mL. There was no significant difference in the AUC between cTnI, Mb and CK-MB and the new variable PRE ($P>0.05$). However, the correlation between cTnI, Mb, CK-MB and PRE was statistically significant ($P<0.05$), as shown in Fig. 1 and Table II.

Diagnostic value of serum cTnI, Mb and CK-MB for HIE myocardial damage

Three indicators (cTnI, Mb and CK-MB) were used as independent variables, and the incidence of myocardial damage (1 for patients with disease and

0 for those without disease) was used as dependent variables. Logistic regression analysis method was used to establish a diagnostic model combining the three indicators, and a new variable (PRE) was generated. The three original indicators (cTnI, Mb and CK-MB) and the new variable (PRE) were used as the test variables, and the incidence of myocardial injury (1 for patients with disease and 0 for those without disease) was used as the classified variables for ROC curve analysis. The area under the ROC curve (AUC) of serum cTnI level in the diagnosis of HIE myocardial injury was 0.767 (95% confidence interval: 0.617 - 0.915), and the optimal diagnostic boundary point was >0.0159 ng/ml. The AUC of serum Mb level in the diagnosis of HIE myocardial damage was 0.679 (95% confidence interval: 0.521 - 0.834), and the best diagnostic boundary was >161.47 ng/ml. The AUC of serum CK-MB level in the diagnosis of HIE myocardial damage was 0.798

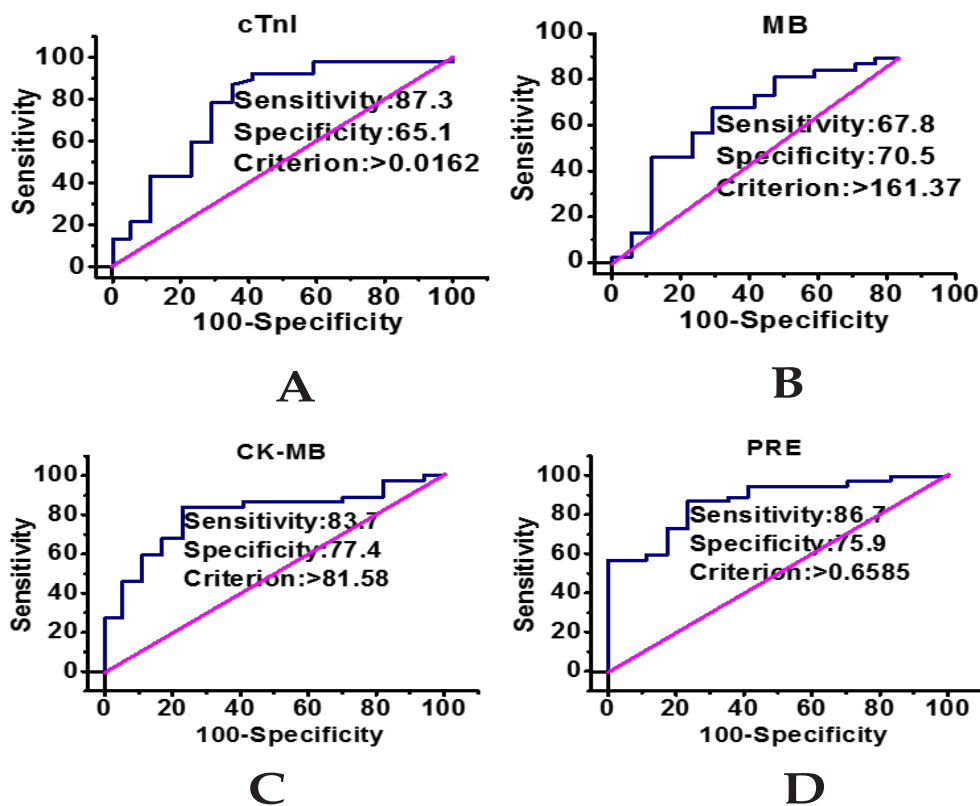


Fig. 2. ROC curve analysis of the diagnostic value of four indicators (cTnI, Mb, CK-MB and PRE) for myocardial injury. A) cTnI; B) Mb; C) CK-MB; D) Joint diagnostic model (PRE).

(95% confidence interval: 0.672 - 0.922), and the best diagnostic boundary was >81.48 U/L. There was no significant difference between cTnI, Mb and CK-MB ($P>0.05$), and the difference between cTnI, CK-MB and PRE were not statistically significant ($P>0.05$). But the difference between Mb and PRE was statistically significant ($P<0.05$), as shown in Fig. 2.

DISCUSSION

Neonatal hypoxic ischemic encephalopathy (HIE) is an important cause of perinatal asphyxia due to hypoxia and cerebral blood flow reduction or suspension determined by various perinatal factors (10). Studies have shown that factors such as gestational hypertension, irregular prenatal examination, abnormal placenta, amniotic fluid pollution, abnormal labor process, and low Apgar score are risk factors for HIE (11). The results of this study showed that the levels of cTnI, Mb, and CK-MB in the moderate and severe HIE group were significantly higher than those in the mild HIE group, the non-HIE group and the control group ($P<0.05$). In addition, serum cTnI, Mb, and CK-MB were positively correlated with each other, and there was a positive correlation between the general pathogenic conditions. Therefore, it can be speculated that the levels of cTnI, Mb and CK-MB in umbilical cord blood serum can reflect the severity of HIE at an early stage. Serum troponin I is a marker with the strongest specificity and highest sensitivity in the diagnosis of myocardial injury (12). 20% of the risk factors for HIE are prenatal factors that include maternal factors, placental abnormalities, and fetal causes. According to the results of this study, the comparison between cTnI, Mb, CK-MB and PRE can help in the diagnosis and treatment of HIE. For the accurate diagnosis of HIE associated with myocardial injury, only the difference between Mb and PRE counts. Therefore, it can be speculated that early detection of cTnI and CK-MB in cord blood can predict the occurrence of HIE myocardial injury.

In summary, the early detection of cTnI, Mb and CK-MB in umbilical cord blood could predict the occurrence of HIE at an early stage and effectively

evaluate its severity, providing a theoretical basis for clinical early intervention and improvement of prognosis.

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LETTER TO THE EDITOR

LONG-TERM BOND STRENGTH OF A MULTIMODE ADHESIVE TO DENTIN

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To the Editor,

The long-term survival of composite resin restorations depends on the bonding efficiency and the ability to achieve a satisfactory marginal seal at the cavosurface (1). The durability of the adhesive layer is therefore of considerable concern. The challenges of achieving predictable adhesion to dentin differ greatly from the challenges of achieving adhesion to enamel due to the histologic differences between the two surfaces (2-4). Manufacturers have recently introduced universal adhesives (multimode one-bottle adhesives), which have gained popularity due to their versatility in usage strategies/modes. The most studied adhesive, Scotchbond™ Universal adhesive (SU; 3M ESPE, St. Paul, MN, USA), penetrates the demineralized dentin more deeply in the etch-and-rinse (ER) mode than in the self-etch (SE) mode (5). The quality of SU is partly due to 10-methacryloyloxydecyl-dihydrogen-phosphate (10-MDP), a phosphate monomer that provides acidity to the adhesive, enables it to etch, and bonds ionically to dentin more effectively than other functional monomers (6). Conflicting reports are found in the literature regarding the immediate (3-4, 7) and long-term performance of these multimode adhesives (3-4). These, together with limited clinical

data, suggest that the performance of universal adhesives has not yet been fully validated. A systematic review revealed a large number of papers related to the static bond strength of these systems to enamel, and only few studies have examined the bond strength to dentin. However, the most important questions of long-term performance and possible deterioration were not addressed (6, 8-9).

The aim of the current study was to evaluate the shear bond strength (SBS) of SU to dentin, using either SE or ER modes, after short- and long-term incubation periods, relative to a two-step ER adhesive used as a control.

MATERIALS AND METHODS

Tooth preparation and study materials

Forty-eight caries-free, freshly extracted third molars from healthy, non-smoking patients were selected. The study protocols were approved by the Tel Aviv University Ethics Committee (# 160514), and all patients signed an informed consent form. The teeth were cleaned with hand curettes to eliminate soft tissue remnants and/or calculus and then stored in tap water containing 0.01% thymol (Sigma-Aldrich, Rehovot, Israel) at 5°C until the experiment was performed. The teeth were embedded

*Key words: dentin; durability; etch-and-rinse; multimode adhesive; self-etch**Corresponding Author:*

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parallel to the long axis using a custom-designed alignment apparatus. Each tooth was suspended in the middle of an aluminum ring (25 mm × 20 mm) and mounted 2 mm apical to the cement enamel junction (CEJ) in poly methyl methacrylate (PMMA) resin (Quick Resin, Ivoclar, Schaan, Liechtenstein) (Fig. 1A).

The lingual and buccal superficial enamel was removed using a rigidly secured high-speed hand piece equipped with a diamond bur under air-water irrigation (F1-Strauss, Ra'anana, Israel) to expose a flat dentin surface. The high-speed hand piece was mounted on a custom-designed surveyor-like apparatus (6.5° taper) (Fig. 1, B and C). A new bur was used for each tooth. SU multimode adhesive (3M ESPE, St. Paul, MN, USA) was used in both the ER and SE modes. The control adhesive was the two-step ER adhesive Adper Single Bond 2 (SB; 3M ESPE, St. Paul, MN, USA).

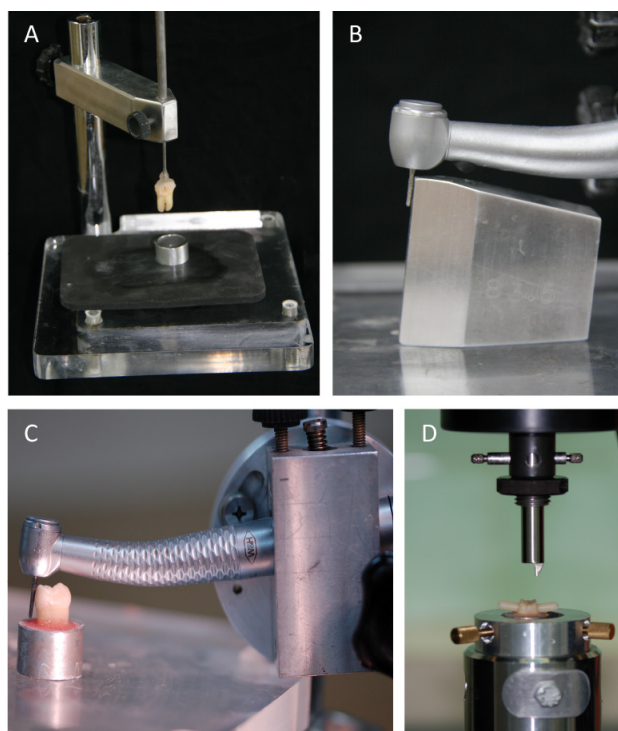


Fig. 1. Sample preparation. *A)* Teeth were suspended in the middle of an aluminum ring and mounted 2 mm apical to the CEJ in a PMMA resin. *B)* A high-speed hand piece was mounted on a custom-designed surveyor-like apparatus with a jig to ensure a 6.5° taper. *C)* The lingual and buccal superficial enamel was removed, exposing a flat dentin surface. *D)* The specimen was loaded into a universal loading machine.

Experimental design

The 48 prepared teeth (96 surfaces) were randomly assigned to 2 experimental and 1 control groups according to the adhesive used and its mode of application. Adhesive systems were applied to the dentin surfaces in accordance with the manufacturer's instructions (Table I) and light-cured using a LED light-curing unit (Elipar FreeLight 2, 3M ESPE, St. Paul, MN, USA) with a light intensity of 600 mW/cm². The light intensity was monitored with a curing radiometer (model 100, Demetron, Kerr, Danbury, CT, USA). Gelatin capsules with an inner diameter of 6 mm (10 mm long) (Torpac, Inc. NJ, USA) were incrementally filled with resin composite (Filtek Supreme XT, 3M ESPE, St. Paul, MN, USA) to 1 mm short of their margins and light-cured for 40 s from each side. The remaining 1 mm of the capsule was filled with the resin composite and was approximated to the adhesive-treated dentin surface. The specimens were clamped in a bonding jig to ensure that the capsule was oriented at 90° to the dentin surfaces. Excess material was removed, and the final curing was conducted for 20 s from 3 different directions. All specimens were incubated in 37°C distilled water. Half of the specimens in each group (8 specimens, 16 surfaces) were tested after one week, while the other half was tested after 6 months. The latter group was subjected to thermocycling for 10,000 cycles between 5 and 55°C with a 10 s dwell time (TC-2000 automatic device, Y Manes, Tel Aviv, Israel).

Shear bond strength testing

SBS testing was performed according to ISO 29022 (10) (using the notched-head SBS test). The specimens were loaded in a universal loading machine (Instron, Model 4502, Buckinghamshire, England) with a crosshead speed of 1.0 mm/min until failure (Fig. 1D). The SBS (in MPa) was calculated as the force exerted at the breaking point divided by the surface area: $(MPa) = \frac{FB + FL}{2\pi R^2}$

Failure analysis

After testing, the debonded dentin surfaces and resin composite cylinders were observed under an optical microscope at 18× magnification (M8 stereo microscope, Wild, Heerbugg, Switzerland). The modes of failure were classified as follows: cohesive failure (either within the composite resin or within the adhesive layer), adhesive failure (either between

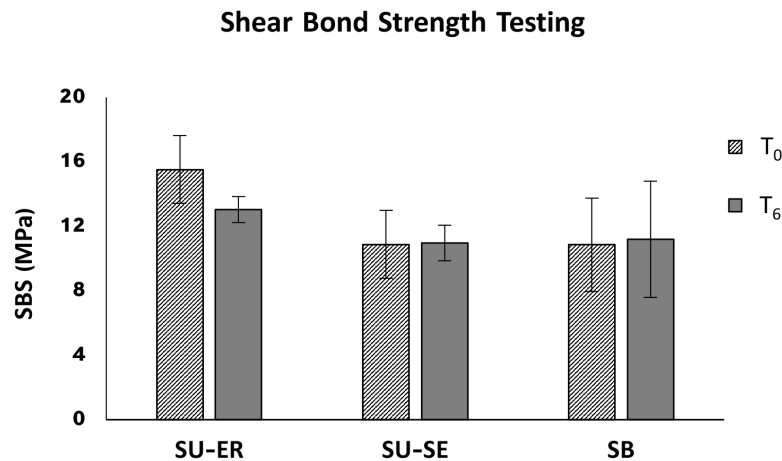


Fig. 2. Shear bond strength (MPa) after one week (T₀) and 6 months (T₆). SU: Scotchbond™ Universal; ER: etch-and-rinse; SE: self-etch; SB: Adper Single Bond 2.

the adhesive and dentin or between the adhesive and composite) or mixed failure. For each category, the number of surfaces was summed, and the results are presented as a percentage of all surfaces exposed to the specific treatment (11).

Selected representative specimens were examined by scanning electron microscopy (SEM, JSM 840A, JEOL) using a Scandium SEM imaging platform (Olympus). The samples were fixed in 2.5% glutaraldehyde in PBS, washed, and dehydrated by ethanol treatment. After reaching the critical drying point, the samples were mounted on aluminum stubs and sputter-coated (E5100, Polaron) with gold. Images were captured at an operating voltage of 10 kV and a working distance of 8 mm.

Statistical analysis

Statistical analyses were performed using SPSS. A paired *t*-test showed no statistically significant difference between the SBS of the buccal and lingual surfaces ($p=0.365$). Accordingly, the average SBS of the two surfaces of each tooth was recorded as the representative value.

Two-way ANOVA was conducted. The dependent variable was the SBS, and the independent variables were the adhesive and time. Tukey's post hoc test was performed to analyze subgroup differences. The level of significance was set at 0.05.

RESULTS

Shear bond strength

The SBS results are shown in Fig. 2. Two-way ANOVA revealed a significant difference between the adhesive systems ($p=0.001$) and a nonsignificant difference between the incubation times ($p=0.305$). The interaction of time X material was also nonsignificant ($p=0.178$). The highest SBS value at T₀ was obtained with SU-ER (15.5 ± 2.1 MPa) and was significantly higher than the values obtained with SU-SE (10.87 ± 2.1 MPa) and SB (10.85 ± 2.92 MPa). No significant difference was observed between the latter two methods. A similar pattern was obtained at T₆. SU-ER yielded the highest SBS value (13.02 ± 0.83 MPa), which was significantly higher than the values obtained with the SU-SE and SB adhesive strategy (10.94 ± 1.09 and 11.17 ± 3.59 MPa, respectively). No significant difference was observed between the latter two methods.

Mode of failure analysis

The frequencies of the failure modes for the different adhesive systems at T₀ and T₆ are presented in Fig. 3A. For SU-ER, mixed failures were dominant, and very few pure adhesive failures

Table I. Application protocol for the tested adhesives.

Adhesive system	Application protocol
SB	The etchant was applied to the dentin for 15 s, followed by 10 s of water spray and medium air spray. Two coats of adhesive were then applied to the etched dentin with a rubbing action for 15 s without light curing between coats, followed by a gentle air stream (3 bar) for 5 s. Light irradiation was applied for 10 s.
SU-SE	The adhesive was applied to the air-dried dentin surface with a rubbing action for 20 s, followed by gentle air pressure (3 bar) for 5 s. Light irradiation was then applied for 10 s.
SU-ER	The etchant was applied to the dentin for 15 s, followed by water spray for 10 s and medium air spray for 2 s. Adhesive was then applied to the dentin surface with a rubbing action for 20 s, followed by medium air pressure for 5 s. Light irradiation was then applied for 10 s.

SB: Adper Single Bond 2; SU-SE: Scotchbond™ Universal/self-etch mode; SU-ER: Scotchbond™ Universal/etch-and-rinse mode

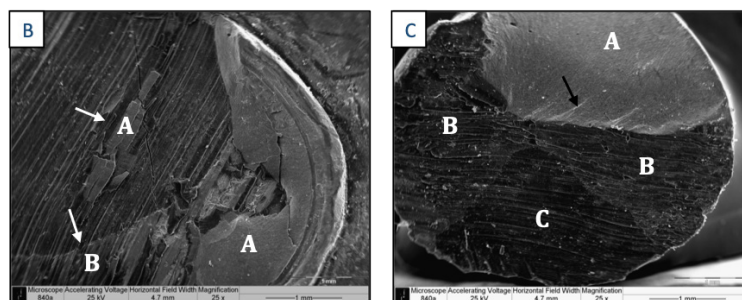
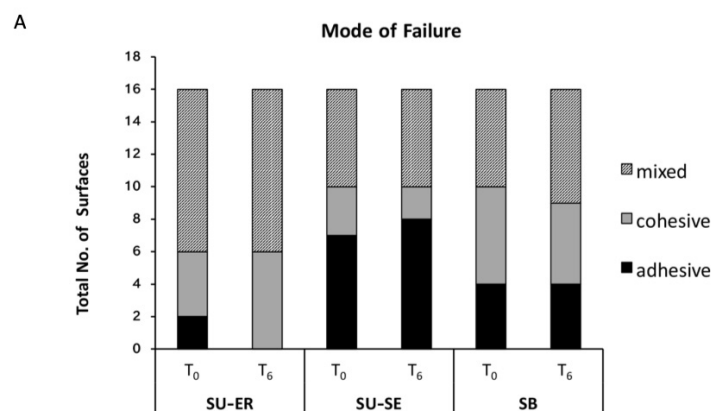


Fig. 3. *A) Frequencies of failure modes for the different adhesive systems determined by light microscopy at one week (T0) or 6 months (T6). SU=Scotchbond™ Universal; ER: etch-and-rinse; SE: self-etch; SB: Adper Single Bond 2. Representative SEM micrographs of resin-dentin interfaces treated with SU-ER at T0 indicating a mixed mode of failure, determined at a magnification of 25×. B) Dentin surface. A (and arrow) clearly denote parts of the composite. B indicates a region partially coated by SU, and the remaining area is clear dentin with intense polishing tracks, which is consistent with some of the topographical features found in Figure 4B. The dentin polishing tracks are continuous, thus reducing the possibility of cohesive dentin failure. C) Composite surface of the gelatin capsule. Three regions can be identified: A: Cohesive composite failure. Note the material striations (black arrow). Two levels seem to be present: an inclined level and a more horizontal level. B: Cohesive failure of SU. C: Exposed composite due to adhesive debonding from the SU.*

occurred (2/32 surfaces). When the same adhesive was used in the self-etch mode (SU-SE), adhesive failure was dominant (47% of surfaces), followed by mixed failure (38%). The SBS failure rates were approximately 40%, 35% and 25% for mixed, cohesive and adhesive failures, respectively. The incubation time had a minor effect on the mode of failure; for all adhesives, the trends at T0 and T6 were similar.

Mixed failure, which was more frequent in the SU-ER and SB groups, is illustrated in an SEM image at a magnification of 25× in Fig. 3, B and C.

DISCUSSION

We found, in the current study, that the SBS to dentin for SU applied in the ER mode was significantly higher (~30%) than the values obtained for the SE mode and for the control, SB. The higher SBS values in the ER mode were accompanied by only a few pure adhesive failures, and the majority of the failures were of mixed mode, whereas in the SE mode and the SB condition, adhesive and mixed modes of failure occurred with similar frequencies. We also found that the bond strength did not deteriorate in either mode after 6 months aging. Our findings regarding the superiority of the ER mode are consistent with those of Vermelho et al. (4). A recent finding showed that pre-etching the dentin before the application of SE adhesives increased the bond strength and did not increase the nanoscale leakage. This result was emphasized by the fact that the universal adhesives penetrated both demineralized and non-demineralized dentin when used in the ER mode, leaving behind no detectable freezable water (5). Lack of deterioration after aging was also shown by Manfroï et al. (3) and was supported clinically over a period of 24 months for both the ER and SE modes (2). The durability of the bond strength might be explained by the bonding of 10-MDP to calcium, which creates a hydrolytically stable salt (MDP-Ca) that protects against hydrolysis (12). The chemical bonding between the carboxylic groups of the methacrylate-modified polyalkenoic acid copolymer and the calcium ions within the hydroxyapatite also contributes to the stability of the adhesive layer (3).

The results of the current study suggest that etching did not disrupt the bonding strength of SU to dentin, either at baseline or after six months of aging. Further clinical studies are required to validate the results of this *in vitro* study.

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LETTER TO THE EDITOR

CLINICAL NURSING INTERVENTION ON SEVERE PANCREATITIS AFTER
SURGICAL TREATMENTL. WANG¹, XY. SUN¹, CL. ZHANG², LJ. ZHOU¹, FY. YANG¹ and MH. SHAN³

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To the Editor,

Pancreatitis is a common digestive system disease. Mild pancreatitis can recover spontaneously under the immune function of human body and assistance of drugs, but severe pancreatitis has a high fatality rate because of damage to organs and tissues and a large number of complications (1). The pathogenesis of severe pancreatitis is complex, and the causes are various, but all eventually lead to excessive secretion of pancreatic enzyme and a series of complications. However, although current surgery causes only mild damage to organs and tissues, patients usually have negative emotions and have little cooperation with doctors because of insufficient understanding of pancreatitis and the influence of pain. Therefore, in addition to conventional nursing, dedicated nursing is also needed for these patients (2, 3). In nursing intervention, the nurses give disease-related information to patients and their families before and after surgery and inform them about the curative effect of surgery; in addition, they make adjustments according to the progress of treatment course while providing nursing services.

MATERIALS AND METHODS

Subjects

One hundred patients with severe pancreatitis in the Second Affiliated Hospital of Harbin Medical University, Heilongjiang, China, were selected. All the patients had a complete medical record and signed informed consent, and the study was reviewed and approved by the ethics committee of the hospital. The 100 patients were randomly divided into a control group and an experimental group of 50 cases each. In the control group the patients were aged 25-75 years (average 45.11±10.12 years). The experimental group patients were aged 24-76 years (average 44.15±11.03 years). The independent *t*-test suggested that the basic data of the two groups showed no significant difference, hence the results of the two groups were comparable.

Control group

Patients in the control group were given conventional nursing. They were asked to stay in bed after surgery and to take medicine on time in accordance with doctor's orders. Nurses always paid attention to the related physiological

Key words: severe pancreatitis; nursing intervention; surgical treatment; satisfaction

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signs of patients to prevent shock. Nurses paid attention to the abdominal clinical symptoms of patients and contacted doctors immediately when abnormal physical signs were found. The stable and smooth flow of transfusion tubes and catheters connected to the body of patients after operation were paid attention to. Antibiotics were applied to the incision after the operation to ensure that it was not infected, and doctors were notified if bleeding occurred.

Experimental group

Besides conventional nursing, patients in the experimental group were also given other nursing interventions. Firstly the medical records of patients with pancreatitis who were admitted between January 2012 and January 2012 were retrospectively analyzed to summarize factors inducing pancreatitis, and corresponding nursing countermeasures were formulated (4). To prevent recurrence caused by original pancreatitis, patients were examined before surgery to accurately evaluate disease condition. Patients with history of pancreatitis were given some preventive medicines. Injection of contrast agent was kept reasonable during surgery, and was expelled thoroughly after surgery. To prevent pancreatitis induced by repeated imaging of the pancreatic duct, imaging was performed after the contrast agent was thoroughly expelled, and the incision knife and guide wire were kept in a right direction in the pancreatic duct. To prevent injury of surgical incision induced by frequent intubation, the patients took nitroglycerin before surgery, and the surgical tools were checked for any defects which could damage organs, and appropriate tubes were selected. To prevent pancreatitis caused by unreasonable concentration and dose of contrast agent, 25% concentration and a dose no more than 20 mL were ensured, contrast agent was expelled thoroughly as far as possible, and the infusion speed was maintained not to exceed 0.6 mL/s. To prevent infectious factors, surgical tools were sterilized, and sterile operation was strictly followed during surgery.

Nursing intervention not only included nursing countermeasures for the factors causing pancreatitis, but also included the following content. Communication with patients was strengthened to timely understand patients' psychological status, and information on the disease and surgery was given to reduce the negative emotion of patients. Moreover, family members of patients were appealed to increase compliance. Patients who were in

bed for a long time after surgery were turned every two hours. Moreover, nurses paid attention to the comfort of patients and assisted patients with their personal hygiene. The bed and room were kept clean and ventilated. The oral cavity of the patients was timely cleaned after surgery. Different rehabilitation training plans were formulated for patients with different degrees of recovery. Information on rehabilitation training and pathology was given to patients and their family members. Living and dietary habits of patients were standardized. Smoking and drinking were strictly prohibited, and other symptoms of biliary tract diseases after the operation were paid attention to. After discharge from hospital, appropriate dietary plans were formulated for them. Moreover, special nurses were assigned to follow-up for 1~3 months to understand the recovery condition of patients and adjust rehabilitation plans.

Evaluation criteria for efficacy

Whether patients had epigastric pain, fever and emesis within 24 h after surgery was observed; if they had, then there was recurrence of pancreatitis.

Data regarding hospitalization time, complications during hospitalization and reoperation were collected. The satisfaction of patients on the nursing service was investigated through a questionnaire.

Statistical analysis

The collected data were analyzed using SPSS. Measurement data were expressed in the form of $\bar{x} \pm s$ and compared by independent *t*-test; difference was considered as statistically significant if value of *P* was smaller than 0.05. Categorical data were expressed in the form of % and compared using *chi*-squared test; difference was considered as statistically significant if *P* value < 0.05.

RESULTS

Occurrence of pancreatitis within 24 h after surgery

As shown in Table I, within 24 hours after operation, 38% of the patients in the control group and 12% of patients in the experimental group had recurrence. The difference between the two groups was significant ($P=0.001 < 0.05$). The recurrence rate of the experimental group which received nursing

intervention was even smaller. 62% of patients in the control group and 88% of patients in the experimental group had stable disease condition and no recurrence within 24 h after surgery, and the difference was statistically significant ($P=0.002 < 0.05$). This confirms that nursing intervention played a role in the recovery of patients after surgery.

As shown in Table II, 4% in the control group and 2% in the experimental group had recurrence because of original pancreatitis, and the difference was significant ($P < 0.05$); 8% of patients in the control group and 4% of patients in the experimental group had recurrence due to repeated imaging of pancreatic duct, and the difference was significant ($P < 0.05$); 14%

in the control group and 6% in the experimental group had recurrence due to injury of surgical incision caused by frequent intubation, and there was a significant difference between the two groups ($P < 0.05$); 6% in the control group and 0% in the experimental group had recurrence because of unreasonable concentration and dose of contrast agent, and the difference was statistically significant ($P < 0.05$); 6% in the control group and 0% in the experimental group had recurrence because of infectious factors, and the difference was significant ($P < 0.05$).

Effects of nursing intervention during hospitalization

The average hospitalization time of the control

Table I. Occurrence of pancreatitis within 24 h after surgery

Occurrence of pancreatitis	Control group/%	Experimental group/%	P value
Recurrence of pancreatitis	38.00	12.00	0.001
Stable disease condition and no recurrence	62.00	88.00	0.002

Table II. Analysis of factors inducing recurrence of pancreatitis after operation

Recurrence related factors	Control group/%	Experimental group/%	P value
Original pancreatitis	4	2	0.001
Repeated imaging of pancreatic duct	8	4	0.000
Surgical incision caused by frequent intubation	14	6	0.001
Unreasonable concentration and dose of contrast agent	6	0	0.000
Infectious factors	6	0	0.000

group was 45.33 ± 8.25 days, and that of the experimental group was 23.14 ± 6.21 days, and the difference between the two groups was significant ($P < 0.05$) (Table III). During the hospitalization period, 82.15% of the patients in the control group and 24.15% of the patients in the experimental group had complications, and there was a significant

difference ($P < 0.05$). The proportion of patients in the control group who underwent reoperation during hospitalization was 51.22%, and that in the experimental group was 7.13%.

Degree of satisfaction on nursing intervention

As shown in Fig. 1, there were three evaluations

Table III. Comparison of average hospitalization time, incidence of complications and reoperation rate between the two groups.

Recurrence related factors	Control group/%	Experimental group/%	P value
Original pancreatitis	4	2	0.001
Repeated imaging of pancreatic duct	8	4	0.000
Surgical incision caused by frequent intubation	14	6	0.001
Unreasonable concentration and dose of contrast agent	6	0	0.000
Infectious factors	6	0	0.000

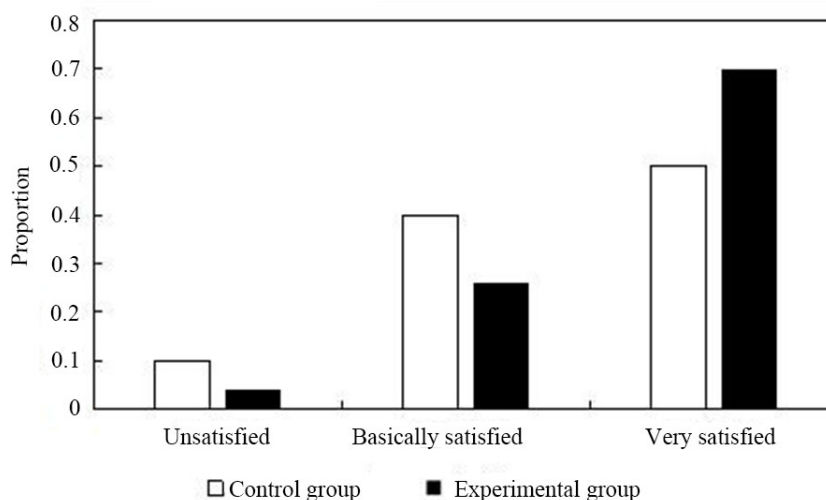


Fig. 1. Satisfaction of the control group and experimental group on nursing service during hospitalization.

of the nursing service during hospitalization: unsatisfied, basically satisfied and very satisfied. 10% of the control group and 4% of the experimental group gave an evaluation of unsatisfied; 40% of the control group and 26% of the experimental group made an evaluation of basically satisfied; 50% of the control group and 70% of the experimental group made an evaluation of very satisfied.

In the calculation of satisfaction, there were two items: basically satisfied and very satisfied; the proportion of patients who made the corresponding evaluation was directly counted as degree of satisfaction. Therefore, the satisfaction degree of nursing service during hospitalization was 90% in the control group and 96% in the experimental group; there was a significant difference ($P < 0.05$).

DISCUSSION

In this study, 100 patients with severe pancreatitis were randomly divided into a control group and an experimental group. The control group received conventional nursing, while the experimental group received targeted nursing interventions. The results showed that the existence of original pancreatitis, repeated pancreatic duct imaging, surgical incision injury caused by frequent intubation, irrational dose and concentration of contrast agent and infectious factors could affect the recurrence of pancreatitis after surgery, but nursing intervention could effectively

reduce the impact of the above factors. Moreover, nursing intervention could significantly shorten the hospitalization time of patients, reduce the incidence of complications and secondary surgery rate, and improve patient satisfaction of nursing.

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LETTER TO THE EDITOR

EXPRESSION OF miR-26R IN OVARIAN CANCER TISSUES AND ANALYSIS OF ITS CLINICAL SIGNIFICANCE

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To the Editor,

Ovarian cancer is one of the most common malignant tumors in the reproductive system of women, and its mortality rate is the highest among all the female malignant tumors (1). In recent years, the prevalence of ovarian cancer in women in China has shown an obvious upward trend year by year, which poses a great threat to women's health (2). Ovarian cancer has no obvious symptoms in the early stage. Most cases are in the late stage when diagnosed, and if treated early, the 5-year survival rate can be significantly improved. Therefore early diagnosis of ovarian cancer is very important (3, 4). At present, detection of serum carbohydrate antigen 125 (CA125) or human epididymal protein 4 (HE4), pelvic examination, and imaging examination are still the main methods of clinical diagnosis, but these methods have their limitations.

MiRNA is a non-coding small RNA with a length of 19 ~ 25 nucleotides, and it can negatively regulate the expression of relevant genes after transcription (5, 6). miR-26b is reported as a tumor suspension miRNA and can be abnormally expressed in multiple cancer tissues such as lung cancer (7), breast cancer (8), colon cancer (9) and glioma (10). miR-26b acts as a tumor suppressor gene in these cancers. However, the expression and clinical significance of miR-26b in epithelial ovarian cancer are not clear. In order to provide a new marker for the diagnosis and prognosis evaluation of ovarian cancer, the

expression of miR-26b in ovarian cancer and its relationship with clinicopathological parameters and prognosis was explored in this study.

MATERIALS AND METHODS

Seventy patients with ovarian cancer who underwent surgical treatment in our hospital from August 2016 to December 2017 and were diagnosed by postoperative pathological diagnosis were retrospectively enrolled as study subjects (ovarian cancer group). They had not undergone radiotherapy or chemotherapy treatment prior to surgery. The age of the patients ranged from 39 to 71 years (average 50.72 ± 6.11 years), and there were 18 cases of endometrioid ovarian carcinoma, 8 cases of mucinous ovarian carcinoma and 44 cases of serous ovarian carcinoma. Twenty-nine patients had no more than 300 mL of ascites and 41 patients had more than 300 mL of ascites. Ascetic cytological examination suggested 31 negative cases and 39 positive cases. According to the International Federation of Gynecology and Obstetrics (FIGO) stages, there were 26 cases of stage I~II and 44 cases of stage III~IV. There were 10 cases of high tissue differentiation, 24 cases of moderate tissue differentiation and 36 cases of low tissue differentiation. There were 23 cases of distant metastasis, 47 cases of non-distant metastasis. In the same period, 50 cases of benign epithelial ovarian tumors (benign group) and 40 cases of uterine fibroid (normal group) were selected as controls. The fifty patients with benign epithelial ovarian tumors

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were aged 38 to 69 years, average (53.03±4.18) years. The specimens were confirmed to be benign ovarian tissues by postoperative pathology. The specimens of the forty patients with uterine fibroid [aged 39 to 70 years, average (51.49±5.38) years] were normal ovarian tissues obtained after hysteromyomectomy and adnexal resection. The

study was approved by the ethics committee of our hospital and informed consent was obtained from all patients.

Detection methods

Real-time fluorescence quantitative PCR was used.

Table I. Expression level of miR-26b in different ovarian tissues.

Group	Number of cases	Level of miR-26b
Ovarian cancer group	70	0.28±0.08
Benign group	50	1.08±0.14
Normal group	40	1.07±0.12
F	42.297	
P	<0.05	

Table II. Relationship between the expression of miR-26b and the clinicopathologic features of ovarian cancer.

Clinicopathologic parameters		Number of cases	Expression level of miR-26b	<i>t</i>	P
Age (year)	≥50	40	0.27±0.09	0.956	P>0.05
	<50	30	0.28±0.10		
Pathological type	Endometrioid ovarian cancer	18	0.35±0.09	0.473	P>0.05
	Mucous ovarian cancer	8	0.31±0.08		
	Serous ovarian cancer	44	0.34±0.11		
Ascites volume (mL)	≤300	29	0.17±0.14	4.174	P<0.05
	>300	41	0.46±0.21		
Ascites cytological examination result	Negative	31	0.21±0.09	3.965	P<0.05
	Positive	39	0.48±0.11		
FIGO stage	I~II stage	26	0.37±0.13	5.032	P<0.05
	III ~ IV stage	44	0.16±0.09		
Differentiation degree	High	10	0.34±0.10	8.417	P<0.05
	Moderate	24	0.27±0.11		
	Low	36	0.14±0.07		
Lymphatic metastasis	Yes	23	0.20±0.07	6.784	P<0.05
	No	47	0.38±0.14		

RNA was separated and extracted following the operation instructions on the TRIzol RNA isolation and extraction kit (Shanghai Sangon Biotech Company, China). The purity of RNA was detected by an ultramicro-spectrophotometer (Beckman Company, USA). The samples with OD260/OD280 value between 1.8 and 2.0 were taken and the RNA was reversely transcribed into complementary DNA (cDNA) according to the instructions of Prime Script™ reverse transcription kit (TaKaRa Company, Japan). After the reaction, the synthesized cDNA sample was diluted and stored in a refrigerator. U6 snRNA was taken as the internal reference in the real-time fluorescence quantitative PCR; the upstream primer of microRNA-

26b was 5'-CAAAGTCCAGTAGCAAGGGGGT-3', and the downstream primer was 5'-GCGACCTTGTCATGTTTAG-3'. The upstream primer of U6 was 5'-CTCGCTTCGCAGCAGCAGCA-3', and the downstream primer was 5'-AACGCTTCACGAATGTTGT-3'. It was pre-denatured at 95°C for 5 min, then denatured at 90°C for 15 s, and annealed and extended at 60°C for 30 s, for 40 cycles. The relative expression quantity of miR-26b was expressed by $2^{-\Delta\Delta C_t}$. The experiment was repeated 3 times, and the average value was regarded as the result. The relationship between the expression of miR-26b and clinicopathological parameters of ovarian cancer,

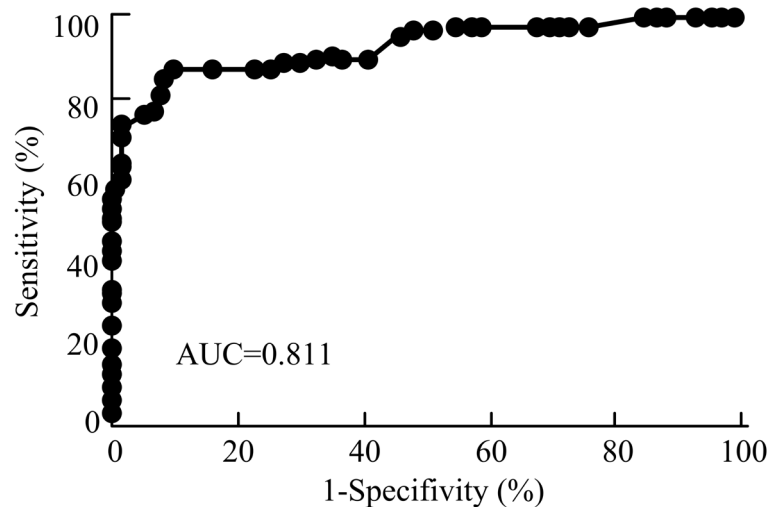


Fig. 1. Efficiency of miR-26b in the ovarian tissues in the diagnosis of ovarian cancer.

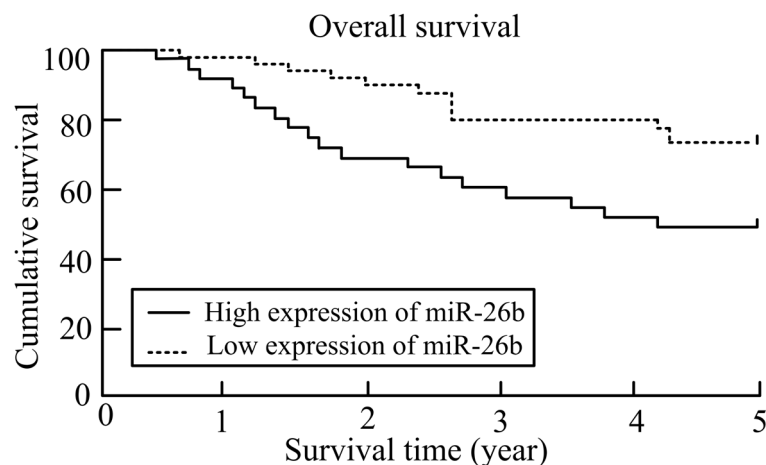


Fig. 2. Correlation between the expression of miR-26b in the ovarian tissues and the 5-year survival rate of the patients.

including age, pathological type, ascite volume, ascite cytological examination result, FIGO stage, tissue differentiation and distant metastasis were examined.

Follow-up

All the subjects were followed up for 8~70 months. The average follow-up time was 54.46 ± 2.06 months. The survival rate of the patients was analyzed using Kaplan-Meier method.

Statistical analysis

Statistical analysis was performed using SPSS 20.0. Measurement data were expressed as mean $\pm$ standard deviation; pairwise comparison was performed by *t*-test, and comparison between three groups was performed using analysis of variance. Categorical data was processed by *chi*-squared test. The diagnostic value of miR-26b was analyzed by receiver operating characteristic (ROC) curve. The survival curve of the patients was drawn using Kaplan-Meier analysis method. The difference of survival between groups was analyzed using Log-Rank test. Difference was considered as statistically significant if $P < 0.05$.

RESULTS

Expression level of miR-26b in different ovarian tissues

The expression level of miR-26b in the ovarian cancer group was significantly lower than that in the benign group and normal group ($P < 0.05$); there were statistically significant differences ($P < 0.05$). No significant difference was observed in the expression level of miR-26b between the benign group and normal group ($P > 0.05$, Table I).

Relationship between miR-26b and the clinicopathologic features of ovarian cancer

The relative expression quantity of miR-26b in the ovarian cancer tissues in the ovarian cancer group was not significantly correlated with age and pathological type ($P > 0.05$), but was correlated to ascite volume, ascite cytological examination result, FIGO stage, tissue differentiation and distant metastasis ($P < 0.05$, Table II).

miR-26b expression in ovarian tissues in the diagnosis of ovarian cancer

The ROC curve demonstrated that the area under the ROC curve was 0.811, and the sensitivity of diagnosing ovarian cancer was 86.56%, the specificity of diagnosing ovarian cancer was 85.27%, and the corresponding critical value was 0.72 (Fig. 1), indicating that the expression of miR-26b had high value in diagnosing ovarian cancer.

Relationship between expression of miR-26b in ovarian tissues and prognosis of ovarian cancer

The survival curve was drawn using Kaplan-Meier analysis method. The results suggested that the overall 5-year survival rate of the patients with high expression of miR-26b was 78.57%, which was significantly higher than the 54.29% in the patients with low expression of miR-26b. The difference was statistically significant ($\chi^2 = 0.575$, $P < 0.05$, Fig. 2).

DISCUSSION

miR-26 is a member of the miRNA family. The results of this study showed that the expression level of miR-26b in the ovarian cancer group was significantly lower than that in the benign group and normal group, suggesting that the expression of miR-26b decreased in ovarian cancer tissues and played an oncogene-like role in ovarian cancer. The relative expression of miR-26b in the ovarian cancer tissues was correlated with the amount of ascites, negative and positive ascite cytological results, FIGO stage, differentiation degree of cancer tissues and distal metastasis of ovarian cancer cells. It indicated that the expression of miR-26b might be correlated to the differentiation, proliferation and migration ability of ovarian cancer cells, which is similar to the conclusions of previous studies (11, 12).

In addition, the ROC curve showed that the sensitivity and specificity of miR-26b expression in the diagnosis of ovarian cancer were 86.56% and 85.27%, respectively, suggesting that miR-26b expression had high diagnostic value. The follow-up results demonstrated that the 5-year overall survival rate of patients with high expression of miR-26b was significantly higher than that with low expression,

indicating that miR-26b was closely correlated to the prognosis of ovarian cancer.

In conclusion, the expression of miR-26b is low in ovarian cancer tissues, and is related to the disease condition, metastasis and prognosis. miR-26b can therefore be a new marker for early diagnosis and prognosis of ovarian cancer.

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LETTER TO THE EDITOR

EXPRESSIONS, FUNCTION AND SIGNIFICANCE OF IL-10 AND RANTES IN RATS WITH ALLERGIC RHINITIS

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To the Editor,

As a common type of rhinitis, allergic rhinitis (AR) mainly refers to type I allergic diseases that are involved and mediated by various inflammatory cells such as eosinophils, T lymphocytes, and mast cells (1, 2). The main clinical manifestation of AR patients is the abnormal level of inflammatory responses in the nasal mucosa. The prevalence rate of AR is high and continues to increase with the increasing environmental pollution in recent years (3).

The specific pathogenesis of AR has not yet been fully understood, and it is generally thought to be related to Th1/Th2 imbalance (4). Histologically, AR is characterized by plasma protein exudation in nasal cavity, infiltration of epithelial tissues and eosinophils, mast cells, basophils, dendritic cells and other inflammatory cells (5). RANTES is the first chemokine of eosinophils (EOS), which mediates the chemotaxis of inflammatory cells to target tissues by binding to the corresponding receptors on the surface of effector cells. IL-10 has strong anti-inflammatory and immunosuppressive activities. It can inhibit the production and release of IL-2, IFN γ and other pro-inflammatory factors at the level of RNA (6). However, there are few studies on the mechanism of AR. In this study, ovalbumin-sensitized rats were used to prepare AR animal models. AR symptoms and pathological changes in the model group were

observed. EOS counts and expressions of RANTES and IL-10 in nasal mucosa were detected. The role of RANTES and IL-10 in the pathogenesis of AR was explored in order to provide a theoretical basis for clinical treatment.

MATERIALS AND METHODS*Animals*

Twenty healthy male Sprague Dawley (SD) rats (aged 6-8 weeks old, weighing 200-220 g, purchased from Kaisheng Biotechnology (Shanghai) Co., Ltd., China, were randomly divided into a model group and a control group, with ten rats in each group. The rats were given free access to feed and drinking water before the study. The animals were housed at 25 $\pm$ 1 $^{\circ}$ C. The animal experiment procedures were in accordance with the International Regulations on Animal Protection and Management and was approved by the ethics committee of Cangzhou Central Hospital.

Preparation of the AR rat model and animal treatment

The rats in the model group were sensitized using 1 ml of saline suspension containing 0.1% OVA (ovalbumin, Sigma, USA) and 0.43% aluminum hydroxide gel (Tianjin Damao Reagent Company, China). Each animal in the model group was subcutaneously injected with the drug into the abdominal wall once a day for a total of 7 days. At

*Key words: AR; IL-10; RANTES; nasal mucosa**Corresponding Author:*

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the same time, the animals in the model group were given by ultrasonic atomization a normal saline solution (20 ml) containing 0.25% OVA for 15 min per day for 5 days to enhance the sensitization. After an interval of 1 week, the nasal cavity of animals in the model group was attacked with 100 μ l of 1% OVA once a day for 20 consecutive days to trigger typical AR symptoms and signs (sneezing, scratching, runny nose, etc.). The rats in the normal control group were given normal saline instead of OVA using the method described above. The behavioral score of rats was recorded, with more than 5 points for each rat. The model was successfully constructed when a large number of EOS was found on nasal secretion smears (Table I).

Behavioral observation of rats

After the last nasal stimulation, the rats were observed for 15 min. The number of rats scratching their nose and sneezing was counted, while their nasal secretion was collected on a piece of filter paper. The size of the filter paper strip was 1 x 25 mm so that it could be placed in the nasal cavity after the last nasal cavity activation. After 5 min, the length of the wetted portion of the filter paper was measured and the amount of nasal secretion (mg) was calculated.

Collection of specimens

The blood near nasal cavity was collected in an Ep tube 48 h after the last stimulation and centrifuged at 3000 r/min. The supernatant was taken 10 min later and stored at -80°C for subsequent experiments.

Subsequently, the rats were anesthetized by an intraperitoneal injection with 1% chloral hydrate (Tianjin

Da Mao Reagent Co., Ltd., China) (3.4 to 3.6 ml/kg). Tracheotomy was performed between the 3rd and 4th tracheal rings. A soft plastic pipe with a diameter of about 2 mm was inserted above and below the location of tracheotomy, respectively. The wire was then ligated at the first tracheal ring and between the fifth and sixth tracheal rings, and the sheath was fixed. A micro pump (300 μ l/min) was then used for 10 min of nasal irrigation before nasal lavage fluid was collected. The amount of liquid in this operation was recorded. After centrifugation of the collected liquid, cell pellets were obtained for eosinophil count. Collection of nasal mucosae: The rats were sacrificed by cervical dislocation and their nose hair was cut off immediately before their turbinate and nasal septum were collected in a Trizol solution (Chengdu Dongsheng Kechuang Technology Co., Ltd., China) for testing.

Then, the nasal mucosa on both sides of the nasal septum was taken and fixed with 10% paraformaldehyde (Chengdu Dongsheng Kechuang Technology Co., Ltd., China) for 24 h. After conventional paraffin embedding, continuous slices were made for conventional HE (hematoxylin-eosin) staining and observed under a microscope.

Count of EOS in nasal lavage fluid

The cell pellet was collected by centrifugation and resuspended in 0.5 ml Hank solution (Shanghai Youyu Biotechnology Co., Ltd., China). After mixing, the suspension was adjusted to 20 μ l. Then, 380 μ l of diluted Ethanol-Eosin (Chengdu Dongsheng Kechuang Technology Co., Ltd., China) was added and the solution

Table I. *Symptom scoring criteria of AR.*

Symptoms	Mild	Moderate	Severe
Nasal itching	Times of rubbing nose	Between the two	Scratching nose and face and rubbing around.
Nasal mucus	Flowing to the front nostril	Flowing beyond the front nostril	Tears streaming down the face
Sneezing	1-3 times	4-10 times	More than 11 times
Scores	1	2	3

was gently mixed. After the mixture was allowed to settle down for 30 min, the eosinophils were stained and then placed into a cell counting dish and were manually counted.

Detection of RANTES in the nasal lavage fluid by ELISA

100 μ l of the test sample was taken and measured using a RANTES test kit (Shanghai Lianmai Bioengineering Co., Ltd., China) according to the instructions. The absorbance value of the reaction substrate was measured at a wavelength of 450 nm using a microplate reader. The concentration of the test sample (pg/ml) was automatically calculated based on the concentration of the standard and the dilution factor.

Detection of serum IL-10 by ELISA

An IL-10 (ELISA) kit (RD, USA) was used to perform the assay according to the instructions. In brief, an ELISA plate was coated with an anti-rat IL-10 monoclonal antibody before the IL-10 in the standards and samples were bound to the monoclonal antibody. Biotinylated anti-rat IL-10 antibody was then added to the plate to generate immobilized immune complexes. After enzyme substrate o-Phenylenediamine (OPD) was added following the addition of horseradish peroxidase-labeled streptavidin and biotin, a yellow color appeared in the samples.

Then, the stop solution (30%~40% sodium hydroxide solution) was added and the OD₄₉₂ value was measured at a wavelength of 492 nm. The concentration of IL-10 in the sample was obtained by using the standard curve.

Detection of the target protein expression by Western blotting

The volume of supernatant samples in each well was calculated by the protein concentration measured by BCA protein concentration detection. The total protein content of supernatant samples in each well was equal. After the sample was added to each well, it was added to the buffer and boiled, then, was placed in SDS- polyacrylamide gel for electrophoresis. The electrophoresis condition: first 80V under electrophoresis for 0.5 h, and then 110V for 1 h. After electrophoresis, the protein in the supernatant was transferred to the membrane by wet transfer method, and the protein on the polyacrylamide gel was transferred to the nitrocellulose membrane at a constant pressure 100V for 1.5 h. The membrane was washed with TBST buffer after membrane transfer and soaked in utensils for use. The supernatant samples were sealed at room temperature for 60 min with TBST 20 mL, BSA 0.75g and 3 spoons skimmed milk powder. The blocked membrane was incubated overnight in a shaking bed with anti-rabbit anti-human HO-1 polyclonal antibody (1: 200), anti-rabbit

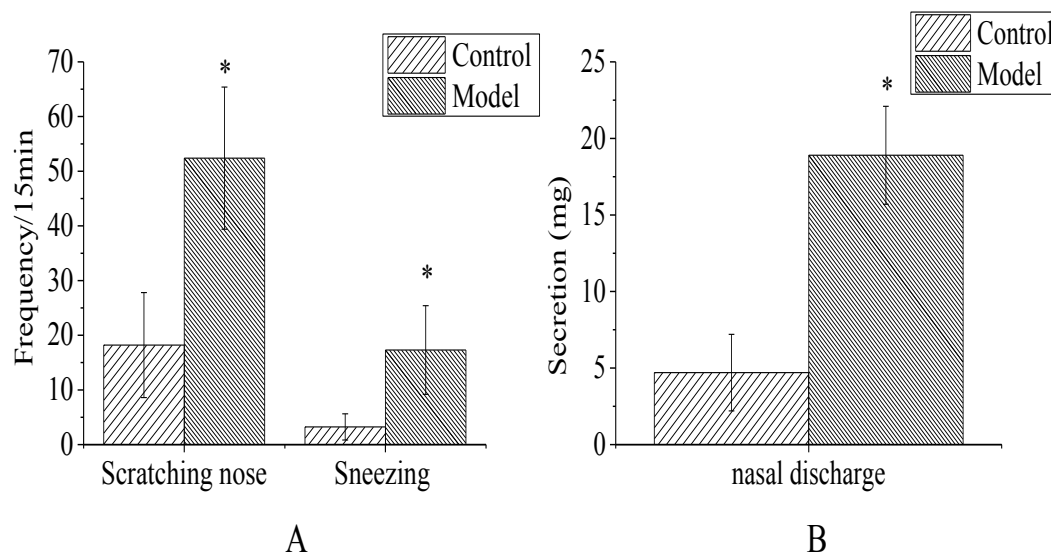


Fig. 1. Determination of behavioral indexes of rats in each group. **A)** weekly frequency statistics of sneezing and nose scratching in the model and normal control groups. **B)** Quantity statistics of nasal secretion in the model and normal control groups (compared with the control group, * $P < 0.05$).

anti-human TNF- α polyclonal antibody (Biyun Tian Biology Co., Ltd., China) (1:1000) and anti-rabbit anti-human IL-1 β polyclonal antibody (1:1000) at 4°C. On the second day, the phosphate buffer solution (PBS) was washed 15 min x 4 times. After cleaning, the membrane was added with 20 mL rabbit monoclonal antibody (Biyun Tian Biology Co., Ltd., China) (1:2000), incubated for 3 h, and then washed 15 min x 4 times with PBS. After these operations were successfully completed, the bands of cellulose nitrate membrane samples were analyzed by image pro plus (BIO-RAD, USA). The gray value of NC film (BIO-RAD, USA) was compared with the gray value of β -actin protein band, and then the ratio of the two was compared. The mean and standard deviation of the ratio were calculated after repeated three times.

Statistical analysis

The measured indicators were expressed as mean $\pm$ standard deviation. Data analysis was performed using SPSS11.0 for Windows statistical software and one-way ANOVA. Significance tests were performed to compare the difference in mean values between pairwise samples in different groups.

RESULTS

Biological behavior of rats in each group

In the excitation phase, the number of sneezes per 15 min was 18.2 ± 9.6 in the model group and 3.23 ± 2.4 in the normal control group, and the difference was obvious ($P < 0.05$). The number of nose scratches per 15 min was 52.4 ± 13 in the model group and 18.2 ± 9.6 in the normal control group, with significant difference ($P < 0.05$). The amount of nasal secretion was 18.9 ± 3.2 mg in the model group and 4.7 ± 2.5 in the normal control group, and the difference was statistically significant ($P < 0.05$), as shown in Fig.1.

Pathological observation of HE staining in rats and EOS counting in lavage fluid

As seen in Fig. 2, HE staining and EOS counting in the two groups of rats. HE staining showed that there were more basophils, fewer eosinophils and no glandular hyperplasia in the control group (Fig. 2A). HE staining showed that there were fewer basophils and more eosinophils infiltration in the model group

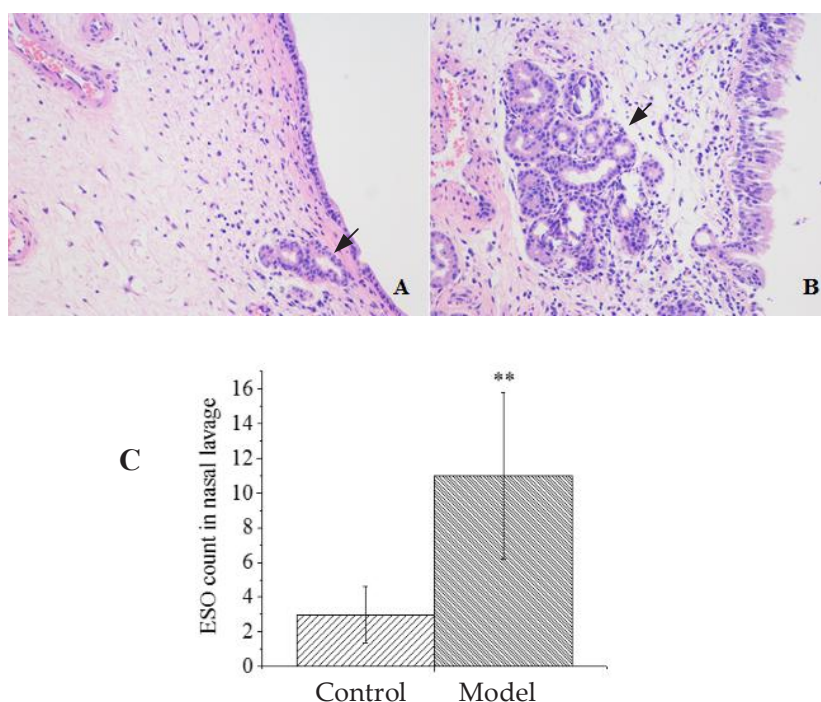


Fig. 2. Pathological examination results of three groups of rats (HE x 400). **A)** control group; **B)** model group (the arrow indicates the nasal mucosal glands); **C)** EOS count in nasal lavage fluid (compared with control group, ** $P < 0.01$).

(Fig. 2B), and the glandular hyperplasia in the nasal mucosa of rats was obvious. From the EOS count of HE staining of nasal lavage fluid in Fig. 2C, it was found that the number of EOS in the control group was 2.98 ± 1.63 , and that in the model group was 11.02 ± 4.78 , with significant difference between the two groups ($P < 0.01$).

Expression levels of RANTES in nasal lavage fluid and IL-10 in serum of rats

Fig. 3. showed the expression of RANTES in nasal lavage fluid and IL-10 in serum detected by ELISA and Western blot. From Fig. 3A, the electrophoretic patterns of RANTES in lavage fluid and IL-10 in serum of rats in both groups were obtained. The results of Fig. 3B showed that the expression of RANTES in nasal lavage fluid of the control group was 11.4 ± 5.9 pg/mL, significantly higher than 82.3 ± 11.2 pg/mL in the model group ($P < 0.05$). The level of IL-10 in the control group was 48.74 ± 3.49 ng/mL, significantly higher than 38.15 ± 4.89 ng/mL in the model group ($P < 0.05$) (Fig. 3C).

DISCUSSION

Symptom observation, on the one hand, can determine the success of AR rat models and ensure the reliability of the animal model established. On the other hand, through the comparison of specific symptoms, we can also observe the effect of intervention treatment. Local EOS infiltration of nasal mucosa is one of the characteristics of AR, which is mainly characterized by symptoms of itching, sneezing, runny nose and nasal obstruction (7). EOS is an important target cell of AR, which participates in the persistent allergic reaction after exposure to allergens. Especially in the late stage of AR, EOS increases the sensitivity of respiratory tract to allergens and promotes the sensitization of non-specific stimulants (8, 9). In this study, ovalbumin sensitized rats showed typical AR symptoms, indicating that the model was successfully established. By observing the difference of symptoms and pathological features between the model group and the control group, the AR model was successfully prepared. EOS counts in nasal lavage fluid of the

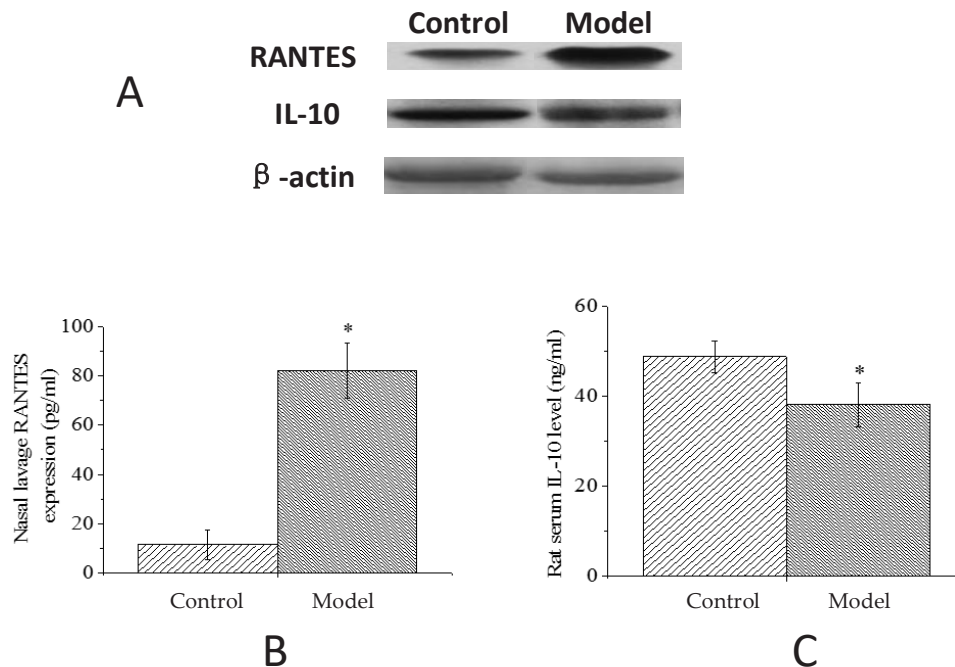


Fig. 3. Analysis of the expression levels of RANTES and IL-10 in rat nasal lavage fluid. **A)** Detection of electrophoresis patterns of RANTES, IL-10 and β -actin protein by Western blot; **B)** Expressions of RANTES in nasal lavage fluid; **C)** Serum IL-10 levels in rats (compared with control group, * $P < 0.05$).

two groups were observed, and it was found that the number of EOS in the model group was significantly higher than that in the control group.

RANTES can induce and activate basophils, mast cells and monocytes in AR by binding to CCR1, CCR2, CCR4 and CCR5 receptors (10). IL-10 is a cytokine secreted by both Th2 cells and Th1 cells (11), which has strong anti-inflammatory and immunosuppressive activities. It also has strong inhibitory effects on the activation of other inflammatory cells such as monocytes, macrophages, neutrophils and CD4⁺ T cells. IL-10 can inhibit the production and release of IL-2, IFN γ and other pro-inflammatory factors at the level of mRNA, and can reduce the surface expression of immune receptor MHC II (12). AR rat models were established to observe the changes of RANTES in nasal mucosa and nasal lavage fluid and IL-10 in serum. The results showed that the expression of RANTES in nasal mucosa and nasal lavage fluid was up-regulated and the level of IL-10 in serum was significantly decreased in the model group. It can be inferred that the dynamic changes of the expression levels of RANTES and IL-10 are the important mechanisms of AR.

In summary, RANTES and IL-10 are key factors involved in the pathogenesis of AR. Further research on RANTES and IL-10 will help to better understand the pathogenesis of AR.

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LETTER TO THE EDITOR

THE ROLE OF NSD2 AND EZH2 IN THE PATHOGENESIS OF ENDOMETRIAL CARCINOMA

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To the Editor,

Endometrial carcinoma (EC) is one of the most common malignant tumors in gynecology (1). According to the clinical characteristics and biological behavior, EC can be divided into type I and type II (2). Type I accounts for about 80% of all EC cases, which also include a small part of mucinous cancer. The prognosis of 80% patients in this group is good (3). On the other hand, while the onset age of type II is later than that of type I, most of type II, which also includes a small part of clear cell carcinoma, is serous (4).

As a histone lysine methyl transferase with a SET domain, NSD2 is involved in the regulation of chromatin. It has been shown that NSD2 may play a role in cancer by catalyzing the methylation of H3K36 (5). As a methylation catalytic enzyme of H3K27, EZH2 is involved in the modification of histone methylation and plays an important role in the growth and development of cells (6). Abnormal overexpression of EZH2 and NSD2 was shown to have a synergistic effect in malignant tumors, such as diffuse large B cell lymphoma (7), prostate cancer (8), B cell lymphoma (9), and breast cancer. Although EZH2 and NSD2 may promote the occurrence and progression of tumor lesions, the underlying mechanisms remain unclear and need further study.

In this study, the expression levels of NSD2 and EZH2 in EC were measured to provide a reference for clinical diagnosis and prognosis of this disease.

MATERIALS AND METHODS

Subjects

From June 2015 to June 2018, 42 paraffin-embedded tissue samples were collected from patients with EC who were pathologically diagnosed through surgical resection or diagnostic dilatation and curettage in the Second Affiliated Hospital of Harbin Medical University. The samples included 32 cases of type I EC (group A) and 10 cases of type II EC (group B). Clinical information of the patients was collected, including age, ethnicity, differentiation degree, vascular infiltration, lymph node metastasis, clinical and pathological stages, and muscle infiltration degree. According to the WHO (World Health Organization) classification of female reproductive organs in 2014, EC can be divided into two groups: type I EC and type II EC. In this study, cases with missing wax chunks, insufficient tissue volume, and plausible diagnosis were removed, while surgical and pathological staging was reperformed in accordance with the criteria of the International Federation of Gynecology and Obstetrics (2014).

A total of 28 cases of paraffin-embedded specimens of endometrial tissues obtained during the same period

Key words: NSD2; EZH2; EC

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from hysterectomy or diagnostic curettage of other uterine diseases (such as uterine leiomyoma, etc.) were selected as the control group, which included 12 cases of atypical hyperplasia or complex hyperplasia of the endometrium (group C) and 16 cases of normal endometrial tissues (group D). The experiment was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University and all patients signed an informed consent to participate in the study.

Each case was diagnosed as a single type of endometrial disease, atypical hyperplasia, complex hyperplasia, type I EC, or type II EC. All patients in the experimental group had no history of other tumors or other related diseases; prior to admission, all patients in the experimental group had not undergone other treatments, including surgical treatment (excluding diagnostic surgery), radiotherapy, and chemotherapy. After pathological diagnosis, HE stained tissue sections were screened under an optical microscope.

Slides

Slides were soaked in a washing buffer for 12 h, rinsed with running water, dried in an electric thermostatic drying oven, incubated in a 3-aminopropyltrimethoxysilane (APES) solution (Shanghai Youyu Biotechnology Co., Ltd., China) for 30 s, washed again in distilled water for 30 s, dried, and stored under a constant temperature.

Each of the wax specimens was sliced into 5 mm sections, placed on APES-treated slides, and baked in a 65°C electrothermal incubator for 120 min.

Immunohistochemistry assay

Paraffin sections were immersed sequentially in a xylene I solution, a xylene II solution, and a xylene III solution (Shanghai Kanglang Biotechnology Co., Ltd., China) for 10 min each using an Envision TM two-step method. Then, the slices were soaked in a 100% alcohol I solution and a 100% alcohol II solution (the 100% alcohol was packed separately, which was named alcohol I liquid and alcohol II liquid, respectively) for 10 min each, 95% alcohol for 5 min, and 75% alcohol for 2 min, respectively, to remove toluene residue. Thereafter, the sections were immersed in distilled water for 10 min to hydrate. After being soaked in methanol (10 min), hydrogen peroxide (10 min), distilled water (5 min), and phosphate buffer saline (PBS) (5 min) (Shanghai Kanglang Biotechnology

Co., Ltd., China) to block the endogenous enzymes, the sections were immersed in 1000 ml of antigen repair solution (EDTA) (Shanghai Kanglang Biotechnology Co., Ltd., China) in a pressure cooker for 3 min and 40 s to repair the antigen. After being cooled to room temperature and dried, the sections were incubated in a constant temperature humidified chamber at 37°C for 2 h or in a refrigerator at 4°C (Haier, China) overnight with anti-NSD2 or anti-EZH2 primary antibodies (Abcam, UK). Then, the sections were washed with PBS for 2 min $\times$ 3 times to remove residual primary antibody, dried, and then incubated at room temperature for 30 min with the secondary antibody (Dako, Denmark). Finally, the sections were washed with PBS for 2 min $\times$ 3 times, dried, developed using a DAB (3,3'-Diaminobenzidine) developer (Dako, Denmark), and observed by microscopy.

Immunohistochemical staining results (10): positive NSD2 and EZH2 signals were a tan or brownish-yellow color and were localized in the nucleus. Under a high-power microscope, ten representative fields were selected from each slide to count the proportion of positive cells. When the number of positive cells was $\geq 10\%$ of total cells, the slide was judged to be positive (when the number of positive cells was $50\% > N \geq 10\%$ of total cells, the slide was considered positive; when the number of positive cells was $75\% > N \geq 50\%$ of total cells, the slide was considered strong positive. When the number of positive cells was $\geq 75\%$ of total cells, the slide was considered extremely positive). When the number of positive cells was $< 10\%$ of total cells or none of the tumor cells were stained, the slide was judged as negative.

Statistical analysis

Statistics SPSS 17.0 software was used for data analysis. All count data were subjected to the χ^2 test and Fisher's exact probability test. The level of significance was set as $\alpha=0.05$. $P<0.05$ indicated that the results were statistically significant. Correlation analysis was performed using Spearman Rank correlation.

RESULTS

Expression of NSD2 in EC tissues

In endometrial tissues, the arrows in Fig. 1A indicate the NSD2 positive cells. In the experimental group, the positive expression rate of NSD2 in 42

cases of EC (EC) tissues was 61.90% (26/42). In addition, the positive rates in group A and group B were 59.38% (19/32) and 70% (7/10), respectively. The positive rate of NSD2 in 28 cases of endometrial tissues in the control group was 10.71% (3/28), while the positive rates in group C and group D were 16.67% (2/12) and 6.25% (1/16), respectively. Therefore, the expression of NSD2 was significantly higher in EC than that in the control group ($P=0.000$). The expression of NSD2 gradually increased in the order of normal endometrium, atypical hyperplasia or complex endometrial hyperplasia, and EC ($P = 0.000$). The results are shown in Fig. 1B and Table I.

Relationship between NSD2 and clinicopathological features of EC

The positive expression rates of NSD2 in patients of different age groups (≥ 50 years and < 50 years) were 67.86% (19/28) and 50% (7/14), respectively, and the difference was statistically significant ($P=0.031$). The positive expression rates in patients with different pathological stages (Ia stage and Ib stage) were 56.67% (17/30) and 75% (9/12), respectively, and the difference was significant ($P = 0.000$). The positive expression rates in patients with different degrees of differentiation (highly differentiated, moderately

Table I. Expression of NSD2 and EZH2 in endometrial tissues of groups A, B, C and D and their correlation in EC.

Protein	Group	Cases	Number of positive cases	Positive rate (%)	χ^2	P value
NSD2	Group A (type I EC)	32	19	59.38	23.091 ^{AC}	0.000
	Group B (type II EC)	10	7	70.00	27.846 ^{BD}	0.000
	Group C (atypical hyperplasia or complex endometrial hyperplasia)	12	2	16.67	14.000 ^{BC}	0.003
	Group D (normal endometrium)	16	1	6.25	31.167 ^{AD}	0.000
					7.143 ^{AB}	0.067
					39.500 ^{CD}	0.000
EZH2	Group A (type I EC)	32	20	62.50	13.636 ^{AC}	0.003
	Group B (type II EC)	10	7	70.00	22.923 ^{BD}	0.000
	Group C (atypical hyperplasia or complex endometrial hyperplasia)	12	3	25.00	10.364 ^{BC}	0.016
	Group D (normal endometrium)	16	2	12.50	22.667 ^{AD}	0.000
					2.762 ^{AB}	0.430
					11.570 ^{CD}	0.001

Note: χ^{2AB} referred to the χ^2 value between group A and group B. P was the value between group A and group B. $P < 0.01$ and $P < 0.05$ suggested that the difference was statistically significant. $P > 0.05$ suggested that the difference was not statistically significant.

differentiated, and poorly differentiated cancer) were 56% (14/25), 70% (7/10), and 71.43% (5/7), respectively, and the difference was significant ($P = 0.001$). The positive expression rates in patients with different degrees of myometrial invasion ($<1/2$ muscle layer and $\geq 1/2$ muscle layer) were

58.82% (20/34) and 75% (6/8), respectively, and the difference was significant ($P = 0.000$). The positive expression rates in patients with or without vascular invasion/lymph node metastasis were 50% (1/2) and 62.5% (25/40), respectively, and the difference was significant ($P > 0.05$). The

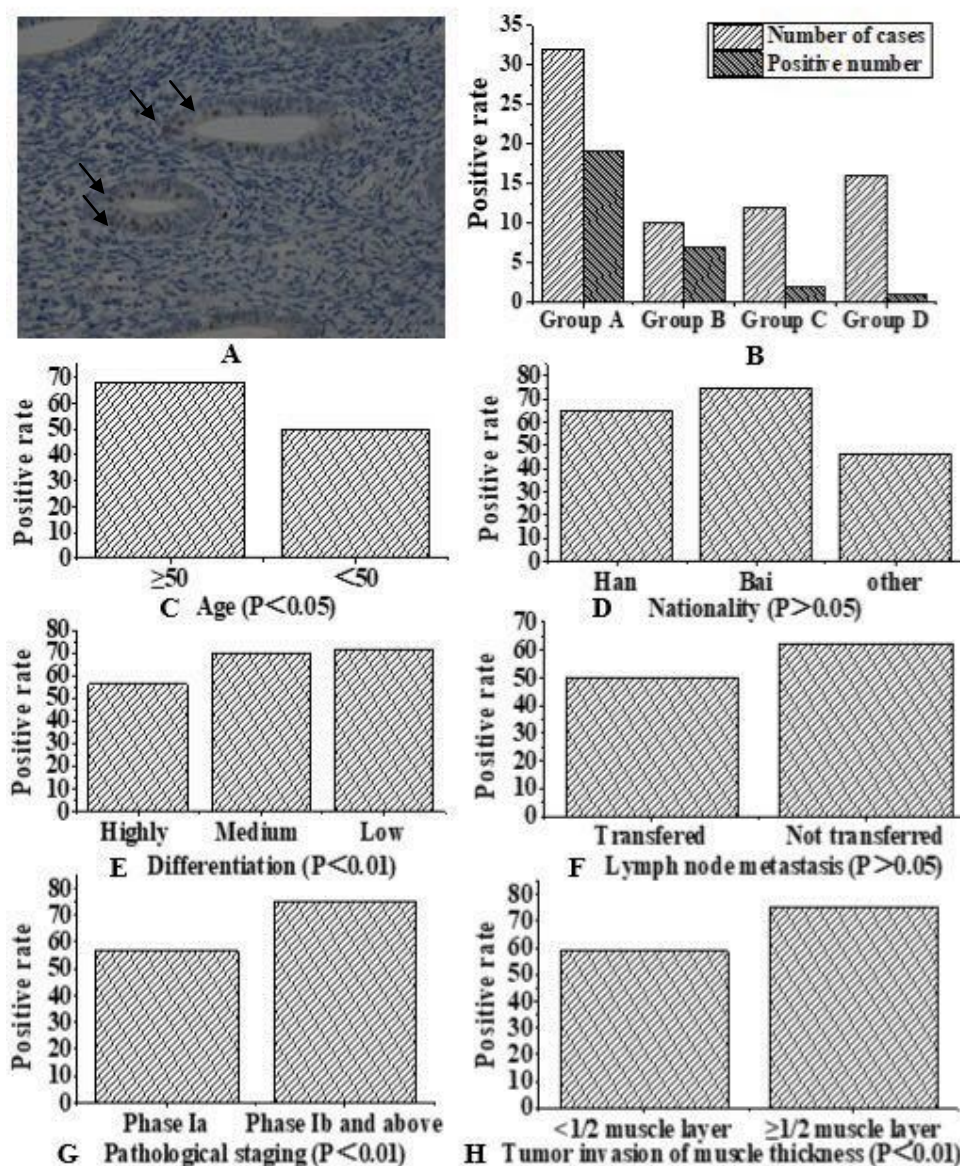


Fig. 1. Expression of NSD2 in endometrial tissues and its relationship with clinicopathological features of EC. **A)** positive expression of NSD2 in the nucleus of endometrial cells ($\times 400$); the cells indicated by the arrow are NSD2 positive cells; **B)** expression of NSD2 in different endometrial tissues; **C)** the relationship between NSD2 and age; **D)** the relationship between NSD2 and nationality; **E)** the relationship between NSD2 and degree of differentiation; **F)** the relationship between NSD2 and vascular invasion or lymph node metastasis; **G)** the relationship between NSD2 and surgical pathological stage; **H)** the relationship between NSD2 and the degree of muscle infiltration).

positive expression rates in patients of different ethnic groups (Han ethnicity, Bai ethnicity, and other ethnicity) were 64.17% (11/17), 75% (9/12), and 46.15% (6/13), respectively, but the difference was not statistically significant ($P=0.607$). The detailed results are shown in Fig. 1, C-H.

Expression of EZH2 in EC tissues

In endometrial tissues, the cells indicated by the arrow in Fig. 2 are NSD2 positive cells. The positive expression rate of EZH2 in 42 cases of EC tissues in the experimental group was 64.29% (27/42). In addition, the positive expression rates in group A

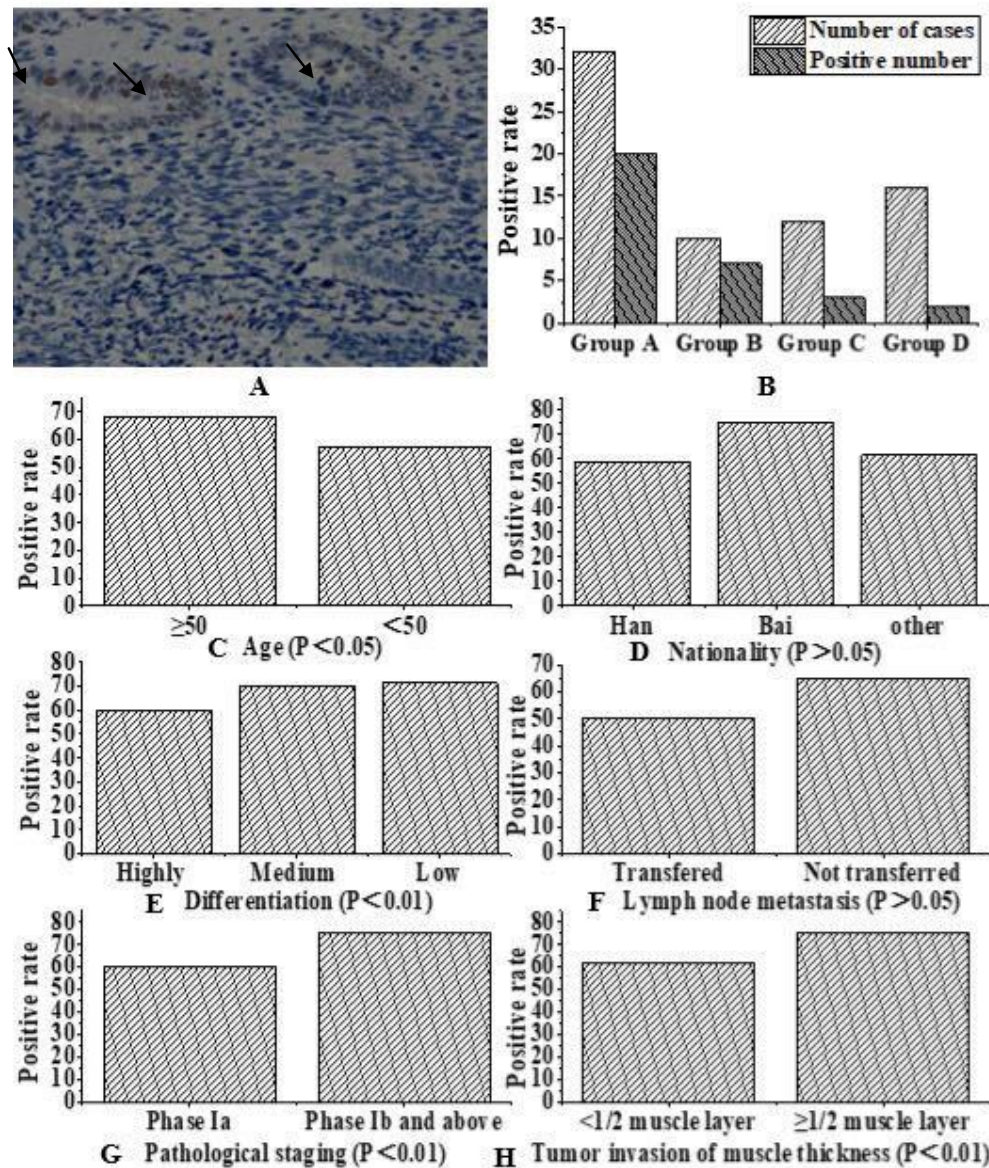


Fig. 2. Expression of EZH2 in endometrial tissues and its correlation with clinicopathological features of EC (A: positive expression of EZH2 in the nucleus of endometrial cells ($\times 400$); the cells indicated by the arrow are NSD2 positive cells; B: expression of EZH2 in different endometrial tissues; C: the relationship between EZH2 and age; D: the relationship between EZH2 and nationality; E: the relationship between EZH2 and degree of differentiation; F: the relationship between EZH2 and vascular invasion or lymph node metastasis; G: the relationship between EZH2 and surgical pathological stage; H: the relationship between EZH2 and the degree of muscle infiltration)

and group B were 62.5% (20/32) and 70% (7/10), respectively. The positive expression rate of EZH2 in 28 cases of endometrial tissues in the control group was 17.86% (5/28). In addition, the positive expression rates in group C and group D were 25% (3/12) and 12.5% (2/16), respectively. The expression of EZH2 in EC was significantly higher than that in the control group ($P=0.000$). The expression of EZH2 gradually increased in the order of normal endometrium, atypical hyperplasia or complex endometrial hyperplasia, and EC ($P=0.000$). The specific results are shown in Fig. 2B and Table I.

Relationship between EZH2 expression and clinicopathological features of EC

The positive rates of EZH2 in patients of different age groups (≥ 50 years and < 50 years) were 67.86% (19/28) and 57.14% (8/14), respectively, and the difference was significant ($P=0.031$). The positive expression rates in patients with different pathological stages (stage Ia and stage Ib) were 60% (18/30) and 75% (9/12), respectively, and the difference was significant ($P=0.000$). The positive expression rates in patients with different degrees of differentiation (highly differentiated, moderately differentiated, and poorly differentiated cancer) were 60% (15/25), 70% (7/10), and 71.43% (5/7), respectively, and the difference was significant ($P=0.001$). The positive expression rates in patients with different degrees of myometrial invasion ($< 1/2$ muscle layer and $\geq 1/2$ muscle layer) were 61.76% (21/34) and 75% (6/8), respectively, and the difference was significant ($P=0.000$). The positive rates of expression in patients with or without vascular invasion or lymph node metastasis were 50% (1/2) and 65% (26/40), respectively, but the difference was not statistically significant ($P>0.05$). The positive expression rates in different ethnic groups (Han ethnicity, Bai ethnicity, and other ethnicity) were 58.82% (10/17), 75.00% (9/12), and 61.54% (8/13), respectively, but the difference was not statistically significant ($P=0.607$). The detailed results are shown in Fig. 2, C-H.

Correlation analysis of NSD2 and EZH2 expression in EC

The positive expression rates of NSD2 and EZH2 in all 42 patients of EC were 61.9% and

64.29%, respectively. The expression of NSD2 alone accounted for 9.52%, while the expression of EZH2 alone accounted for 11.9% of the factors involved in the onset of EC. In addition, the values of negative expression and positive expression of both NSD2 and EZH2 were 26.19% and 52.38%, respectively. The results of the Spearman Rank correlation analysis suggested that the expression of NSD2 and EZH2 was positively correlated with the onset of EC in the experimental group ($r=0.773$, $P=0.000$). Similarly, the positive expression rate of EZH2 in the control group was 17.86%, and the positive expression rate alone was 10.71%. The positive expression rate of NSD2 was 10.71%, and the positive expression alone accounted for 3.57%. In addition, the values of negative expression and positive expression of both NSD2 and EZH2 were 78.57% and 7.14%, respectively. The results of the Spearman Rank correlation analysis suggested that the expression of EZH2 and NSD2 ($r=0.419$, $P=0.026$) in the control group also had a positive correlation.

DISCUSSION

Histones are an important component in the basic structural nucleosomes of chromatin (11). In addition, EZH2 can change the memory system of cells and regulate their transcription via gene silencing, thus promoting the malignant formation of premalignant tumors (12).

This study found that NSD2 and EZH2 were mainly expressed in the nucleus of cancer cells. The positive expression rates of NSD2 and EZH2 in EC patients were significantly higher than those of the control group. In addition, the expression levels of NSD2 and EZH2 were related to the age, pathological stage, degree of differentiation, and degree of muscle infiltration of the patients, but not related to ethnicity. Furthermore, the high expression of NSD2 and EZH2 could promote the invasion and metastasis of EC. Interestingly, the expression of NSD2 and EZH2 is highly consistent in many tumors, suggesting that they are positively correlated and act on the same pathway. In synergy, NSD2 and EZH2 play an important role in the development of EC. Therefore, the expression levels of NSD2 and

EZH2 in tumor tissues can be used as indicators for predicting the metastasis of EC, thus facilitating early diagnosis.

In summary, the abnormal expression of NSD2 and EZH2 may affect the occurrence and development of EC. However, the mechanism underlying the synergy between EZH2 and NSD2 in promoting the occurrence and development of EC remains unclear and requires further research.

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LETTER TO THE EDITOR

DRUG-INDUCED ECZEMATID-LIKE PURPURA OF DOUCAS-KAPETANAKIS

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To the Editor,

We present the case of an 80-year-old man in good general condition. The patient was hospitalized for a complaint about the appearance of highly itchy plaques in the area of both lower legs for several months. In 2001, the patient suffered arterial thrombosis of the lower left limb, and subsequently in 2018 arterial thrombosis of the right limb, for which he took acetylsalicylic acid 100 mg (0-0-1) and cilostazol 100 mg (1-0-1) for 1 year. In addition, there was systemic therapy with bisoprolol 5 mg (0-0-1) regarding the concomitant arterial hypertension, which also dates back to 1 year. In the framework of the dermatological examination, we found the presence of erythematous plaques with a clear edge, petechial changes, hypo- and hyper-pigmentation zones and areas of lichenoid and eczematous character, located in the area of both lower legs (Fig. 1, a-b). According to anamnestic data, the cutaneous changes appeared 3 months after the introduction of the current cardiac therapy. Based on the clinical data and concomitant information, there was a suspicion of drug-induced eczematid-like purpura of Doucas-Kapetanakis, as in the differential diagnostic aspect the possibility of eczema nummularis was also considered. A skin biopsy was taken, and

the histological examination confirmed the initial diagnosis, namely that it was eczematid-like purpura of Doucas-Kapetanakis. Due to the possibility of medication triggering, the administration of acetylsalicylic acid and bisoprolol was discontinued after consultation with a cardiologist and a vascular surgeon, and therapy was optimized with intake of clopidogrel 75 mg (1-0-0) and moxonidine 0.2 mg (0-0-1). Systemic and topical antihistamine therapy was performed with desloratadine 5mg x 2 daily and dimetindene maleate 0.1% gel for 7 days, resulting in a significant improvement of the clinical picture.

The patient signed informed consent for publication of this report.

Eczematid-like purpura of Doucas and Kapetanakis is a subtype of the pigmented purpuric dermatoses (PPD) for which there are currently no clearly identified etiological factors (1). By definition PPD are represented as chronic and relapsing cutaneous lesions, and according to some authors the cases of eczematid-like purpura are more extensive than the other types. (1). However, according to the literature, they can also be assigned to the group of drug-induced diseases and a number of medications are included in the list of possible PPD inducers, such as the widely used

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Fig. 1. a-b) Clinical finding: presence of erythematous plaques with petechial changes, hypo- and hyper-pigmentation zones and areas of lichenoid and eczematous character, located in the area of both lower limbs.

acetylsalicylic acid, nonsteroidal anti-inflammatory drugs and diuretics (2). That is why particular attention should be paid to patients with a variety of concomitant diseases and diverse systemic medication. It is believed that this group of diseases is quite resistant to treatment, as there is currently a set of therapeutic options such as psoralen and ultraviolet A, oral antihistamines, oral colchicine or tetracycline antibiotics, topical steroids, topical tacrolimus or pimecrolimus, but none of these are demonstrably superior for their treatment (1,2). In cases of suspected drug induction, discontinuation of medication is sufficient for reversal of the process (2). Although rarely, these patients need to be monitored due to the existing risk for progression

of pigmented purpura-like eruptions to mycosis fungoides (3).

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LETTER TO THE EDITOR

**RELATIONSHIP BETWEEN TRANSMEMBRANE 6 SUPERFAMILY MEMBER 2
rs58542926 POLYMORPHISM AND CHRONIC NON-INFECTIOUS LIVER DISEASE:
A META-ANALYSIS**C. ZHU¹, S. ZHANG¹, C. CHEN¹ and Z. CHEN²

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To the Editor,

Chronic non-infectious liver disease poses an enormous health threat worldwide (1), and obesity and excessive alcohol intake are already verified to be pathogenic factors. Nevertheless, the huge difference observed in odds of developing chronic non-infectious liver disease in people exposed to these pathogenic factors suggests that genetic factors also play a key role in its pathogenesis (2-3).

In 2014, two genome-wide association studies conducted by Kozlitina et al. (4) and Liu et al. (5) found that the transmembrane 6 superfamily member 2 (*TM6SF2*) rs58542926 polymorphism was relevant to higher liver fat levels, elevated serum levels of alanine transaminase and progressed liver fibrosis. To date, several genetic association studies have been performed on diverse populations to analyze potential associations between rs58542926 polymorphism and chronic non-infectious liver disease. However, the results of these studies were not consistent, therefore, we carried out a meta-analysis of all relevant studies to evaluate the effects of *TM6SF2* polymorphisms on individual susceptibility to chronic non-infectious liver disease in a larger pooled population.

MATERIALS AND METHODS

This meta-analysis was conducted according to the guidelines recommended by the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) (6).

Literature search and inclusion criteria

Studies were selected from Web of Science, PubMed, Embase and CNKI using the following terms: (transmembrane 6 superfamily member 2 OR *TM6SF2*) AND (polymorphism OR variant OR variation OR mutation OR genome-wide association study OR genetic association study) AND (non-alcoholic fatty liver disease OR non-alcoholic fatty liver OR non-alcoholic steatohepatitis OR alcoholic liver disease OR liver cirrhosis OR hepatocellular carcinoma). The literature search was conducted from November 2018 to February 2019. We also screened the references of all retrieved articles to evaluate whether there were other potential relevant studies.

Included studies met the following standards: i) genetic association studies related to *TM6SF2* polymorphisms and chronic non-infectious liver

Key words: transmembrane 6 superfamily member 2 (TM6SF2); rs58542926 polymorphisms; chronic non-infectious liver disease; meta-analysis

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disease in humans; ii) genotypic/allelic frequencies of investigated polymorphisms in cases and controls were provided; iii) available full text in English or Chinese. The following studies were excluded: i) not about *TM6SF2* polymorphisms and chronic non-infectious liver disease; ii) case reports or case series; iii) reviews and comments.

Data extraction and quality evaluation

We extracted the following data from the included studies: the surname of the first author, publication year, country and ethnicity of study subjects, sample size and genotypic/allelic distributions of *TM6SF2* polymorphisms in cases and controls. The probability value (*p* value) of Hardy-Weinberg equilibrium (HWE) was also calculated.

If needed, we contacted the corresponding authors to gain extra information. The Newcastle–Ottawa scale (NOS) was used to assess the quality of relevant studies (7). The score of this scale ranged from 0 to 9, and studies with a score > 7 were considered to be of high-quality. Data extraction and quality evaluation were performed by two independent reviewers. Any divergence raised by two reviewers was resolved by collective discussion until reaching an agreement.

Statistical analyses

We analyzed relevant data by using the Review Manager version 5.3.3 (Cochrane Collaboration, Software Update). Odds ratios (ORs) and 95% confidence

Table I. The characteristics of included studies.

First author, year	Country	Ethnicity	Type of disease	Sample size	Genotype distribution		<i>P</i> -value for HWE
					Cases	Controls	
Bale 2017	India	Mixed	NAFLD	250/232	171/66/13	190/40/2	0.947
Buch 2015	Germany	Caucasian	ALD	712/1426	NA	NA	NA
Coppola 2015	Italy	Caucasian	Cirrhosis	101/47	85/16/0	45/2/0	0.882
Donati 2017	Italy	Caucasian	HCC	132/633	109/19/4	538/88/7	0.121
Eslam 2016	Australia	Caucasian	NAFLD	502/228	391/100/11	197/30/1	0.901
Falletti 2016	Italy	Caucasian	Cirrhosis	511/228	443/66/2	203/25/0	0.381
Falletti 2016	Italy	Caucasian	ALD	226/228	188/36/2	203/25/0	0.381
Falletti 2016	Italy	Caucasian	NAFLD	150/228	123/26/1	203/25/0	0.381
Goffredo 2016	Italy	Caucasian	NAFLD	158/296	135/22/1	270/26/0	0.429
Grove 2018	UK	Caucasian	NAFLD	186/439	NA	NA	NA
Koo 2018	Korea	Asian	NAFLD	365/96	306/57/2	87/9/0	0.630
Krawczyk 2016	Germany	Caucasian	NAFLD	143/180	110/30/3	152/28/0	0.258
Krawczyk 2016	Germany	Caucasian	Cirrhosis	90/205	68/20/2	164/38/3	0.641
Kruk 2018	Poland	Caucasian	Cirrhosis	55/123	50/5/0	107/16/0	0.440
Li 2019	China	Asian	NAFLD	201/239	188/13/0	234/5/0	0.870
Liu 2014	UK	Caucasian	NAFLD	349/379	271/70/8	328/49/2	0.908
Manchiero 2017	Brazil	Mixed	Cirrhosis	58/232	46/12/0	215/17/0	0.562
Musso 2017	Italy	Caucasian	NAFLD	60/60	37/21/2	40/20/0	0.121
Raksayot 2018	Thailand	Asian	HCC	132/105	78/46/8	89/15/1	0.682
Sagnelli 2016	Italy	Caucasian	Cirrhosis	31/136	26/5/0	124/12/0	0.590
Sookoian 2015	Argentina	Mixed	NAFLD	226/135	184/37/5	120/15/0	0.494
Stickel 2018	Switzerland	Caucasian	HCC	751/1165	558/164/29	957/193/15	0.143
Teng 2018	China	Asian	Cirrhosis	239/179	209/29/1	161/18/0	0.479
Wang 2016	China	Asian	NAFLD	367/366	302/65/0	333/33/0	0.366
Wang 2018	China	Asian	Cirrhosis	677/364	602/74/1	331/33/0	0.365
Wang 2018	China	Asian	HCC	418/364	363/55/0	331/33/0	0.365
Yue 2018	China	Asian	NAFLD	118/122	111/7/0	114/8/0	0.708
Zhang 2018	China	Asian	ALD	507/645	435/65/7	559/83/3	0.966

Abbreviations: *TM6SF2*: transmembrane 6 superfamily 2; HCC: hepatocellular carcinoma; NAFLD: Nonalcoholic fatty liver disease; ALD, alcoholic liver disease; HWE: Hardy-Weinberg equilibrium; NOS: Newcastle-Ottawa scale; NA: not available.

intervals (CIs) were calculated to estimate the relevance in dominant, recessive, over-dominant and allele genetic models. Statistical significance of pooled analyses was determined the by Z test, and statistically significant threshold was defined as a p value ≤ 0.05 . I^2 statistics were calculated to assess the between-study heterogeneity. If I^2 was $>50\%$, random-effect models were used to pool the data on account of significant heterogeneity. Otherwise, fixed-effect models were used for analyses. Subgroup analyses were conducted according to ethnicity of participants and types of disease. Sensitivity analyses were used to evaluate the stability of synthetic results, and we used funnel plots to evaluate publication biases.

RESULTS

Characteristics of included studies

The initial literature search found 256 relevant articles. Of these, 24 were articles that satisfied the inclusion criteria and were included in this meta-analysis (Fig. 1). Baseline characteristics of relevant studies can be seen in Table I.

Overall and subgroup analyses

The main results of this meta-analysis can be seen at Table II. In brief, 8,398 cases and 9,444 controls were analyzed, and the pooled analyses showed

that rs58542926 polymorphism was significantly associated with chronic non-infectious liver disease in the overall population (dominant model: $p < 0.0001$, OR = 0.63, 95%CI 0.57-0.69, $I^2 = 24\%$; recessive model: $p < 0.0001$, OR = 3.62, 95%CI 2.45-5.34, $I^2 = 0\%$; over-dominant model: $p < 0.0001$, OR = 1.47, 95%CI 1.33-1.62, $I^2 = 0\%$; allele model: $p < 0.0001$, OR = 0.62, 95%CI 0.57-0.67, $I^2 = 15\%$), and these significant findings were also confirmed in both Asians and Caucasians. Further stratified analyses according to type of disease showed similar positive results in hepatocellular carcinoma (HCC), cirrhosis, nonalcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) (Table II).

Sensitivity analyses

Sensitivity analyses were conducted to determine the effects of individual studies on pooled results. No altered results were observed in sensitivity analyses, indicating that our findings were statistically robust.

Publication biases

Funnel plot were used to evaluate publication biases. Obvious asymmetry of funnel plots did not present in any comparisons, indicating that our findings were not likely to be affected by severe publication biases.

Table II. Results of pooled analyses.

Population	Sample size	Dominant comparison			Recessive comparison			Overdominant comparison			Allele comparison		
		P value statistic	OR (95%CI)	I^2	P value statistic	OR (95%CI)	I^2	P value statistic	OR (95%CI)	I^2	P value statistic	OR (95%CI)	I^2
Overall	8398/9444	<0.0001 24%	0.63 (0.57-0.69)		<0.0001 0%	3.62 (2.45-5.34)		<0.0001 17%	1.47 (1.33-1.62)		<0.0001 15%	0.62 (0.57-0.67)	
Asian	3707/2844	0.0005 55%	0.63 (0.49-0.82)		0.01 0%	3.16 (1.27-7.88)		0.001 51%	1.51 (1.18-1.93)		0.0002 51%	0.64 (0.51-0.81)	
Caucasian	4157/6001	<0.0001 0%	0.64 (0.56-0.72)		<0.0001 0%	3.42 (2.17-5.39)		<0.0001 0%	1.44 (1.26-1.64)		<0.0001 0%	0.63 (0.57-0.69)	
HCC	1433/2267	0.003 65%	0.58 (0.40-0.83)		<0.0001 0%	3.29 (1.92-5.66)		0.01 59%	1.55 (1.10-2.18)		0.0007 62%	0.58 (0.42-0.79)	
Cirrhosis	1762/1514	0.006 25%	0.72 (0.57-0.91)		0.37 0%	1.79 (0.50-6.37)		0.01 27%	1.36 (1.08-1.71)		0.004 18%	0.73 (0.58-0.90)	
NAFLD	3075/3000	<0.0001 0%	0.54 (0.46-0.64)		<0.0001 0%	5.20 (2.43-11.13)		<0.0001 0%	1.69 (1.44-1.98)		<0.0001 0%	0.55 (0.48-0.63)	
ALD	1445/2299	0.18 40%	0.82 (0.62-1.10)		0.06 0%	3.33 (0.97-11.44)		0.41 42%	1.13 (0.84-1.51)		<0.0001 26%	0.71 (0.60-0.84)	

Abbreviations: HCC: hepatocellular carcinoma; ALD: alcoholic liver disease; NAFLD: nonalcoholic fatty liver disease; OR: odds ratio; CI: confidence interval; NA: not available. The values in bold represent there is statistically significant differences between cases and controls.

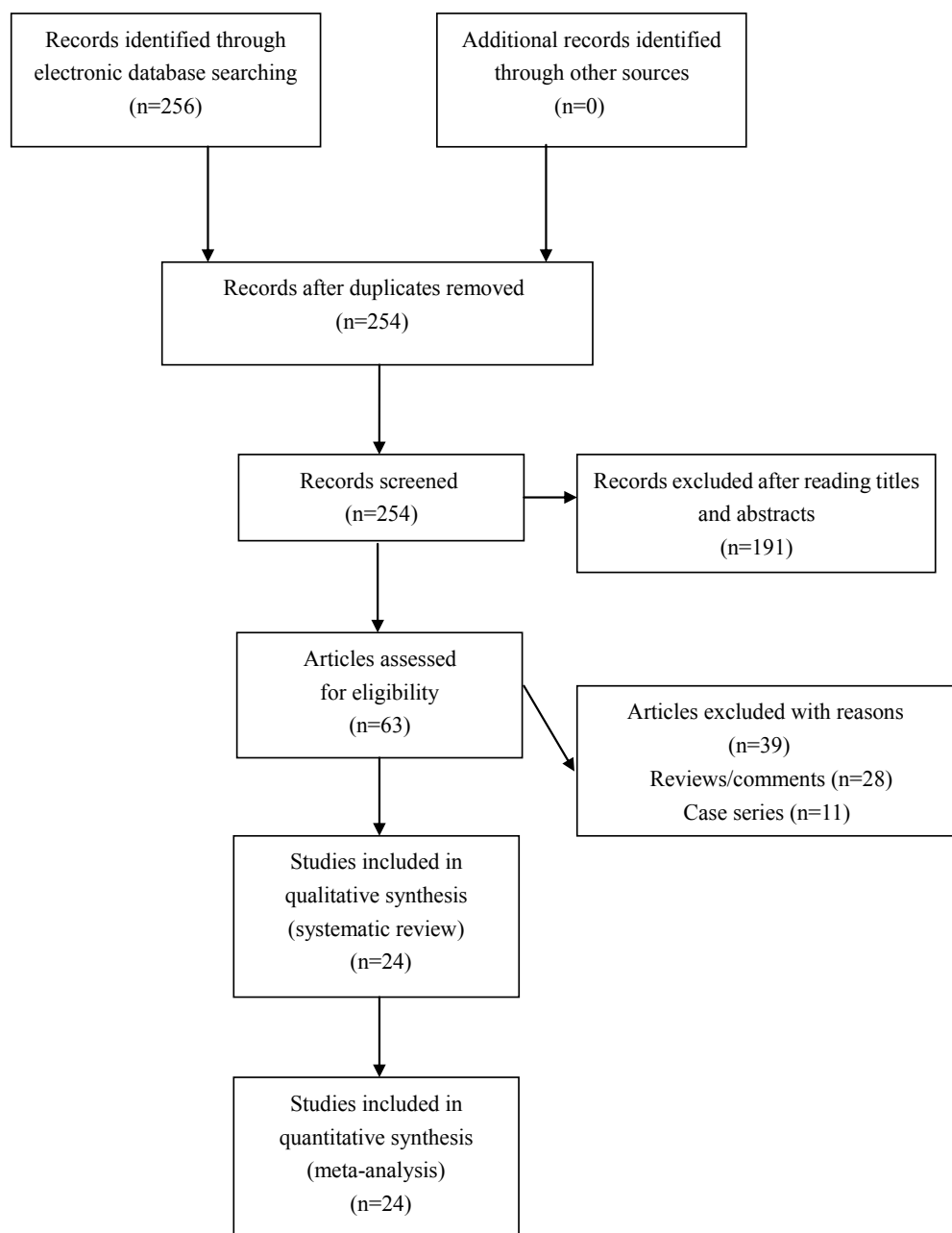


Fig. 1. Flowchart of study selection for the present study.

DISCUSSION

To our knowledge, this is the first meta-analysis on relevance between *TM6SF2* polymorphisms and chronic non-infectious liver disease. In our

study, the results suggested that rs58542926 polymorphism had a significant association with chronic non-infectious liver disease in both Asians and Caucasians. Further stratified analyses revealed similar positive results in HCC, cirrhosis, ALD and

NAFLD. There are two possible explanations for underlying molecular mechanisms of our positive findings. Firstly, rs58542926 polymorphism may alter gene expression or protein structure of *TM6SF2*, which may affect biological functions of *TM6SF2* and ultimately impact individual susceptibility to chronic non-infectious liver disease. Secondly, it is also possible that rs58542926 polymorphism may be linked to other *TM6SF2* polymorphisms or even other genes, which may jointly form a genetic network and impact individual susceptibility to chronic non-infectious liver disease.

Similar to all other meta-analysis, this study also has some limitations. Adjusted analyses were not applicable due to lack of original data, and potential confounding factors might affect the reliability of our findings (8), furthermore gene-environmental interactions might also modify associations between rs58542926 polymorphisms and chronic non-infectious liver disease. However, we failed to conduct relevant analyses because most studies did not investigate these associations (9-10). Considering the above-mentioned limitations, our findings should be cautiously interpreted.

In our meta-analysis, the results suggested that *TM6SF2* rs58542926 polymorphism might affect individual susceptibility to chronic non-infectious liver disease in both Asians and Caucasians. These results suggested that this polymorphism could be used to identify individuals at higher susceptibility to chronic non-infectious liver disease.

ACKNOWLEDGEMENTS

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LETTER TO THE EDITOR

CEREBRAL SMALL-VESSEL DISEASE IN THE PROGNOSIS OF ACUTE CEREBRAL HEMORRHAGE IN NORTHEASTERN CHINA: A RETROSPECTIVE STUDY

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To the Editor,

Cerebral small-vessel disease (CSVD) is the major result of various factors involving cerebral arterioles, micro-arteries, capillaries, veins and small veins. Clinical symptoms include lacunar infarction, cerebral hemorrhage, subcortical white matter lesions (WML), cerebral microbleeds and micro-cerebral infarction (1). Studies have shown that CSVD has varying degrees of influence in osteoporosis, ischemic cerebral infarction and acute cerebral hemorrhage (ACH) (2, 3). The impact of SVD on ACH is poorly known. A recent study of CSVD with a bleeding volume less than 30 ml showed that pre-existing CSVD involving WML and brain atrophy predicted a poor outcome for acute intracerebral hemorrhage (ICH) (4). In contrast, according to the INTERACT2 regimen, there was no significant risk of death or injury resulting from the early reduction of blood pressure (BP) in patients with underlying CSVD (4).

In the northeastern region of China, the incidence of cerebrovascular disease is among the highest known to exist worldwide, therefore the prognostic factors associated with the occurrence of ACH in this region are of great interest (5). A baseline computed tomography (CT) assessment of the prognosis of patients with ischemic stroke has shown that WML and brain atrophy were associated

with poor prognosis, and the relationship between cerebral hemorrhage and underlying CSVD was stronger than the relationship with ischemia (6). In the present study, our aim was to verify whether CSVD affected the prognosis for ACH. Although the importance of early intensive BP reductions has been emphasized in recent years (7), in more severe cases of CSVD, less blood flow, and perfusion during BP changes may lead to adverse outcomes via reduced perfusion of advanced WML regions (8). Thus, the characteristic manifestations of CSVD which can be observed by baseline CT may be independent predictors of poor outcome in patients receiving BP-lowering treatments. This study aimed to determine the relationship between CSVD and the prognosis of patients with ACH as observed on CT scan and to assess the relationship between BP-lowering treatment and outcomes in patients with CSVD.

MATERIALS AND METHODS

This retrospective study analyzed 2,006 out of 25,59 patients with ACH admitted to The Second Affiliated Hospital of Harbin Medical University between January 2014 and August 2016 from the electronic medical record system. Patients who were treated for spontaneous ICH within 6 h of onset and with a bleeding volume < 60ml and could be contacted were selected for further analysis.

*Key words: CSVD, white matter lesions, acute cerebral hemorrhage**Corresponding Author:*

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We excluded the patients whose CT scan was difficult to measure because of poor imaging quality or potentially biasing anatomic features. Baseline brain CT scans were performed to measure CSVD, including WML, lacunes and brain atrophy (using two linear measurements and a visual rating). For the 552 patients that were excluded, 122 were secondary, 96 lacked information on 1-year functional outcomes, 97 were functionally dependent at baseline, 124 had baseline CT scan >6 h, and 113 had poor imaging quality or potentially biasing anatomic features. The research program was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. Informed consent was received from all patients included in the analysis.

We recorded the patients' demographic and clinical characteristics which were provided on admission. Patients with elevated systolic BP (150–220 mmHg) were randomly assigned to receive intensive BP reduction treatment (target systolic BP <140 mmHg within 1 h) or contemporaneous guideline-recommended BP-lowering therapy (target systolic BP <180 mmHg). Surgery was performed when required, and hemostatic medicines were also used when appropriate. Baseline non-controlled head CT scans (recommended slice thickness, 5–8 mm) were performed on patients with ACH. Three professional neuroscientists who had read the CT imaging information were blinded to the clinical data, and half of the CT imaging information was regularly re-analyzed

by the “gold standard” doctor to ensure the reliability of the assessments. The prognosis of the patients was provided by the patient or by an appropriate substitute by telephone or visits from January 2015 to August 2017. If the information was provided by an appropriate substitute, we asked them the MRS (modified Rankin Scale) first: MRS=0 means completely asymptomatic; MRS=1 means despite the patient having symptoms, no obvious dysfunction, able to complete all daily work and life; MRS=2 means mild disability, cannot complete all activities as before the disease, but does not need to help to take care of daily affairs; MRS=3 means moderately disabled, needs some help, but can walk independently; MRS=4 means moderately severe disability, cannot walk independently, needs help from others in daily life; MRS=5 means severe disability, bed rest, incontinence, and dependence on others in daily life; MRS=6 means dead. Patients living or deceased or major disability were defined as scores 3–6 on the modified Rankin Scale.

WML ratings were assigned according to the levels described by van Swieten et al. (9); the entire WML score range from 0 to 4 was employed. Lacune was defined as a diameter of 3–15 mm in a round or oval, subcortical, fluid-filled cavity (similar to cerebrospinal fluid), consistent with a past acute small deep brain infarct or hemorrhage in the area of one perforating arteriole, or perivascular space. Brain atrophy was assessed in two ways, using linear measurements and visual rating on the contralateral side

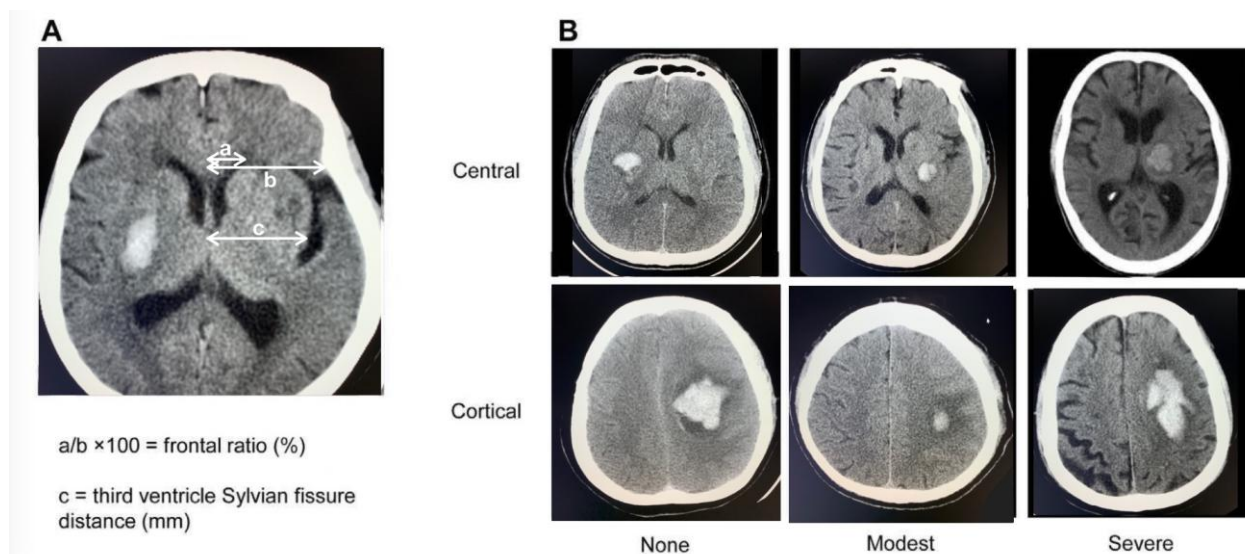


Fig. 1. Brain atrophy assessment procedure. Linear measurements (A) and the template of brain atrophy (B).

of the ICH. Linear measurements consisted of the frontal ratio and the third ventricle Sylvian fissure distance (Fig. 1A). A higher frontal ratio and a shorter third ventricle Sylvian fissure distance indicated more advanced brain atrophy. According to visual presentation templates obtained from previous studies (Fig.1B), central and

cortical brain atrophy were categorized into three grades (none, modest and severe).

If the measurement was difficult due to poor imaging quality or appeared to be biased toward anatomical characteristics, the case was considered unreliable and eliminated from the study.

Table I. Patient characteristics.

	Death or Major Disability (n=712)	No Death or Major Disability (n=1294)	P
Demographic			
Age	61.57±12.23	54.31±10.70	<.0001
Women	260(36.52)	419(32.38)	0.0610
Life style			
Smoke	316(44.38)	548(42.35)	0.3790
Drink	186(26.12)	289(22.33)	0.0561
Medical history			
ICH	115(16.15)	104(8.04)	<.0001
Ischemic stroke	165(23.17)	166(12.83)	<.0001
Hypertension	504(70.79)	907(70.09)	0.7448
Diabetes mellitus	93(13.06)	116(8.96)	0.0040
Medication history			
Antihypertensives	148(20.79)	272(21.02)	0.9021
Oral anticoagulants	15(2.11)	8(0.62)	0.0027
Antiplatelets	69(9.69)	75(5.80)	0.0012
Antithrombotics	29(4.07)	24(1.85)	0.0030
Lipid-lowering agents	35(4.92)	16(1.24)	<.0001
Hemostatic medicines	540(75.84)	956(73.88)	0.3339
Clinical features			
Onset to randomization time, h	3.00(2.00-5.00)	4.00(2.00-5.00)	0.0061
Onset to CT time, h	2.00(1.00-3.00)	2.00(1.00-3.00)	0.0006
Systolic BP, mm Hg	163.70±25.56	161.10±25.59	0.0280
Diastolic BP, mm Hg	94.86±14.42	96.61±15.11	0.0116
Admission blood glucose	7.87±3.04	7.88±3.11	0.9970
NIHSS score	17.00(13.00-22.00)	12.00(7.00-17.00)	<.0001
ICH findings			
Volume, mL	21.95(8.95-42.54)	11.28(5.68-22.61)	<.0001
Deep location	672(94.38)	1170(90.42)	0.0019
Intraventricular extension	330(46.35)	385(29.75)	<.0001
Randomized intensive BP lowering	214(30.06)	396(30.60)	0.7990
Surgery	266(37.36)	496(38.33)	0.6680

Data are n (%), mean±SD, or median (interquartile range). BP: blood pressure; CT: computed tomography; ICH: intracerebral hemorrhage; NIHSS: National Institutes of Health Stroke Scale.

Statistical analysis

Categorical and quantitative variables were described by numbers (percentages) and means (standard deviation) or by median and interquartile range, respectively. Baseline differences between patients living or deceased or major disability (defined as scores 3–6 on the modified Rankin Scale) were assessed using *t*-tests for quantitative variables, Kruskal–Wallis tests for continuous variables and χ^2 tests for categorical variables. WML grades 3 and 4 were grouped together, and frontal ratio and third ventricle Sylvian fissure distance were divided into quartiles based on patient numbers. Logistic regression models were used to estimate odds ratios with 95% confidence intervals for association with outcomes (death or major disability), after first adjusting for potential confounding variables (age, sex, smoking, alcohol intake, ICH, ischemic stroke, hypertension, diabetes mellitus, use of anti-hypertensives, oral anti-coagulants, anti-platelets, anti-thrombotics, lipid-lowering agents or hemostatic medicine, onset to randomization time, onset to CT time, systolic BP, diastolic BP, blood glucose, National Institutes of Health Stroke Scale (NIHSS) scores, ICH findings, volume, deep location, intraventricular extension, randomized intensive BP lowering and surgery). Comparisons of the effects of intensive BP-lowering treatment or contemporaneous guideline-recommended BP-lowering therapy on the outcome across CSVD findings were assessed by logistic regression models. Trend tests utilized *chi*-squared tests. GraphPad Prism 7 was used for graphics editing. All analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Two-tailed $P < 0.05$ was adopted as the level of statistical significance for all tests.

RESULTS

Of the 4,559 patients who provided consent for the use of all data, 2,006 were included in analyses utilizing available baseline DICOM format CT scan data and information based on a modified Rankin Scale at 1 year. Patients with a poor outcome were more likely to be older, have a history of ICH and ischemic stroke and diabetes, or were taking anti-thrombotic or lipid-lowering agents (Table I). They also had shorter times from ICH onset to randomization and CT, higher systolic BP and lower diastolic BP, higher NIHSS scores, larger ICH volume, and were more likely to have a deep location

of ICH with intraventricular extension. However, the prognosis of patients with acute cerebral hemorrhage in the northeastern region of China was not affected by smoking, drinking, hemostatic medicines, admission blood glucose levels or surgery (Table I).

Patients with a poor outcome at 1 year had a more severe WML grade, lacunes and worse measurements of brain atrophy (Table II). WML grade and all four measurements of atrophy were associated with death or major disability in the crude models (all P for trend < 0.001) (Fig. 2A and Table II). After adjustment for potential confounders, the associations of WML grade, lacunes, frontal ratio, third ventricle Sylvian fissure distance, central atrophy and cortical atrophy remained significant for death or major disability (P values for trend for the previous respective factors were < 0.001 , < 0.0001 , < 0.0001 , < 0.001 , < 0.001 and < 0.001 , respectively); adjusted odds ratios for WML (grades 1–4 versus 0) were 1.35 (95% confidence interval, 1.04–1.77), 1.9 (1.4–2.59), 2.91 (1.98–4.29), and third ventricle Sylvian fissure distance (most versus least severe atrophy quartile) were 1.37 (95% confidence interval, 1.01–1.86); for central and cortical atrophy, 2.97 (2.04–4.32) and 2.44 (1.68–3.55), respectively (all P for trend < 0.0001). There was no association between lacunes, frontal ratio, and outcomes with adjusted odds ratios of 1.16 (95% confidence interval, 0.93–1.45) and 1.27, 1.03 and 1.17 (95% confidence intervals, 0.94–1.71, 0.76–1.4 and 0.86–1.58, respectively) (Fig. 2B).

The effect of lowering BP was not significantly different in each subgroup of CSVD abnormality, all $P > 0.1$. Similarly, there were no significant trends in patients with underlying CSVD who received intensive BP-lowering therapy and achieved mean systolic BP in the initial 24 h.

DISCUSSION

In this study, we found that WML and brain atrophy were predictors of poor outcome in acute ICH at 1 year in northeastern China. There was no effect of lowering BP on the outcomes in patients with CSVD. CSVD associated with poor outcome in acute ICH, WML and brain atrophy has been shown to be significantly associated with both cognitive decline and depression

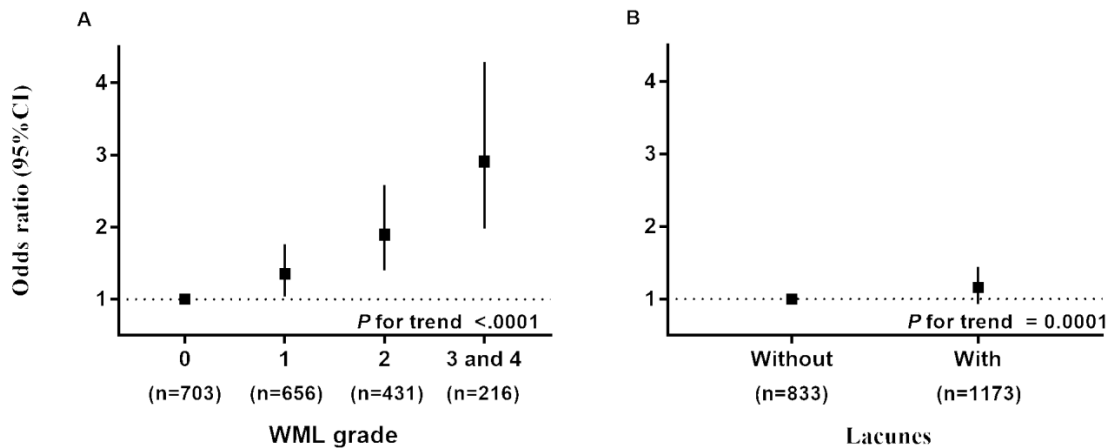


Fig. 2. Association of white matter lesions (WML) and lacunes with death or major disability at 1 year. Models were adjusted for potential confounding variables (age, sex, smoking, alcohol intake, ICH, ischemic stroke, hypertension, diabetes mellitus, use of antihypertensives, oral anticoagulants, antiplatelets, antithrombotics, lipid-lowering agents or hemostatic medicine, onset to randomization time, onset to CT time, systolic BP, diastolic BP, blood glucose, National Institutes of Health Stroke Scale (NIHSS) scores, ICH findings, volume, deep location, intraventricular extension, randomized intensive BP lowering and surgery). **A)** WML grade. **B)** Lacunes. CI: confidence interval.

Table II. Baseline cerebral small-vessel disease findings.

	Death or Major Disability (n=712)	No Death or Major Disability (n=1294)	p
WML			
Grade 0	161(22.61)	542 (41.89)	<.0001
Grade 1	222(31.18)	434 (33.54)	
Grade 2	197(27.67)	234 (18.08)	
Grade 3 and 4	132(18.54)	84 (6.49)	
Lacunes			
Without	255(35.81)	578(44.67)	0.0001
With	457(64.19)	716(55.33)	
Atrophy: linear measurements			
Frontal ratio, %	32.13±5.24	31.10±5.06	<.0001
Third ventricle Sylvian fissure distance, mm	38.62±4.18	39.74±3.97	
Atrophy: visual inspection			
Central			
None	184(25.84)	535(41.34)	<.0001
Modest	346(48.60)	657(50.77)	
Severe	182(25.56)	102(7.88)	
Cortical			
None	261(36.66)	664(51.31)	<.0001
Modest	297(41.71)	543(41.96)	
Severe	154(21.63)	87(6.72)	

Data are n (%) or mean±SD. WML: white matter lesions.

in elderly individuals (10). Moreover, several studies have shown that SVD in stroke is associated with cognitive impairment after 90 days (11). This suggests that CSVD reflects the potential vulnerability of the brain and increased susceptibility to hemorrhagic brain injury. Since China is a developing country where the cost of magnetic resonance imaging (MRI) is relatively high, CT scan may offer an economical and practical alternative for patients. Thus, we used baseline CT to examine both linear measurements and visual presentation template methods on the contralateral side of the ICH, which may be a simple and reliable method. CSVD may cause loss of structure in arteriolar walls, narrowing of vessel lumens and thickening of vessel walls in patients (10). Our study found no statistically significant effect on death or major disability from lowering BP in each subgroup of CSVD patients, irrespective of preexisting CSVD conditions. These findings are supported by previous studies (12). Therefore, lowering BP is likely to be a safe and generalizable treatment strategy across a broad range of patients with acute ICH (7).

There are some limitations to our study. Firstly, MRI is more sensitive than CT when evaluating WML. Secondly, this is the first study to assess the relationship between CSVD and the prognosis of patients from northeastern China with ACH, therefore selection bias may have occurred. Thirdly, recommended slice thickness was 5–8 mm for CT scans, so smaller lacunes may have been missed. Finally, our findings may be biased because of a younger study population and may suffer from a larger volume of ICH.

In conclusion, WML and brain atrophy were predictors of poor outcome in acute ICH in a population of patients from northeastern China. There was no significant risk involved with the early reduction of BP in patients with underlying CSVD.

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LETTER TO THE EDITOR

IMPROVED METHOD FOR THE EVALUATION OF STRESS HYPERGLYCEMIA IN ACUTE PULMONARY THROMBOEMBOLISM

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To the Editor,

Pulmonary thromboembolism (PTE) is important for several reasons, especially its high prevalence. PTE is the third most common cardiovascular disease [after acute myocardial infarction (AMI) and stroke]. It is believed that the annual incidence of venous thromboembolism (VTE) is as high as 250,000 among whites in the USA. The importance of PTE also stems from the high mortality rate.

Moreover, in PTE there is great potential to improve survival by improved prevention, early diagnosis and therapy. This potential is likely to be much higher than in AMI, which is easier to recognize clinically. PTE often manifests insufficiently specific symptoms and signs, making its diagnosis difficult. The importance of stress hyperglycemia (SH) in PTE has not been elucidated sufficiently, in contrast to AMI, where dozens of publications have been published (1).

SH, i.e. the raised blood glucose concentration, resulting from neurohormonal activation in an organism exposed to stress, is usually observed in patients hospitalized because of critical illness. SH is important as a marker (and probably a factor) of

poor prognosis in critical illnesses, such as stroke, sepsis, pneumonia, AMI, unstable angina pectoris, and acute heart failure (2).

In addition, a significant percentage of these patients had no previous diagnosed abnormalities of glycemic control. Given the paucity of published papers on this subject (very few references in PubMed), the aim of the present study was to analyze a possible association of hyperglycemia with poor in-hospital outcome in PTE.

MATERIALS AND METHODS

The analysis included 478 successive patients with PTE, hospitalized in the Department for Cardiovascular Diseases, Clinical Center Nis, between 2006 and 2013. They were diagnosed and treated according to current PTE Guidelines by the European Society of Cardiology at that time. The definitive diagnosis was given using pulmonary angiography by computed tomography (CTPA), unless the patient was in shock, when an echocardiogram was performed prior to eventual fibrinolysis. All subjects gave informed, signed consent to participate in the study. The methodology and patient characteristics (including

Key words: stress hyperglycemia; pulmonary thromboembolism; admission blood glucose; mortality

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symptoms, comorbidities and treatment) have been more extensively explained elsewhere (3). The protocol consisted of 35 parameters, including demographic characteristics, the results of laboratory analysis and basic echocardiographic findings. The already published threshold for in-hospital hyperglycemia in critically ill patients was used (150 mg/dL, i.e., 8.3 mmol/L) (4).

Statistical analysis

Statistical analysis was carried out with a commercial software SPSS (version 21, SPSS, Chicago, IL, USA). Student's *t*-test, *chi*-squared test and linear correlation, univariate and multivariate analyses were used. The level of statistical significance was set at 0.05.

RESULTS

Female gender was more prevalent (60.67%) and the average age was 65.67 ± 13.88 years. The admission blood glucose (BG) was above the threshold for in-hospital hyperglycemia in critically ill patients (≥ 150 mg/dL, ≥ 8.3 mmol/L) in approximately one-third of the PTE patients: 173 (36.19%). Admission BG concentration was a significant predictor of mortality: patients who did not survive had higher admission BG (9.01 ± 4.29 vs 8.09 ± 4.61 , $p=0.00366$).

In the analysis of tertiles of admission BG, mortality was highest in third tertile (31.18%), statistically significantly higher than in the first tertile (12.65%, $p=0.00043$) and second tertile (15.88%, $p=0.000903$). The difference between the first and the second was not significant. Female gender was more frequent in the third tertile (with the highest admission BG). In the third tertile 65.35% of patients were females, which

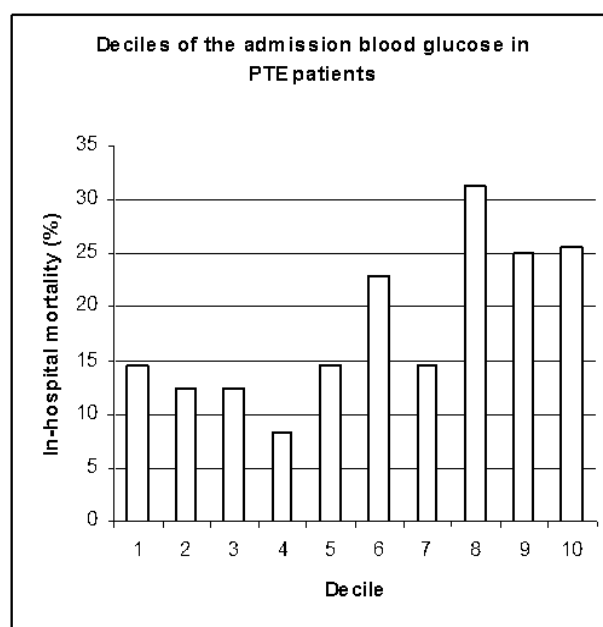


Fig. 1. Admission BG (presented in deciles) and in-hospital mortality in PTE, pulmonary thromboembolism.

is significantly more than in the first tertile (53.12%, $p=0.041$). In the second tertile the prevalence of female patients was 57.84%, which was not significantly different from the first and third tertiles. The in-hospital mortality showed “J-shaped curve” or “U-shaped curve” for admission BG (Fig. 1).

The differences between IV and VIII deciles was statistically significant, ($p=0.0025$), as well as between IV and X ($p=0.0293$), but it was not statistically significant between I and IV deciles. In patients without diabetes mellitus (DM), ROC curve analysis found the best glycemic cut-off for

Table I. Multivariate analysis for predictors of death in PTE patients, adjusted for pulmonary artery systolic blood pressure and age.

Variable	Odds ratio	Confidence intervals	P value
Shock	31.52	5.59 – 177.69	<0.0001
Infection	0.141	0.047 – 0.422	<0.0001
Heart rate	0.98	0.966 – 0.995	=0.009

in-hospital mortality 7.2 mmol/L (Fig. 2A)

The mortality of patients with admission serum glucose concentration ≤ 7.2 mmol/L was 30/243 =12.346%. On the other hand, the mortality of patients with admission BG >7.2 mmol/L was 39/146 =26.712%, OR= 0.3929, 95%CI (0.2633-0.6343), $p<0.001$. There was not such cut-off value for DM patients to distinguish between patients who will survive from those who will not (Fig. 2B). A multivariate analysis for mortality predictors was carried out (Table I). Patients with previously diagnosed DM (57 patients), newly diagnosed DM (22 patients) and without DM (427 patients), had admission BG 12.29 ± 7.18 mmol/L, 14.81 ± 8.63 mmol/L, and 7.34 ± 2.90 mmol/L, respectively; $p<0.0001$ for the difference between patients without DM and other groups); in-hospital mortality was 21.05%, 31.82% and 19.20%, respectively ($p=0.074$ between newly diagnosed DM and non-DM group).

DISCUSSION

To the best of our knowledge, this is the first study

which demonstrated “J-shaped curve” in patients with PTE for in-hospital mortality in relation to admission BG. Such relationship has been demonstrated in AMI and stroke (5, 6). This “J-shaped curve” relation may decrease the probability of admission BG being an independent predictor of in-hospital mortality as assessed by multivariate analysis. Furthermore, this is the first study which confirmed significance of SH in predicting in-hospital mortality, using recently suggested methodology for SH: both tertiles and deciles, as well as ROC curves (2). Few studies have investigated SH in PTE: PubMed search (04/25/2019) for the key words “pulmonary embolism glucose” revealed only 146 studies, most of which are not directly related to the topic, with only three related (7-9).

Most of the studies on the relationship between abnormal glucoregulation and PTE or VTE refers to the suggestion that dysglycemia is a risk factor for VTE. It was demonstrated for the admission glucose level, DM, metabolic syndrome and abdominal obesity. Some papers had the primary goal to recognize the importance of glycemic disorders in

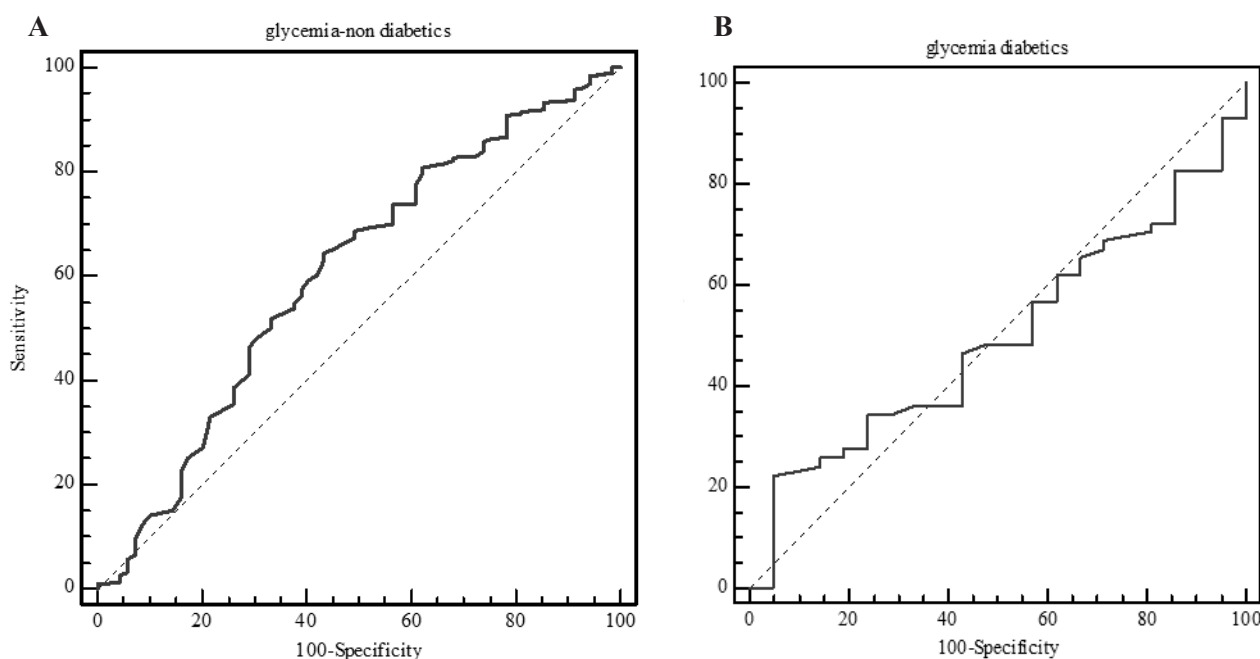


Fig. 2. *A) A receiver operating characteristic (ROC) analysis in PTE patients without DM. B) ROC curve analysis in PTE patients with DM.*

patients with VTE (7-10). Scherz et al. examined the association between glucose levels and mortality in 13,621 patients with PTE. Their results showed the independent prognostic role of admission BG for short-term mortality, which they explain by the procoagulant effect of hyperglycemia (10). Statistically significant correlation between admission BG and mortality in our study was registered only in patients without DM, as in the paper of Scherz et al. (10). Analyzing a total of 22 PTE patients with the submassive form of the disease, Gohbara et al. demonstrated insufficient improvement in mean pulmonary artery pressure in patients with high admission blood concentration (7). In 567 patients with VTE (PTE and DVT combined), Akirov et al. were able to confirm raised mortality for markedly increased admission blood glycemia in those without DM (8). One group demonstrated higher in-hospital mortality in advancing quartiles of admission glucose level in 183 patients with acute PTE (9).

The SH, i.e., increase of glycemia in critical condition of the patient, is explained essentially in two ways. Some patients had previous disturbance of glycemic control, which was not recognized, and became obvious only during hospitalization due to the current illness. Another reason for SH is the increased secretion of stress hormones (such as adrenaline and cortisol) and activation of the sympathetic nervous system, resulting in hyperglycemia.

Admission hyperglycemia can contribute to a higher death rate in PTE by various mechanisms. For example, hyperglycemia and/or hyperinsulinaemia can have both procoagulant action and decreased fibrinolysis. Moreover, microvascular obstruction may promote thrombosis. Admission BG was above the recognized threshold for in-hospital hyperglycemia in critically ill patients (≥ 8.3 mmol/L, ≥ 150 mg/dL) in a significant percentage of our PTE patients (36.19%). In PTE, just as in AMI, stress hyperglycemia should be analyzed with different cut-offs for DM and non-DM patients, because of differences in usual glucose concentration. The blood glucose level on admission was significantly higher in PTE patients who died in the hospital, making admission BG a potential candidate for risk stratification in this important disease.

Glycemia is a simple, fast, widely available and inexpensive laboratory parameter, with the potential to become a prognostic marker in PTE. "J-shaped curve" was demonstrated for the first time in PTE patients for in-hospital mortality related to admission BG concentrations. This "J-shaped curve" relation may decrease admission BG's probability of being an independent predictor of in-hospital mortality.

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LETTER TO THE EDITOR

TRAUMATIC BONE CYST OF THE JAWS: AN OVERVIEW

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To the Editor,

The World Health Organization (WHO) defines the traumatic bone cyst (TBC) as an intra-osseous cavity surrounded by a tenuous lining of connective tissue devoid of epithelium. Therefore, from a pathological perspective the most appropriate definition of this pathosis is “pseudocyst”. The scientific literature refers to TBC with a great variety of synonyms suggesting a lack of understanding of its nature, especially of its etiology and pathogenesis. It has been reported as: simple bone cyst, hemorrhagic bone cyst, solitary bone cyst, idiopathic bone cyst, unicameral bone cyst, extravasation cyst and progressive bone cavity (1). Therefore, the aim of the present study is to summarize the best available and up-to-date knowledge concerning this topic.

Epidemiology

TBCs are rare lesions affecting 1% of the general population. TBC occurs most commonly during the first two decades of life. Sex distribution is reported to be statistically quite even, with some studies reporting a slight male predilection (2).

Location

Over 90% of TBCs are discovered in long bones. The typical location is the metaphysis adjacent to growth plate of the tubular bones, mostly the humerus and femur; other locations include the pelvis, ribs vertebrae, and the tarsal bones, especially calcaneus.

Facial TBCs are less frequent and almost exclusively of the mandible: the posterior mandibular body between canine and third molar is the most common site, followed by the mandibular symphysis. Fewer cases have been reported in the maxilla, mandibular ramus and condyle. Sporadically, the presence of multiple TBCs as well as lesions crossing the middle line of the mandible have been documented (3).

Clinical features

Usually TBC is asymptomatic and is discovered during a routine radiographic examination (*i.e.* incidentaloma) and, when symptomatic, tenderness or pain are the most common presenting symptoms. Despite its name, a clear medical history of trauma is variable or either questionable in most of the cases. Other unusual complaints include tooth sensitivity, paresthesia, failure of eruption of permanent teeth and tooth displacement. In some cases, TBC was discovered after a pathologic fracture of the mandible, which is a rare complication of this lesion (1, 2).

Imaging features

The traditional radiographic appearance on panoramic X-rays is that of a central, unilocular, well circumscribed, radiolucent lesion with or without sclerotic margins, rarely showing a multilocular pattern because of the presence of bony septa within the lesion. A characteristic radiological feature of

Key words: traumatic bone cyst; simple bone cyst; pseudocyst; oral diseases; oral surgery

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Fig. 1. Panoramic radiograph showing the scalloping effect of a TBC at the apex of the involved teeth.

the TBC is the scalloping effect when extending between the roots of the teeth (Fig. 1). However, this scalloped outline can be detected also in edentulous areas. In most cases the lamina dura of the teeth adjacent to the lesion is preserved as well as the positive response to pulpal sensibility tests. This aspect is of primary importance since TBC locating in the periradicular area can potentially mimic apical periodontitis. Some radiological features, such as root resorptions, nodular expansion and multiple lesions, are occasional and associated with a more aggressive behavior and a higher rate of recurrence.

Computed tomography (CT) findings reveal a radiolucent cyst-like low density lesion which can be characterized by a bucco-lingual bone expansion. Both CT scans and occlusal films are able to depict the thinning or the erosion of the buccal and lingual cortical plates. MRI analysis of TBCs showed a homogeneous intermediate signal intensity on the T1-weighted images and a homogeneous high signal intensity on the T2 weighted images with a clear border indicating a fluid-filled lesion (1, 4-7).

Diagnosis

The diagnosis of TBC is made during surgical exploration due to the pathognomonic presentation of this pseudocystic cavity and the lack of tissue that can be collected for histologic diagnosis. In some cases, fine-needle biopsy is reported to be both a diagnostic and a treatment procedure. After surgical exposure, TBC appears as a bone cavity that can be completely empty or it can contain gas, a serous clear or straw-colored fluid or it can be filled with blood (especially when the lesion is located in jaw bones

(Fig. 2). It has been hypothesised that the different content of the cavity may represent different stages in its pathological development (2, 4, 8).

Histopathology

A small amount of connective fibrous tissue can occasionally be collected during the curettage of the "cystic" cavity for biopsy. Microscopic evaluation reveals the absence of epithelium, the presence of connective tissue with numerous collagen fibers and fibroblasts, vascularity, occasional chronic inflammatory cells and areas of new bone formation. In addition, pieces of trabecular reactive bone surrounded by osteoblasts, hemosiderin, scattered giant cells adjacent to the bone surface and cementum-like tissue can be observed. Suei et al. (as well as other Authors) found no evidence of a relationship between histological findings and the patient prognosis (3, 7, 9).

Etiopathogenesis

The etiology and pathogenesis of TBC still remains unclear and controversial although several theories have been formulated. The main etiopathogenetic theories are reported below:

i) The traumatic-hemorrhagic theory is that most supported by the literature. This states that a trauma, including a tooth extraction, can result in an intramedullary hemorrhage. The compromised vascular supply, the edema and the aseptic bone necrosis caused by the trauma can lead the blood clot to liquefy, therefore not promoting the healing pathway. Consequently, lytic enzymes are released in

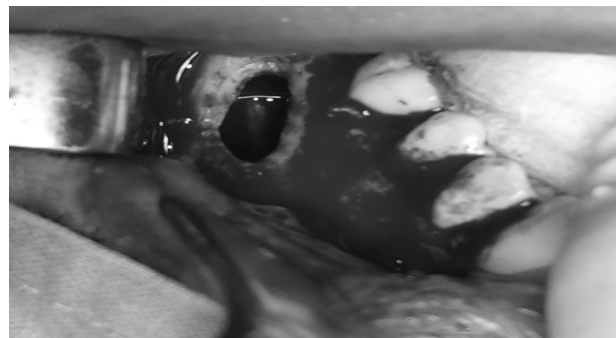


Fig. 2. Intraoperative picture of a TBC showing a sero-sanguineous content.

the area and osteoclastic bone resorption is activated. In addition, the expansion of the cavity seems to be sustained by the edema and the osmotic pressure caused by the persistent blood extravasation (2).

ii) The tumor-degeneration theory states that a developing tumor or lesion (i.e. hemangioma, lymphoma, fibrous-osseus dysplasia, central giant cell granuloma) can undergo liquefactive degeneration, leaving behind an empty cavity. This theory is supported by the frequent association of TBC with other intra-osseus lesions such as the contemporary presence of cemento-osseus dysplasia (3).

iii) The infective theory supports that a small, low grade chronic infection of bone marrow is involved in the pathogenesis of TBCs. This theory is supported by the occasional presence of a mild inflammatory infiltrate in the connective tissue surrounding the bone cavity. However, this theory remains mostly speculative since insufficient evidence has been collected for its support (4).

iv) The local-thrombosis theory suggests that a local thrombosis can generate a local ischemia with necrosis of bone marrow and blockage of interstitial fluid drainage, leading to the formation and expansion of an intra-osseus cavity (2,10).

Treatment

TBC may resolve without any treatment, but the widely recommended approach is surgical exploration followed by curettage of the bony walls, since this serves as both diagnostic and therapeutic procedure. Persistence following curettage may occur and require additional treatment. The grafting of allogenic bone with platelet-rich plasma (PRP) and the injection of a mixture of blood, hydroxyapatite and bone fragments were reported to produce good results to support the healing of the residual bone cavity. The rationale of this technique stands behind the fact that an incomplete filling of the cavity after curettage and primary closure can engender a possible reason for recurrence (6, 10, 11).

Prognosis

Several authors reported recurrences of these pseudocysts, therefore a careful clinical and radiographic follow-up of at least 3 years after

its treatment is strongly recommended by the literature (9, 12).

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LETTER TO THE EDITOR

CARBON FIBER SCREWS IN SPINAL TUMOR AND METASTASIS: ADVANTAGES IN SURGERY, RADIO-DIAGNOSTIC AND HADRONTHERAPY

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To the Editor,

In primary or metastatic tumors involving the spine, the best treatment option is a multidisciplinary approach that is the combination of surgery with permanent metallic implant and associated (adjuvant) radiotherapy (1, 2).

The traditional implant materials used in orthopedic surgery are usually stainless steel or titanium. These materials are an obstacle for accurate radiotherapy planning, because the CT images of patients with metallic implants often result distorted due to the artifacts, which reduce the accuracy in dose calculation of radiotherapy with subsequent errors that can be beyond a clinically acceptable range. Furthermore, these materials are not compatible with high-energy hadrontherapy, because of the high level of reflection of the proton ions, with consequent wide and severe damage of the surrounding tissue (3-5).

Carbon-Fiber Reinforced Polyetheretherketone (CFR-PEEK) material is now broadly accepted as an alternative to metallic biomaterials for the production of pedicle screws, offering the advantages of radio-transparency and full compatibility with high-energy radiotherapies. Moreover, recent studies indicate CFR-PEEK as safe and comparable with

the commonly used titanium implants in terms of intraoperative complications, stability at weight bearing and at functional recovery (6, 7).

In medicine, 3D printing is making an increasing contribution to modern therapeutic planning. It has become more important in recent decades with applications in orthopedics, spinal surgery and other various disciplines (8).

Here, we report our initial experience with the use of CFR-PEEK pedicle screw and rod fixation for the treatment of spinal metastases and a primary spinal tumor, after accurate planning on the 3D printed model, which also allowed the production of customized spinal implants. The subjects involved signed an informed consent before being enrolled.

Case Report 1

A 71-year-old male had been operated three years previously for the removal of a clear cell cancer of the right kidney. He did not comply with any regular oncologic follow-up, nor adjuvant therapy. After three years, a sciatic pain started in the right leg and it was associated with a motor deficit of the extensor hallucis longus. MRI revealed a vertebral lesion at the segment L5, with significant vascularization,

Key words: 3D printing; carbon fibre screws; hadrontherapy; spine surgery; spine tumours

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compressing the cauda equina and the root L5 on the right side. The results of the biopsies diagnosed “clear cell tumor metastasis (vimentin +, CD10 +, racemase +, cytokeratin 7-)”, and the staging with CT scan confirmed it as isolated metastasis at L5.

In order to plan the surgery, 3D printing of the L3-S1 vertebral segment was carried out. The reconstruction of the vessels was also included in the 3D printed model to investigate the blood supply to the metastatic tissue. The surgical treatment allowed the debulking of the vertebral lesion and fixation with a carbon fiber system (screws and rods).

CT and MRI control and postoperative 3D printing highlighted the removal of the mass and the correct positioning of the screws and rods. After surgery, the patient had immediate pain and neurological deficiency relief. The wound healed completely in four weeks, so he began carbon-ion radiotherapy (CIRT) using active scanning beam delivery system at CNAO (National Center of Oncological Hadrontherapy). The total dose prescribed to the tumor target volume was 55.2 Gray equivalents (GyE). CIRT was performed once daily, 4 days per week, in 12 fractions. The treatment was well tolerated without neurological symptoms and only a

skin erythema (grade 1, G1) was observed caused by acute toxicity during treatment. A trimestral and then bi-annual clinical and radiological monitoring has been undertaken. The 18-month follow-up did not show progression of the disease or local recurrence, with good clinical response to the treatment.

Case 2

A 60-year-old male had been diagnosed with a large intra-extracanalicular expansive lesion at L2 level in 2013. The biopsy allowed to formulate the diagnosis of chordoma. Radical excisional surgery was not feasible, therefore the patient was treated the first time in March 2014 with CIRT once daily, 4 days per week, for a total of 16 fractions. The total dose was 70.4 Gray equivalents (GyE). The treatment was well tolerated. An erythema (grade 2, G2) was observed during treatment, while all the pre-existing neurological symptoms were improved at the end of the treatment. The patient was followed-up for radiologic response every three months after CIRT with clinical visits and high field magnetic resonance (MRI). Post-treatment MRI demonstrated a notable reduction in tumor volume. The baseline clinical target volume of the tumor compared with MRI after

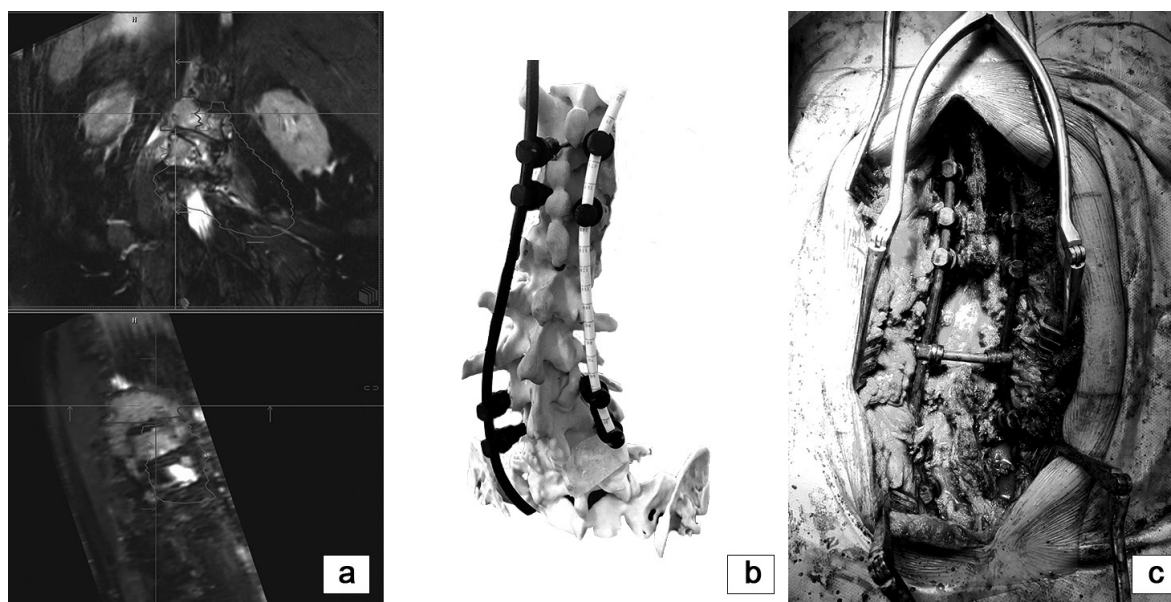


Fig. 1. a) Local relapse appeared after 31 months; b) screws and phantom rods on the 3D model; c) intra-operative image showing fixation of the carbon fiber screws and rods.

12 months was 74x49x70 mm vs 54x45x50 mm. After 31 months of stable condition, both clinically and radiologically, the patient experienced worsening low-back pain, muscle weakness in the right leg and difficulty walking. The MRI showed a progression of the mass at D12 level (Fig. 1a) and weight-bearing X-rays showed post-radiation scoliosis (25° according to Cobb at D12-L4 level). In order to plan the intervention and evaluate its feasibility, a 3D model of the D8-sacrum section was printed (Fig. 1b). We placed the screws on the printed model, and shaped and customized the rods on the phantom. We carried out a D9-L4 fixation with carbon system implanting the customized rods during surgery to cover the scoliosis curve, medullary decompression and debulking of the high-grade lesion (Fig. 1c).

After the surgery, the patient had immediate pain relief, and four weeks later he began a new cycle of hadrontherapy without relevant side effects. In the first nine-month phase the patient's follow-up was satisfying because he had no pain or only mild hip pain with reduction in analgesic use and his walking was possible without an orthopedic corset. Later, a disease progression (local and distant recurrence) was detected so the patient underwent salvage chemotherapy with tyrosine kinase inhibitor imatinib mesylate.

DISCUSSION

The two cases show that carbon instrumentation devices allow three main post-surgical steps compared with other systems. The first is that these devices avoid CT-scan imaging artifacts (typical of traditional titanium fixation device). The second is the possibility of performing conventional radiotherapy without interference. The third consideration is the possibility to use hadrontherapy, which is not feasible in the presence of metal. Therefore, from a radiotherapy point of view, CFR-PEEK in these cases gave more flexibility in the treatment planning of patients receiving an accurate dose in post-operative particle therapy, decreasing integral radiation to normal tissues (9, 10). The carbon screws show some technical difficulties of connection between the rod and the screw, which are more complicated

in long fixation. For this reason, it is mandatory to have a 3D image planning and a 3D printing service. In the first case, we were able to estimate the mass vascularization, location and relationships with other structures with extreme accuracy. In the second case, the preoperative rod customization on a 3D model made it possible to by-pass the difficulty of using carbon screws in the presence of a severe scoliosis deformity. The 3D models permitted a direct observation and measurement of the spine deformities, which helped the surgical team to perform assessment and communicate with the patient and colleagues. Moreover, printed models provided us with the opportunity to perform these procedures as efficiently and safely as possible while at the same time hadrontherapy grants a less invasive surgery. The only limit for these procedures lies in costs (11). According to other similar experiences with CFR-PEEK in spine surgery (6, 7), these combined technologies optimize pre-operative planning, surgical strategy and post-operative adjuvant therapy. Further studies are needed to confirm the increase of likelihood of healing in case of spinal tumors.

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LETTER TO THE EDITOR

DAMAGE QUANTIFICATION OF MANDIBULAR CONDYLE IN JUVENILE IDIOPATHIC ARTHRITIS: 3D MORPHOLOGICAL STUDY BY CONE BEAM COMPUTED TOMOGRAPHY

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To the Editor,

Juvenile idiopathic arthritis (JIA) is a chronic joint disease the etiology of which is still unknown. Its typical onset occurs before the age of 16 years and persists for at least 6 weeks. JIA prevalence is 0.07-4.01/1000 children, while its annual incidence is 0.008-0.226/1000 children. The disease affects mainly females with a 3:1 female:male ratio. Joints affected by JIA display chronic synovitis and inflammatory cell infiltration that leads to cartilage and bone destruction. When JIA affects the temporomandibular joint (TMJ) it usually causes condylar lesions. Early minimal margin erosion later evolves into articular surface flattening, with consequent articular space reduction, and condyle head destruction (1, 2). TMJ is more frequently involved in female patients with an earlier onset of JIA. Published studies indicate that 17-87% of subjects affected by JIA show also a TMJ involvement. Moreover, as arthritis involving the TMJ is often asymptomatic, its diagnosis is easily delayed. Because of this, both the incidence

of JIA and the TMJ involvement might be currently underestimated. Thanks to its high sensitivity, magnetic resonance imaging (MRI) is considered as the gold standard in the diagnosis of TMJ involvement even in the early phases of JIA. However, MRI has high maintenance costs and is badly tolerated by young patients, often requiring conscious sedation or even general anaesthesia for its application. Panoramic radiograph and ultrasonography (US) have a lower sensitivity than MRI and do not allow the early diagnosis of condylar damage (3). Given its significantly lower risk in radiation exposure compared to conventional computed tomography (CT), also cone beam computed tomography (CBCT) has been proposed as an alternative tool for early diagnosis of JIA and assessment of TMJ damage. Its high sensitivity allows accurate condylar cortical bone visualization in TMJ images. Indeed, Koos et al. have recently proposed a new classification of TMJ damage in patients affected by JIA, which involves applying both MRI and CBCT (4).

Key words: juvenile idiopathic arthritis; temporomandibular joint; cone beam computed tomography; diagnostic imaging; mandibular condyle

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

MATERIALS AND METHODS

Record selection

Records for the retrospective analysis of the present study were selected among those of 42 patients affected by JIA according to Durban criteria referring to the Fondazione I.R.C.C.S. Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Department of Orthodontics, Milan, Italy. This was purely an observational retrospective study. The patient management and outcome were unaltered using retrospective analyses of their imaging studies. An ethical approval was required for this study. Date 03/09/2016, N. 421. Written informed consent was obtained from the patients for the publication of their data and images. Inclusion criteria were age between 9 and 14 and the clinical record including an orthodontic evaluation and a complete CBCT scan of the TMJ. Patients with cardiac pacemakers, metal implants or clips and/or dental braces were excluded. Patients were also excluded if presenting intra-oral affections or other symptoms suggesting potentially additional concomitant clinical issues. Additional 20 records concerning non-arthritis subjects (control group) were selected. These were young patients, mean age 11.45 years, having CBCT performed in order to assess tooth migration for possible orthodontic correction. None of them presented any historical or clinical evidence of craniomandibular dysfunction or TMJ associated symptoms.

CBCT data analysis

CBCT data were captured using an I-CAT™ Cone Beam 3-D imaging system (Imaging Sciences International, Hatfield, PA, USA) and processed using the I-CAT Vision viewing software (Croall Radiography, San Jose, CA, USA), which allows 3D image visualization and measurement: images are shown as a three-dimensional rendering and in their three spatial projections (frontal, lateral and axial). Analysis of CBCT images first involved identifying the horizontal, mediolateral and posteroanterior condylar axes as well as the horizontal condylar angle, and then measuring – by appropriate software functions – the mediolateral and posteroanterior axis on the axial projection, at the greatest mediolateral diameter, and the horizontal condylar angle with the parasagittal axis of the skull (Fig. 1)

Condylar damage was evaluated qualitatively on the panoramic radiographs according to the Pedersen score (grade 0: no radiological abnormalities; grade 1:

defects in the condylar border, minor erosions; grade 2: flattening of the condyle; grade 3: total destruction of the condyle) (3). The same operator repeated both the software measurements and the condylar damage scoring 15 days apart to reduce possible assessing errors,

Statistical analysis

Differences between the two groups concerning the parameters under investigation were evaluated by means of t-tests. In the JIA group, the correlation between condylar damage and radiographic data was assessed by a non parametric test. The Mann-Whitney Test was used to identify the group differences. Differences and correlations were regarded as significant when $p < 0.05$. All values are given as mean $\pm$ Standard Deviation (SD). Analysis was performed with a standard statistical software (Origin 9.0; Microcal, USA).

RESULTS

Records retrieved

Records concerning the JIA patients were 42, including 36 females (82.61%) and 6 males (17.39%). Their mean age was 12.78 years and the mean duration of the disease was 6.61 years. The control group included 20 subjects, 9 females (45%) and 11 males (55%), whose mean age was 11.45 years.

TMJ damage frequency

CBCT revealed TMJ damage in 35 (83.3%) out of the 42 JIA subjects. Of these, 36 (85.71%) were females and 6 (14.29%) were males. The joints were involved bilaterally in 33 cases (71.74%) comprising 29 females (63.04%) and 4 males (8.70%). The two condyles showed a similar involvement in 16 cases (34.78%; 14 females – 30.43%, and 2 males – 4.35%), while in 17 cases (36.96%; 15 females – 32.61%, and 2 males – 4.35%) the two condyles were involved differently. Nine patients (19.57%; 7 females - 15.22%, and 2 males - 4.35%) showed an unilateral involvement of the TMJ.

Damage score according to Pedersen

Data concerning the damage score of all 42 patients having TMJ involvement are provided in Table I. Patients displaying a similar bilateral involvement of the joints showed, in the majority



Fig. 1. CBCT multiplane reconstruction image showing axial view through the mid region of the mandibular condyle: (a) angular; (b) mediolateral and posterior anterior diameters measurement; (c) CBCT coronal view: cortical erosion of the right condyle and normal left condyle; (d) CBCT coronal view: flattening of the right condyle.

of cases (43.75%), a total destruction of the condyle (grade 3). When the bilateral involvement was not similar, a higher value of flattening was observed on the right side (52.94%) than on the left side (11.76%), while there was a similar value of erosion and total destruction on the left side (47.06 and 41.18%, respectively). When the TMJ was involved unilaterally, the left side was more affected than the right side and the damage belonged equally to all Pedersen's grades. When data were analysed according to the patient's gender, female patients showed a similar incidence of erosion (25%), flattening (36.11%) and total destruction (25%) in the right condyle, while on the left side there was a prevalence of erosion (36.11%) and total destruction (41.67%) over flattening (16.67%). In female patients showing a similar bilateral involvement of TMJ, total destruction and flattening (35.71%) were the most common damage observed. If the bilateral involvement was dissimilar, a prevalence of flattening (53.33%) was observed on the right side. On the left one, the incidence of erosion (46.67%) and total destruction (40.00%) was comparable. The left side showed worse damage than the right side in unilateral cases.

In male patients damage was unequal on the two sides, the left side being more affected by total destruction (50%) than the right one (33.33%). In males with unilateral involvement no damage were observed on the right side.

Condylar measurements

The overall mediolateral (ML) diameter means were smaller in JIA subjects than in the control group. Both right and left condyles were significantly smaller in the JIA subjects than in the control group (left, 15.11 vs 17.20, $p = 0.02$; right, 14.63 vs 17.05, $p = 0.002$). When comparing female subjects only with the respective controls, difference in the right diameter was significant, while that of the left side was not (left, 14.74 vs 16.33, $p = 0.07$; right, 14.26 vs 16.22 $p = 0.03$). Difference was not significant for both sides when male subjects were compared to male controls (left, 16.88 vs 17.91, $p = 0.18$; right, 16.38 vs 17.73, $p = 0.14$).

Concerning the posteroanterior diameter, no

significant differences were observed between the left side of the JIA patients and the controls (7.80 vs 7.55, $p = 0.26$) and between their right sides (7.52 vs 7.75, $p = 0.28$). The right PA diameter was significantly greater in JIA males than in controls (8.50 vs 7.73, $p = 0.04$), while the left one was similar (8.00 vs 7.91, $p = 0.28$). No differences were observed concerning both the right (7.66 vs 7.33, $p = 0.45$) and the left side of the female patients and controls (7.42 vs 7.56, $p = 0.38$).

The control group had condylar angles means that were smaller than those of JIA subjects. On the right side the angle measured 33.59° in JIA subjects and 23.57° in the control group, the difference being significant ($p < 0.001$). On the left side the angle measured 34.70° in JIA subjects and 25.11° in the control group, but the mean was not significant ($p = 0.07$). In both groups the right condylar angle was lower than on the left condylar angle. Also considering females and males separately, average condylar angles were smaller in the control group than in the JIA subjects, but in the JIA females the right angle was greater than the left angle. In males there was a significance ($p = 0.003$) in left condylar angle (Table II).

Correlation between condylar measurements and condylar damage

Condylar measurements were compared with radiographic signs as erosion, flattening and total destruction. Considering all patients, a certain degree of correlation was observed at the right side between the condylar damage and the right mediolateral diameter ($p = 0.016$) and between the condylar damage and the right condylar angle ($p = 0.004$). Correlations at the left sides were, instead, weaker. Regarding the female patients, a certain degree of correlation was observed between the condylar damage and the right condylar mediolateral diameter ($p = 0.014$), and between the condylar damage and the right condylar angle ($p = 0.005$). Correlations at the left side were weaker. For the male patients, a strong correlation was observed between the condylar damage and the condylar mediolateral diameter at the right side ($p = 0.004$). Correlations were also observed between the condylar damage and the left condylar angles ($p = 0.020$).

Table I. Condylar damage according to the Pedersen classification.

Total					
Grade	Damage	Right side		Left side	
1	Erosions	10	23.81%	15	35.71%
2	Flattening	14	33.33%	7	16.67%
3	Total destruction	11	26.19%	18	42.86%
0	No damage	7	16.67%	2	4.76%
Total		42		42	
Bilateral equal					
Grade	Damage				
1	Erosions	5	31.25%	5	31.25%
2	Flattening	4	25.00%	4	25.00%
3	Total destruction	7	43.75%	7	43.75%
Total		16		16	
Bilateral different					
Grade	Damage	Right side		Left side	
1	Erosions	4	23.53%	8	47.06%
2	Flattening	9	52.94%	2	11.76%
3	Total destruction	4	23.53%	7	41.18%
Total		17		17	
Unilateral					
Grade	Damage	Right side		Left side	
1	Erosions	1	11.11%	2	22.22%
2	Flattening	1	11.11%	2	22.22%
3	Total destruction	0	0.00%	3	33.33%
0	No damage	7	77.78%	2	22.22%
Total		9		9	

Grade 0: no radiological signs; Grade 1: defects in the condylar border-minor erosions; Grade 2: flattening of the condyle; Grade 3: total destruction of the condyle.

Table II. Condylar diameters, angles and condylar damage.

Patients	Right ML condylar diameter	Left ML condylar diameter	Right PA condylar diameter	Left PA condylar diameter	Right condylar angle	Left condylar angle	Right condylar damage	Left condylar damage
JIA	14.63	15.11	7.80	7.52	33.59	34.70	1.57	1.80
Control	17.05	17.20	7.55	7.75	23.57	25.11		
JIA females	14.26	14.74	7.66	7.42	33.92	32.39	1.66	1.84
JIA males	16.38	16.88	8.50	8.00	32.00	45.63	1.13	1.63
Control females	16.22	16.33	7.33	7.56	25.04	27.70		
Control males	17.73	17.91	7.73	7.91	22.36	22.99		

(ML=mediolateral; PA=posteroanterior)

DISCUSSION

In patients with JIA, TMJ involvement is often asymptomatic and can lead to severe craniofacial growth disturbances and facial deformities if not treated at the initial stage. CBCT has been included together with MRI as diagnostic tool for the new classification of TMJ damage in JIA subjects proposed by Koos et al. (4).

Considering the localization of the damage, the majority of TMJ damage detected in this study was bilateral, in accordance with other studies (1, 3, 5, 6). When the TMJ was involved bilaterally, a prevalence of flattening in the right condyle and a prevalence of erosions and total destruction on the left condyle was observed. In this study unilateral involvement was observed more frequently in the left condyle, in agreement with Pearson and Ronning (7), and differently from what was reported by Sidiropoulou-Chatzigianni (8), where the right condyle was found to be affected more frequently than the left. This might be associated with some habits or differential muscle activity of masticatory muscles or different pattern of mastication in the patients.

The values of condylar angle obtained in subjects with JIA were higher than in the control group. Values obtained in this study for the JIA group were significantly greater than the data available in the literature. This could be due to the young age of subjects included in the study and to the damage on TMJ in growing subjects (9). The results obtained in this work confirmed how CBCT represented an improvement in quantifying the morphological changes of the condyle and mandible. CBCT is a valuable alternative to MRI for diagnosis of TMJ disorders. This is particularly significative considering the limitation of MRI application in young patients together with the importance of an early detection of TMJ disorders in JIA patients in order to avoid serious bone lesions and deformities (10-12).

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LETTER TO THE EDITOR

PREVENTION OF BACTERIAL COLONIZATION ON SUTURE THREADS AFTER ORAL SURGERY: COMPARISON BETWEEN PROPOLIS- AND CHLORHEXIDINE-BASED FORMULAE

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To the Editor,

Tooth extraction is the most common procedure in oral surgery, directly followed by implant surgery, muco-gingival surgery, and peri-apical surgery (1). The standard operative protocol in oral surgery includes the use of suture threads in order to facilitate wound healing and to prevent surgical site dehiscence. The biological rationale behind the use of sutures after surgery is due to the possibility of obtaining first intention healing facilitating the contractile action of myofibroblasts during the first step of repair phase (2).

Nevertheless, it is also known that bacteria have more adherence properties on suture threads, affecting oral hygiene and the tissue healing itself. To prevent wound-infections around suture threads, several antimicrobial agents have been tested to be incorporated in suture materials or administered by the patients at home. Nowadays, chlorhexidine is still the most recommended chemo-therapeutic agent for domiciliary care after tooth-extraction, due to its properties in terms of reducing inflammation and

bacterial colonization (3). It is known that prolonged use of chlorhexidine may have some side effects, such as tongue discoloration, tooth staining, and oral mucosa desquamation (4). For these reasons, researchers and clinicians are opening to alternative therapies including herbal extracts and probiotics. The use of propolis (a phyto-therapeutic agent) has been largely discussed both in medicine and in dentistry in recent years (5). Propolis is a resinous mixture produced by honeybees which contains a mixture of plants, Vitamin C, and Vitamin E. These components have been reported as having antimicrobial, anti-inflammatory, and immunomodulating properties (6).

In the present study, a gingival gel containing propolis [bio-adhesive Nano-Bio Fusion (NBF)] was tested for its anti-microbial efficacy on suture threads and was compared with a gingival gel containing 0.2 chlorhexidine. The null hypothesis was that there would be no differences in terms of bacterial growth and relative presence between the two groups.

Key words: oral surgery; teeth extraction; propolis; chlorhexidine; microbial load

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MATERIALS AND METHODS

Population of the study

The present clinical case-controlled double blinded study was performed at the Tuscan Stomatologic Institute (Forte dei Marmi, Lucca, Italy). All the included patients were informed about the study protocol and were asked to sign an informed consent form, explaining the absence of risk and the absence of conflicts of interests. The present clinical trial was conducted in accordance to the Declaration of Helsinki of 2008.

The study population was derived from patients who needed at least one tooth extraction and who satisfied all following inclusion criteria: aged between 30 and 60 years; good oral hygiene; and compliance with the study protocol and willingness to adhere to the post-operative instruction.

Exclusion criteria were addiction to drugs, alcohol, or smoking (>5 cigarettes/ day), pregnancy, severe systemic pathologies, severe psychiatric disorders, severe physical handicaps, immunosuppressive therapy, radiotherapy or bisphosphonate therapy in the previous 36 months, need of antibiotic therapy for the present tooth extraction.

Surgery

A periapical radiograph was taken before surgery with the paralleling-cone technique. Mepivacaine with adrenaline (1:100 000) was administered as local anesthetic agent. All patients were asked to rinse their mouth for 1 min with ozonized water (Aquolab EB2C Srl), for decontamination purposes, immediately prior to surgery. Teeth were extracted using forceps and elevators, performing a mini-invasive flap. In case of ankylosed teeth, an osteotome was used. After the surgical procedures, non-absorbable silk sutures (Sweden & Martina, Due Carrare, Italy) were used and a non-steroidal anti-inflammatory drug was prescribed twice a day for 3 days.

Study design

Patients were asked to not rinse for one day, and the day after surgery to start local application of a gingival gel for 10 days after domiciliary oral hygiene with a soft toothbrush and without any other toothpaste, gel or mouthwash. At the end of the screening procedure, 40 patients were included in the study and were equally divided into two groups by a computer program generating

the letter A or B.

In the Control group (letter A; n=20), a gingival gel with chlorhexidine 0.2% (n=20) [Curaden Healthcare S.p.A. - Saronno (VA), Italy] was used. The Test group (letter B; n=20) was assigned a gel containing propolis bio-adhesive NBF (Sungwon Pharmaceutical Co., Ltd, South Korea). NBF gingival gel ingredients were: sorbitol, water, hydrated silicates, glycerine, propolis extract, PEG-1500, cellulose gum, sodium monofluorophosphate, menthol, ascorbic acid, tocopherol acetate, mentha piperita, oil, steviol glycoside, triclosan 0.1% p/p, methyl paraben, Maltodextrin, xylitol and may contain Fluoro 526 ppm ions.

The suture was removed after 12 days, collected, and sent to the laboratory to be stored at -20°C until microbiological analysis.

A flow-chart summarizing the study protocol is represented in Fig. 1.

Microbiological test and bacterial scoring

The microbial load was assessed after suture removal and stored at -20°C until microbiological test.

Each sample of silk suture threads was cut into 3 segments of similar length and analyzed with three different growing Petri plates:

- One plate contained agar-based broth media (TSA= Tryptone Soy Aar) and (MRS= De Man, Rogosa and Sharpe) were incubated at 36±1°C for 48 h to determine the microbial growth of mesophilic bacteria;
- The second plate contained MRS agar-based broth media and was incubated at 36±1°C for 48 h to determine the microbial growth of *Lactobacillus* species;
- The other plate contained Bacteroides bile esculin (BBE) agar and brucella blood agar (BRU) with vitamin K and hemin, and was incubated at 36±1°C for 7 days under anaerobic conditions for the qualitative analysis of specific anaerobic strains.

The first two plates were placed in a glass tube (Erlenmeyer-Kolben) for the enumeration of bacterial colonies containing a diluent solution (buffered peptone water; BPW) for 1 h, then, further serial decimal dilutions were carried out. The first plate was seeded in plates with the same agar-based broth media and incubated for 48 h at 36±1°C in order to quantify the microbial load. Bacterial colonies were then counted and expressed as colony-forming units per mL (cfu/mL).

For the other plate, it was determined whether microbial load was present on the thread surface after the period of observation, as previously described. The plates were visually inspected and the bacterial load accordingly classified as 'absent' or 'present'. In positive cases, individual colonies were reseeded to achieve a pure bacterial culture suitable for biochemical recognition. For evaluation of the growth of specific anaerobic bacteria, the classes of 'growth' and 'no growth' were established, depending on whether bacterial growth was observed.

Statistical analysis

Data were expressed as mean±standard deviation (SD) of mL (cfu/mL) values, where available. The student's *t*-test for independent samples was used to evaluate the difference between the Control group and Test group. A value of $P \leq 0.01$ was taken as statistically significant. In the case of categorical data, that is growth/no growth of bacterial species, descriptive statistic was used because for the Yates's corrected version of Pearson's *chi*-squared-test there was insufficient sample for a significant

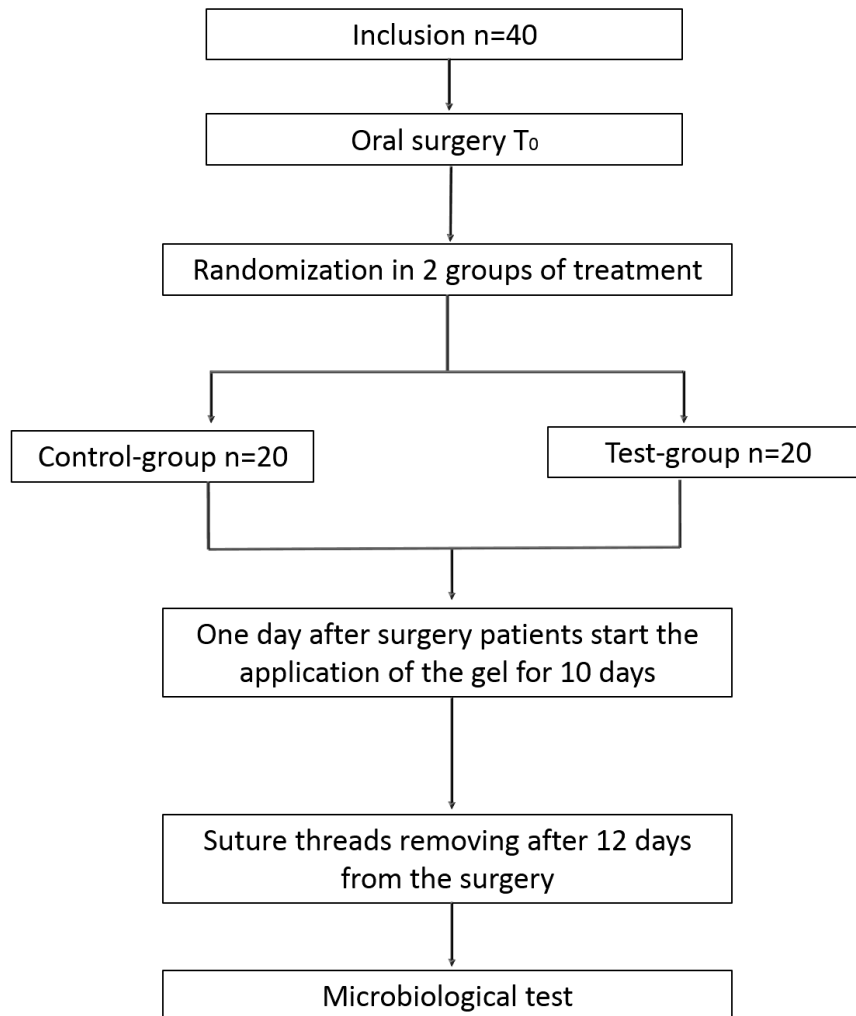


Fig. 1. Flow-chart illustrating the study protocol.

analysis, and the data were organized in graphs and tables. Statistical analysis was performed using Excel (Microsoft – windows 2018).

RESULTS

Demographic data

Forty patients were enrolled in the present study (15 males, 21 females) after screening of all consecutive patients in need of a single dental extraction. Suture samples were collected from all the patients. The mean age of the entire cohort was 55.12 ± 11.04 years, with no significant differences between the two groups: Control group (55.55 ± 11.27) and Test group (54.70 ± 11.08). The gender distribution was homogeneous within each group of treatment. All patients carried out the clinical study, and post-surgical severe complications were not registered in any patient, nor was antibiotic therapy prescribed after the extractions. The patient drop-out was 0%.

Microbiological analysis

The results of the microbiological analysis are summarized in Table I, showing the mean $\pm$ standard deviation (SD) values for Bacterial colonies counted and expressed as colony-forming units per mL (cfu/

mL) in different culture conditions. The mean $\pm$ SD score of bacteria seeding calculated on TSA was 551.3 ± 709.4 and 1575.4 ± 1113.3 in the Test group and Control group, respectively. The mean $\pm$ SD score of bacteria-seeding calculated on MRS was 417.7 ± 607.9 and 1054.9 ± 725.7 in the Test group and Control group, respectively.

In both plates (TSA and MRS), the P values showed significant statistical differences with a $P \leq 0.01$; the Test group was shown to have less mesophilic bacteria than the Control group after 10 days of gel application.

For the evaluation of specific bacteria, the classes of 'growth' and 'no growth' were established, depending on whether bacterial growth was observed. For the presence of fungal species isolated from biofilms, there was no observation of growth in either of the groups (Table I).

In the Control group there was a larger number of Petri dishes containing *Pseudomonas spp.*, *Staphylococcus spp.* and *Streptococcus spp.* than the Test group, but the P values were not statistically significant with Yates's corrected version of Pearson's *chi*-squared test, meaning that no statistically significant differences were observed between groups, probably because of the small

Table I. The total number of Petri dishes observed with specific colonies in the Control group and Test group with *p*-value; and bacterial colonies (cfu/mL) with mean values $\pm$ standard deviation for the two groups with *p*-value.

	Fungal spp.	<i>Pseudomonas spp.</i>	<i>Staphylococcus spp.</i>	<i>Streptococcus spp.</i>
Control group chlorhexidine	0 / 20	7 / 20	1 / 20	11 / 20
Propolis Nano-Bio Fusion (NBF)	0 / 20	4 / 20	0 / 20	8 / 20
P value	n.a.	0.48	1	0.53
		SEEDING ON TSA cfu/mL $\pm$ SD	SEEDING ON MRS cfu/mL $\pm$ SD	
Control group chlorhexidine 0.2		1575.4 $\pm$ 1113.3	1054.9 $\pm$ 725.7	
Propolis Nano-Bio Fusion (NBF)		551.3 $\pm$ 709.4	417.7 $\pm$ 607.9	
		0.0013	0.0046	

sample size. In detail, the growth of *Pseudomonas spp.* was observed in 4 of 20 samples in the Test group, and 7 of 20 samples in Control group. The growth of *Staphylococcus spp.* was observed in 0 of 20 samples in Test group and 1 of 20 samples in the Control group. The growth of *Streptococcus spp.* was observed in 8 of 20 samples in the Test group and 11 of 20 samples in the Control group. Fungus species were not observed in any of the samples of either group (Fig 2).

DISCUSSION

Suture threads in oral extraction

In dentistry, despite the modern improvement in

oral health and dental treatments, tooth extraction is still a procedure with a high prevalence (1). Suture is an important tool to prevent complications, but suture threads could be considered an iatrogenic factor in increasing plaque accumulation and imposing a risk for post-surgical complication.

Gingival gel or mouthwashes with chlorhexidine are commonly recommended for domiciliary post-surgical care, but natural products may have a better patient compliance, and several researchers have focused on this topic (7).

Literature on propolis-based formulae

The gel containing propolis Nano-Bio Fusion (NBF) is a natural powerful antibiotic and healing substance,

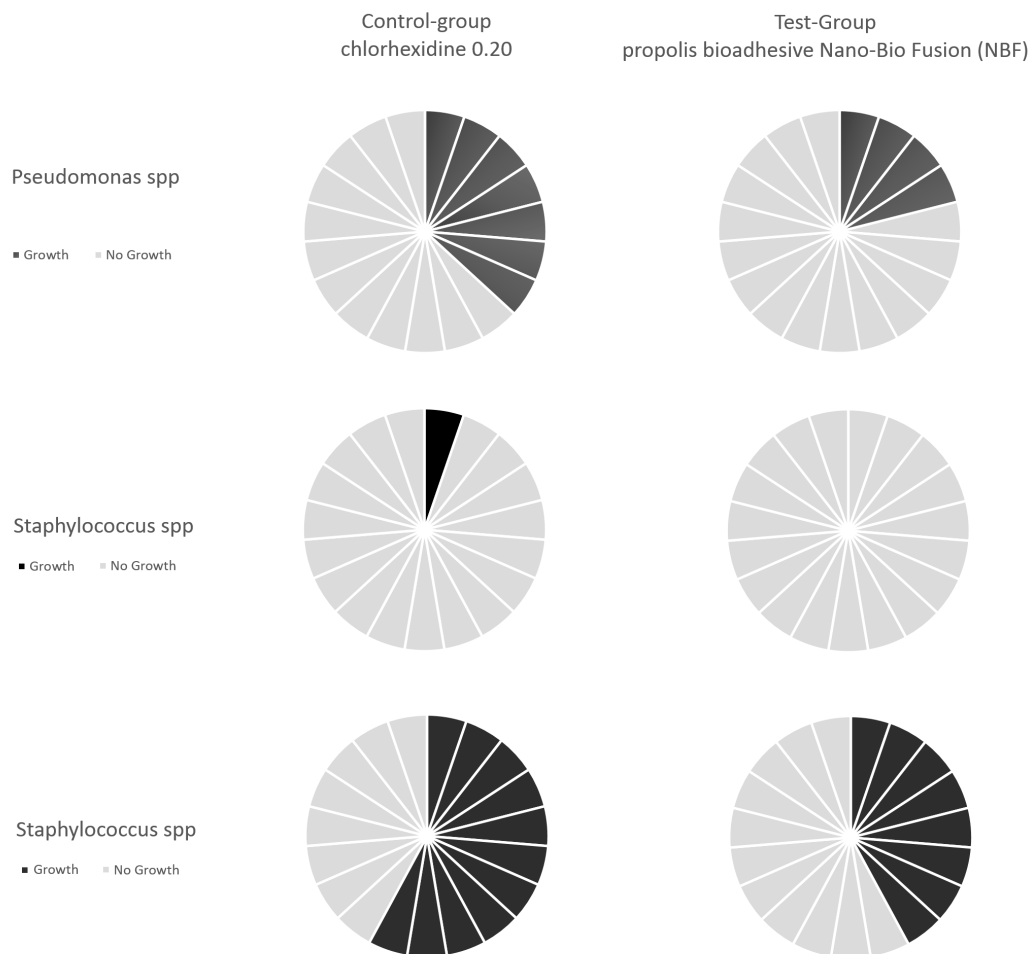


Fig. 2. Search for specific anaerobic bacteria: the black section represents presence of growth of a specific kind of microorganism in the Control or Test group.

it is a botanical product produced by honeybees (*Apis mellifera*). Several *in vivo* studies have shown various antioxidant, anti-inflammatory, and also anticancer properties of propolis (8). Vitamin C has been widely studied because of its antioxidant and anti-inflammatory power, therefore it may improve and promote gingival health and avoid the colonization of suture threads (9). Vitamin E improves soft tissue growth and may also have a role in tissue healing (10).

Other clinical studies on propolis reported that the NBF gel is an adjuvant therapy to oral inflammation and chronic periodontitis and is a safe product not showing allergic reactions (11).

In this case-control pilot study, the findings show significative differences between the two groups in terms of bacterial load, with a lesser growth of bacteria in the test group using propolis NBF gel. Also, according to the qualitative analysis, in the Control group, there was a larger proportion of samples containing pathogen bacteria than in the Test group, even if the difference did not reach significance.

These results suggest that natural products such as propolis gel have anti-microbial efficacy that should be considered by clinicians and researchers. In uninfected post-surgical sites, this formulae represents a valid alternative therapy to chlorhexidine which must be prescribed only when it is absolutely necessary. Propolis gel has anti-inflammatory and immuno-stimulant properties and does not manifest the typical side effects of chlorhexidine, such as alteration of taste perception, tooth staining, tongue discoloration, desquamation and soreness of the oral mucosa (12).

On the other hand, these positive results might be related to the flavonoids contained in the NBF formula that have bio-stimulating or anti-inflammatory properties. Other limitations of the present study were the small sample size and the microbiological test used may have been too unspecific, as well as the lack of a patient-related outcome regarding pain, quality of life after surgery, and the taste of the gingival gel.

Further studies with larger samples and different microbiological methods are required to support the results of the present pilot study.

DISCLOSURE STATEMENT: Dr. Lorenzi has a continuative collaboration with IDS Spa., Savona,

Italy. The other Authors have no conflicts of interest regarding the present research.

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LETTER TO THE EDITOR

SECONDARY SINONASAL HEADACHE IN CHILDREN: AN EMPIRIC APPROACH

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To the Editor,

Headache is considered one of the most common symptoms during childhood and is usually long-lasting and frequent enough to need special care (1). The lifetime prevalence of headache in childhood and adolescence is estimated to be approximately 40% in the general paediatric population, as compared to 4–20% in preschool children and 38–50% in school-aged children (2). There are considerable differences between the headaches of children and adults in terms of pain duration, laterality, and other factors. In preschool children, the attack duration is usually shorter with lower symptom association, and shows greater male gender prevalence and earlier onset of the attacks (3). The so called “rhinogenic headaches” are an important aetiology of secondary headaches, and as a diagnostic group include headache due to acute rhinosinusitis, chronic or recurrent rhinosinusitis, or other sinonasal pathologies believed to cause headache (such as mucosal contact points). “Sinus headache” is a commonly referred diagnosis for general practitioners, otolaryngologists, and neurologists when headaches are thought to be of rhinogenic origin (4). However, the term “sinonasal headache” could be nebulous, and there is no well-defined diagnosis at present. It is typically a self-diagnosis used by patients to describe a history of recurring headaches associated with classical

sinusitis symptoms, including facial pressure or pain, nasal congestion, or rhinorrhoea (5). Because the presentation of sinonasal symptoms can overlap with primary headache disorders, such as migraine, trigeminal autonomic cephalalgia (including cluster headaches), and tension headaches, it can be difficult for clinicians and patients to differentiate between these diseases. In patients with a complaint of sinus headache and a normal examination, it is clinically relevant that the distinction between potential aetiologies is made.

Sinonasal headache recognizes a primary rhinogenic source, including chronic, sub-acute and acute rhinosinusitis, as well as mucosal contact point headache sustained by turbinate hypertrophy (6). The importance of considering these items lies in the number of paediatric patients who could avoid being subjected to unnecessary treatments or surgical procedures when presenting to the clinic with a primary complaint of chronic headaches and associated nasal complaints. There is a paucity of studies on sinus headache or mucosal contact point headaches in the paediatric population.

Sinonasal headaches in children and adults, typically defined by recurrent headaches with facial pain, nasal congestion, rhinorrhoea, and a normal work-up, are generally the result of an undiagnosed primary headache disorder such as

Key words: headache; empiric treatment; sinonasal disorders; pirometaxine®; diet; children

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migraine, tension-type headache, or trigeminal autonomic cephalalgia. However, there are many subjects who could have sinonasal disorders without clear symptoms. In this regard, we report the data belonging to an observational retrospective study conducted on paediatric patients suffering from secondary headache.

MATERIALS AND METHODS

One hundred children (46 males and 54 females, aged between 4 and 12 years, mean age 9.5 years) were consecutively enrolled in the current observational study. All children were evaluated by a neuro-paediatrician. The data were retrospectively analysed.

The inclusion criteria were: i) history of headache symptoms for at least 6 months; ii) pain duration between 2 and 12 hours; iii) pain regarding the vertex or diffused without laterality, iv) high frequency of attacks (>2/month); and v) separate presence of phono- and photophobia.

Exclusion criteria were: i) primary headache and well-defined secondary headache; ii) abnormal neurological examination; iii) fixed pain; and iv) pain duration >12 hours.

The study was conducted according the Helsinki Conference Document and the parents or legal tutor, after receiving information on the study, gave their written informed consent to participate.

All children were treated with a standard treatment approach, usually adopted in children with rhinosinusal complaints and headache, including:

- i. nasal aerosol containing PirometaxineTM [pyrrolidone carboxylic acid (PCA) and copper sulphate (CS)] (Narlisim[®]) bottle 10 drops and isotonic saline 2 mL twice daily for 1 week, followed by short courses (4 days) every 2 weeks for 2 months, followed by Pirometaxine[®] and copper sulphate (Narlisim[®] spray) 1 puff per nostril twice daily for 1 month;
- ii. Pidolate magnesium (Mag 2[®]) 1 sachet daily for 15 days;
- iii. diet excluding fizzy drinks, egg, milk (if documented allergy), chocolate, red fruit, dry fruit, preserved tomato, food colouring, sausage, and fried food for 6 months;
- iv. paracetamol 500 mg *per os* as needed;

- v. limited exposure to TV, play station, tablet, smartphone, etc;
- vi. exerting physical activity;
- vii. keeping correct position while studying or reading.

Pirometaxine[®], a new patent combination of 4% Pyrrolidone Carboxylic Acid (PCA), and 0.002% CS, is contained in the NarlisimTM (a Medical Device) bottle. The last component adds to the well-known mechanical activities of nasal washing other benefits, such as the hypertonicity of the solution (2.4%) with a decongestant effect, the low pH of the solution that, together with the restoring effect of CS, creates an unfavourable environment for virus and bacteria proliferation in the upper airways (7, 8).

PCA contained in Pirometaxine[®] is responsible for the acidic pH of NarlisimTM; an early application of low pH nasal spray controls the severity of influenza A Sydney/5/97 virus as compared with saline control (9). It was shown that nasal mucosal pH ranges from 5.5 to 6.5 in healthy subjects, but during rhinitis it rises to 7.2–8.3 (10, 11), thus the low pH of NarlisimTM may be considered a “local stressor” for viruses and bacteria. Furthermore, PCA is universally considered a “compatible osmolyte” with cellular osmo-protecting activities, improving the osmotic balance of the nasal mucosa, and exerting a moistening activity in the nose (12).

Pidolate magnesium 2.25 g (Mag 2[®], a NutraceuticalTM, is a trace element, potentially able to improve physiological functions of the nervous system.

All children were visited at baseline, and after 8 months. The study was conducted between October 2017 and May 2018.

The primary outcome was the number of headache episodes, the secondary outcome was the severity of headache, both of which were recorded during the observation period of 5 months, lasting from the end of the 3-month treatment until the final visit. Headache severity was assessed by the visual analogue scale (VAS) where 0 = no pain and 10 = the worst pain or by the Wong-Baker Pain FACES scale according to the age of children (3).

Sensitisation was documented by skin prick test. An otorhinolaryngological visit was carried out on all children. Neuroimaging, such as magnetic resonance, was performed in very selected patients. The diagnostic procedure was conducted according to validated criteria defined by the 3rd edition of the International Classification of Headache

Disorders (18). The patients were classified as responder, partial responder and non-responder. Responder patients had no headache episodes during the observation period. Partial responder patients had up to 3 headache episodes during the observation period; non-responder patients had more than 3 headache episodes. Safety was assessed by monitoring any adverse events.

RESULTS

Baseline characteristics

The family history showed atopy, mainly maternal, in 45% of children as well as familiar headache, always prevalent in the mother, in 39%. Personal history demonstrated that 40% of the episodes had acute onset and 60% subacute onset, mainly in the morning. Pain duration was < 2 hours in 34% of attacks and > 2 hours in 30%. The pain severity was such that it blocked the activities. The pain characteristics were: cumbering in 49%, pulsating in 47%, striking in 1%, tensive in 1% and not well-defined in others.

Concerning the localization of headache, 47% had pain in the frontal area, 25% had widespread

pain, 12% in the temporal area, 9% in the occipital area, and 7% in the nuchal area. For the frequency of episodes, 31% had weekly attacks, 53% had biweekly or monthly attacks. Neuro-vegetative symptoms were associated in 60% of children. The most important triggers were stress, mainly emotive, physical, and mental, seldom acoustic or visive triggers. On the other hand, resting and sleeping were relieving factors. Recurrent respiratory infections, including upper and lower airways, and otitis occurred in 75% of children.

Considering sensitization, 52% of children were sensitized to inhalant allergens (mainly mites and grass pollen), 26% to food allergens (mainly milk, egg, and wheat), and 11% to both inhalant and food allergens. The clinical examination showed that 60% of children had the Dennie-Morgan fold, pressure pain on sinus points, and halitosis. However, the ENT examination, imaging, and inflammation biomarkers were negative in all children. Gender was not associated with particular clinical difference.

Observation period

Ten children did not complete the therapeutic

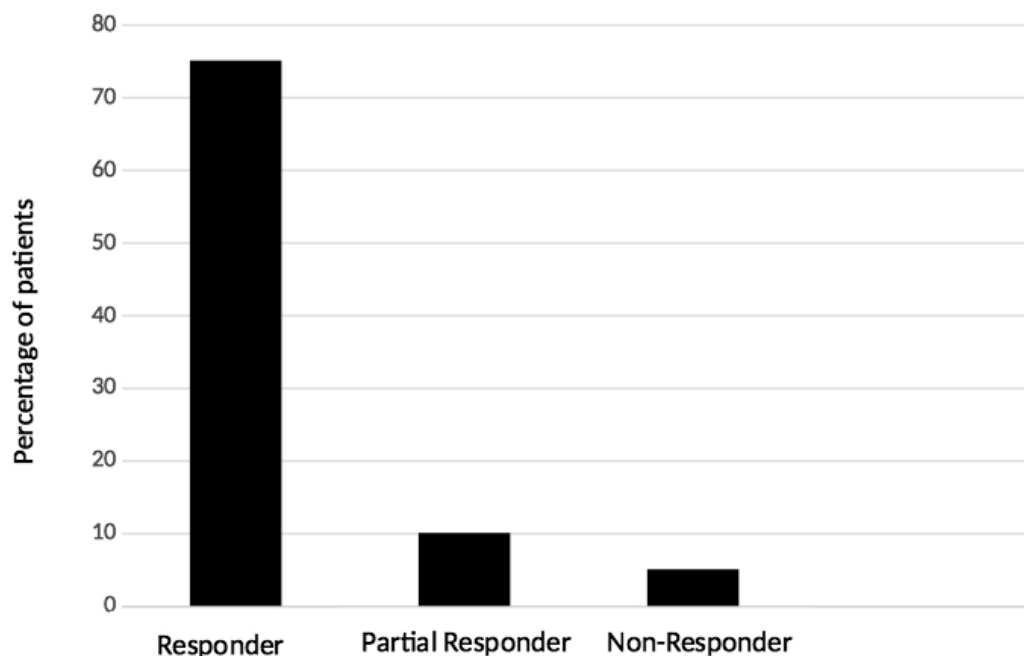


Fig. 1. Distribution of frequencies concerning the responder, partial responder, and non-responder children after the treatment. Data are expressed as percentage.

program and were excluded from the final evaluation. Ninety children completed the treatment and were evaluated at the final visit. Notably, 75 children (83.3%) no longer had headache attacks and were classified as responder; 10 children (11.1%) had a reduced number of episodes (9 of them also had a reduction of symptom severity) and were classified as partial responder (Fig. 1). On the other hand, 5 children were non-responders to the treatment. The prescribed treatment was safe and well tolerated in all children.

DISCUSSION

Headache is a very common disorder in childhood. The work-up of headache is well defined by the guidelines (3). In clinical practice, the main aim is to differentiate between primary and secondary headache. However, it may occur that a precise differential diagnosis cannot be performed because of lack of clear-cut symptoms, signs, and/or imaging findings. As pharmacologic treatment of primary headache is demanding and long-lasting, and side effects are not rare, an empiric approach may be attempted in patients with secondary headache. The current experience is based on an *ex juvantibus* pragmatic way to demonstrate that upper airways disorders may cause secondary headache in some cases.

The main outcome of the present study is that the vast majority (about 95%) of enrolled children significantly improved after an upper airways-oriented treatment. Interestingly, headache disappeared in more than 80% of the treated children.

Allergic, including asthma, and respiratory infection comorbidities were frequently associated in this group of patients, as recently pointed out (13). Indeed, according to different studies these diseases may coexist in 35-65% of affected children. In particular, a persistent Th2-driven airway inflammation, dominated by eosinophilic infiltrate, may be commonly involved in the same pathogenic mechanisms. In this regard, the link between allergy and headache has been investigated and there is agreement that an accurate diagnosis of headache and allergic diseases is critical in clinical practice (14). In the current experience, concomitant allergic diseases were however parsimoniously treated only on demand as many subjects had mild allergic complaints and other

children were symptomless at that time. This issue is well known and the term rhinogenic or sinonasal headache has been coined and commonly used (4, 5). However, no pathognomonic signs, symptoms, or imaging findings were detected in the subjects evaluated in this study. The paucity of clinical features had not discouraged however from suspecting upper airway disorders. Indeed, pharmacologic, dietetic, and behavioural approach was successful in these children.

PirometaximeTM is a new patent compound including 4% PCA and 0.002% CS. This compound exerts decongestant, osmo-protective, and restoring properties in the upper airway mucosa (7, 8). Copper sulphate exerts an ancestral anti-infective activity. Magnesium is an oligo-element that may exert a relevant role in the physiological function of the nervous system, and its defect is very frequent.

The present pharmacological strategy was important in this subset of children with secondary headache. However, a holistic approach was fundamental in the present experience as headache is very frequently caused by multiple, complex, and complicated triggers (3). Therefore, this empiric pragmatism may lessen some concepts: i) physical examination, specialist visits, imaging may be of little use in some patients; ii) history, on the contrary, may be very fruitful, mainly in inducing the suspect that family history, allergy and infection could be crucial triggers for secondary headache; iii) *ex juvantibus* approach may be tried in this setting.

Of course, the present study has some limitations, including the open design, the absence of a control group, the lack of objective parameters, the lack of biomarker assessment, and the absence of statistical associations. Thus, further studies should be designed and conducted to confirm these preliminary results. However, the obtained outcome was very impressive and underlines the relevance of considering the possible diagnosis of secondary headache, namely sinonasal, in subjects with subclinical feature suggesting upper airways involvement.

In conclusion, sinonasal disorders, including allergy and infections, may be relevant causal factors in children with secondary headache and their empirical treatment could be fruitful in clinical practice.

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LETTER TO THE EDITOR

SYSTEMIC NICKEL ALLERGY SYNDROME: TIPS AND TRICKS ON HOW TO BE SUSPECTED AND TREATED

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To the Editor,

Nickel is a metal among the most common contact allergens (1). Nickel allergic contact dermatitis (ACD) is not the exclusive phenotype of nickel sensitization as it is not only contained in metal objects but is also an essential nutrient in most foods. Nickel-induced clinical manifestations other than ACD include systemic contact dermatitis (SCD) and systemic nickel allergy syndrome (SNAS). SCD may occur in nickel sensitized individuals after ingesting nickel-containing foods, with a flare-up of earlier eczema lesions caused by previous exposure to the metal or in the form of pompholyx, vesicular hand eczema, baboon syndrome, papular macular exanthema, flexural eczema, itching, skin rash (2). SNAS is considered the association of gastrointestinal symptoms after ingestion of nickel-containing foods with cutaneous symptoms; gastrointestinal symptoms usually include bloating, diarrhea or constipation, vomiting, nausea, and abdominal pain (3).

Nickel-containing foods are mostly plant-derived foods. The nickel content of specific foods can vary widely, depending on the climate and season and the content in the soil or water. Foods recognized as particularly rich in nickel include peanuts, beans, lentils, peas, soybeans, oats, cocoa,

chocolate, nuts, whole wheat, pears and mushrooms (4). Gastrointestinal involvement in nickel allergic subjects has been reported by Di Gioacchino et al. (5) who described a marked inflammatory infiltrate of the duodenum mucosa in patients with nickel related ACD after an oral nickel challenge. Although there is no general concordance in literature on the efficacy of diet in nickel allergy, a low nickel specific diet called “BraMa-Ni” has been suggested for the work-up of the diagnosis of SNAS (6). Rizzi et al. studied the role of a low nickel diet in patients with irritable bowel syndrome (IBS) and nickel allergy, claiming that the almost complete avoidance of nickel sulfate with the adoption of a low nickel diet could explain the clinical benefits through a reduction of the pro-inflammatory state induced by nickel (7).

SNAS pathophysiology

SNAS pathophysiology involves both Th1 and Th2 cells; stimulation with nickel has been shown to induce specific patterns of cytokine secretion in lymphocyte cultures from metal-allergic patients, involving both Th1 and Th2-type cytokines (8).

Oxidative stress (OS) has also been reported to occur in patients who are diagnosed with SNAS as a consequence of the chronic inflammatory status

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Fig. 1. Cutaneous systemic reaction after Nickel oral provocation test with 1.25 mg of nickel sulfate.

provoked by systemic nickel sensitization. Signs of OS, such as higher serum levels of nitrosylated proteins (NPs), biomarkers of OS, were shown to be present in nickel-allergic patients affected by SNAS. These signs of OS are absent in patients with nickel ACD only (9).

Diagnostic work-up of SNAS: the nickel oral provocation test

SNAS can be suspected in subjects with Nickel ACD, positive to nickel patch-testing, with systemic cutaneous and gastrointestinal symptoms which improve after four weeks of low nickel diet (Table I). An Oral Nickel Provocation Test (OPT) is therefore conducted according to the following scheme: a dose of 1.25 mg of nickel sulfate (Lofarma S.p.A, Milan, Italy) is orally given on the first day in an open challenge fashion. After 24 h in the absence of symptoms another two single doses of 1.25 mg of nickel sulfate are given and the patient is evaluated the day after. The maximum cumulative dose of 3.75 mg of nickel sulphate was chosen on the basis of previous studies showing that this amount is approximately 10 times greater than the estimated normal daily dietary intake of nickel (10); usually SNAS patients react after a single dose of 1.25 mg.

The Nickel OPT is considered positive if cutaneous systemic symptoms occur with or without gastrointestinal symptoms. Typical cutaneous manifestations are flare-up of previous sites of contact dermatitis, flare-up reactions in previously positive nickel patch-test sites, itching or generalized erythema (Fig. 1). Typical gastrointestinal manifestations are bloating and diarrhea.

Nickel oral hyposensitizing treatment

Nickel desensitization can be proposed for the treatment of SNAS in patients with a compromised quality of life. Nickel oral hyposensitizing treatment (NiOHT) (Lofarma, Milan, Italy) consists in the administration of an up-dosing of nickel sulfate from 1 ng to 10, 100 and up to 500 ng. Oral nickel doses are taken three times per week and the maintenance dose of 500 ng is reached in 4 weeks and, in our experience, continued up to 2-3 years. A one-year double-blind, randomized, placebo-controlled trial evaluating the efficacy of NiOHT in patients with SNAS was carried out on patients from different Allergy centers in Italy (11). The study concluded that NiOHT is effective in SNAS, in particular, on gastrointestinal manifestations, but also on cutaneous symptoms, and allows patients with SNAS to safely

Table I. *Low nickel diet: food allowed and food not allowed.*

Food allowed		Food not allowed	
SPICE AND DRESSING	Paprika, pepper, olive oil, vinegar, mayonnaise, tomato sauce, salt.	VEGETABLES	Asparagus, broccoli, artichokes, carrots, cabbage, cauliflower, onions, green beans, peas, fennel, mushrooms, lettuce, tomatoes, rocket salad, celery, spinach, Brussels sprouts.
DRINKS	Frozen drinks, beer (not canned), coffee, wine.	FRUIT	Apricots, pineapples, watermelon, avocado, cherries, figs, berries, kiwi, pears, plums, grapes.
CEREALS	Plain flour, bread, pasta, pizza, rice.	HERBS AND DRESSING	Bay leaves, basil, parsley, ketchup, seed oil, mustard.
VEGETABLE AND FRUITS	Citrus fruits, bananas, apples, melons, peaches, cucumbers, aubergines, peppers, potatoes, garlic, pumpkins, courgettes.	CEREALS	Wholemeal products, buckwheat oat, barley, maize, malt, rye, soy.
FISH	Sword-fish, tuna.	SEAFOOD	Cod, shell-fish, salmon, octopus, squid, plaice.
SWEETS	Choux-pastry, biscuits, custard, honey, pastry, sponge cakes, sugar.	CHOCOLATE	All chocolate sweets.
OTHERS	Meat, eggs, salami, ham, dairy products.	NUTS	All of them
		DRINKS	Tea

re-introduce Ni-rich foods, improving their quality of life.

Tolerance to nickel has been reported to be associated with the increase of T regulatory cells. A two-year treatment with NiOHT induced a significant reduction of serum IL-2 levels while IL-10 levels increased (12). The interplaying balance between the two cytokines, IL-2 and IL-10, induced by oral tolerance to NiOHT, is supposed to have an important role in provoking anergy in T cells responsible for SNAS.

CONCLUSIONS

Although, the presence of nickel in foods is nowadays well known, the possibility of SNAS occurring is still greatly debated; the mechanism

inducing a systemic reaction only in a subset of Ni-ACD patients is yet to be understood. Information on how to clinically suspect SNAS and which is the correct diagnostic and treatment management is of great interest not only for allergists but also for dermatologists and internists as well as for nutritionists and dieticians.

NiOHT is the only current available treatment for SNAS which can restore the immunoregulatory mechanisms altered by nickel systemic sensitization.

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LETTER TO THE EDITOR

HLA-DRB1 HAPLOTYPE ASSOCIATES WITH SELECTION OF LIPID TRANSFER PROTEIN VARIANTS AS TARGETS OF FOOD ALLERGY

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To the Editor,

Lipid transfer proteins (LTP) constitute a protein family widely distributed throughout the plant kingdom, and play a role in plant defense for their antifungal and antibacterial activities (3).

Allergenic LTP has been identified in tree and weed pollens, vegetables, fruit and latex. LTPs are stable molecules, predominantly present in fruit peel, and some LTP-sensitized individuals can tolerate fruit without peel (1, 2).

Patients positive for LTP-specific IgE differ in the list and number of foods for which a specific response can be demonstrated. Thus, in view of a personalized management of LTP+ patients, it is of interest to understand whether a pattern for specific response exists, and whether (genetic) risk factors can be identified that predict or exclude, at the individual level, the possibility of developing IgE specific for a given food.

Previous studies examined the role of MHC class II genes (HLA-DR, -DQ and -DP families) in the context of food allergies. The environment-dictated exposure to allergens and the distribution of HLA

alleles together may explain the contradictory results reported regarding the association between HLA and allergy to foods.

In the present study, we examined the distribution of HLA-DRB1 alleles in a cohort of 96 patients suffering from food allergy, comparing 61 patients positive for IgE specific for LTP to 35 patients who were negative for IgE specific for LTP.

MATERIALS AND METHODS*Patients*

Sixty-one patients with history of adverse reactions after the ingestion of plant-derived foods who referred to the Allergy Unit of the Policlinico “A. Gemelli” in Rome and were diagnosed with allergy to lipid transfer protein (obtained through the tests described below) were enrolled in the study. The study protocol was approved by the Ethics Committee of the Università Cattolica del Sacro Cuore of Rome (17642/14). All subjects provided signed informed consent.

The group of patients positive for IgE specific for LTP (LTP+) were compared with a control group of patients

Key words: food allergy, genetics, HLA, lipid transfer protein, panallergens

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negative for IgE specific for LTP (LTP-) (35 subjects with at least one diagnosed allergy to allergens different from LTP: latex, profilin, milk, egg, codfish). A medical interview was performed to identify the foods involved in the reactions and the triggered symptoms.

According to Sampson et al. (6), symptoms were divided into five degrees of severity based on symptoms reported by patients.

Skin test

All the patients underwent skin prick test (SPT) with commercial extracts (Alk-Abellò, Milan, Italy) of allergens involved in the clinical history and with fresh fruit/vegetables (prick by prick method) referred in anamnesis. All SPT were performed according to the EAACI guidelines (4), wheal reactions equalling or exceeding a diameter of 3 mm after 15 minutes were considered positive. SPT with histamine (10 mg/ml) and saline solution were carried out as positive and negative control, respectively.

Serological tests

Quantification of specific IgE to the allergens positive in the skin tests was performed according to the manufacturer's instructions (UniCAP System; Pharmacia, Uppsala, Sweden). Only the samples with specific IgE concentrations of ≥ 0.35 KU/l were regarded as positive.

Genotyping

Genomic DNA was extracted from peripheral whole blood samples stored at -20°C until DNA extraction. The extraction process was performed with QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany): DNA was isolated from 200 μl of peripheral blood according to manufacturer's instructions and then eluted in 50 μl of ddH₂O. The DNA samples obtained were subsequently amplified by polymerase chain reaction and used for HLA typing. HLA typing was performed using a reverse line blot assay kit (INNO-LiPA HLA-DRB1 Plus Decoder; Innogenetics, Dartford, UK) at the level of allelic groups from DRB1*01 to DRB1*16, according to the manufacturer's instructions (7).

Statistical analysis

Statistical analysis was performed by using the Statistical Package for the Social Sciences (SPSS, Version 11.0, SPSS

Inc, Chicago, Illinois). For comparisons between the groups, the *chi*-squared test, MannWhitney U test and Student's *t*-test were used, as appropriate. To account for multiple comparisons, the significance of the *p* value for the χ^2 tests was adjusted using the Bonferroni method.

RESULTS

Patients

According to the grading of symptoms previously described, in the LTP+ group 19/61 patients referred symptoms classified as grade I, 21/61 grade II, 14/61 grade III and 6/61 grade IV. Only one patient reported a history of reactions attributable to grade V. Among patients of the LTP- group, 9/35 patients referred symptoms classified as grade I, 16/35 grade II, 8/35 grade III and 2/35 grade IV. No patient reported a history of reactions attributable to grade V.

Table I. Usage frequency of HLA-DRB1 alleles in LTP+ and LTP- groups and in the population of Central Italy (<http://www.allelefrequencies.net/>).

DRB1* Allele	LTP-	LTP+	Central Italy	p value
All patients (n=96)	n=35	n=61		
	Allele frequency (%)			
DRB1*01	8.11	9.09	7.40	ns
DRB1*03	6.76	2.48	6.10	ns
DRB1*04	8.11	11.57	8.20	ns
DRB1*07	24.32	12.40	15.80	0.04 #
DRB1*08	5.41	3.31	2.00	ns
DRB1*09	1.35	0.83	0.10	ns
DRB1*10	1.35	0.83	0.60	ns
DRB1*11	21.62	30.58	27.00	ns
DRB1*12	1.35	1.65	1.70	ns
DRB1*13	8.11	15.70	10.50	ns
DRB1*14	4.05	1.65	7.00	0.03 °
DRB1*15	8.11	6.61	6.30	ns
DRB1*16	1.35	3.31	2.20	ns

Significant correlation obtained comparing LTP+ vs LTP-. ° Significant correlation obtained comparing LTP+ vs Central Italy.

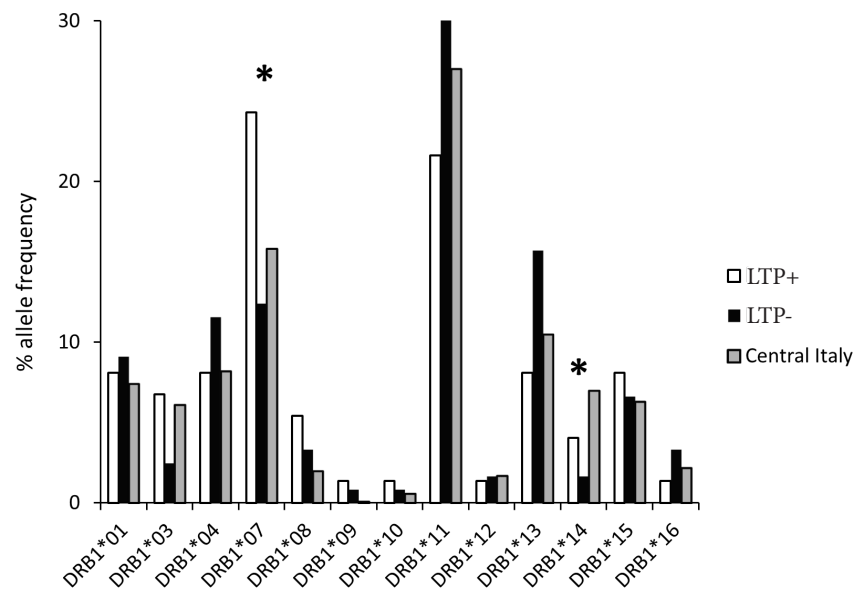


Fig. 1. Frequency of usage of HLA-DRB1 alleles. The histogram shows the different allele frequency of each HLA-DRB1 alleles for the group LTP+ (black bars), LTP- (white bars) and Central Italy (<http://www.allelefreqencies.net/>) (grey bars). Significant positive correlation was found between LTP + and LTP- groups for the DRB1*07 allele ($p=0.04$) and between LTP+ group and reference population for DRB1*14 allele ($p=0.03$).

Table II. Multivariate statistical analysis of the association of the HLA alleles with food in LTP+ (A) and LTP- (B) groups.

Allele	Food	% HLA-DRB1 negative	% HLA-DRB1 positive	P value
DRB1*01	hazelnut	37	80	0.017
DRB1*03	chestnut	0	33	0.049
	mango	0	33	0.049
	papaya	0	33	0.049
DRB1*04	yeast	2	21	0.035
	peach	83	50	0.029
DRB1*08	kiwi	16	75	0.022
DRB1*09	watermelon	0	100	0.016
DRB1*11	corn	42	13	0.021
DRB1*13	peanut	34	65	0.044
	cherry	5	24	0.046
	almond	5	24	0.046
	walnut	25	76	0.0001
	peach	68	94	0.047
DRB1*16	fennel	2	50	0.010
	celery	7	50	0.045

B

Allele	Food	% HLA-DRB1 negative	% HLA-DRB1 positive	P value
DRB1*14	corn	6	67	0.029

HLA-DRB1 alleles distribution between LTP+ and LTP- groups

A cohort of 96 patients suffering from food allergy was divided into LTP+ (61 patients) or LTP- (35 patients) according to positivity of SPT and RAST (when available) for LTP, as described in Materials and Methods.

We examined the distribution of HLA-DRB1 alleles between LTP+ and LTP- patients (Table I). Fig. 1 shows the frequency of usage of HLA-DRB1 alleles in LTP+ and LTP- allergic patients and the published frequency of each allele in the population of Central Italy (<http://www.allelefrequencies.net/>). The results indicate that the HLA-DRB1 haplotype had a limited effect of on the development of an allergic response to LTP, when considering LTP as a single allergenic target. We found that frequency of allele HLA-DRB1*14 was significantly ($p=0.03$) decreased in LTP+ patients in respect to LTP- patients who appeared to have the same frequency of the reference population. Allele HLA-DRB1*07 is significantly more frequent in the LTP-group compared to the LTP+ group ($p=0.04$) where it had the same frequency of the reference population.

In LTP+ patients, individual HLA-DRB1 alleles associate to response to individual foods

As a next step, we examined whether any association between response to individual foods and HLA-DRB1* alleles could be identified within the LTP+ group of patients, as described in Materials and Methods. As shown in Table II A, several associations were found, some of which were particularly strong, for instance, response to walnut and HLA-DRB1*13. In most cases, we observed a positive association, suggesting that, within the LTP+ population, a given HLA-DRB1* allele predisposed to sensitization to a restricted group of foods. However, also some negative associations resulted (in particular, HLA-DRB1*04 and peach, and HLA-DRB1*11 and corn) indicating that other HLA-DRB1* alleles may exert a protective role for the sensitization to LTP of some foods. When the same analysis was carried out in the LTP- group, only one association was found that reached significance, namely that of HLA-DRB1*14 with corn (Table II B).

It was also observed that, 15/16 HLA-DRB1*13+ LTP+ patients tested positive for serum IgE specific for

peach, and 12/16 for walnut; on the contrary, only one out of 4 HLA-DRB1*13+ LTP- patients was positive for peach and one other for walnut. Thus, the associations reported in Table II A appear to be specifically restricted to the population responsive to LTP.

Multivariate analysis for DRB1 alleles and foods showed a significant association for hazelnut and DRB1*01 (p 0.017) and for walnut and DRB1*13 (p 0.0001).

HLA-DRB1 alleles appear co-dominant in determining the association of individual food-specific IgE and HLA-DRB1* allele*

In a limited number of patients, the HLA-DRB1* haplotype comprised 2 alleles that were both associated with responses to individual foods (Tab II A and II B). The 2 larger subgroups were 6 patients having the HLA-DRB1*11, 13 haplotype and 4 patients having the HLA-DRB1* 04, 11 haplotype. Patients belonging to the former group were mostly negative for reactivity to wheat and corn (5/6, associated with HLA-DRB1*11) and positive for reactivity to peach (6/6, associated to HLA-DRB1*13). Patients belonging to the latter group were negative for reactivity to wheat and corn (4/4, associated with HLA-DRB1*11) and mostly negative to peach (3/4, associated to HLA-DRB1*04). This observation suggested that associations between HLA-DRB1* alleles and responsiveness to individual foods within the LTP+ group of patients were inherited independently and co-dominantly without evidence for interference between alleles.

*A 6-mer peptide is shared amongst LTPs of foods eliciting allergic responses patients carrying the DRB1*13 allele*

The association between the LTPs and HLA-DRB1* alleles as a phenomenon of co-dominance suggested that selection of distinct short peptides derived from the LTPs by HLA-DRB1* encoded is a central event in the determination of the pattern of foods to which an individual allergic to LTP will produce specific IgE. To address this hypothesis, we compared all the available sequences of LTPs from various foods. We found that there are eight

short sequences (6-12mer) that were shared among the various LTPs (Table III). Each LTP sequence enclosed from a minimum of one (pineapple, raspberry, walnut, barley, sunflower seeds, spinach and grape) to a maximum of seven (apricot and almond) of these motives. Intriguingly, LTPs from 4 out of 5 foods (cherry, almond, walnut and peach) that are associated to the HLA-DRB1*13 allele (Table II A), shared sequence PGKCGV.

It must be pointed out that not all HLA-DRB1*13⁺ patients respond to all LTPs sharing this sequence. Thus, we found no evidence of production of IgE specific for chestnut, bean and raspberry in HLA-DRB1*13⁺ patients. At the same time, while more than 50% of HLA-DRB1*13⁺ patients have IgE specific for apple, peach, peanut and walnut, only 13 to 25% have IgE specific for almond, apricot, cherry, lettuce, pear, plum and grape. Thus, in addition to

Table III. LTP aminoacidic sequences shared among the LTPs from various foods.

Food	s eq1* PGKCGVN	seq2* VNIPYKIS	seq3#* ACNCLK	seq4# PGKCGV	seq5#* IPYKIS	seq6#* NNAAALPGKCGV	seq 7# VPPACCNGI	seq 8 ACCSGV
apricot	●	●	●	●	●	●	●	
pineapple					●			
orange			●		●			
cabbage		●			●			●
carrot		●			●			
chestnut	●			●				
cherry	●		●	●		●	●	
bean	●	●		●	●			
wheat			●					●
raspberry				●				
lettuce	●			●	●			●
almond	●	●	●	●	●	●	●	
apple	●		●	●			●	
walnut				●				
barley								●
pear	●		●	●			●	
peach			●	●	●	●	●	
red pepper		●			●			
tomato		●			●			
plum	●		●	●		●	●	
rice	●		●					●
sunflower seed					●			
sesame		●			●			
spinach			●					
grape				●				

Black circles ● indicate the presence of the specific sequences in that food. #: seq3, seq4, seq5, seq6 and seq7 are predicted to bind to HLA-DRB1*04. *: seq1, seq2, seq3, seq5 and seq6 are predicted to bind to HLA-DRB1*13.

LTP sequence and HLA-DRB1 haplotype, other so far unknown factors play a role in determining at the individual level the allergic response to foods.

Finally, we used an online server (<http://crdd.osdd.net/raghava/mhc2pred/index.html>) to predict binding of HLA-DRB1 alleles to the shared LTP short sequences. The results obtained from the predictive analysis confirm specific bindings between HLA-DRB1, in particular DRB1*04 and DRB1*13, with some short sequences from LTPs as described in Table II. Interestingly, sequence 4, i.e. the one shared by all LTPs of foods associated with HLA DRB1*13 was not among those predicted to have high affinity for HLA DRB1*13. Nevertheless, we showed that sequence 6 enclosed the PGKCGV motif, suggesting that this “core” motif may need some flanking amino acid residues to be effectively complexed with HLA DRB1*13.

DISCUSSION

In the present report, we described the distribution of HLA-DRB1* alleles in a cohort of patients allergic to foods, and examined the relationship between HLA-DRB1* alleles and presence of IgE specific for LTP. We found a weak specific association between response to LTP and HLA-DRB1* alleles 07 and 14, as well as several associations between HLA-DRB1* alleles and food-specific IgE responses within the sub-cohort of LTP+ patients.

The observations reported here confirmed the difficulty of describing a relationship between HLA-DR haplotype and allergy to food allergens. We limited our study to the major allergen LTP. We previously reported that HLA-DR alleles can select a subset of epitopes even within a small allergen such as β -lactoglobulin (5).

The management of patients allergic to pan-allergens is made difficult by the overall poor predictability of the foods that will generate an adverse reaction in individual patients. Here, we described the association of HLA-DR alleles to presence of IgE specific for single foods, specifically within the LTP-responsive patients, and distinct from those associated with IgE specific for single food in the LTP- patients.

This observation suggested that associations between HLA-DRB1* alleles and responsiveness to individual foods within the LTP+ group of patients were inherited independently and co-dominantly without interference between alleles.

Our data therefore provide evidence that, once expanded to a larger population, HLA typing may prove helpful for the clinical counseling of patients suffering from allergy to pan-allergens.

In conclusion, propensity to develop allergy to food appears not related to HLA-DR haplotype; propensity to focus the immune response onto LTP is weakly associated to HLA-DR; finally, the propensity to select only a few of the various LTPs appears more strictly determined by HLA-DR haplotype.

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LETTER TO THE EDITOR

FAST RECOVERY BREAST AUGMENTATION: THE SMART MASTOPLASTY

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To the Editor,

Statistics reported by the American Society for Aesthetic Plastic Surgery show that breast augmentation in 2017, as for 2016, was the most performed procedure in aesthetic plastic surgery with 333,392 procedures being performed (1).

Year after year, several techniques with the purpose of breast augmentation with implants have been proposed, although no single technique has been recognized as a the gold standard (2-4).

The "perfect technique" in breast augmentation should be safe and with a short recovery time; it has already been shown in standardized surgical techniques that less is the time of surgery, faster is the recovery (5).

During the last 3 years the authors standardized, in their own private practice, the surgical procedure of breast augmentation with implants to get an optimal result and a really fast recovery after surgery.

Two hundred and eighteen (218) consecutive female patients looking for breast augmentation with implants were operated from 2015 to 2018; the present case series did not consider those patients operated for breast symmetrization with implants, mastopexy augmentation or any other breast surgery not requiring only implant based augmentation. Different breast implant brand were used (Nagor, Allergan, Polytech, Motiva and Mentor), with a round shape in approximately 60% of the cases and

an anatomical shape in the remaining 40%.

In all cases, the incision was placed at the new infra mammary fold (IMF) planned pre-operatively; the new IMF fold was identified by placing 50% of the implant under the nipple when the anatomical-shaped implants were used, and placing 55% of the implant under the nipple when round-shaped implants were used (Fig. 1).

The technique

After the incision at the new IMF, a suprafascial dissection is performed up to 1 cm above the "old" IMF, then the pectoralis major muscle is incised as for dual plane type I described by Tebbetts (complete division of pectoralis origins across the inframammary fold, stopping at the medial aspect of the inframammary fold) and a sub-muscular pocket is harvested with the index finger (3). The pocket harvested is a little bit undersized compared to the planned implant to be inserted. As soon as the pocket is harvested, accurate hemostasis is performed, and then an antibiotic solution (a vial of gentamicyn 80 mg/2 mL and cefazolin 1g/4 mL above 500 cc of saline) is used to wash it; similar pocket antibiotic irrigations have already been proposed in literature (6). At this point the surgeon changes his surgical gloves and new sterile surgical instruments are opened to insert the implants. Once the implant is introduced, the pocket is tailored with the index

Key words: breast augmentation; fast recovery; safety; breast implant

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finger of the surgeon, releasing muscle attachments if present. Finally, a 3-layer suture is performed. Drainage was never placed. Only macro and micro-textured implants were used; no differences were performed surgically regardless of which implant surface was used, nor were subjective differences noted by the surgeons during implant insertion.

In our experience, OR time has been 28 minutes on average, ranging from 22 and 41 minutes. Major

complications such as bleeding were never observed; in 5 cases (all mono lateral) wound dehiscence without implant exposure requiring further surgery has been recorded; also these 5 patients healed without complications after the revision surgery.

All the patients were discharged the same day of surgery, on average 6 hours after surgery, with antibiotics and painkiller oral therapy for 5 days post-operatively. The patients were instructed to wear

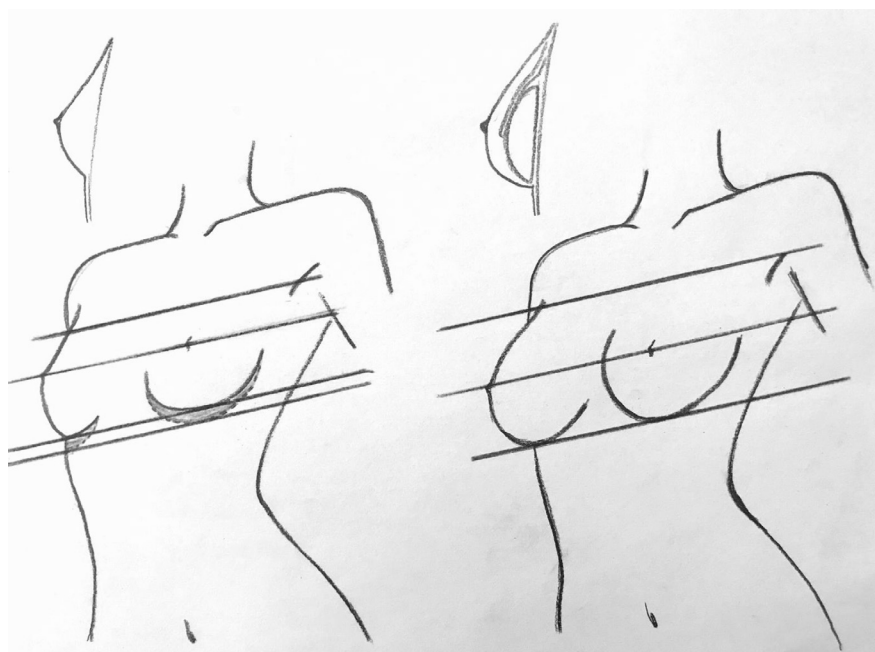


Fig. 1. A schematic view of the IMF lowering when an implant is inserted.



Fig. 2. A 36-year-old patient operated with the breast augmentation technique described: on the left side the pre operative picture, on the middle the 24h post op, on the right the 3-month follow-up.

a supportive bra in the first 60 days after surgery, and to avoid strenuous activity of the upper thorax and arms during the first 3 weeks. The patients were specifically told not to drive, not to use safety belt, to avoid sports involving the upper thorax, avoid housework, not to raise the arms above shoulders, and to generally avoid any strenuous activity.

All patients were followed-up for at least for 3 months, ranging 3-24 months (Fig. 2).

This is a large case series of breast augmentation with different implant brands performed with a standardized technique:

- placing the incision at the “new IMF”
- harvesting a dual plane type I submuscular pocket
- washing the pocket with antibiotic solution
- changing the gloves and using new retractors (not the same used to harvest the pocket) to insert the implant
- performing a 3-layer suture to close the wound
- never inserting drainages

The surgical technique performed by the authors is carried out mixing several issues already raised in the published literature, however the standardization of the surgical procedure, as shown by the results, reduces the OR time and, consequently, also the recovery time, this is why we called it “smart mastoplasty”. Another advantage of this technique is represented by the absolute “no touch” of the breast parenchyma during surgery thanks to the IMF approach and by the separation of the glandular itself from the implant due to the interposition of the whole pectoralis major muscle. However, the surgical approach proposed, as stated earlier, is suitable only for women looking for breast augmentation with implant, and is not suitable for other clinical situations such as breast asymmetries, in these cases

an inferior periareolar approach could be preferred to better control the different lengths of the IMF bilaterally.

In this “letter to the editor” we reported the preliminary results of our large case series that looks interesting in terms of reducing the post-operative recovery time after implant-based breast augmentation, although, to give a scientific meaning to the present report, large randomized blinded studies are required; only further investigations can determinate the effectiveness of this single center standardized technique.

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LETTER TO THE EDITOR

HYALURONIC ACID VERSUS HYALURONIC ACID AND DOLATROX®: A PROSPECTIVE, DOUBLE-BLIND RANDOMIZED CONTROLLED PILOT STUDY FOR THE TREATMENT OF KNEE OSTEOARTHRITIS

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To the Editor,

Osteoarthritis (OA) is the most common form of arthritis, affecting millions of people worldwide. It is characterized by destruction of articular cartilage and subchondral bone associated with synovial changes that cause pain and disability (1). Despite the availability of a wide spectrum of non-pharmacologic and pharmacologic options, chronic pain is not adequately controlled and leads to reduced activity and significantly diminished quality of life (2). It has been well-established that in clinical practice, the intra-articular use of hyaluronic acid (HA) represents one of the most common options in the management of patients affected by knee OA with persistent pain who do not respond to oral treatment, or who are not candidates for joint replacement (3). In randomized controlled studies conducted in the last years, oral chondroprotection with nutraceuticals and dietary supplements have proven efficient in modulating OA and an excellent safety profile in long-term use (4). Indeed, nutraceuticals could relieve some of OA symptoms such as pain and disability (4). For OA, glucosamine, chondroitin sulfate, methylsulfonylmethane (MSM), avocado/soybean unsaponifiables (ASU) and S-adenosyl methionine (SAME) are the most commonly used supplements (5-7). The objective of this first pilot study is to evaluate the efficacy and the safety of Dolatrox®, a

new nutraceutical product provided by Kolinpharma [Lainate (MI), Italy], containing MSM, ASU, SAME, vitamin C, and copper and manganese bisglycinate. In this study, we compared the efficacy and safety of intra-articular viscosupplementation (hyaluronic acid) and Dolatrox® to intraarticular hyaluronic acid and placebo for the treatment of knee OA using clinical outcome measures. Our outcome measures were the visual analog scale (VAS) for pain (0-100, with 100 denoting worst possible pain) and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) pain, stiffness and functional subscales.

MATERIALS AND METHODS

This is a prospective, randomized, double-blind, controlled clinical trial. This study was conducted with the highest respect for individual participants, the latest version of the Declaration of Helsinki and the Good Clinical Practices. Before the beginning of any study-related activities, each study participant signed an informed consent. The protocol, site-specific informed consent forms (local language and English versions), participant information leaflets, recruitment materials, other requested documents and any subsequent modifications were reviewed and approved by the Policy of Clinical Orthopedics, Ancona, Italy.

Key words: osteoarthritis, nutraceutical, hyaluronic acid, randomized controlled study

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Patient selection

Between 2017 and 2018, a total of 125 patients were screened for participation. All patients with a diagnosis of knee OA were screened. Of these, 40 patients did not meet the inclusion criteria and/or fulfilled an exclusion criterion. Twenty-five patients declined to participate or specifically requested one of the treatments. Inclusion criteria were: history of chronic (4 months) pain or knee swelling, limitation of daily activities, radiographic imaging findings of degenerative joint changes (Kellgren-Lawrence grade I-IV). Exclusion criteria were: use of intra-articular

corticosteroids or viscosupplementation from 3 months prior to the start of the study through the entire follow-up period, use of non-steroidal anti-inflammatory drugs or other nutraceutical supplements in the 3 weeks before treatment and during the follow-up period, osteonecrosis, systemic disorders such as diabetes, rheumatic diseases, severe cardiovascular diseases and infections.

Treatment and evaluation

Patients who met the inclusion criteria were randomized via an electronic randomization process into

Table I. Patient data and characteristics.

		Group A	Group B	p
N° of patients		24	30	
Mean ages (SD) year		56 (7.0)	54 (3.5)	0.1477
Female		18	15	0.611
Body mass index (BMI) (SD)		25.91 (4.58)	25.63 (4.80)	0.827
Kellgren-Lawrence (KL)	KL 1-2	16	21	0.793
	KL 3-4	8	9	

Table II. Mean VAS and WOMAC scores for group A and B at T0, T1, T2, T3.

		T ₀	T ₁	T ₂	T ₃
VAS	Group A	78±14 (54-100)	32±19 (0-87)	30±17 (0-77)	35±29 (0-91)
	Group B	76±15 (43-100)	41±25 (12-89)	40±24 (0-100)	54±30 (0-94)
WOMAC 1 (pain)	Group A	238±31 (175-300)	100±68 (50-270)	103±66 (50-270)	147±79 (10-280)
	Group B	246±33 (175-300)	149±69 (10-300)	150±68 (10-300)	180±81 (10-300)
WOMAC 2 (stiffness)	Group A	100±31 (50-150)	20±40 (0-120)	19±37 (0-130)	38±40 (0-130)
	Group B	107±32 (50-150)	39±34 (0-120)	47±39 (0-130)	66±52 (0-130)
WOMAC 3 (function)	Group A	673±162 (425-925)	265±210 (150-800)	278±206 (150-800)	402±256 (150-800)
	Group B	696±158 (425-925)	366±215 (150-800)	406±220 (150-800)	470±261 (150-900)

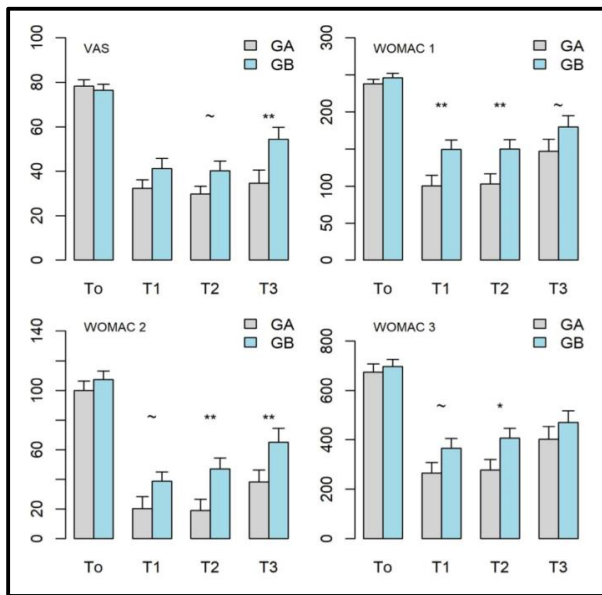


Fig. 1. Trend of the four response variables in the two experimental groups at times T0, T1, T2, T3. Symbols represent highly significant differences ($P < 0.01$, **), significant ($P < 0.05$, *), and at the limits of statistical significance ($P < 0.10$, ~).

2 groups: group A received intra-articular HA (Synovial forte® 1.6%, IBSA Farmaceutici Italia) and Dolatrox® (Kolinpharma, Lainate [MI], Italy) (5-g sachet); group B received intra-articular HA and placebo. Each 5 g sachet of Dolatrox® contains 2.5 g MSM, 150 mg ASU, 100

mg SAME, 250 mg vitamin C, and 0.9 mg copper and 5 mg manganese as bisglycinate. Each patient from both groups received 3 injections of HA at 14-day intervals (0-14-28 days) and no further injection during the follow-up period. The skin was sterilely dressed, and the infiltration was performed through a classic lateral approach with a 22-gauge needle under ultrasound guidance (7.5 MHz transducer). All patients received a series of instructions after each injection. In case of knee pain during the treatment cycle, cold therapy and rest were recommended for at least 24 hours. Mild activities and a gradual resumption of normal sport or recreational activities were allowed as tolerated.

Regarding the nutraceutical supplement, patients enrolled in group A received 2 single-dose sachets (5 g) of Dolatrox® per day (after lunch and after dinner) for the first month of follow-up; in the following 30 days, each patient received one single dose per day (after lunch). Patients enrolled in group B received placebo with the same schedule. All patients were prospectively evaluated at 0 (T0), 14 (T1), 30 (T2) and 60 (T3) days. Equal VAS (EQ VAS) scores and WOMAC pain (WOMAC 1), stiffness (WOMAC 2) and functional (WOMAC 3) subscales were used for clinical evaluation. Adverse events and patient satisfaction were also recorded. Non-clinical staff performed the randomization, clinical staff administered the injections, and the data collection and analyses were performed by the primary research team. Patients and the

Table III. Differences between Group A and Group B in relation to the 4 variables (VAS, WOMAC 1, WOMAC 2, WOMAC 3).

VARIABLE		t	p
VAS	T0	0.295	0.769
	T1	1.438	0.154
	T2	1.669	0.098
	T3	3.170	0.002
WOMAC 1 (pain)	T0	0.478	0.634
	T1	2.765	0.007
	T2	2.671	0.009
	T3	1.861	0.067
WOMAC 2 (stiffness)	T0	0.692	0.491
	T1	1.758	0.083
	T2	2.662	0.010
	T3	2.536	0.013
WOMAC 3 (function)	T0	0.389	0.698
	T1	1.715	0.091
	T2	2.182	0.032
	T3	1.158	0.251

primary research team performing analyses were blinded to assignments.

Statistical analysis

Preliminary analyses showed that variables were normally distributed, and no outliers were detected.

Differential responses of groups A and B to the experimental treatments were assessed by a linear mixed model including group (A vs B), time (T0 to T3) and their interaction as explanatory variables. We performed 4 independent analyses, one for each response variable (VAS, WOMAC 1, WOMAC 2 and WOMAC 3). Analyses were performed using the lme4 package in R ver. 3.2.4.

RESULTS

A total of 60 patients (37 women and 23 men) were enrolled. The participants' mean age was 55 (45–77) years. During the follow-up period, between 2017 and 2018, 6 (10%) patients were lost to follow-up or were unwilling to complete the study. The final study population consisted of 24 patients in group A and 30 patients in group B. There were no significant differences between the two groups in terms of age, sex, body mass index, Kellgren-Lawrence grade for OA or laterality. No severe adverse events were

recorded. Five patients (9%), 3 in Group A and 2 in Group B, complained of abdominal pain with nausea, but they continued the study. The patient data and characteristics are summarized in Table I.

The mean VAS and WOMAC scores for both groups at T0, T1, T2 and T3 are presented in Table II, with standard deviations (SD).

VAS

During the follow-up period, patients enrolled in group A had lower VAS scores at T1, T2 and T3 compared to group B (Fig. 1). No significant differences were found between the two groups at baseline, T1 and T2 ($P=0.77$, $P=0.15$ and $P=0.098$, respectively). In contrast, the differences at T3 were found to be statistically significant ($P=0.002$), as described in Table III. In group A, VAS decreased progressively from T0 to T1, T2 and T3, although the difference was found significant only from T0 to T1 ($P<0.001$; T1-T2: $P=0.54$ and T2-T3: $P=0.23$) (Table IV).

WOMAC 1

During the follow-up period, patients enrolled in group A had lower WOMAC 1 scores compared to group B at T0, T1, T2 and T3 (Fig. 1). No significant differences were found between the two groups at

Table IV. Comparison of the values of the response variables between the baseline T0 and T3 within both groups.

VARIABLE			P
VAS	GROUP A	T0-T3	<0.001
	GROUP B	T0-T3	<0.001
WOMAC 1 (pain)	GROUP A	T0-T3	<0.001
	GROUP B	T0-T3	<0.001
WOMAC 2 (stiffness)	GROUP A	T0-T3	<0.001
	GROUP B	T0-T3	<0.001
WOMAC 3 (Function)	GROUP A	T0-T3	<0.001
	GROUP B	T0-T3	<0.001

T0 and T3 ($P=0.63$ and $P=0.067$, respectively). However, differences at T1 and T2 were statistically significant, with P -values of 0.007 and 0.0009 (Table III). In both groups, WOMAC 1 decreased progressively to T2, although the decrease was statistically significant only from T0 to T1 ($P<0.001$). From T2 to T3, symptoms worsened in both groups, with the differences being statistically significant ($P<0.0001$) (Table IV).

WOMAC 2

During the follow-up period, patients enrolled in group A had lower WOMAC 2 scores compared to group B at T0, T1, T2 and T3 (Fig. 1). No significant differences were found between the 2 groups at T0 and T1 ($P=0.49$ and $P=0.083$, respectively). In contrast, the differences at T2 and T3 were found to be statistically significant ($P=0.010$ and $P=0.013$, respectively) as described in Table III. It should be noted that in both groups WOMAC 2 decreased progressively from T0 to T2 although the difference was found significant only from T0 to T1 ($P<0.001$) in each group. From T2 to T3, we observed a worsening of symptoms in both groups. The differences reached statistical significance ($P<0.001$) (Table IV).

WOMAC 3

During the follow-up period, patients enrolled in group A had lower WOMAC 3 scores compared to group B at T0, T1, T2 and T3 (Fig. 1). The differences between the 2 groups were not statistically significant at T0 ($P=0.70$), T1 ($P=0.091$), and T3 ($P=0.25$), in contrast to the difference at T2 ($P=0.032$) (Table III). In both groups, WOMAC3 scores decreased progressively from T0 to T1; however, only the difference from T0 to T1 was found to be statistically significant ($P<0.001$), in each group. A worsening of symptoms was observed from T2 to T3, with the differences reaching statistical significance in both groups ($P<0.05$) (Table IV).

DISCUSSION

To our knowledge, this is the first prospective randomized controlled trial to compare the efficacy and safety of intra-articular HA and Dolatrox®

with intra-articular HA plus placebo, considering subjective outcomes (WOMAC subscales and VAS). The most significant finding of this study was that Dolatrox® combined with intra-articular HA injection was effective in relieving pain and improving daily activities in the short-term. The combination was more efficient than intra-articular injection HA and placebo. For these reasons, in light of our results, we believe that the significant short-term (T1) clinical improvement in both groups was mainly due to HA. In addition, we reported that a lower pain and a better articular function were more durable in group A compared to B. Therefore, we hypothesize that Dolatrox® may be able to prolong and enhance the clinical effect of HA. Nowadays, we need to consider that a complete cycle of HA injections represents an important cost for the patients. Furthermore, the intra-articular administration can cause acute reactions such as arthralgia or infection (8). For these reasons, having a safe and efficacious oral supplement available for the patients that maintains the clinical effect of HA for a longer period might be very useful both in terms of cost and safety. Based on current knowledge in the field and our results, we believe that HA represents the most important and essential conservative treatment for OA, capable of reducing pain and restoring articular function (9), whereas the association of Dolatrox® and HA seems to be more effective in long-term use through the synergy of the nutraceutical and HA. To date, a growing amount of evidence supports the use of nutraceuticals and dietary supplements in the treatment of OA, especially for early OA, which might still be characterized by some regenerative ability of the articular cartilage. Indeed, pain reduction and improvement of joint function are the primary treatment goals. However, the slowing of the progression of OA represents an important secondary goal. Nutraceuticals consist of a large variety of products with a wide range of effects. They open new and broad horizons for the treatment of degenerative joint diseases. Many nutraceuticals and dietary supplements are claimed to provide pain relief in OA, but there is a need for more substantial data. However, these results have limitations. Firstly, the small cohort of patients and the very short-term follow up represent the main

limits of the research. Secondly, a wide range of OA severity was included in this study.

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LETTER TO THE EDITOR

PHYSICO-CHEMICAL CHARACTERIZATION OF A SMART THERMO-RESPONSIVE FLUORIDE-RELEASING POLOXAMER-BASED GEL

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To the Editor,

The effective role of fluoride in the prevention of dental demineralization has been widely documented over the last 80 years (1, 2). Many studies report substantial reductions in dental caries in developed countries after the use of both systemic and topical fluoride applications became widespread (3-5). In 2011, the Scientific Committee on Health and Environmental Risks (SCHER) stated that water fluoridation, as well as topical fluoride applications appear to prevent caries, primarily on permanent dentition.

Systemic fluoride intake during the pre-eruptive phase appears to give remarkable benefits on dental tissue mineralization, preventing decay in dental grooves and interproximal areas. Topical fluoride application during the tooth eruption period also appears to have good results (2). Fluoride remains in the oral cavity after topical application, in the form of deposits of calcium-fluoride complexes covered by a protein layer. These deposits are detectable in the saliva, on tooth surfaces, and also in bacterial biofilms. When the oral pH decreases, the slow release of fluoride from protein-covered deposits begins. Augmented fluoride concentration allows the

re-mineralization of tooth surfaces, preventing caries.

For the above reasons, in the last 20 years, the topical use of fluorides has become more popular than systemic use (4). The paper presents an innovative thermo-responsive fluoride-releasing dental material, based on a smart poloxamer, capable of releasing fluoride when applied to the teeth; it is “quasi-solid” at the temperature of the oral cavity, but “semi-liquid” at lower temperatures typical of washing steps.

Due to their temperature-dependent physical state, poloxamers doped with fluoride might be an interesting product for delivering topical fluoride.

MATERIALS AND METHODS

Three different fluoride-releasing gel materials were studied: a poloxamer-based gel (P1) containing sodium monofluorophosphate (Na₂PFO₃), a poloxamer-based gel (P2) containing sodium fluoride (NaF), and a commercial gel (C, Neutral Fluorine, Dental Medical) for comparison purposes.

Gel preparation and different formulations

The gels were prepared by mixing the Poloxamer 407 (Poly(ethylene glycol)-block-poly(propylene glycol))-

Key words: thermo-responsive material; poloxamers; pluronic; fluoride-releasing dental material

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block-poly(ethylene glycol); reference standard according to the European Pharmacopeia and the National Formulary of the United States Pharmacopeia - USP/NF) with water at different weight percentages of the organic fraction (see Table I, first column). After preliminary physico-chemical characterization (see below), the formulation containing 22% of organic fraction (P), which presented the proper balance between high viscosity and good solubility of the doping agents, was chosen for the subsequent doping studies.

The doping was carried out on two aliquots of P formulation, obtaining the two fluoridating devices:

- Σ P1: 1.14% in weight of sodium monofluorophosphate (Na_2PFO_3), corresponding to 0.15% of NaF;
- Σ P2: 0.15% in weight of sodium fluoride (NaF, Fluka, $\geq 99.0\%$).

The prepared gels were kept in a refrigerator, prior to being used in the fluoridating tests.

Gel formulation characterization

The different gel formulations (before doping) were characterized by rheological measurements, to study their viscous behavior in function of the temperature. The measurements were carried out using a Rotational Rheometer Physica MCR300, equipped with 25 mm parallel plates. Complex viscosity was measured from 5°C to 45°C using a plate gap of 1 mm, constant strain set at 5%, and a constant frequency of 10Hz.

In order to evaluate the temperature at which the different polymeric formulations presented the pseudo-transition phase (from liquid to “semi-solid”, gel), the magnetic stirrer method was adopted. The formulation in a 100 ml beaker containing a magnetic bar and a thermometer was placed on a magnetic stirrer. The temperature was raised until the magnetic bar instantaneously stopped moving, indicating that the “semi-solid, gel” state had been achieved. The values obtained with this method were compared with those obtained by rheological measurements.

Fluoride release measurements in water

In this study, the measurements were performed by direct potentiometry under constant agitation, on an Amel

338 potentiometer, using a Fluoride Ion Selective Electrode (F-ISE, Amel, Italy) with a single crystal of LaF_3 doped with EuF_2 , as solid-state membrane, and a saturated calomel as reference electrode. The working solution comprised 20 ml TISAB IV (Total Ionic Strength Adjustment Buffer) in 80 ml of deionized water (Milli-Q, Millipore). TISAB IV consisted of sodium tartrate dihydrate (Sigma Aldrich, Primary Standard, 23 g), TRIS (Fluka, SRM, 24.2 g) and hydrochloric acid (Fluka, 37%, 8.4 ml) in 100 ml deionized water. The ISE calibration was run using a 0.005 M sodium fluoride (Fluka, $\geq 99.0\%$) standard solution, obtaining an experimental slope of 54.9 mV.

The release of fluoride over time from different gel materials was monitored by placing a small container filled with gel directly in the working solution at 37°C, under continuous stirring. The container had a volume of 1.65 ml and the surface of gel exposed to the solution was 50.24 mm².

Ex-vivo fluoride release measurements

Teeth (T1 and T2) were obtained from two young female patients (22 and 20 years old) who, for orthodontic treatment, required the surgical removal of a third molar under local anesthesia. Both subjects gave informed, signed consent to participate in the study. The teeth presented integrity of the enamel surfaces, and absence of traumatic injuries, cavities, and erosion of the enamel. Access to the third molar was from the buccal aspect, and bone removal, where required, was achieved with a round bur under continuous irrigation, preserving tooth integrity.

After rinsing with deionized water and a toothbrush, T1 and T2 were each divided with diamond bur under continuous irrigation into three fragments: T1F1 (0.4288 g), T1F2 (0.2567 g) and T1F3 (0.2331 g); T2F1 (0.2515 g), T2F2 (0.3740 g) and T2F3 (0.1937 g).

Analysis and fluoridating treatment of tooth T1

Fragments T1F1 and T1F2 were characterized by XPS analysis, to determine the surface quantity of fluoride (and other elements) before and after the fluoridating treatments. After a preliminary analysis, the two fragments were immersed for 15 min in two different gels: P1, the new poloxamer-based gel doped with sodium monofluorophosphate (Na_2PFO_3), and C, the commercial gel by Neutral Fluorine, Dental Medical. After treatment, the two fragments were washed with deionized water and

a toothbrush and dried by rapid immersion in acetone.

The three fragments, treated (T1F1 and T1F2) and untreated (T1F3), were then dissolved by immersion in a stirred solution of 5 ml concentrated perchloric acid (60%) for 24 h, in a 25-ml polyethylene flask. After complete dissolution, the flasks were filled with deionized water. One ml of each sample was added to a solution containing 5 ml of TISAB IV and 19 ml of deionized water and the resulting solution was analyzed by potentiometric method, with the standard addition technique, using a 0.005 M sodium fluoride standard solution.

Analysis and fluoridating treatment of tooth T2

All the fragments were characterized by XPS analysis before and after treatment, to determine the surface quantity of fluoride (and other elements) and subsequently dissolved in perchloric acid, for potentiometric analysis as described above.

Fragment T2F1 was untreated; fragment T2F2 was immersed for 15 minutes in the fluoridating gel P2, the new poloxamer-based gel doped with sodium fluoride (NaF); fragment T2F3 was immersed for 15 min in the fluoridating gel P2, rinsed with deionized water and then again treated by immersion for a further 15 min in the P2 gel. After treatment, the fragments were washed with deionized water and a toothbrush, and dried by rapid immersion in acetone.

XPS analysis

XPS measurements were performed in an M-Probe Instrument (SSI) equipped with a monochromatic Al K α source (1486.6 eV) with a spot size of 200 $\times$ 750 μ m and a pass energy of 25 eV, providing a resolution for 0.74 eV.

The energy scale was calibrated with reference to the 4f7/2 level of a freshly evaporated gold sample, at 84.00 $\pm$ 0.1 eV, and with reference to the 2p3/2 and 3s levels of copper, at 932.47 $\pm$ 0.1 and 122.39 $\pm$ 0.15 eV, respectively.

With a monochromatic source, an electron flood gun was used to compensate the buildup of positive charge on the insulator samples during the analyses: a value of 10 eV was selected for measurements on these samples. For all samples, the C1s peak level was taken as internal reference at 284.6 eV. The accuracy of the reported binding energies (BE) may be estimated as $\pm$ 0.2 eV.

The quantitative data were also carefully checked, and were reproduced several times; the percentage error is estimated at $\pm$ 1%

RESULTS

Physico-chemical characterization

All gels show sigmoidal thermoresponsive behavior, characterized by minimum viscosity (the formulation behaves as a liquid) at temperatures below 18°C, and maximum viscosity (the formulation behaves as a gel) for temperatures above 33°C. Repeated heating and cooling of the formulations does not affect the shape of the curves. The typical sigmoidal shape of the rheological curves enables a “phase pseudo-transition” (gelation point, T_{PT}) temperature to be established, by determining the position of the maximum of the first derivative curve.

The gelation point temperatures were also determined using the new empirical method described above; the two methods gave similar results (Table I). They indicated that the linear dependence of T_{PT} decreases as the percentage of organic fraction in the gel increases (*i.e.* with decreasing percentages of water).

In particular, all the test formulations presented maximum viscosity values at the temperature of the oral cavity (37°C), allowing them to be used as smart fluoridating matrixes in dental practice. Among the different compositions studied, the second part of the research examined the formulation containing 22% of organic fraction, which presented the optimal balance between high viscosity values and good solubility of the doping active agents.

Fluoride release in water

The fluoride release over time in water from the P2 gel, which contains NaF as source of fluoride ions, was characterized by an activation period (7 min), after which the gel began to release fluoride into the solution (Fig. 1). After approximately 11 minutes, the fluoride concentration reached a plateau, followed by a second slow growth period. A period of 15 minutes thus appeared an appropriate exposure time in dental treatment.

P1 gel release was one order of magnitude

lower than that of P2, indicating the poor fluoride dispensing capability of this material, containing Na_2PFO_3 as fluoride source. Indeed, Na_2PFO_3 is usually added to toothpastes as long-term fluoride-releasing matter, whereas for the present purpose it is of no use. It was not possible to study the release in water from the commercial gel, as it dissolves in aqueous matrixes.

Ex-vivo fluoride release

The ex-vivo experiments of fluoride release from the gel, and fluoride intake by the tooth, were studied by an ad-hoc protocol that took into account the initial results of this study. The procedure was described in detail above. The results of ISE electrochemical analysis and XPS measurements are summarized in Table II. The gel containing sodium monofluorophosphate had a very slight fluoridating effect, confirming the results of release in water. Of the gels containing NaF, the new polymer (P2) doubled the fluoride concentration in the tooth. Moreover, the fluoride released by the P2 gel easily entered inside the tooth, as confirmed by the low percentage of surface fluoride measured by XPS. On C2 gel treatment, the result was the opposite. Lastly,

repetition of the treatment (with P2 gel) further increased the fluoride content

DISCUSSION

The poloxamer family comprises more than 30 non-ionic, amphiphilic ABA-type block copolymers, in which A is poly(ethylene glycol) (PEG) and B is poly(propylene glycol) (PPG). Their physical state (liquid, paste, solid) is governed by their molecular weight and block ratio. Poloxamers are well tolerated (non-toxic), although at high concentrations some side-effects, including hypercholesterolemia and hypertriglyceridemia, suggest that the polymer concentration should be kept to a minimum (6-10). Poloxamer 407 (Pluronic® F127) has a lower critical solubility temperature (LCST) at biologically-relevant temperatures (25°C at 20% wt.), a feature making it the most popular candidate of the series for biomedical applications. Drug loading is readily achieved by simple mixing (9). Polymeric gels capable of absorbing and releasing cations and anions for different purposes are widely used, and have been studied with electroanalytical techniques. (11) These gels are “solid” and adhere to the tooth surface at

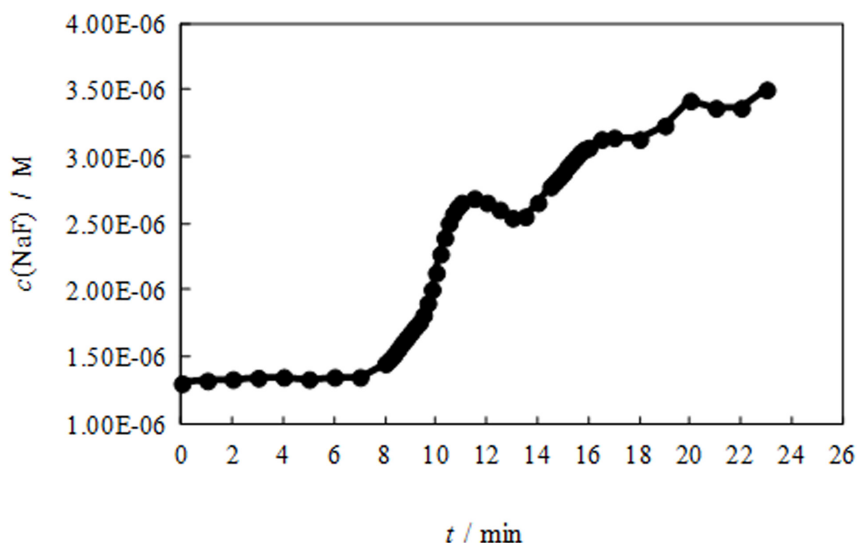


Fig. 1. Release of fluoride ions in water from P2 gel, containing NaF as source of fluoride ions.

Table I. Phase transition temperature at different percentages of organic fraction in the polymeric gel material, determined by magnetic stirrer method and by rheological measurements.

Organic fraction %	Phase Transition Temperature / °C	
	Magnetic stirrer	Rheology
16	32.1	31.8
17	28.7	
18	27.6	26.0
19	25.0	
20	23.9	22.8
21	22.7	
22	22.1	21.8
23	20.0	
24	19.4	19.3
25	17.7	18.7

Table II. Amount of fluoride (and other elements) determined in different samples by potentiometric method (ISE) and XPS measurements.

SAMPLES	ISE	XPS					
	[F ⁻] /ppm	% F	% Ca	% C	% N	% O	% Others
T1F1 untreated	< LOD	< LOD	5.3	57.8	8.6	24.7	3.6
T1F2 untreated	< LOD	< LOD	6.5	54.7	7.4	26.0	5.4
T1F1 treated with P1	22	< LOD	7.3	50.7	8.0	27.5	6.5
T1F2 treated with C	182	12.3	13.5	37.0	4.1	26.9	6.2
T2F1 untreated	< LOD	< LOD	1.0	60.3	15.9	22.8	0
T2F2 treated once with P2	353	0.7	4.3	53.7	6.0	28.1	7.2
T2F3 treated twice with P2	625	0.4	2.0	67.6	7.9	22.1	0

37°C, thus ensuring perfect and continuous contact between gel and the oral part to be treated, and avoiding accidental ingestion. Furthermore, the polymeric materials can be rapidly removed by simply rinsing with cold water during mechanical oral suctioning. (12) The strong fluoridating capability of a smart thermo-responsive material has been demonstrated, through analytical measurements performed on ex-vivo tooth samples. The biocompatible polymeric gel presented here can not only increase the fluoride content on the surface and within the treated tooth, but also presents important features able to facilitate dental practice and enhance efficiency. The thermo-responsive behavior offers the dental practitioner a material that is easily manipulated outside the oral cavity (when it is in its liquid form), and that sticks to the tooth surface at the oral cavity temperature, as the fluoridating process takes place, avoiding the risk of dispersion and ingestion of the active material.

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