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Aspirin Combined with Curcumin on Lgr5 Signaling Pathway Model of Colorectal Cancer through Warburg Effect by TAp63α Ubiquitination

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Background: The changing lifestyles and dietary patterns in recent years have led to an increased incidence rate of colorectal cancer, posing a significant threat to the well-being and safety of patients. Therefore, the objective of this study was to investigate the therapeutic efficacy of the combination treatment of Aspirin and curcumin in a colorectal cancer model. The aim is to provide insights into potential approaches for managing this disease.

Methods: All mice were randomly assigned to different groups, including sham, dextran sulphate sodium (DSS) + azoxymethane (AOM), Aspirin, Curcumin, and Union group, with six mice in each group. *In vitro* experiments utilized the human colon adenocarcinoma cell lines (HCT-116) as a model. For HCT-116 cells, TAp63alpha (TAp63α), si-TAp63α, negative control, or si-negative were transfected using Lipofectamine 2000, with three replicates per group. Quantitative polymerase chain reaction (qPCR) was performed to assess the expression levels of Lrg5 and TAp63α after treatment with Aspirin combined with curcumin, both *in vivo* and *in vitro*. The Cell Counting Kit-8 (CCK8) assay and 5-ethynyl-2′-deoxyuridine (EDU) staining were used to evaluate the proliferation of colorectal cancer cells following combination treatment.

Results: The combination treatment of Aspirin and curcumin resulted in a significant reduction in tumor numbers and tumor size in the colorectal cancer mouse model ($p < 0.05$). Moreover, this combination treatment led to reduced mRNA expressions of interleukin-6 (*IL-6*), *IL-17*α, and Prostaglandin-Endoperoxide Synthase 2 (*Ptgs2*) in the tumor tissue of the mouse model ($p < 0.05$). In HCT-116 cells, the combination treatment of Aspirin and curcumin demonstrated inhibitory effects on cell proliferation and migration rates. It also reduced the rate of EDU-positive cells and increased Caspase-3/9 activity levels ($p < 0.05$). Additionally, this combination treatment resulted in decreased glucose consumption, lactate production, and adenosine triphosphate (ATP) quantity in HCT-116 cells ($p < 0.05$). Furthermore, it reduced extracellular acidification rate and increased oxygen consumption relative to basal levels (OCR, oxygen consumption rate) in HCT-116 cells ($p < 0.05$). The combination treatment of Aspirin and curcumin suppressed the mRNA expressions of *TAp63*α and *Lgr5* in both the mouse model and *in vitro* models ($p < 0.05$). It also downregulated the protein expressions of TAp63α, Lgr5, and p-β-catenin in both models ($p < 0.05$). Notably, Aspirin had no effect on TAp63α ubiquitination, while curcumin promoted TAp63α ubiquitination in HCT-116 cells. Furthermore, compared to curcumin alone, the combination treatment of Aspirin and curcumin further enhanced TAp63α ubiquitination in HCT-116 cells ($p < 0.05$). The regulation of TAp63α played a crucial role in mediating the effects of Aspirin combined with curcumin on colorectal cancer cell growth and the progression of the Warburg effect.

Conclusions: The combination treatment of Aspirin and curcumin effectively suppresses the proliferation and migration of colorectal cancer cells both *in vitro* and *in vivo* by targeting the Warburg effect. This modulation of the Warburg effect is mediated through the TAp63a/Lgr5 signaling pathway. These findings highlight the potential therapeutic value of Aspirin combined with curcumin for the treatment of colorectal cancer.

Keywords: aspirin; curcumin; colorectal cancer; TAp63α; Lgr5

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Introduction

As colorectal cancer is a frequently occurring malignant tumor, its incidence has been observed to rise steadily over the years [1]. The development of colorectal cancer follows a progressive sequence from normal intestinal gland epithelium to adenoma and eventually to adenocarcinoma [2]. For patients diagnosed with stage I~III colon cancer, radical surgery remains the primary clinical treatment option. By surgically removing the affected tissue, the survival time of patients can be significantly extended. Nonetheless, the substantial rates of post-operative metastasis and cancer cell recurrence are significant contributors to mortality [3–5].

Like other family members, Lgr5 possesses seven transmembrane domains that are involved in cell membrane transport [6]. Since the 21st century, Lgr5 has been extensively studied as a mature intestinal stem cell marker. In normal tissues, Lgr5 is predominantly expressed in actively proliferating intestinal stem cells (ISCs) located at the base of the crypt. These ISCs can differentiate into all types of intestinal cells and can be transformed along with static ISCs [7]. Deletion of the *Lgr5* gene leads to crypt damage and dysplasia of the intestinal mucosa, while overexpression of Lgr5 causes excessive proliferation of crypts ultimately resulting in tumor formation [8]. It has been observed that overexpression of Lgr5 can induce the formation of a structure called "cell filament" on the cell membrane. This structure enables the independent transmission of signals in a concentration gradient-dependent manner, and it is associated with myosin. Although myosin serves as a marker for tumor invasion, its movement along the "cell filament" does not fully elucidate the underlying mechanism by which Lgr5 promotes tumor formation. However, it is widely accepted that Lgr5 promotes excessive proliferation of intestinal crypts through the Wnt/β-Catenin signaling pathway, thereby contributing to tumor development [9]. Lgr5 belongs to the class of seven-fold alpha-helix transmembrane glycoprotein hormone receptors and is classified as a member of the rhodopsin subfamily of GPR49 [10]. Its distribution spans various human tissues, including the esophagus, gastrointestinal tract, breast tissue, ovaries, eyes, and brain [11].

Aspirin has been shown to potentially reduce the production of PGE2 by inhibiting the activity of COX-1 and COX-2. Additionally, it can decrease the expression of VEGF and epidermal growth factor receptors, thereby inhibiting tumor angiogenesis. Aspirin also can suppress NF-κB activity and the Wnt/β-Catenin signaling pathway, while promoting the transcription of apoptosis-related genes. Furthermore, it has been observed that aspirin can regulate the structure of the intestinal flora [12]. These multifaceted effects of aspirin have led to its investigation as adjuvant chemotherapy for colon cancer, making it a prominent research focus in recent years [12]. Curcumin, derived from turmeric, is a potent compound with various beneficial properties [13]. It exhibits anti-inflammatory, lipid-lowering, antibacterial, antioxidant, and radical-scavenging effects [14,15]. Notably, curcumin has been found to possess significant anti-oral cancer properties by inducing cell apoptosis, inhibiting cell proliferation, reducing tumor cell invasion, promoting cell autophagy, and exerting antioxidative, anti-inflammatory, and anti-oral fibrosis effects. Furthermore, curcumin has been shown to enhance the efficacy of radiotherapy and chemotherapy in oral cancer treatment [13]. In the context of colorectal cancer, curcumin demonstrates promising preventive and therapeutic effects through various mechanisms. However, the specific underlying mechanisms are still not fully understood and require further investigation. The potential of Aspirin in combination with curcumin for preventing colorectal cancer remains uncertain. In this study, we aim to investigate the anticancer effects of this combination using an *in vitro* model. Specifically, we will assess the impact on Warburg effect progression, conduct a proliferation assay, and perform 5-ethynyl-2′-deoxyuridine (EDU) staining. The primary objective of this research is to explore the therapeutic efficacy of Aspirin combined with curcumin in a colorectal cancer model.

Materials & Methods

Vivo Model of Colon Cancer Cancer

All mice were randomly assigned to different groups, including sham, dextran sulphate sodium (DSS) + azoxymethane (AOM), Aspirin, Curcumin, and Union group, with six mice in each group. The mice in the Aspirin group received a dose of 30 mg/kg/day (n = 6) [16], while those in the Curcumin group received a dose of 100 mg/kg/day (n = 6) [17]. The Union group received both Aspirin (30 mg/kg/day, n = 6) and Curcumin (100 mg/kg/day, n = 6). The sham group consisted of six mice that were injected with normal saline. Ethical approval for the study was obtained from the Animal Care and Use Committee of Guiyang Public Health Clinical Center (Approval No. 2020061317852). The DSS + AOM-induced colon cancer model was established based on the reference [18]. The mice were initially injected with 5 mg/kg of AOM (A5486, Sigma-Aldrich LLC., Shanghai, China). Then, they were exposed to 3.0% DSS (CAT NO: 160110, MP, Santa Ana, CA, USA) in their drinking water for 7 days, followed by a period of normal water for 14 days. This cycle was repeated three times. The mice were weighed weekly and were sacrificed on day 56 using 50 mg/kg pentobarbital sodium for anesthesia. Colon tissue samples were collected for hematoxylin and eosin (HE) staining to confirm the successful construction of the model. Additionally, the colon tissue was dissected open, and the intestinal cavity was exposed. If a noticeable tumor was present in the intestinal cavity, it indicated a successful tumor construction. As-

pirin (BP617) and curcumin (C1386) were purchased from Sigma-Aldrich LLC. (Shanghai, China). Before cell culturing, mycoplasma testing was conducted to ensure the sterility of the cell culture. The laboratory maintained a strict sanitary environment through regular cleaning and disinfection of laboratory equipment and worktops. Sterile techniques and reagents were used during cell cultivation to prevent contamination. Regular testing of cell cultures for sterility was performed, and any mycoplasma infections detected were promptly addressed. In cases of mycoplasma infection, infected cells were immediately isolated, the infected culture was disposed of, and thorough cleaning and disinfection measures were carried out. Mice were anesthetized with 50 mg/kg pentobarbital sodium.

Cell Culture and Transfection

Human colon adenocarcinoma cell lines (HCT-116, SCSP-5076, National Model and Characteristic Experimental Cell Resource Library) were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (10099141C, FCS, Gibco, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO_2 at 37 °C. All cell lines were identified by STR identification and no mycoplasma contamination was found in routine mycoplasma detection. TAp63alpha (*TAp63*α) (5′-CCGTTTCGTCAGAACACACAT-3′ and 5′-GAGTGGAATGACTTCAACTTT-3′), si-*TAp63*α (sc-37477, Santa Cruz Biotechnology, Inc., Shanghai, China), negative control, or si-negative were transfected into HCT-116 using Lipofectamine 2000 (ThermoFisher Scientific, Shanghai, China). For the Cell Counting Kit-8 (CCK8) experiment, 3000 nM of Aspirin [16], 30 μM of curcumin [17], or a combination of 3000 nM of Aspirin and 30 μM of curcumin were administered at 48 h, while for another experiment, the same treatments were given at 24 h.

Quantitative Polymerase Chain Reaction (qPCR)

Total RNAs were extracted using the RNA isolator total RNA extraction reagent (9769S, Takara, Dalian, China) and cDNA was synthesized using the PrimeScript RT Master Mix (RR047Q, Takara, Dalian, China). Subsequently, qPCR was conducted with the ABI Prism 7500 sequence detection system using the following thermocycling parameters: 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 30 seconds. The relative levels of mRNA expression in the samples were calculated and expressed as $2^{-\Delta\Delta Ct}$. The primer sequences used were as follows: primer sequences used were as follows: expression in the sample: *Lgr5*: 5′-GAGTTACG TCTTGCGGGAAAC-3′ and, 5′-TGGGTACGTGTCTT AGCTGATTA-3′; *TAp63*α: 5′-GTGTGCCACCCTACA GTACT-3′ and 5′-GCAGCTCATCATCTGGGGAT-3′; interleukin-6 (*IL-6*): 5’-ACAACCACGGCCTTCCCTAC-3’ and 5’-CATTTCCACGATTTCCCAGA-3’; *IL-17*α: 5′-GAAGGCCCTCAGACTACCTC-3′ and 5′-CAGCATCT TCTCGACCCTGA-3′; Prostaglandin-Endoperoxide Synthase 2 (*Ptgs2*): 5′-TGAGTACCGCAAACGCTTCTC-3′ and 5′-TGGACGAGGTTTTTCCACCAG-3′; *GAPDH*: 5 ′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-C ATGTAGGCCATGAGGTCCACCAC-3′.

Microarray Analysis

Total RNA was extracted from serum samples (n = 3 per group), and the RNA quantity was determined using the NanoDrop 1000 (Hs00997627, ThermoFisher Scientific Inc., Shanghai, China). Subsequently, the total RNA from each sample was subjected to reverse transcription using the Invitrogen SuperScript double-stranded cDNA synthesis kit (D7168S, Beyotime, Shanghai, China). The resulting double-stranded cDNA was labeled using the NimbleGen one-color DNA labeling kit (Hs0329729, ThermoFisher Scientific Inc., Shanghai, China) and then used for array hybridization with the NimbleGen hybridization system and washed using the NimbleGen wash buffer kit (Dm01823793, ThermoFisher Scientific Inc., Shanghai, China). The Axon GenePix 4000B microarray scanner (20455, Molecular Devices, Shanghai, China) was employed for scanning.

Warburg Effect Progression

The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using the Seahorse XFe96 analyzer and Seahorse XF Pro for the determination of OCR (Seahorse Bioscience, Agilent, Beijing, China). Glucose levels were determined using a glucose assay kit (GAGO20, Sigma, St-Louis, MO, USA). The progression of the Warburg effect was analyzed based on Reference [19].

Proliferation Assay and EDU Staining

After 48 hours of transfection, approximately 2×10^3 cells/well were seeded in a 96-well plate for the Cell Counting Kit-8 (CCK8, C0037, Beyotime, Shanghai, China). For the 5-ethynyl-2′-deoxyuridine (EDU, ST067, Beyotime, Shanghai, China) incorporation assay, 10 mM EDU was added to each well and the cells were fixed with 4% formaldehyde for 30 minutes.

Transwell Invasion Assay

After 48 hours of transfection, a cell suspension was added to the upper compartment of the Transwell chamber. Specifically, 200 μL of cell suspension in serum-free medium was added to the upper chamber, while 500 μL of culture medium containing 15% FBS was added to the lower chamber of the 24-well plate. The plates were then placed in a CO_2 incubator at 37 °C for 48 hours. Subsequently, the cells on the upper layer of the chamber’s membrane were fixed using 4% paraformaldehyde and stained with crystal violet solution.

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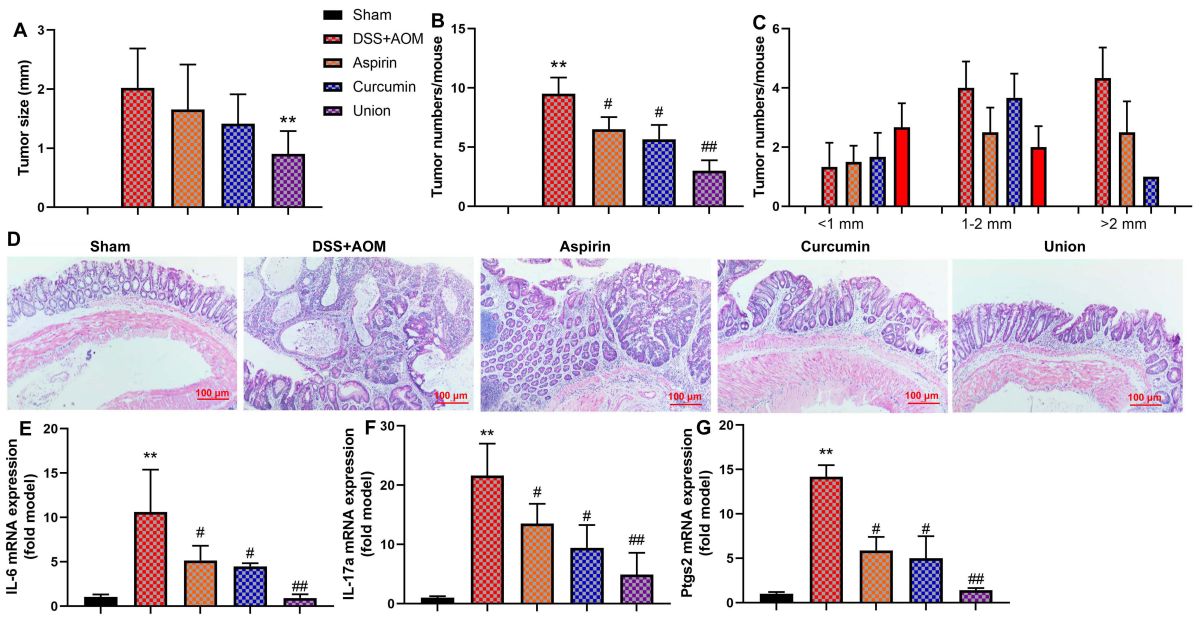

Fig. 1. Aspirin combined with curcumin prevented colorectal cancer in mice model. Tumor size (A), Tumor numbers (B,C), colorectal cancer (hematoxylin and eosin (HE) staining, scale bar = 100 μm) (D), interleukin-6 (*IL-6*), *IL-17α* and Prostaglandin-Endoperoxide Synthase 2 (*Ptgs2*) mRNA expressions (E–G). Sham, sham control group; dextran sulphate sodium (DSS) + azoxymethane (AOM), mice model of colorectal cancer group; Aspirin, colorectal cancer mice by Aspirin group; Curcumin, colorectal cancer mice by Curcumin group; Union, colorectal cancer mice by Aspirin combined with curcumin. $^{**}p < 0.01$ compared with sham group; $^{\#}p < 0.01$ compared with mice model of colorectal cancer group; $^{\#\#}p < 0.01$ compared with colorectal cancer mice by Curcumin group. Number = 6. Data between groups were followed using analysis of variance (ANOVA).

Western Blot

Tissue or cell samples were lysed using ice-cold RIPA buffer supplemented with complete protease and phosphatase inhibitors. The total proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (IPVH0011, Millipore, Shanghai, China). After blocking with 5% BSA in TBS, the membranes were incubated with primary antibodies including TAp63α (ab124762, 1:2000, Abcam, Shanghai, China), Lgr5 (ab75850, 1:2000, Abcam, Shanghai, China), p-β-catenin (ab75777, 1:2000, Abcam, Shanghai, China), and β-Actin (1:5000, 1:1000, Santa Cruz Biotechnology, Shanghai, China). Subsequently, the membranes were incubated with peroxidase-conjugated secondary antibodies (sc-2354, 1:5000, Santa Cruz Biotechnology, Shanghai, China). The signals were detected using an ECL system and visualized using the ChemiDoc XRS system with Image Lab software (6.1, Bio-rad, Shanghai, China).

Statistical Analyses

Statistical significance was defined as $p < 0.05$. GraphPad Prism 6 software (GRAPHPAD SOFTWARE, LLC., San Diego, CA, USA) was utilized for the statistical analysis. Group comparisons were conducted using either Student's *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's post hoc test.

Results

Aspirin Combined with Curcumin Prevented Colorectal Cancer in Mice Model

We initially assessed the efficacy of a combination therapy involving Aspirin and curcumin in a murine model of colorectal cancer. The results revealed a significant reduction in both tumor numbers ($p < 0.05$) and tumor size ($p < 0.05$) when Aspirin was combined with curcumin (Fig. 1A–D). Moreover, the combination treatment demonstrated a substantial decrease in the expression of *IL-6*, *IL-17α*, and *Ptgs2* mRNA (all $p < 0.05$) within the tumor tissue of the mouse model (Fig. 1E–G). Collectively, these findings suggest that the combined administration of Aspirin and curcumin exerts an anti-cancer effect in colorectal cancer.

Aspirin Combined with Curcumin Reduced Colorectal Cancer Cell Growth and Warburg Effect Progression

We investigated the impact of combining Aspirin with curcumin on cancer progression in colorectal cancer. Our results demonstrated that the combination treatment significantly reduced cell proliferation and migration rate, inhibited the rate of EDU positive cells, and increased the activity levels of Caspase-3/9 (all $p < 0.05$) in HCT-116 cells (Fig. 2).

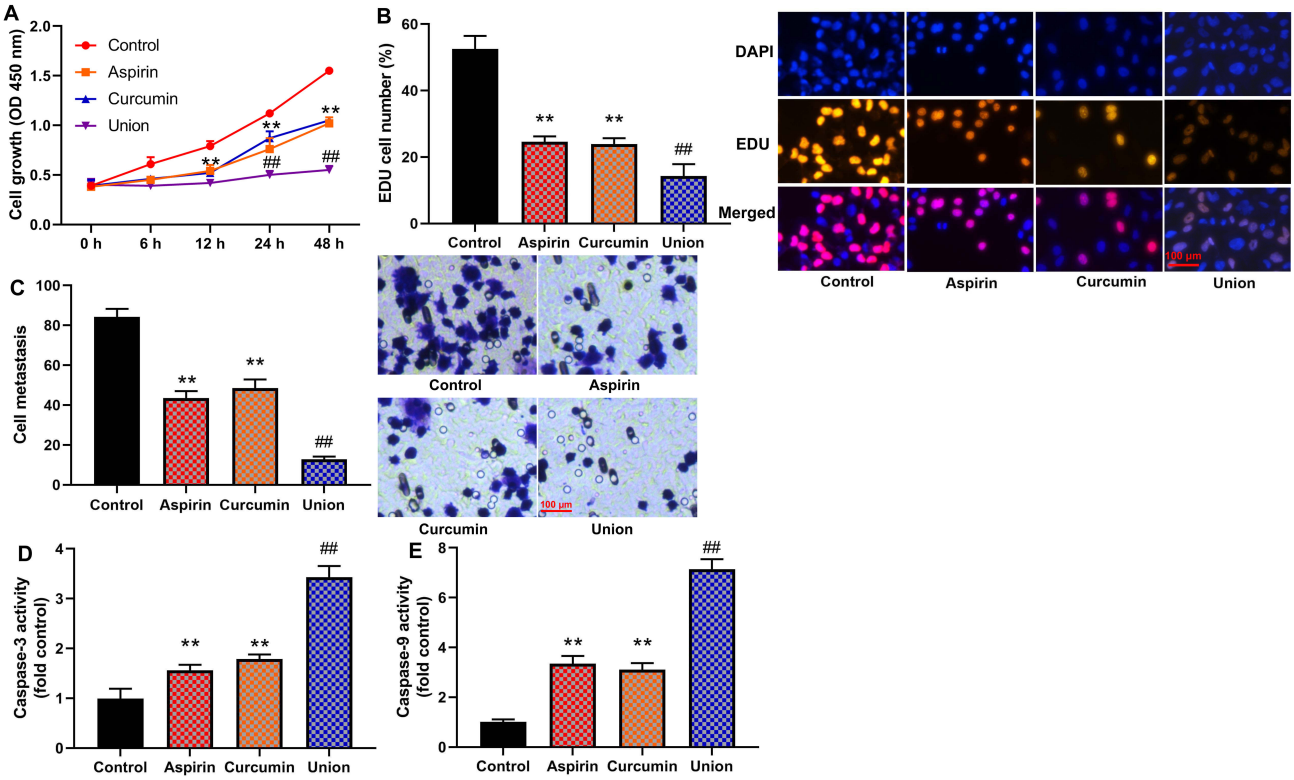

Fig. 2. Aspirin combined with curcumin reduced colorectal cancer cell growth. Cell proliferation (A), 5-ethynyl-2′-deoxyuridine (EDU) positive cell (B) (scale bar = 100 μm), migration rate (C) (scale bar = 100 μm), Caspase-3/9 activity level (D,E) *in vitro* model. Control, human colon adenocarcinoma cell lines (HCT-116) group; Aspirin, HCT-116 cell by Aspirin group; Curcumin, HCT-116 cell by Curcumin group; Union, HCT-116 cell by Aspirin combined with curcumin. $^{**}p < 0.01$ compared with control group; $^{\#\#}p < 0.01$ compared with HCT-116 cell by Aspirin group. Number = 3. Data between groups were followed using ANOVA.

The combination of Aspirin and curcumin significantly decreased glucose consumption, lactate production, and adenosine triphosphate (ATP) quantity (all $p < 0.05$) in HCT-116 cells (Fig. 3A–C). Additionally, the combination treatment resulted in a reduction in the extracellular acidification rate and an increase in OCR relative (all $p < 0.05$) in HCT-116 cells (Fig. 3D,E). These findings suggest that the combination of Aspirin and curcumin effectively inhibits colorectal cancer cell proliferation and the Warburg effect.

Aspirin Combined with Curcumin Suppressed Lgr5/Wnt/β-Catenin Signaling Pathway by TAp63α

The present study investigated the underlying mechanism by which the combination of Aspirin and curcumin influences cell progression in colorectal cancer cells using Microarray experiments. Our findings revealed that the combination treatment significantly reduced the expressions of *TAp63α* and *Lgr5* (all $p < 0.05$) (Fig. 4A–C). Furthermore, both in mice models and *in vitro* studies, Aspirin combined with curcumin effectively suppressed the mRNA expressions of *TAp63α* and *Lgr5* (all $p < 0.05$) (Fig. 4D–G).

Subsequently, the combination of Aspirin and curcumin exerted a notable inhibitory effect on the protein expressions of TAp63α, Lgr5, and p-β-catenin (all $p <$ 0.05) in both mice models and *in vitro* studies (Fig. 5A–F). Interestingly, Aspirin alone did not affect TAp63α ubiquitination, while curcumin promoted TAp63α ubiquitination in HCT-116 cells (Fig. 5G). Remarkably, when Aspirin was combined with curcumin, it further enhanced TAp63α ubiquitination (all $p < 0.05$) in HCT-116 cells compared to curcumin alone (Fig. 5G). These findings suggest that the combined treatment of Aspirin and curcumin suppresses the Lgr5/Wnt/β-Catenin signaling pathway by promoting TAp63α ubiquitination in colorectal cancer.

The Regulation of TAp63α Affected the Effects of Aspirin Combined with Curcumin on Colorectal Cancer Cell Growth and Warburg Effect Progression

The study investigated the involvement of TAp63α in the effects of Aspirin combined with curcumin on the growth of colorectal cancer cells. si-TAp63α treatment resulted in a significant reduction in the mRNA expressions of *TAp63α*, *Lgr5*, and *p-β-catenin* (all $p < 0.05$) in HCT-116 cells treated with Aspirin combined with curcumin (Fig. 6A–C). On the other hand, overexpression of TAp63α promoted cell proliferation and migration rates, increased the proportion of EDU-positive cells, and decreased Caspase-3/9 activity levels (all $p < 0.05$) in HCT-116 cells

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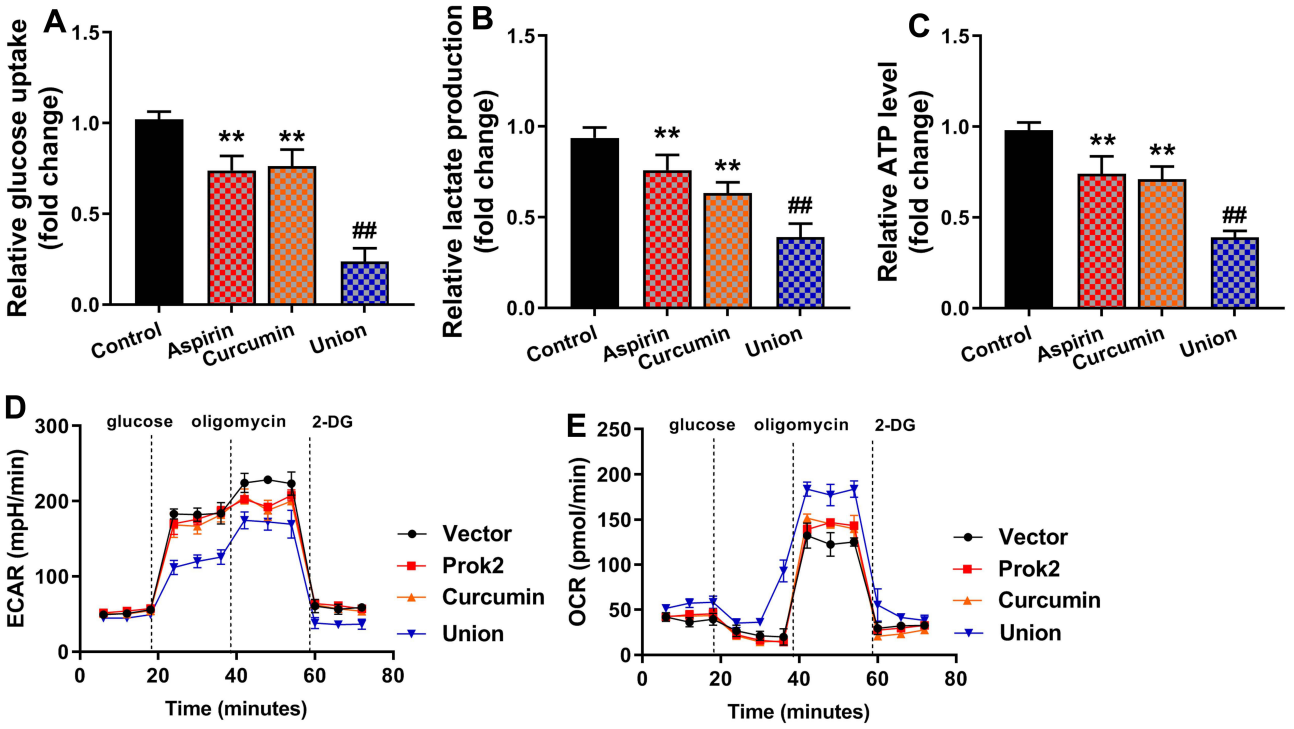

Fig. 3. Aspirin combined with curcumin reduced colorectal cancer cell Warburg effect progression. Glucose consumption (A), lactate production (B), adenosine triphosphate (ATP) quantity (C), extracellular acidification rate (ECAR) analysis (D), oxygen consumption rate (OCR) analysis (E). Control, HCT-116 cell group; Aspirin, HCT-116 cell by Aspirin group; Curcumin, HCT-116 cell by Curcumin group; Union, HCT-116 cell by Aspirin combined with curcumin. $^{**}p < 0.01$ compared with control group; $^{\#\#}p < 0.01$ compared with HCT-116 cell by Aspirin group. Number = 3. Data between groups were followed using ANOVA.

treated with Aspirin combined with curcumin (Fig. 6D–H). Conversely, si-TAp63α reduced cell proliferation and migration rates, suppressed the proportion of EDU-positive cells, and increased Caspase-3/9 activity levels (all $p < 0.05$) in HCT-116 cells treated with Aspirin combined with curcumin (Fig. 6D–H).

Finally, the overexpression of TAp63α in HCT-116 cells treated with Aspirin combined with curcumin resulted in a significant increase in glucose consumption, lactate production, and ATP quantity (all $p < 0.05$) (Fig. 7A–C). Conversely, si-TAp63α reduced glucose consumption, lactate production, and ATP quantity (all $p < 0.05$) in HCT-116 cells treated with Aspirin combined with curcumin (Fig. 7A–C). Moreover, TAp63α overexpression led to an increase in extracellular acidification rate and a decrease in OCR relative in HCT-116 cells (all $p < 0.05$) under treatment with Aspirin combined with curcumin (Fig. 7D,F). On the other hand, si-TAp63α decreased extracellular acidification rate and increased OCR relative (all $p < 0.05$) in HCT-116 cells treated with Aspirin combined with curcumin (Fig. 7E,G). These results indicate that Aspirin combined with curcumin reduces proliferation of colorectal cancer cells and diminishes the Warburg effect by regulating the TAp63α/Lgr5 signaling pathway.

Discussion

The development of colorectal cancer, like other malignant tumors, is attributed to the uncontrolled expression and regulation of multiple genes. However, the exact mechanism behind this process remains largely unknown. Currently, the combined mutation of oncogenes and tumor suppressor genes is recognized as the molecular biological foundation of colorectal cancer [20]. Surgical intervention continues to be the primary approach for treating colon cancer [18,21]. Therefore, it holds significant importance to investigate early diagnostic markers and prognostic factors associated with colon cancer to enhance patient prognosis. Colorectal cancer ranks third in terms of incidence rate and fifth in terms of mortality among all malignant tumors, with its prevalence still on the rise. Radical resection is currently the primary treatment method for patients with stage III colon cancer. However, postoperative recurrence rates are alarmingly high, leading to treatment failure and patient mortality. This directly impacts the survival of patients post-operation. Hence, it is crucial to deepen our understanding of the factors contributing to tumor recurrence after surgery to mitigate its occurrence, decrease recurrence rates, and extend patient survival time [22]. In the medical community, there is a consensus regarding the treatment

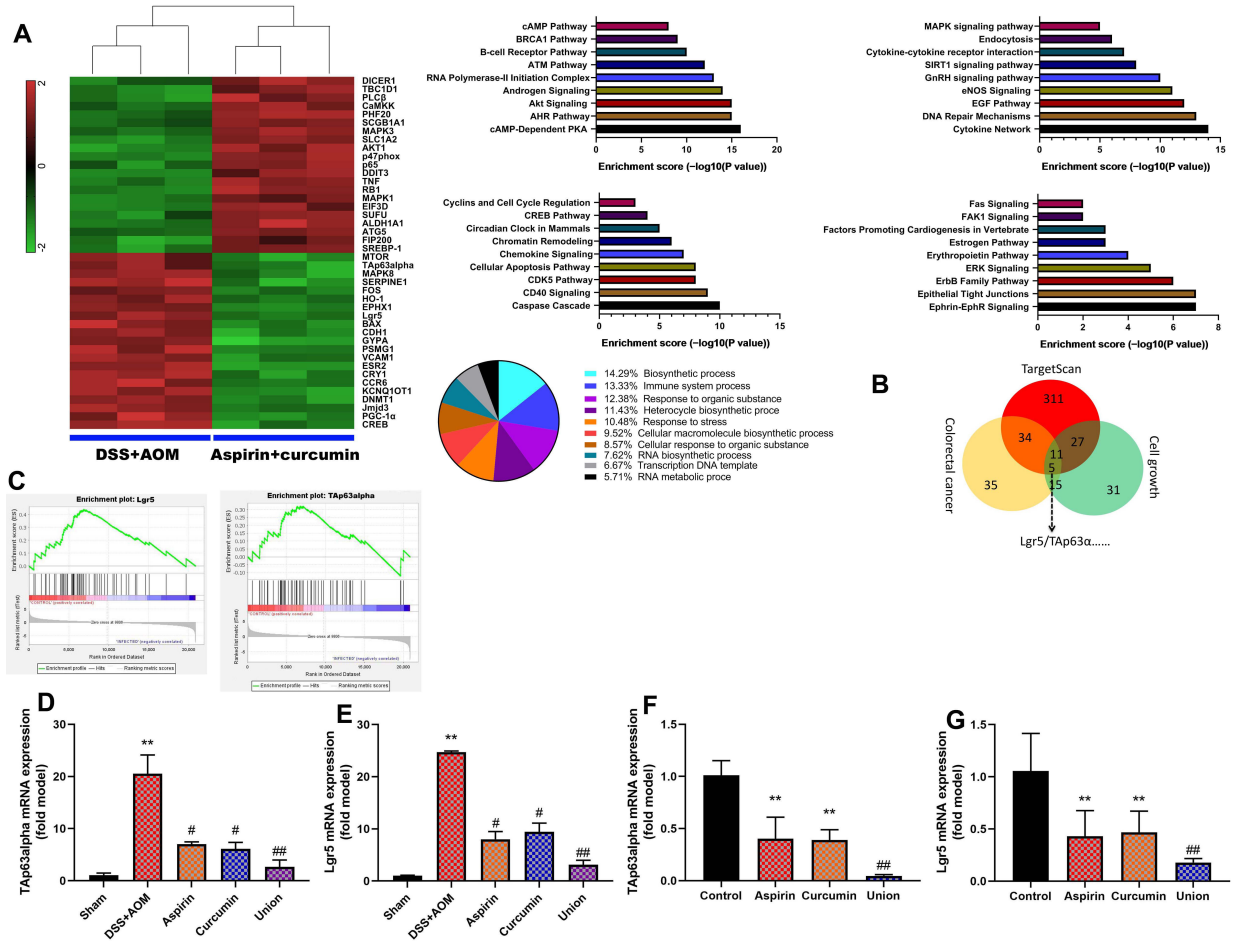

Fig. 4. Aspirin combined with curcumin regulated Lgr5 and TAp63alpha (TAp63α) expression. Heart map (A), result analysis (B), KEGG terms (C), *TAp63*α and *Lgr5* mRNA expressions (D,E) in mice model, *TAp63*α and *Lgr5* mRNA expressions (F,G) or *vitro* model. Sham, sham control group; DSS + AOM, mice model of colorectal cancer group; Aspirin, colorectal cancer mice by Aspirin group; Curcumin, colorectal cancer mice by Curcumin group; Union, colorectal cancer mice by Aspirin combined with curcumin. Control, HCT-116 cell group; Aspirin, HCT-116 cell by Aspirin group; Curcumin, HCT-116 cell by Curcumin group; Union, HCT-116 cell by Aspirin combined with curcumin. $^{**}p < 0.01$ compared with sham control group or control group; $^{\#}p < 0.01$ compared with mice model of colorectal cancer group; $^{\#\#}p < 0.01$ compared with colorectal cancer mice by Curcumin group or HCT-116 cell by Aspirin group. Number = 3. Data between groups were followed using ANOVA.

of colon cancer, which involves combining postoperative chemotherapy with fluorouracil following radical surgery. Nonetheless, there is a paucity of research investigating the factors influencing colon cancer recurrence after surgery.

The worldwide incidence rate of cancer is ranked third [23]. Colon cancer, specifically, has been on the rise in most countries, including China [24]. Examining the relationship between Aspirin and curcumin, our current data demonstrates their combined effectiveness in treating colorectal cancer in a DSS + AOM-induced mice model. Thakkar *et al*. [25] also demonstrated that combinations of aspirin, curcumin, and sulforaphane inhibited cell growth in pancreatic cancer. However, while these findings suggest that Aspirin and curcumin offer potential benefits for colorectal cancer treatment, the underlying mechanism remains unclear. Additionally, our research indicates that the combination of Aspirin and curcumin reduces *IL-6*, *IL-17*α, and *Ptgs2* mRNA expressions in the tumor tissue of the mice model. This reduction in inflammation levels supports the anti-inflammatory and anticancer effects of Aspirin and curcumin in the colorectal cancer model.

Curcumin, an ingredient commonly found in traditional Chinese medicine, possesses various beneficial properties such as anti-inflammatory, lipid-lowering, sterilizing, anti-oxidation, and scavenging of oxygen free radicals [26]. Furthermore, curcumin has demonstrated its anti-cancer effects [27] and has been shown to exert these effects across various types of tumors [28–30]. One possible mechanism through which curcumin may exhibit its anti-cancer effect is by inhibiting tumor cell proliferation [31]. In our study, we observed a noteworthy reduction in colorectal cancer cell growth *in vitro* when Aspirin was combined with cur-

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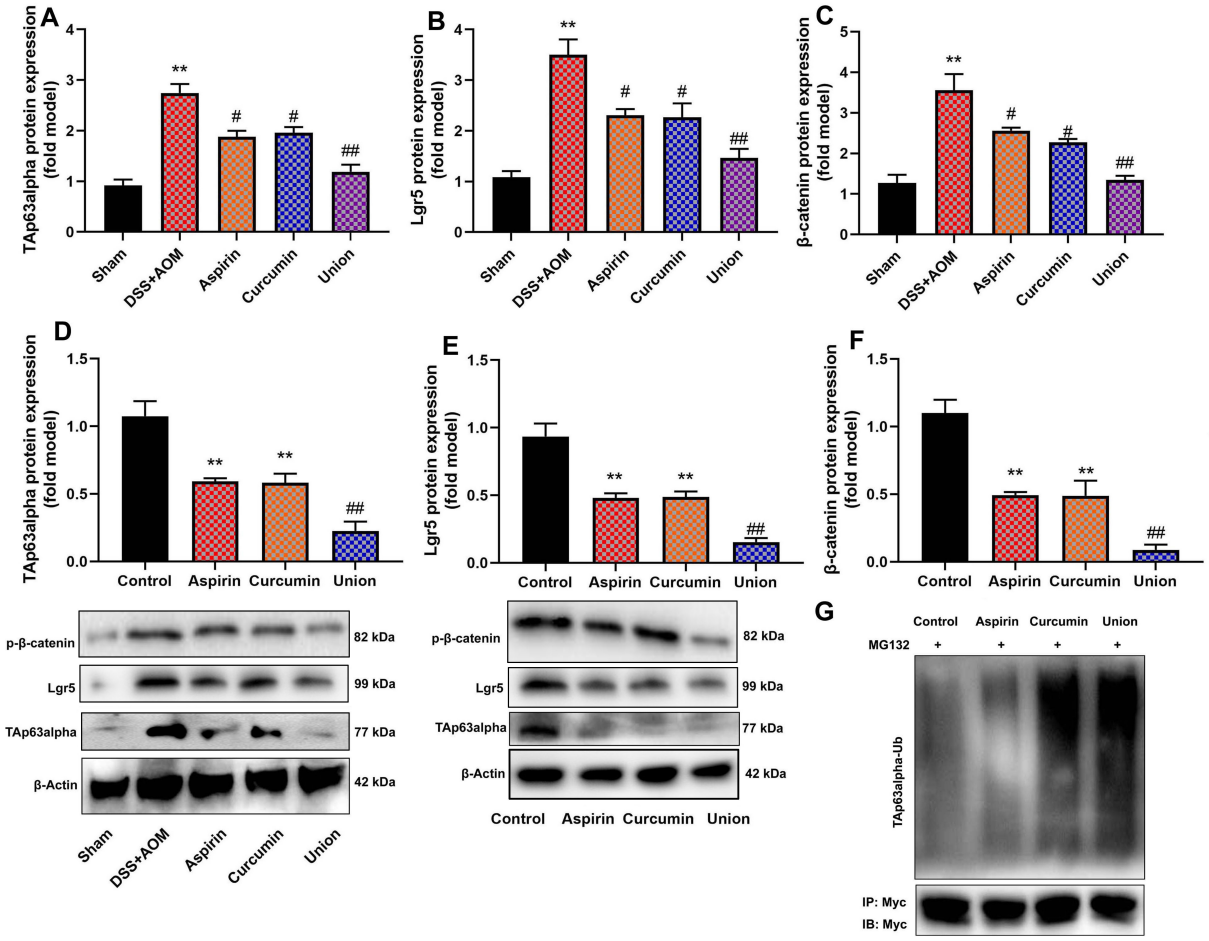

Fig. 5. Aspirin combined with curcumin suppressed Lgr5/Wnt/β-Catenin signaling pathway by TAp63α. TAp63α, Lgr5 and p-β-catenin protein expressions (A–C) in mice model, (D–F) *in vitro* model, TAp63α protein Ubiquitination by Aspirin combined with curcumin (G). Sham, sham control group; DSS + AOM, mice model of colorectal cancer group; Aspirin, colorectal cancer mice by Aspirin group; Curcumin, colorectal cancer mice by Curcumin group; Union, colorectal cancer mice by Aspirin combined with curcumin. Control, HCT-116 cell group; Aspirin, HCT-116 cell by Aspirin group; Curcumin, HCT-116 cell by Curcumin group; Union, HCT-116 cell by Aspirin combined with curcumin. $^{**}p < 0.01$ compared with sham control group or control group; $^{\#}p < 0.01$ compared with mice model of colorectal cancer group; $^{\#\#}p < 0.01$ compared with colorectal cancer mice by Curcumin group or HCT-116 cell by Aspirin group. Number = 3. Data between groups were followed using ANOVA.

cumin. This finding aligns with the work of Srour *et al.* [32], who also reported potential antitumor effects of the aspirin-curcumin combination. Based on these results, it can be concluded that the combination of Aspirin and curcumin effectively reduces the growth of colorectal cancer cells.

Curcumin exhibits substantial potential in combating cancer through diverse mechanisms, including the induction of cell apoptosis, inhibition of cell proliferation, reduction of tumor cell invasion, induction of cell autophagy, anti-oxidation, anti-inflammatory effects, mitigation of oral fibrosis, and enhancement of the efficacy of radiotherapy and chemotherapy.

Despite the multifaceted potential of curcumin in combating oral cancer through mechanisms such as inducing cell apoptosis, inhibiting cell proliferation, reducing tumor cell invasion, inducing cell autophagy, providing anti-oxidative and anti-inflammatory effects, mitigating oral fibrosis, and enhancing the efficacy of radiotherapy and chemotherapy, its clinical efficacy is hindered by challenges related to its low drug absorption, rapid metabolism, and limited bioavailability. Therefore, it is imperative to conduct further clinical trials to uncover its full therapeutic potential. Currently, research on curcumin for oral cancer primarily remains in the basic research stage. The use of curcumin monomer or compound preparations in clinical practice is still limited. Consequently, additional research is warranted to investigate the preventive and therapeutic applications of curcumin in the context of oral cancer.

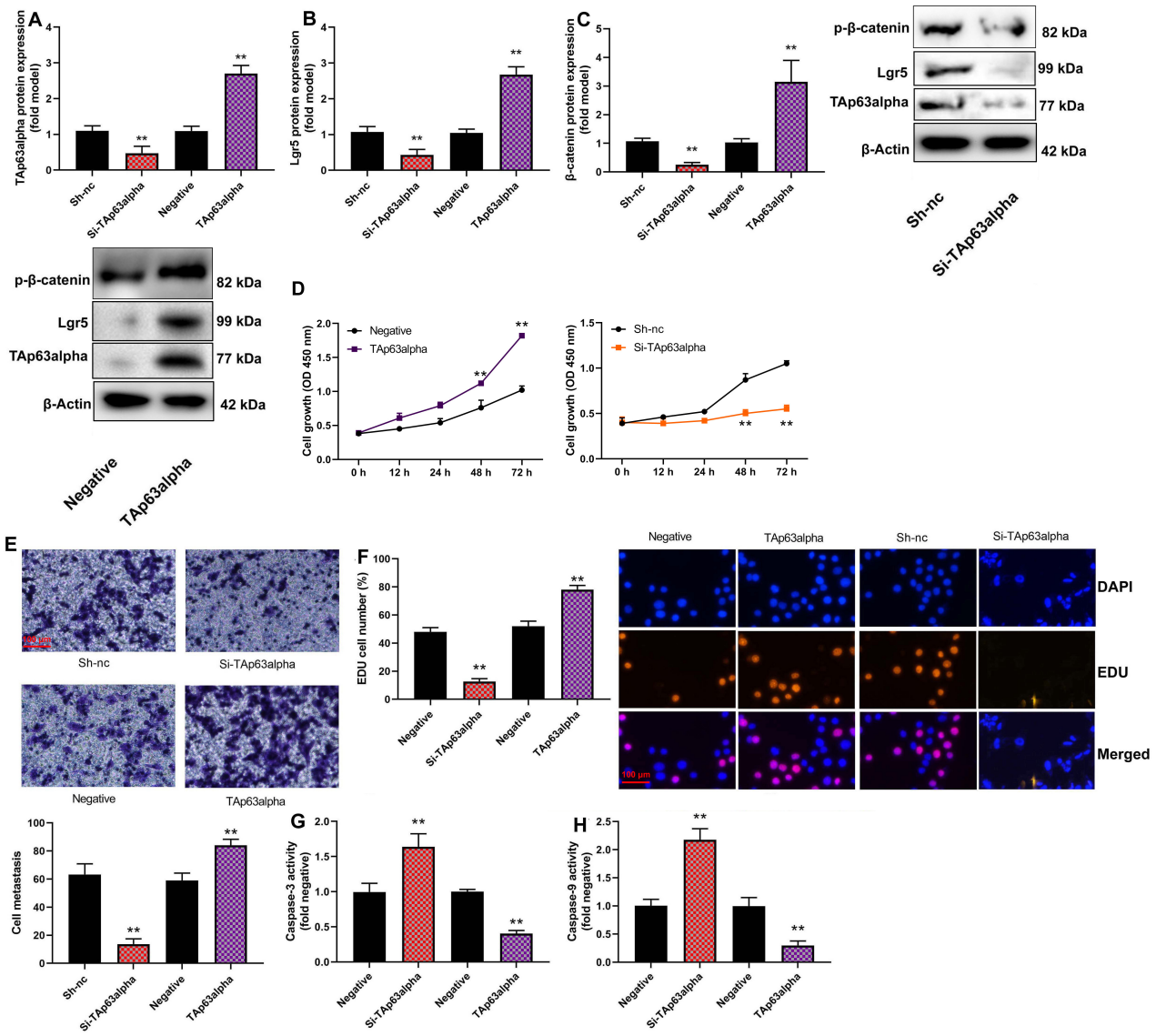

Fig. 6. The regulation of TAp63α affected the effects of Aspirin combined with curcumin on colorectal cancer cell growth. TAp63α, Lgr5 and p-β-catenin protein expressions (A–C); Cell proliferation (D), EDU positive cell (scale bar = 100 μm, E), migration rate (scale bar = 100 μm, F), Caspase-3/9 activity level (G,H) *in vitro* model. Negative, HCT-116 cell by Aspirin combined with curcumin, negative group; TAp63α, HCT-116 cell by Aspirin combined with curcumin, TAp63α plasmid group; si-TAp63α, HCT-116 cell by Aspirin combined with curcumin, si-TAp63α mimics group. $^{**}p < 0.01$ compared with HCT-116 cell by Aspirin combined with curcumin, negative group. Number = 3. Data between groups were followed using ANOVA.

Compared to normal cells, tumor cells undergo significant changes in energy metabolism, a phenomenon known as metabolic reprogramming. Among these alterations, the abnormal shift in glucose metabolism has gained considerable attention and is a complex process. Despite extensive research efforts, there remain numerous unresolved questions at each critical juncture. Studies have demonstrated that tumor cells often rely on glycolysis for energy production, even in the presence of sufficient oxygen. This phenomenon, known as aerobic glycolysis or the "Warburg effect", was first described by the German scientist Otto Warburg. The Warburg effect influences the progression of colorectal cancer by impacting energy metabolism, particularly the glycolytic pathway in cancer cells, as well as participating in the expression of common regulatory factors and regulatory proteins [19,33]. Our study's data indicates that the combination of Aspirin and curcumin reduces the progression of the Warburg effect in colorectal cancer cells. Siddiqui *et al.* [34] also reported that Curcumin can diminish the Warburg effect in cancer cells.

Lgr5 serves as a marker for colorectal cancer stem cells, and current research indicates its association with the Wnt/β-Catenin signaling pathway. Hepatocellular carcinoma with mutations in the β-*Catenin* gene exhibits signifi-

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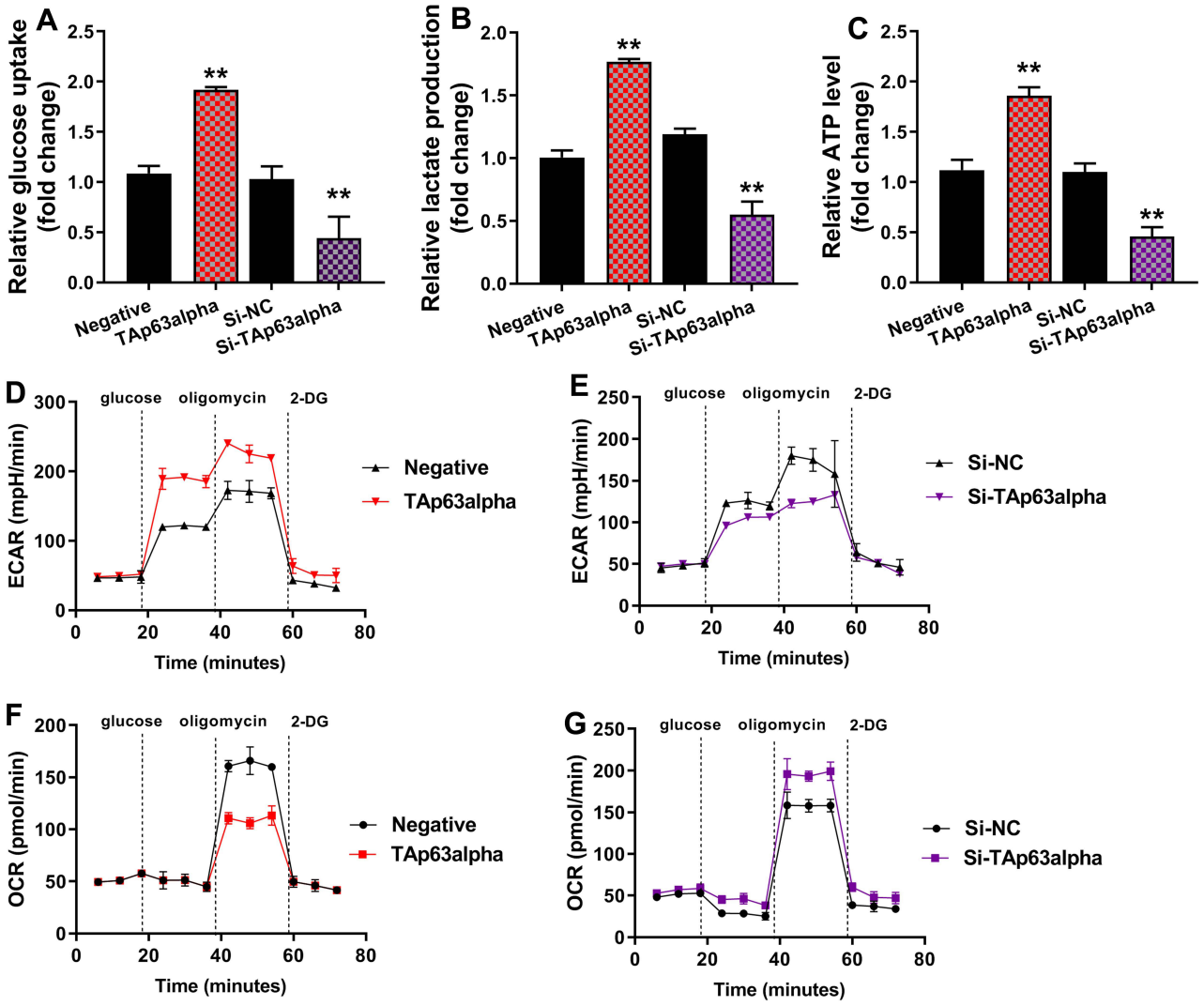

Fig. 7. The regulation of TAp63α affected the effects of Aspirin combined with curcumin on Warburg effect progression. Glucose consumption (A), lactate production (B), ATP quantity (C), ECAR analysis (D,E), and OCR analysis (F,G). Negative, HCT-116 cell by Aspirin combined with curcumin, negative group; TAp63α, HCT-116 cell by Aspirin combined with curcumin, TAp63α plasmid group; si-TAp63α, HCT-116 cell by Aspirin combined with curcumin, si-TAp63α mimics group. $^{**}p < 0.01$ compared with HCT-116 cell by Aspirin combined with curcumin, negative group. Number = 3. Data between groups were followed using ANOVA.

cantly elevated expression of Lgr5. Transferring the mutant *β-Catenin* gene into mouse hepatocytes leads to increased mRNA expression of *Lgr5*, confirming that *Lgr5* is a target gene regulated by the Wnt pathway. On the other hand, in cholangiocarcinoma, breast cancer, nasopharyngeal carcinoma, glioma, and other tumors, elevated expression of *Lgr5* continuously activates the Wnt/β-Catenin signaling pathway. In HT29 colon cancer cells, downregulating the *Lgr5* gene using si-RNA reduces the expression of APC and β-Catenin while inhibiting cell proliferation. *Lgr5* is widely distributed in the human gastrointestinal tract, breast tissue, ovary, eyes, and brain [35–37]. Currently, research has shown that Aspirin combined with curcumin can suppress the Lgr5/Wnt/β-Catenin signaling pathway through TAp63α. Mao *et al.* [38] have demonstrated that Curcumin can suppress *Lgr5* colorectal cancer stem cells. Based on these findings, it can be speculated that Aspirin combined with curcumin suppresses the *Lgr5* signaling pathway, thus impacting colorectal cancer presentation through the Warburg effect.

P63 is a member of the p53 family of tumor suppressor genes [12]. While certain subtypes of p63 are considered tumor suppressors, others are regarded as oncogenes [39]. TAp63, a homologue of P63, possesses a transcriptional activation region that regulates tumor apoptosis. Notably, TAp63 has been shown to inhibit distal metastasis in colon cancer [22]. Furthermore, previous reports have indicated the inhibitory role of TAp63 in colorectal cancer [26]. Our study findings reveal that the combination of Aspirin and curcumin promotes TAp63α ubiquiti-

nation in HCT-116 cells. Chen *et al.* [40] have reported on TAp63α's targeting of Lgr5 expression in colorectal cancer. Thus, our results demonstrate that Aspirin combined with curcumin enhances TAp63α ubiquitination to suppress the TAp63α/Lgr5 signaling pathway in the colorectal cancer model.

Conclusions

In conclusion, this study demonstrates that the combination of Aspirin and curcumin effectively reduces the progression of the Warburg effect and suppresses cell growth in colorectal cancer through the TAp63α/Lgr5 signaling pathway. These findings suggest the potential therapeutic application of Aspirin combined with curcumin for treating colorectal cancer and other types of cancers.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

MMZ and HW designed the experiments. MMZ performed the experiments. HW and KZ collected and analyzed the data. KZ drafted the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Ethical approval for the study was obtained from the Animal Care and Use Committee of Guiyang Public Health Clinical Center (Approval No. 2020061317852).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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