Article

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β-Sitosterol Inhibits Osteosarcoma Cell Proliferation through Regulating Caspase-3

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Background: **β-sitosterol, a plant-derived sterol, shows anti-cancer properties in multiple cancers. However, its biological effects on osteosarcoma are unclear. This study aims to decipher β-sitosterol's biological function in osteosarcoma and its regulatory mechanism.**

Methods: **Osteosarcoma cell lines 143B and HOS were used as the *in vitro* models. They were treated with β-sitosterol, and cell counting kit-8 was adopted to assess cell proliferation. Flow cytometry was conducted to examine cell cycle; apoptosis was detected through Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) assay and flow cytometry. The targets of β-sitosterol were predicted in the Traditional Chinese Medicine Systems Pharmacology database, and osteosarcoma-associated genes were analyzed with GeneCards database. Cytoscape software and Search Tool for the Retrieval of Interaction Gene/Proteins (STRING) database were applied to establish a protein-protein interaction network and perform modular analysis to screen hub genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology pathway enrichment analyses were then performed with the hub genes with DAVID database. Caspase-3 mRNA expression level was determined via quantitative real-time polymerase chain reaction (qRT-PCR). Western blot was performed to detect the expression level of cleaved caspase-3 protein.**

Results: **β-sitosterol could inhibit 143B and HOS cell growth, and induce cell apoptosis and cell cycle arrest in G1 phase. Caspase-3 was identified as a target of β-sitosterol in osteosarcoma. Additionally, β-sitosterol could significantly promote cleaved caspase-3 expression, and caspase-3 knockdown could markedly reverse the suppressive effect of β-sitosterol on the malignant phenotypes of 143B and HOS cells.**

Conclusion: **β-sitosterol can suppress osteosarcoma cell proliferation and induce apoptosis by facilitating caspase-3 expression.**

Keywords: osteosarcoma cell; β-sitosterol; proliferation; caspase-3

Introduction

Osteosarcoma is a primary bone malignancy mainly occurring in kids and juveniles, making up about 60% of all bone tumors [1]. Typical clinical symptoms of osteosarcoma include bone pain and swelling [2]. The current clinical treatment strategy for osteosarcoma is surgery, supplemented by chemotherapy and radiotherapy; nonetheless, on account of the complications of surgery, and chemoresistance and radioresistance of cancer cells, the five-year survival rate of patients with non-metastatic osteosarcoma is 60–70%, while that of the patients with metastatic osteosarcoma is merely 11–13% [3,4]. In this context, it is highly significant to explore more effective drugs for osteosarcoma treatment.

Growing evidence shows that the drugs derived from natural products may have anti-tumor properties and show low toxicity and high efficacy [5]. For instance, in osteosarcoma, degalactotigonin extracted from *Solanum nigrum L.* can repress the malignancy of cancer cells through modulating the Hedgehog/Gli1 pathway mediated by GSK3β inactivation; and *in vivo* experiments suggest that degalactotigonin can remarkably reduce the volume of osteosarcoma xenograft and the incidence of lung metastasis [6]. Costunolide derived from multiple medicinal plants can restrain osteosarcoma cell growth and metastasis *in vivo* and *in vitro* by inhibiting the transcriptional activity of STAT3 and the phosphorylation of STAT3 (Tyr-705) [7–9]. Cordyceps is a kind of natural entomopathogenic fungus containing various bioactive components such as sterol, cyclic peptide, flavonoids and alkaloids, and has multiple pharmacological effects such as immunomodulatory effect, antioxidant effect, anti-tumor effect and anti-inflammatory effect, and it is widely applied in traditional Chinese medicine [10]. β-sitosterol is a phytosterol that is extracted from Cordyceps; increasing studies have revealed that β-sitosterol exerts anti-cancer effects by modulating various biological processes [8], and it shows pharmaco-

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logical effects on multiple human diseases, for example, intracranial aneurysm (IA) [11], pancreatic cancer [12] and colorectal cancer [13]. Nonetheless, the regulatory effects of β-sitosterol on osteosarcoma and its regulating mechanism warrant elucidation.

Caspase-3 is a cysteine-aspartic acid protease, and it is one of the dominators of apoptosis, and its dysregulation is involved in tumorigenesis [14,15]. In this study, we treated 143B and HOS cells with β-sitosterol to investigate β-sitosterol's effects on osteosarcoma cells' growth and apoptosis. Furthermore, the possible targets of β-sitosterol in osteosarcoma were identified, and hub genes were screened through bioinformatics analysis, and finally the potential mechanism of β-sitosterol in osteosarcoma cells was investigated.

Materials and Methods

Cell Culture and Treatment

From ATCC (Manassas, VA, USA), osteosarcoma cell lines (MG-63, 143B, HOS and SAOS-2) were obtained. These cell lines were cultured in Dulbecco's modified Eagle's medium (SH30021.FS, HyClone, Logan, UT, USA) containing 1% penicillin/streptomycin (15140122, ThermoFisher, Rockford, IL, USA) and 10% fetal bovine serum (FBS; A5669701, ThermoFisher, Rockford, IL, USA). The cells were placed in a humidified incubator with 5% CO_2 at 37 °C for culturing. The cell lines were authenticated by STR profile. Before the experiments, the cell contamination was evaluated to confirm that the cells were not contaminated by mycoplasma.

BS Preparation and Treatment

BS ($C_{29}H_{50}O$, molecular weight: 414.71, purity $\geq$70%) was bought from Sigma-Aldrich (567152, St. Louis, MO, USA). β-sitosterol was dissolved in dimethyl sulfoxide (DMSO; 472301, Sigma-Aldrich, St. Louis, MO, USA) to prepare 10, 20, 40, 80 and 160 μM solutions, and they were kept at –20 °C until use.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

We extracted the total RNA of 143B and HOS cells utilizing TRIzol reagent (15596-018, Invitrogen, Carlsbad, CA, USA) and reverse-transcribed it into cDNA employing the SuperScript II First-Strand Synthesis System (11904018, Invitrogen, Carlsbad, CA, USA). On the ABI 7900 HT Real-time System (Applied Biosystems, Foster City, CA, USA), the SYBR-Green method was adopted to measure caspase-3 RNA using qRT-PCR, with a SYBR Green kit (Roche, Pleasanton, CA, USA) (Cat. 4913914001). With *GAPDH* expression as the control, the $2^{-\Delta\Delta Ct}$ method was adopted for calculating caspase-3 mRNA amount. The primer sequences are the following: caspase-3 (*CASP3*): Forward, 5′-AATGGATTATCCTGAGATGGG-3′, Reverse, 5′-GACCGAGATGTCATTCCAG-3′; *GAPDH*: Forward, 5′-GGGTGTGAAACCATGAGAAGT-3′, Reverse, 5′-CAGTGATGGCATGGACTGTG-3′. Amplification conditions: 94 °C, 30 s; Next: 94 °C, 60 s; 50 °C, 60 s; 72 °C, 120 s; 30 cycles.

Cell Transfection

Caspase-3 siRNA (si-CASP3) (and the control siRNA) was constructed by GenePharma (Shanghai, China), and when the cells reached 70–80% confluency, 143B and HOS cells were transfected with the oligonucleotides employing Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The efficiency of transfection was verified by qRT-PCR 24 h later.

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation was detected utilizing the CCK-8 kit (C0038, Beyotime, Shanghai, China). The cells were inoculated at 5000 cells/well into 96-well plates. After 24 h of cell culture, we added 10 μL of CCK-8 reagent, and the plates were placed in 5% CO_2 at 37 °C for 2 h. A microplate reader (168-1130, Bio-Rad, Hercules, CA, USA) was utilized to measure the OD value of each well at 450 nm. Cell inhibition rate (%) = (1 – OD value of experimental group/OD value of control group) × 100%.

Cell Cycle Analysis

143B and HOS cells were inoculated at 5 × 10^5 cells/well into 6-well plates, rinsed twice with PBS after culturing for 24 h, fixed with 70% ethanol, and stored overnight at 4 °C. The cells were centrifuged, and the supernatant was removed. We added an appropriate amount of PBS to wash the cells and remove the ethanol. The well plates were added with 40 μg/mL propidium iodide (PI) staining solution (ST1569-250mg, Beyotime, Shanghai, China) and 0.2 mg/mL RNase A (ST577, Beyotime, Shanghai, China) and incubated at 4 °C away from light for 30 min. The cells were then filtered through a 400-mesh filter cloth, DNA content was analyzed by the flow cytometer (FACSAria II, BD Biosciences, San Jose, CA, USA), and Modfit software (version: LT 4.1, Verity Software House, Topsham, ME, USA) was adopted for analyzing the percentages of cells in different phases.

Flow Cytometry Assay

The cells during logarithmic growth were trypsinized and transferred into 6-well plates (1 × 10^5 cells/well), and incubated for 24 h at 37 °C. The cells were subsequently suspended in annexin-binding buffer and stained with PI solution and Annexin V-fluorescein isothiocyanate solution (40302ES20, Yeasen Biotech Co., Ltd., Shanghai, China) for 20 min. After that, after dilution in Annexin binding buffer, the stained cells were detected employing the flow

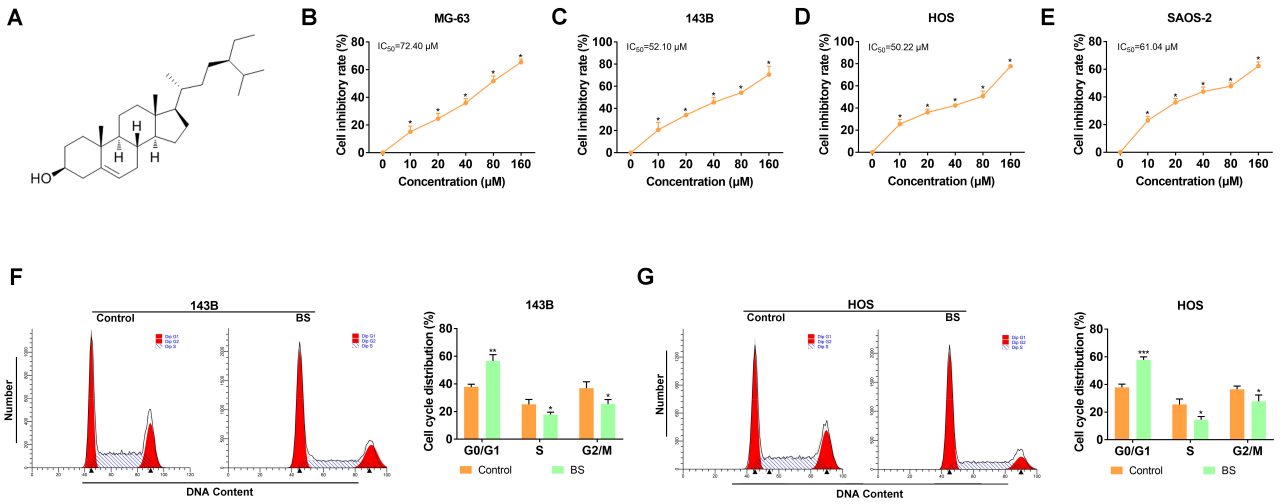

Fig. 1. β-sitosterol inhibits osteosarcoma cell proliferation. (A) Chemical structure of β-sitosterol. (B–E) Cell counting kit-8 (CCK-8) assay was conducted to detect the cell inhibition rates of osteosarcoma cells (MG-63, 143B, HOS and SAOS-2) treated with different concentrations of β-sitosterol. (F,G) Flow cytometry analysis of cell cycle distribution after β-sitosterol treatment of 143B and HOS cells. Control group v.s. BS group, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

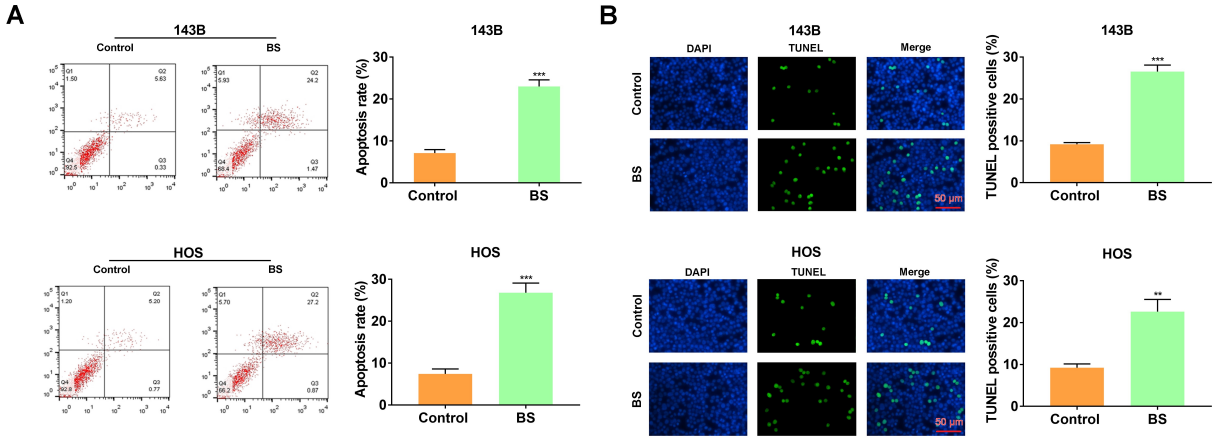

Fig. 2. β-sitosterol can promote osteosarcoma cell apoptosis. (A) 143B and HOS cell apoptosis after β-sitosterol treatment was detected by flow cytometry. (B) Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) assay was applied to detect apoptotic cells after β-sitosterol treatment of 143B and HOS cells. Scale bar = 100 μm. Control group v.s. BS group, $**p < 0.01$; $***p < 0.001$.

cytometer (FACSAria II, BD Biosciences, San Jose, CA, USA). The percentage of apoptotic cells was determined utilizing FlowJo software (version: 10.8.1, TreeStar, Ashland, OR, USA). The percentage of "Q2+Q3" cells was the apoptosis rate.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

The apoptosis rate of 143B and HOS cells was also measured by TUNEL assay. Cells were inoculated in 6-well plates, and apoptosis was examined utilizing a TUNEL detection kit (C1086, Beyotime, Shanghai, China). Subsequently, DAPI was added for nuclear staining; under a fluorescence microscope (BX53, Olympus, Tokyo, Japan), we observed and counted the positive cells, and calculated the apoptosis rate. The apoptosis rate = TUNEL positive cells/total cells × 100%.

Western Blot Assay

Protein was extracted from 143B and HOS cells by RIPA lysis buffer (P0013B, Beyotime, Shanghai, China) and quantified employing the bicinchoninic acid (BCA) kit. 50 μg of sample in each sample was added to each well, and then SDS-PAGE was performed to separate the protein. The protein was then transferred to the polyvinylidene difluoride membrane (BTN101123, Millipore, Bil-

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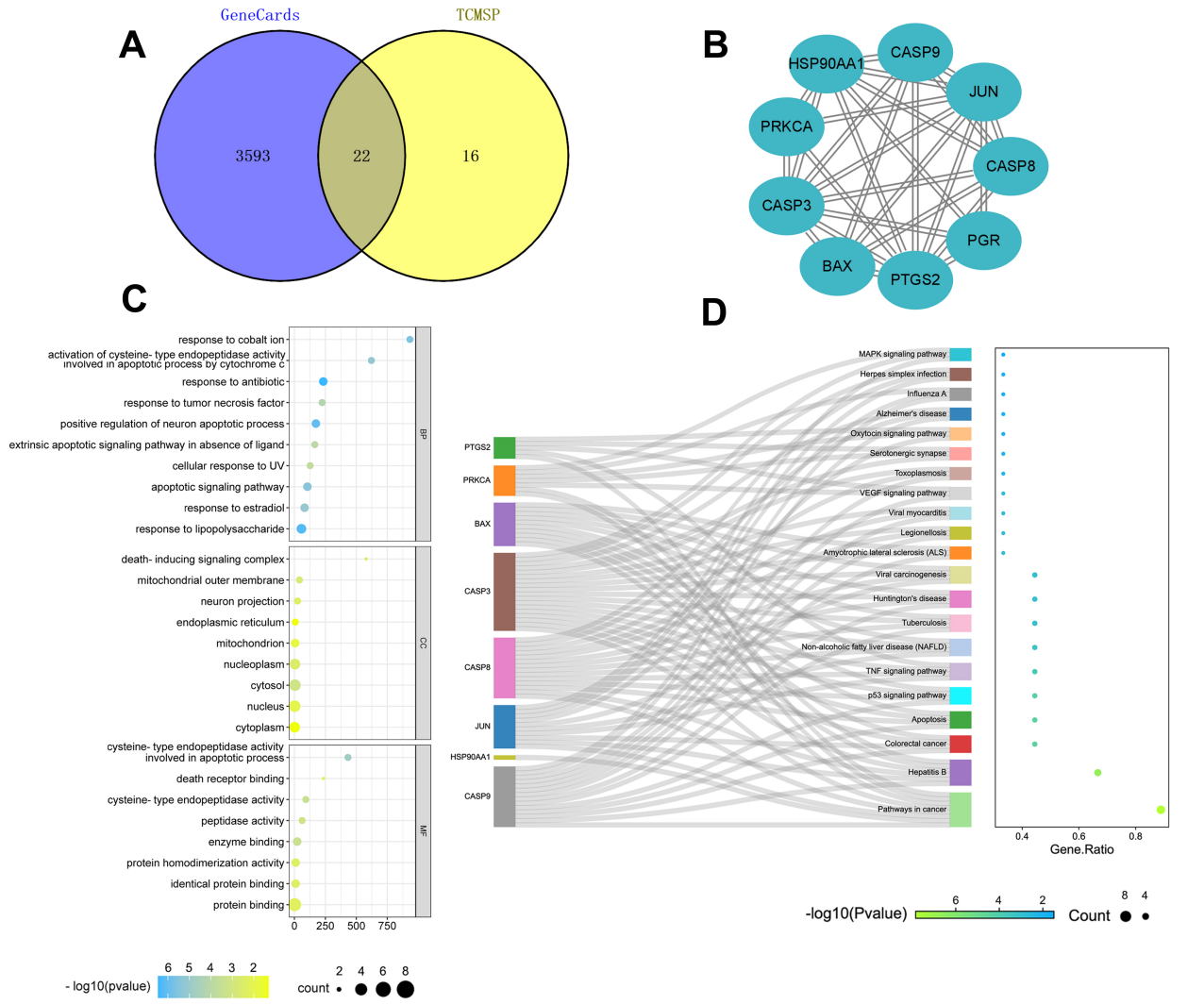

Fig. 3. Identification of β-sitosterol targets in osteosarcoma cells. (A) A Venn diagram was drawn between the β-sitosterol target genes screened out in the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database and osteosarcoma-related genes in the GeneCards database. (B) A protein-protein interaction (PPI) network was constructed by the Search Tool for the Retrieval of Interaction Gene/Proteins (STRING) database and Cytoscape software to screen out hub genes. (C) Gene Ontology (GO) analysis of the hub genes utilizing the DAVID database. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was conducted on the hub genes employing the DAVID database, and a sankey bubble chart was drawn. The left one is the sankey diagram, which represents the genes in each pathway, and the right one is the bubble chart.

lerica, MA, USA). The membranes were then blocked with 5% skim milk for 2 h at room temperature, followed by incubation with primary antibodies overnight at 4 °C. The primary antibodies are the following: cleaved caspase-3 (1:1000, ab13847, Abcam, Cambridge, UK) or GAPDH (1:1000, ab9485, Abcam, Cambridge, UK). Next, horseradish peroxidase-conjugated secondary antibody (1:2000, ab6721, Abcam, Cambridge, UK) was incubated with the membrane for 2 h at room temperature, and the protein bands were detected with the ECL luminescence reagent (abs920, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Relative protein expression levels were analyzed utilizing ImageJ software (version: 1.8.0, NIH, Bethesda, MD, USA).

Bioinformatics Analysis

Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (https://tcmsp-e.com/tcmsp.php) was used to predict the targets of β-sitosterol. Briefly, parameters "OB >30%" and "DL >0.18" were set, to validate the oral avaibility and drug-likeness of BS. Next, related targets of BS were generated. The GeneCards database (https://www.genecards.org/) was adopted to identify target genes associated with osteosarcoma, with the key word "osteosarcoma". β-sitosterol's target genes, screened out in the TCMSP database and the osteosarcoma-related target genes in the GeneCards database, in intersection, were obtained via a Venn diagram.

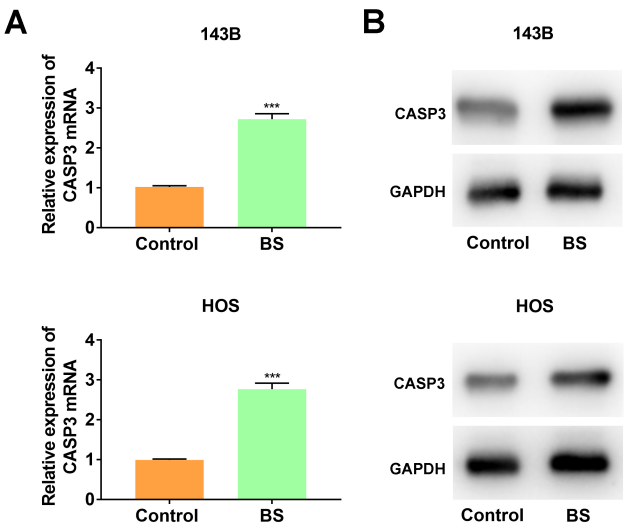

Fig. 4. β-sitosterol can positively regulate caspase-3 expression and activation. (A) Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to detect caspase-3 expression after β-sitosterol treatment (80 μM) of 143B and HOS cells. (B) Western blot was applied to detect cleaved caspase-3 expression after β-sitosterol treatment (80 μM) of 143B and HOS cells. Control group v.s. BS group, ***$p < 0.001$.

Protein-Protein Interaction (PPI) Network

STRING (http://string-db.org), an online database for retrieving interacting genes, was searched to establish a PPI network with interaction score >0.4 as the inclusion criterion. Cytoscape (version 3.7.0, http://cytoscape.org/) is a bioinformatics software to visualize molecular interaction networks. We used the plug-in Molecular Complex Detection (MCODE) in Cytoscape to identify the most important modules in the PPI network, and drew the PPI network graph. The inclusion criteria included MCODE score >4, node score cut-off = 0.2, degree cut-off = 2, k-score = 2, and Max depth = 100. Genes in the PPI network were subsequently subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses employing the DAVID database (https://david.ncifcrf.gov/home.jsp) with "$p < 0.05$" as the inclusion criterion.

Statistical Analysis

GraphPad Prism 7.0 software (version: 7.9, GraphPad, San Diego, CA, USA) and SPSS 24.0 software (IBM, Chicago, IL, USA) were utilized for statistical analysis, and each experiment was performed 3 times, and the results were expressed as "mean ± standard deviation". Student's *t*-test or one-way of variance analysis was utilized for comparison of the data in different groups, and a difference was of statistical significance when $p < 0.05$. Half inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism 7.0 software.

Results

β-Sitosterol Suppresses Osteosarcoma Cell Proliferation

To investigate the biological functions of β-sitosterol (Fig. 1A) on osteosarcoma cells (MG-63, 143B, HOS and SAOS-2), osteosarcoma cells were treated for 48 h with β-sitosterol at concentrations of 10, 20, 40, 80 and 160 μM, respectively, and then the cell viability was evaluated through CCK-8 assay and the cell inhibition rate was calculated. It was revealed that compared with the control group, the cell inhibition rates were all remarkably improved after treatment with β-sitosterol at different concentrations, suggesting that β-sitosterol inhibited cell proliferation in a concentration-dependent manner, and the IC_{50} values of MG-63, 143B, HOS and SAOS-2 cells were 72.40, 52.1, 50.22 and 61.04 μM, respectively (Fig. 1B–E). Given the better suppressive effect of β-sitosterol on 143B and HOS cells, we subsequently selected these two cell lines for experiments, and the concentration of β-sitosterol was selected to be 80 μM. Then we further verified the effects of 80 μM β-sitosterol on the cell cycle of 143B and HOS cells, and it was revealed that as against the control group, the proportion of cells in the G0/G1 phase was markedly elevated in β-sitosterol treatment group (Fig. 1F,G). Therefore, the above findings suggest that β-sitosterol can remarkably restrain 143B and HOS cells' growth and arrest the cells at G1 phase.

β-Sitosterol Promotes Osteosarcoma Cell Apoptosis

To further verify the effects of β-sitosterol on the apoptosis of 143B and HOS cells, we treated the cells with 80 μM β-sitosterol for 24 h. The apoptosis rate of the osteosarcoma cells in β-sitosterol treatment group was significantly increased (Fig. 2A). In addition, TUNEL assay showed that, the number of apoptotic cells in β-sitosterol treatment group was markedly elevated (Fig. 2B). These data suggest that β-sitosterol can dramatically induce 143B and HOS cell apoptosis.

Identification of the Targets of β-Sitosterol in Osteosarcoma Cells

Next, we adopted the TCMSP database for predicting the targets of β-sitosterol and screened out the osteosarcoma-related genes in the GeneCards database, and then analyzed the target genes of these two databases in intersection by drawing a Venn diagram; it was revealed that there were 22 target genes within the overlap (Fig. 3A). Subsequently, a PPI network of these 22 target genes was established through Cytoscape software and the STRING database, and subsequently 9 hub genes of *PGR*, *JUN*, *PRKCA*, *CASP9*, *PTGS2*, *BAX*, *CASP8*, caspase-3 (*CASP3*), and HSP90AA1 were obtained (Fig. 3B). Moreover, we conducted functional enrichment analysis on the 9 hub genes through the DAVID database. GO analysis

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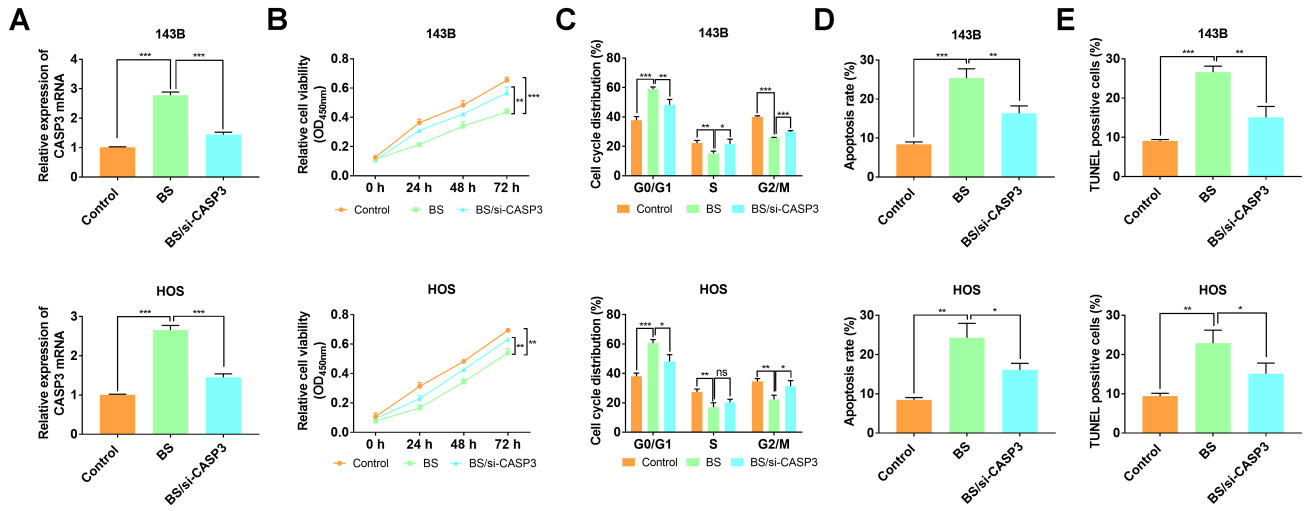

Fig. 5. Caspase-3 knockdown reverses the effects of β-sitosterol on osteosarcoma cell proliferation and apoptosis. (A) For control group, transfection of control siRNA was performed, and dimethyl sulfoxide (DMSO) was used to treat the cells. For BS group, transfection of control siRNA was performed, and β-sitosterol dissolved in DMSO was used to treat the cells. For the BS/si-CASP3 group, transfection of si-CASP3 was performed, and β-sitosterol dissolved in DMSO was used to treat the cells. The transfection efficiency was determined by qRT-PCR assay. (β-sitosterol: 80 μM). (B) CCK-8 was applied to detect the viability of 143B and HOS cells after β-sitosterol treatment and transfection of si-CASP3. (C) Flow cytometry analysis was used to evaluate 143B and HOS cell cycle distribution after β-sitosterol treatment and transfection of si-CASP3. (D) Flow cytometry was applied to detect the apoptosis of 143B and HOS cells after β-sitosterol treatment and transfection of si-CASP3. (E) TUNEL assay was applied to detect 143B and HOS cell apoptosis after β-sitosterol treatment and transfection of si-CASP3. ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

showed that the biological processes of these target genes were mainly enriched in apoptotic signaling pathways, etc.; the cellular components were mainly enriched in cytosol, mitochondrial outer membrane; the molecular functions were mainly enriched in protein homodimerization activity, cysteine-type endopeptidase activity involved in apoptotic process, etc. (Fig. 3C). KEGG enrichment analysis also indicated that these target genes were associated with apoptosis, p53 signaling pathway, TNF signaling pathway, etc., and it showed that caspase-3-enriched pathways were probably the most enriched (Fig. 3D). Therefore, we selected caspase-3 for further analysis.

β-Sitosterol can Positively Regulate CASP3 Expression and Activation

To verify whether β-sitosterol can regulate caspase-3 expression in HOS and 143B cells, we treated the cells with β-sitosterol and detected caspase-3 expression level via qRT-PCR and Western blotting, and caspase-3 expression was remarkably elevated in β-sitosterol treatment group (Fig. 4A,B), indicating that β-sitosterol could facilitate cleaved caspase-3 expression in osteosarcoma cells, which was consistent with the results of bioinformatics analysis.

Caspase-3 Knockdown can Counteract the Impacts of β-Sitosterol on Osteosarcoma Cell Growth and Apoptosis

Given that β-sitosterol could modulate cleaved caspase-3 expression, we next needed to verify whether caspase-3 partakes in the regulatory effects of β-sitosterol on osteosarcoma cells. We transfected si-CASP3 into HOS and 143B cells to construct a caspase-3 knockdown model, which was shown by qRT-PCR to be successful (Fig. 5A). CCK-8 assay indicated that compared with β-sitosterol treatment group, the cell viability of the si-CASP3 transfection group was remarkably elevated (Fig. 5B). The proportion of cells at the G0/G1 phase in the si-CASP3 transfection group was markedly lower compared with that of β-sitosterol treatment group (Fig. 5C). Flow cytometry showed that caspase-3 knockdown could dramatically counteracted the promoting effect of β-sitosterol on 143B and HOS cell apoptosis compared with β-sitosterol treatment group (Fig. 5D). Furthermore, TUNEL assay showed that compared with β-sitosterol treatment group, the number of apoptotic cells after si-CASP3 transfection was markedly lower (Fig. 5E). Thus, these findings indicate that caspase-3 knockdown reverses the suppressing effect of β-sitosterol on the malignant phenotypes of osteosarcoma cells.

Discussion

Mounting evidence suggests that compounds derived from natural products can serve as adjuvant therapy after surgery, chemotherapy, radiotherapy, or other types of treatment, and have the virtues of relatively low toxicity and high efficiency, which can reduce the limitations and disadvantages of chemotherapy and radiotherapy, showing promising therapeutic effect for cancer patients worldwide [16]. In the present work, for the first time, we report that β-sitosterol has tumor-suppressive effects on osteosarcoma. We report that β-sitosterol osteosarcoma represses the viability of osteosarcoma cells in a dose-dependent manner. Additionally, we report that β-sitosterol can suppress the malignancy of cancer cell via modulating the expression of casepase 3.

β-sitosterol is a biologically active compound naturally present in plant cell membranes, and has diverse biological effects such as antibacterial effect, antioxidant effect, anti-inflammatory effect, analgesic effect, immune regulation, and tumor inhibition [17]. In rat model for intracranial aneurysm, β-sitosterol can repress inflammatory responses by decreasing the levels of pro-inflammatory factors including tumor necrosis factor-α, and significantly reduce the size of intracranial aneurysm in rats [11]. Previous research also suggest that β-sitosterol can play an inhibitory role in various tumors. In pancreatic cancer, β-sitosterol may be used in combination with gemcitabine to restrain tumor cell growth and epithelial-mesenchymal transition and induce cell apoptosis by inhibiting the Akt/GSK-3β signaling pathway, therefore exhibiting synergistic anti-pancreatic cancer activity [12]. In colorectal cancer, β-sitosterol can increase the sensitivity of cancer cells to oxaliplatin by repressing the expression of breast cancer resistance protein, and in the xenograft mouse model, β-sitosterol and oxaliplatin exert a synergistic tumor-suppressing effect *in vivo* [13]. In this study, we confirmed that β-sitosterol could restrain osteosarcoma cell proliferation and induce apoptosis through a series of *in vitro* experiments, which is consistent with the previous studies focusing on other types of human malignancies [12,13]. Our data suggest that β-sitosterol can exert antitumor activity and suppress osteosarcoma tumorigenesis and development, and may be a novel adjuvant drug for osteosarcoma.

In this study, we analyzed β-sitosterol's target genes that can play a part in osteosarcoma by bioinformatics, identified hub genes through establishing a PPI network, and then carried out a functional enrichment analysis of these hub genes. GO and KEGG results showed that the hub genes were highly correlated with apoptosis pathways. Apoptosis is considered the most important form of programmed cell death, and inhibition of apoptosis can lead to the tumorigenesis of multiple cancers [18]. We chose caspase-3 as the target of β-sitosterol in the present work. caspase-3, a cysteinyl aspartate specific proteinase, is a pivotal protein in modulating cell apoptosis; caspase-3 can mediate extrinsic and intrinsic cell death signaling pathways and participate in the development of various tumors [19]. Many studies have indicated that caspase-3 plays a crucial role in osteosarcoma progression. Specifically, methyltransferase 14 overexpression facilitates osteosarcoma cell apoptosis by activating caspase-3, thus blocking osteosarcoma progression [20]. Sevoflurane can inhibit cell viability via increasing the activity of caspase-3, thus exerting a tumor-suppressive effect on osteosarcoma cells [21]. GSK343 can induce osteosarcoma cell apoptosis via elevating caspase-3 expression [22]. Our present study found that β-sitosterol could remarkably facilitate cleaved caspase-3 expression, and caspase-3 knockdown could partly weaken β-sitosterol's effects on osteosarcoma cell proliferation and apoptosis. This suggests that β-sitosterol can partake in osteosarcoma progression by positively modulating caspase-3 expression.

There are some limitations of our study. First of all, in the present work, only *in vitro* model of osteosarcoma was established, and to further elucidate the tumor-suppressive role of β-sitosterol in osteosarcoma, the *in vivo* models are required in the following work, and the safety and effectiveness of β-sitosterol should be validated by clinical trials. Secondly, even though we found that caspase-3 was modulated by β-sitosterol, the detailed mechanism by which β-sitosterol regulates caspase-3 is still unclear, molecular docking and high performance liquid chromatography-mass spectrometric technologies (HPLC-MS) are helpful to further investigate whether β-sitosterol modulates caspase-3 directly or indirectly. Notably, our bioinformatics analysis also suggests that β-sitosterol can probably modulate caspase-8 and caspase-9, two other crucial players in apoptosis, and this should be explored in the following work, which will help further clarify the relationship between β-sitosterol treatment and death of cancer cells.

Conclusion

To sum up, our study confirms that β-sitosterol can inhibit osteosarcoma cell proliferation and induce the apoptosis by promoting caspase-3 expression, thus suppressing osteosarcoma progression. β-sitosterol may serve as a potentially effective natural medicine for treating osteosarcoma, and it also provides new perspectives for the clinical treatment of osteosarcoma.

Availability of Data and Materials

All data included in this study are available upon request by contact with the corresponding author (YX).

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Author Contributions

LL designed the experiments. LL, JD, CH, BG, and YX participated in data analysis. LL prepared the draft. 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

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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