Oxyresveratrol induces apoptosis and inhibits cell viability via inhibition of the STAT3 signaling pathway in Saos-2 cells

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Abstract. Oxyresveratrol (ORES) is a natural phenolic compound with multiple biological functions including antioxidation, anti-inflammation and neuroprotection; however, the inhibitory effect of ORES on osteosarcoma remains largely unknown. The present study aimed to determine the effects of ORES on osteosarcoma cell Saos-2. Cell Counting Kit-8 assay was performed to detect Saos-2 cell viability. Annexin-FITC/PI staining and JC-1 staining were used to measure cell apoptosis and the change of mitochondrial membrane potential. In addition, western blotting was conducted to determine the expression levels of apoptotic proteins and the phosphorylation of STAT3. It was found that ORES inhibited cell viability and induced apoptosis of osteosarcoma Saos-2 cells in a concentration-dependent manner. In addition, ORES increased the expression levels of apoptotic proteases caspase-9 and caspase-3 and reduced mitochondrial membrane potential. In response to ORES treatment, the expression levels of pro-apoptotic proteins, Bad and Bax, were enhanced, whereas those of anti-apoptotic proteins, Bcl-2 and Bcl-xl, were reduced. In addition, the phosphorylation of STAT3 was attenuated in Saos-2 cells after treatment with ORES. Inhibition of cell viability and apoptosis induction by ORES were rescued by enhancement of STAT3 activation upon treatment with IL-6. Collectively, the present study indicated that ORES induced apoptosis and inhibited cell viability, which may be associated with the inhibition of STAT3 activation; thus, ORES represents a promising agent for treating osteosarcoma.

Introduction

Osteosarcoma is the most common primary malignant bone tumor of mesenchymal origin that occurs primarily in children and adolescents (1). It is characterized by the production of calcified osteoid extracellular matrix and a high frequency of inducing lung metastases, which directly results in a high mortality rate (2). The 5-year survival rate of patients with local osteosarcoma ranges between 65 and 75%, whereas that of patients with metastatic osteosarcoma is <30% (3). Surgical resection, chemotherapy and radical resection are established strategies for treating patients with osteosarcoma (4,5). Immunotherapy or a combination of radiotherapy and immunotherapy using nanomaterial delivery systems have also been a research hotspot in the field (6-8). However, the 5-year survival rate of patients with osteosarcoma demonstrates little improvement after treatment with chemotherapy drugs and traditional amputation (4). Therefore, it is important to identify new effective agents for treating patients with osteosarcoma.

Certain malignant tumors present an anti-apoptotic phenotype, which allows cancer cells to survive in local tissues (9). Most of the current anticancer medicines target apoptotic signaling pathways (10). Activation of caspases can be initiated via both the extrinsic and the mitochondria-mediated apoptotic signaling pathways (10-12). The extrinsic pathway is triggered by activation of caspase-8 at the plasma membrane upon ligation of the death receptor, and subsequent cleavage of downstream effector caspases, such as caspase-3 (10). The mitochondria-mediated signaling pathway relies on interactions among Bcl-2 family proteins at the mitochondrial outer membrane and the release of cytochrome c (11). Among Bcl-2 family proteins, the activators directly bind both anti-apoptotic proteins and pro-apoptotic effector proteins, while sensitizers such as Bad bind only anti-apoptotic proteins (11,12). By competing for the BH3 binding site, sensitizers displace the binding of activators to anti-apoptotic proteins, including Bcl-2 and Bcl-xL (11). By interacting with the activators, pro-apoptotic effector proteins such as Bax and Bak create openings in the outer mitochondrial membrane and release cytochrome c, which promotes caspase-9 activation, followed by a cascade of apoptotic proteases, including caspase-3, resulting in cell death (12). The increased permeability of the mitochondrial outer membrane also results in mitochondrial depolarization with reduced mitochondrial membrane potential (MMP) and

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the release of apoptotic factors, triggering the downstream cell death processes (13-15). Venetoclax, a direct small molecular inhibitor of Bcl-2, was initially approved by the Food and Drug Administration (FDA) in April 2016 and exhibited impressive outcomes particularly in chronic lymphocytic leukemia, small lymphocytic lymphoma and acute myeloid leukemia (16).

STAT3-mediated signaling pathways have an oncogenic potential in cancer cell proliferation and metastasis (17). The expression levels of STAT3 have been reported to be significantly higher in osteosarcoma tissue compared with those in normal bone or cartilage tissue, and high levels of STAT3 are associated with poor tumor differentiation and metastasis (18). Five-year overall survival and relapse-free survival rates in patients with high STAT3 expression levels are lower compared with those with low STAT3 expression levels (18). Inhibition of STAT3 has also been reported to suppress osteosarcoma cell growth and induce apoptosis (19). Therefore, STAT3 may have an important therapeutic and prognostic value in osteosarcoma. In addition, STAT3 is directly involved in transcriptional regulation of osteopontin (OPN) (20), and increased OPN expression levels serve a critical role in osteosarcoma, which is a potential biomarker and promising drug target for osteosarcoma (21).

As a natural ingredient, which is primarily found in *Morus alba L.*, oxyresveratrol (ORES) has extensive biological effects. Over the previous two decades, ORES has been reported as a powerful tyrosinase activity inhibitor (22,23), and also as having antioxidative (24,25), anti-inflammatory (26,27), anti-cancer (28-30) and anti-lipogenesis properties (31). ORES has also been observed to exert strong neuroprotective effects, as it reduces neuronal oxidative damage (32,33). Notably, ORES and its derivatives have been reported to serve an efficient role against various types of cancer, such as head and neck carcinoma (28), neuroblastoma (29), prostate (30), kidney (34) and lung cancer (35). However, it remains unknown whether ORES has an effect on the inhibition of osteosarcoma cells and the mechanism by which ORES inhibits tumor cell viability.

In the present study, the inhibitory effect of ORES on Saos-2 osteosarcoma cells was determined, which indicates the ORES is a promising agent for treating osteosarcoma.

Materials and methods

**Compound and reagents.** ORES (2',3',4',5'-Tetrahydroxyl-trans-stilbene, C14H12O2; molecular weight: 244.24; purity ≥97.0%; cat. no. 29700-22-9) was purchased from Sigma-Aldrich (Merck KGaA). DMSO was used as control. DMEM, penicillin and streptomycin solution (100 IU/ml; 100 μg/ml), PBS, 0.25% trypsin-EDTA and enhanced chemiluminescent (ECL) substrate were all provided by Thermo Fisher Scientific, Inc. Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. Cell Counting Kit (CCK)-8 assay kit, MMP assay kit with JC-1, biocinchonic acid (BCA) protein assay kit and RIPA lysis buffer (cat. no. P0013B) were acquired from Beyotime Institute of Biotechnology. Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was purchased from MultiSciences (Lianke) Biotech Co., Ltd. Tris, non-fat milk and Tween-20 were purchased from Sangon Biotech Co., Ltd. IL-6 was purchased from PeproTech, Inc. Primary antibodies against cleaved caspase-9 (cat. no. 20750), cleaved caspase-3 (cat. no. 9664), GAPDH (cat. no. 5174), Bcl-2 (cat. no. 4223), Bcl-xL (cat. no. 2764), Bad (cat. no. 9239), Bax (cat. no. 5023), phophorylated-STAT3 (P-STAT3; cat. no. 9145) and total-STAT3 (T-STAT3; cat. no. 12640) were obtained from Cell Signaling Technologies, Inc. An antibody against OPN (cat. no. 7C5H12) was obtained from Thermo Fisher Scientific, Inc. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (cat. no. 115-035-003) and HRP-conjugated anti-mouse antibody (cat. no. 115-035-003) were supplied by Jackson ImmunoResearch Laboratories, Inc. Methanol and ethanol were obtained from Sinopharm Chemical Reagent Co., Ltd.

**Cell culture and cell viability assay.** Saos-2 cells were obtained from the American Type Culture Collection. Cells were grown in DMEM containing 10% FBS and 1% penicillin and streptomycin solution with 5% CO2 at 37°C. Cell passage was performed with 0.25% trypsin-EDTA.

Cell viability was detected using the CCK-8 assay kit according to the manufacturer's instructions. Briefly, cells were seeded in 12-well plates at a density of 4x10⁴ cells/well. After attachment, the cells were incubated with ORES at 0, 5, 15 and 45 μM at 37°C for 48 h. The CCK-8 solution (10 μl) was added to each well and after 1.5 h incubation, the viability of Saos-2 cells was detected using a microplate reader at 450 nm. In certain experiments, the Saos-2 cells were incubated with 30 μM ORES or 30 μM ORES and 20 ng/ml IL-6 at 37°C for 24 h.

**Apoptosis assay.** Early and late apoptosis was detected using the Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instructions. Cells were seeded in 12-well plates at a density of 4x10⁴ cells/well. After attachment, the cells were incubated with ORES at 0, 5, 15 and 45 μM at 37°C for 24 h. The cells were then washed twice with ice-cold PBS. After digestion with 0.25% trypsin-EDTA, cells were collected and washed with PBS. Cells were resuspended in 400 μl Annexin-binding buffer, and then incubated with 5 μl Annexin V-FITC and 10 μl PI at room temperature for 5 min. Apoptosis of Saos-2 cells was detected with a LSRFortessa TM X-20 flow cytometer (BD Biosciences). Given that ORES significantly inhibited Saos-2 cell viability at the concentration between 15 and 45 μM, the Saos-2 cells was incubated with 30 μM ORES or 30 μM ORES and 20 ng/ml IL-6 at 37°C for 24 h. FlowJo 7.6 software (Tree Star, Inc.) was used for data processing.

**MMP measurement.** Changes in MMP were detected using the MMP assay kit with JC-1 staining, according to the manufacturer's instructions. Cells were seeded in 12-well plates at a density of 4x10⁴ cells/well. After attachment, cells were incubated with ORES at 0, 5, 15 and 45 μM at 37°C for 24 h. The cells were then harvested and washed with PBS. After incubation with JC-1 staining solution for 20 min at 37°C in the dark, the cells were washed twice with JC-1 buffer. Finally, cells were resuspended in PBS and detected using a LSRFortessa TM X-20 flow cytometer (BD Biosciences). FlowJo 7.6 software (FlowJo LLC) was used for data processing. The polymer/monomer (red/green) fluorescence intensity ratio was used to quantify the MMP.
Western blotting. Saos-2 cells were seeded in 6-well plates at 1x10^6 cells/well overnight at 37°C, and were then incubated with ORES at 0, 5, 15 and 45 µM at 37°C for 24 h. In some experiments, the Saos-2 cells were incubated with 30 µM ORES or 30 µM ORES and 20 ng/ml IL-6 at 37°C for 24 h. After washing with cold phosphate-buffered saline (PBS) three times, cells were lysed in RIPA lysis buffer and the protein concentration was detected using a BCA protein assay kit, according to the manufacturer’s instructions. Each sample was adjusted to the same concentration with lysis buffer and 20 µg protein was loaded per lane. Proteins were then separated by SDS-PAGE on 10% gels and were transferred to 0.45 µm nitrocellulose filter membranes. The membranes were washed and blocked in blocking buffer (5% non-fat milk) for 1 h at room temperature. Subsequently, the membranes were incubated with primary antibodies against cleaved caspase-9, cleaved caspase-3, GAPDH, Bcl-2, Bcl-xL, Bad, Bax, P-STAT3, T-STAT3 and OPN (diluted at 1:1,000) with gentle agitation overnight at 4°C. After three washes, the membrane was incubated with HRP-conjugated anti-rabbit secondary antibody (diluted at 1:3,000) with gentle agitation for 2 h at room temperature. Finally, the ECL reagent was used to detect the target proteins. Semi-quantification of the bands was performed using ImageJ software (1.48v; National Institutes of Health).

Statistical analysis. The experiments were repeated at least three times independently, and data were presented as the mean ± SEM. One-way ANOVA followed by Tukey's post hoc test was applied to compare the differences between groups, using GraphPad Prism 5 (GraphPad Software, Inc.). *P<0.05 was considered to indicate a statistically significant difference.

Results

ORES inhibits the viability of Saos-2 cells. Although ORES has been shown to inhibit prostate and colon cancer cell viability (30), it remains unknown whether ORES can inhibit osteosarcoma cell viability. As a natural phenolic compound, ORES is a resveratrol analogue that contains an additional hydroxyl group in the aromatic ring (22). The chemical structure of ORES is shown in Fig. 1A. To determine the effect of ORES on the proliferation of osteosarcoma cells, the cell viability following ORES treatment in the Saos-2 cell line was detected. Cells were incubated with ORES at 0, 5, 15 and 45 µM for 48 h, and cell viability was detected using the Cell Counting Kit-8 assay. (c) Saos-2 cells were incubated with ORES at 0, 5, 15 and 45 µM for 24 h. Cells were then collected and underwent Annexin-FITC/PI staining. Apoptosis was measured with flow cytometry and Annexin-FITC positive cells were identified as apoptotic cells. (D) Percentage of Saos-2 apoptotic cells was quantified. The experiments were repeated at least three times and the results are shown as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001. ORES, oxyresveratrol; PI, propidium iodide.

Figure 1. ORES inhibits cell proliferation and induces apoptosis of Saos-2 cells. (A) Chemical structure of ORES. (B) Saos-2 cells were incubated with ORES at 0, 5, 15 and 45 µM for 48 h. Cell viability was detected using the Cell Counting Kit-8 assay. (C) Saos-2 cells were incubated with ORES at 0, 5, 15 and 45 µM for 24 h. Cells were then collected and underwent Annexin-FITC/PI staining. Apoptosis was measured with flow cytometry and Annexin-FITC positive cells were identified as apoptotic cells. (D) Percentage of Saos-2 apoptotic cells was quantified. The experiments were repeated at least three times and the results are shown as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001. ORES, oxyresveratrol; PI, propidium iodide.

A decrease in MMP is a marker of early apoptosis. In the mitochondria of normal cells, JC-1 is present as a polymer with bright red fluorescence. Once the mitochondria-mediated apoptotic pathway is initiated, JC-1 cannot aggregate and the JC-1 monomer generates green fluorescence, according to the manufacturer’s instructions. The relative ratio of polymer/monomer (red/green) fluorescence was used to measure the mitochondrial depolarization. In response to ORES treatment at 0, 5, 15 and 45 µM for 24 h, cells were clearly divided into two groups, with polymer at the top and monomer at the bottom (Fig. 2A). The ratio of JC-1 polymer/monomer significantly decreased in a concentration-dependent manner in response to ORES compared with that of the untreated group (Fig. 2B).

In the mitochondria-mediated pathway, cytochrome c is released from mitochondria and induces the cleavage of caspase-3 and caspase-9, which ultimately triggers apoptosis via proteolysis of several cytoskeletal proteins, as well as the cleavage of DNA via caspase-activated DNase (12). The results of the present study demonstrated that compared with those of the untreated group, the expression levels of cleaved caspase-9 and cleaved caspase-3 were significantly increased by ORES treatment in a concentration-dependent manner (Fig. 3A-C). These results suggested that ORES induces osteosarcoma apoptosis.

ORES modulates apoptotic signaling pathways in Saos-2 cells. Given that ORES was demonstrated to promote apoptosis, the mechanism by which ORES regulates the apoptotic
signaling pathways was further investigated. Western blotting was used to detect the expression levels of Bcl-2 family proteins, including Bcl-2, Bcl-xL, Bad and Bax (Fig. 4A). Mitochondria-mediated apoptosis is regulated by Bcl-2 family proteins (10). Pro-apoptotic sensitizer protein Bad can bind anti-apoptotic proteins Bcl-2 and Bcl-xL, which cause pro-apoptotic activator proteins to dissociate from these anti-apoptotic proteins and then bind to pro-apoptotic effector proteins, such as Bax, to initiate apoptosis (11). Compared with those of the untreated group, following treatment with oreS, the expression levels of anti-apoptotic proteins Bcl-2 (Fig. 4B) and Bcl-xL (Fig. 4C) were significantly decreased, whereas the expression levels of pro-apoptotic proteins Bad (Fig. 4D) and Bax (Fig. 4E) were significantly increased. These results suggested that oreS may regulate the mitochondria-mediated apoptotic signaling pathway in Saos-2 cells.

ORES induces apoptosis and inhibits cell viability via STAT3 phosphorylation in Saos-2 cells. Given that STAT3 serves an important role in the proliferation and apoptosis of osteosarcoma cells (17), STAT3 activation in Saos-2 cells upon treatment with oreS was further examined. Compared with those of the untreated group, after treatment with oreS at 5, 15 and 45 µM for 24 h, the expression levels of P-STAT3 were significantly reduced in a concentration-dependent manner (Fig. 5A and B), whereas no significant differences were observed in the expression levels of T-STAT3 (Fig. 5A and C). OPn is regulated by STAT3 signaling, which is involved in osteosarcoma pathogenesis (21). Compared with those of the untreated group, treatment with oreS significantly decreased the expression levels of OPn (Fig. 5A and D). To determine whether oreS inhibits Saos-2 cell viability via a reduction in STAT3 activation, IL-6 was used to activate STAT3 signaling (36) in Saos-2 cells and reverse the effects of oreS. Upon treatment with 30 µM oreS for 24 h, the phosphorylation of STAT3 was induced by co-stimulation with 20 ng/ml IL-6 (Fig. 6A and B). After incubation with 20 ng/ml IL-6, the effects of oreS on the percentage of apoptotic cells (Fig. 6C and D) and cell viability (Fig. 6E) were reversed. These results indicated that oreS induces apoptosis and inhibits cell viability via partially reducing STAT3 signaling in Saos-2 cells.

**Discussion**

The results of the present study demonstrated that oreS effectively inhibited cell viability and induced apoptosis of Saos-2 cells. Upon treatment with oreS, the expression levels of cleaved caspase-9 and cleaved caspase-3 were significantly decreased, whereas the expression levels of Bcl-2 and Bcl-xL were significantly increased.
increased. Additionally, the expression levels of anti-apoptotic proteins Bcl-2 and Bcl-xL were decreased, whereas the expression levels of the pro-apoptotic proteins Bad and Bax were upregulated. These results indicated that ORES has specific antitumor activity against Saos-2 cells.

ORES is a natural compound derived from Morus alba L., and its roles have been revealed in various disease models. In macrophages, ORES has been proven to suppress LPS-stimulated inflammatory responses by blocking the MAPK signaling pathway (26). In addition to its anti-inflammatory effects, ORES significantly inhibited the proliferation of hepatocellular carcinoma (HCC) cells (37), and head and neck squamous cell carcinoma cells (28). In both cases, the expression levels of vascular endothelial growth factor (VEGF) were inhibited by ORES treatment, which indicates that ORES may reduce micro-blood vessel density and micro-lymphatic vessel density in tumors. As VEGF expression is regulated directly by STAT3 in diverse human cancer cell lines (38), in addition to inhibition of cell viability, ORES may target STAT3-mediated VEGF expression in osteosarcoma. The inhibitory concentrations of ORES in various tumor cells were different. The IC₅₀ value of ORES in T24 bladder cancer cells was 47.46 µM (39). In HCC cells, the IC₅₀ values were nearly 80 µM (37). As for neuroblastoma cells, the IC₅₀ values reached 140 µM, with little inhibitory effect against non-cancer cells, such as Rat-2, HEK293, NIH3T3 and human peripheral blood mononuclear cells (29). Therefore, Saos-2 cells were relatively sensitive to ORES with an IC₅₀ of <15 µM. In view of the broad biological activities of ORES, especially its significant antitumor effects, further research should focus on both cancer cells and tumor-surrounding cells, including endothelial cells and immune cells.

It has been reported that multiple signaling pathways influence the process of osteosarcoma cell growth and anti-apoptosis, which can be interfered with using small molecule compounds (40-42). For instance, caffeine has been shown to induce apoptosis of osteosarcoma cells by inhibiting the AKT/mTOR/ribosomal protein S6 kinase, NF-kB and MAPK pathways (40). Glycogen synthase kinase 3 inhibitors have also been demonstrated to inhibit proliferation in osteosarcoma cell lines U2OS and MG-63 in vitro (41). Messerschmitt et al (42) reported that specific tyrosine kinase inhibitors, such as inhibitors of EGF-R, insulin-like growth factor 1 and Met, decreased motility and colony formation in human osteosarcoma cell lines. Signaling pathways, such as the hedgehog (43), Wnt (44) and PI3K/AKT signaling pathways (45), are all involved in osteosarcoma proliferation. Notably, recently
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discovered types of cell death, such as receptor-interacting protein (RIP)1- and RIP3-dependent necroptosis, as well as Ca2+/calmodulin-dependent protein kinase type II activity-related autophagic degradation, have been considered as efficient anti-ostEOSARCOMA strategies in recent research (46,47). Resveratrol, which is structurally similar to ORES, has been shown to induce the apoptosis of osteosarcoma cells via modulating the microRNA139-5p/NOTCH1 signaling pathway (48). Additionally, resveratrol can inhibit osteosarcoma MG-63 and MNNG/HOS cell proliferation and tumorigenesis via blocking janus kinase (JAK)2/STAT3 signaling (49). Consistent with this, in the present study, as a potent tyrosinase inhibitor, ORES inhibited the growth of osteosarcoma cells and promoted apoptosis by affecting the STAT3 signaling pathway, which indicated that STAT3 may be another drug target for ORES.

STAT3 has already been confirmed to serve a crucial role in selectively inducing and maintaining a carcinogenic inflammatory microenvironment in response to cytokine stimulation (17). The IL-6-JAK-STAT3 signaling axis contributes to the pathogenesis of myeloma cells by preventing apoptosis (50). By blocking the IL-6-OPN-STAT3 signaling pathway, the viability and tumorigenesis of osteosarcoma cells are inhibited (21). This is consistent with the findings of the present study, which demonstrated that compared with in the control group, the phosphorylation of STAT3 was attenuated by ORES treatment in Saos-2 cells. In addition, when P-STAT3 was partially activated by IL-6, the inhibition of cell viability and apoptosis promotion induced by ORES were also rescued. As a major cytokine in the tumor microenvironment, IL-6 protects cancer cells against apoptosis via multiple signaling pathways (51). The rescue of STAT3 phosphorylation may explain in part the reversal effect of ORES. Given that completely blocking STAT3 was demonstrated to be a risk factor causing other diseases, such as hyper-immunoglobulin E syndrome (52), inhibition of STAT3 should be under strict control. Local suppression of STAT3 for a limited period of time may convert inflammation in the tumor microenvironment from promoting tumor growth to inhibition without serious side effects (17). FDA-approved tyrosine kinase inhibitors, such as sorafenib (53) and sunitinib (54), are already in clinical use and can indirectly inhibit STAT3 signaling, resulting in tumor cell cycle arrest and the induction of apoptosis. The development of new STAT3 inhibitors may inhibit the viability and survival of multiple tumor cells.

In conclusion, the present study demonstrated the interaction between ORES and osteosarcoma. ORES induced apoptosis and inhibited cell viability by reducing STAT3 signaling in Saos-2 cells. More comprehensive studies of ORES may establish it as a promising agent for the future treatment of osteosarcoma.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

BY designed the project and drafted the manuscript. TL and ZJ performed the experiments and drafted the manuscript.

Figure 6. STAT3 activation rescues ORES-induced apoptosis and inhibits proliferation of Saos-2 cells. Saos-2 cells were incubated with 30 µM ORES or 30 µM ORES and 20 ng/ml IL-6 for 24 h. (A) P-STAT3 and T-STAT3 expression levels were detected via western blotting. (B) Ratio of P-STAT3 to T-STAT3 was semi-quantified with ImageJ software. (C) Apoptosis of Saos-2 cells was identified by staining with Annexin V-FITC/PI and detected using flow cytometry. (D) Percentage of apoptotic cells was quantified. (E) Cell viability was measured using the Cell Counting Kit-8 assay. The experiments were repeated three times and the results are shown as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001. ORES, oxyresveratrol; P-STAT3, phosphorylated STAT3; T-STAT3, total STAT3; PI, propidium iodide.
DL, RA and XZ participated in data analysis and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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