The cyclooxygenase-2 inhibitor NS-398 inhibits proliferation and induces apoptosis in human osteosarcoma cells via downregulation of the survivin pathway

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Abstract. Cyclooxygenase-2 (COX-2) is frequently overexpressed in human malignancies and plays a crucial role in tumorigenesis and cancer progression. The present study aimed to investigate the expression and clinical significance of COX-2 and survivin (SUV) in human osteosarcomas (OS), and explore the effects and molecular mechanisms of a selective COX-2 inhibitor NS-398 and SUV on tumor proliferation and apoptosis. Fifty cases of human OS and osteochondromas (OC) were collected. The expression of COX-2 and SUV was assessed using immunohistochemical assays in biopsy samples. MG-63 human OS cells were treated with different concentrations of NS-398, used to investigate their effects on cell proliferation and apoptosis. The recombinant small hairpin RNA adenovirus vector rAd5-SUV was constructed, and the effects and molecular mechanisms of knockdown of SUV on proliferation and apoptosis were evaluated in MG-63 cells. A subcutaneous xenograft tumor model was established, validating the effects of rAd5-SUV on tumor growth in vivo.

Based on the results, the expression of COX-2 and SUV in OS showed a higher strong reactivity rate compared with OC (73.3 vs. 25.0%, P=0.001; 63.3 vs. 30.0%, P=0.02), but it did not correlate with the clinicopathological characteristics of OS. NS-398 inhibited proliferation, induced apoptosis and decreased the mRNA expression of COX-2 and SUV in MG-63 cells. Furthermore, adenovirus-mediated knockdown of SUV inhibited proliferation, induced apoptosis, reduced the expression of proliferating cell nuclear antigen (PCNA), increased the expression of caspase-3 (CAS-3) and slowed the growth of xenograft tumors in MG-63 cells. Taken together, the expression of COX-2 and SUV is closely correlated with human OS, and inhibition of COX-2 or knockdown of SUV suppresses tumor proliferation and induces apoptosis, suggesting that COX-2 may be involved in OS cell proliferation and apoptosis through SUV-mediated regulation of PCNA and CAS-3 expression, and provides a potential therapeutic strategy for the treatment of cancer.

Introduction

Osteosarcoma (OS) is a malignant bone tumor that typically occurs in children, adolescents and young adults. Incorporation of chemotherapy into initial treatment significantly increases the cure rate. However, ~40% of patients still die from lung metastases (1,2). So, it is very important to develop biomarkers that can inform therapy and provide prognostic insight, especially into identifying poor prognosis patients who should be offered more aggressive therapy at an early time-point in the clinical continuum (3,4). Cancer is also a genetic disease developing from a multi-step process. Single or multiple mutations in genes related to growth control, invasion and metastasis form the molecular genetic basis of malignant transformation and tumor progression (5). Therefore, identification of key genes and targets related to tumorigenesis is crucial for OS prevention and treatment.

Cyclooxygenase-2 (COX-2) is an enzyme catalyzing the conversion of arachidonic acid and O₂ to prostaglandin H₂, the committed step in prostanoid biosynthesis. The major final end product is prostaglandin E₂ (PGE₂). COX-2 expression is induced by various stimuli, and the overexpression is closely related to the pathogenesis of some degenerative diseases including cancer (6). COX-2 expression is found increased in metastatic rhabdomyosarcoma, leiomyosarcoma and OS, and can be considered as a prognostic value and a target for adjuvant therapy (7-9). COX-2 is highly expressed in high grade OS and application of COX-2 inhibitors may improve the tumor response to chemotherapy and the outcome of OS patients (10). Moreover, COX-2 is directly associated with the proliferation, migration and invasion in human OS cells, and the therapeutic value of COX-2 inhibitors should be evaluated continuously (11). COX-2 expression correlates inversely with disease-specific survival in
patients with OS lung metastases, indicating that COX-2 expression in metastatic OS may have prognostic significance (12).

Intriguingly, researchers hold different views towards the prognosis of COX-2 in OS. COX-2 overexpression in the primary tumor correlates with the occurrence of distant metastasis in patients with OS; COX 2 is a valuable diagnostic marker for OS (13-15). However, it has been proven that there is no significant relationship between COX-2 expression and clinical outcome (16). COX-2 expression does not correlate with outcome of OS or rhabdomyosarcoma (17).

Therefore, it is indispensable to further elucidate the function and molecular regulatory mechanisms of COX-2 in OS. In the present study, the expression and clinical significance of COX-2 and SUV were assessed using immunohistochemical (IHC) assay in biopsy samples. Human MG-63 OS cells were treated with different concentrations of NS-398, used to investigate its effects on cell proliferation and apoptosis. Recombinant small hairpin RNA adenovirus vector rAd5-SUV was constructed, and the effects and molecular mechanisms of knockdown of SUV on proliferation and apoptosis were evaluated in MG-63 cells, attempting to find the potential therapeutic target for the treatment of OS.

Materials and methods

Materials. MG-63 cell line used in the experiment was from the Laboratory of Second Affiliated Hospital of Xi’an Jiaotong University; 6-week-old female immune-deficient nude mice (BALB/c-nu) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai Laboratory Animal Center of Chinese Academy Sciences). Adenovirus-mediated SUV small hairpin RNA vector, negative control vector and virion-packaging elements were from Genechem (Shanghai, China); the primers of COX-2, SUV, PCNA and CAS-3 were synthesized by ABI Co., Ltd. (USA). All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Drugs and reagents. NS-398 was purchased from Cayman Co., Ltd. (USA); 3-(4,5)-dimethylthiahiazo(-z-yl)-3,5-di-phenytrezoliumbromide (MTT) was from Dingguo Biology (Shanghai, China); Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were from Thermo Fisher Scientific Inc. (Waltham, MA, USA); TRIZol reagent and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA, USA); M-MLV Reverse Transcriptase was from Promega (Waltham, MA, USA); pCMV-β-Gal was from Clontech Lab., Inc. (USA). rAd5-SUV virus vectors transfected was named as rAd5-SUV and negative control rAd5-GFP were transfected into MG-63 cells. Cells were subcultured at a 1:5 dilution in 300 µg/ml G418-containing medium. Positive stable transfectants were selected and expanded for further study. The clone in which the rAd5-SUV virus vectors transfected was named as rAd5-SUV group, the negative control vectors transfected was named as GFP group and MG-63 cells as CON group.

Cell culture and adenovirus transfection. MG-63 cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin. They were all placed in a humidified atmosphere containing 5% CO₂ at 37°C. Recombinant adenovirus vector rAd5-SUV and negative control rAd5-GFP were transfected into MG-63 cells. Cells were subcultured in a 1:5 dilution in 300 µg/ml G418-containing medium. Positive stable transfectants were selected and expanded for further study. The clone in which the rAd5-SUV virus vectors transfected was named as rAd5-SUV group, the negative control vectors transfected was named as GFP group and MG-63 cells as CON group.

RT-PCR. To quantitatively determine the mRNA expression level of COX-2, SUV, PCNA and CAS-3 in MG-63 cells, RT-PCR was used. Total RNA of each clone was extracted with TRizol according to the manufacturer’s instructions. Reverse-transcription was carried out using M-MLV and cDNA amplification was carried out using SYBR Green Master Mix kit according to the manufacturer’s instructions. The genes were amplified using specific oligonucleotide primer and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control. The PCR primer sequences were as follows: COX-2, 5’-GAAGTACCAAGCTGTGGTTGAATAA-3’ and 5’-GGCTTGATTCCAATGCACCTA-3’; SUV, 5’-ACCAGGTGAGAAGTGAGGGA-3’ and 5’-AACAGTAGGGGAGCGGCAAGGA-3’; PCNA, 5’-CCATCCTCAAGAAGGTATTCTTGAATGACC-3’ and 5’-TTGTTACCCATTTGTGTTTTA-3’; GAPDH, 5’-CAACGAATTTTGCGTCACAGCA-3’ and 5’-AGGGTTCTACATGGCAACTG-3’. Data were analyzed using the comparative Ct method (2^ΔΔCt). Three separate experiments were performed for each clone.

IHC staining. Formalin-fixed tissue samples were prepared as paraffin-embedded sections and stained with hematoxylin and eosin. Unstained sections were deparaffinized and incubated overnight at 4°C with primary antibodies against COX-2 and SUV and with biotinylated secondary antibody at room temperature for 1 h, followed by incubation with ABC peroxidase and 3,3’-diaminobenzidine (DAB; 30 mg dissolved in 100 ml Tris-buffer containing 0.03% H₂O₂). Sections were counterstained with hematoxylin. Expression of COX-2 and SUV in each specimen was scored according to the percentage of positive-stained cells counted in five randomly selected high magnification fields: 0, no expression; 1, positive cell ratio <25%; 2, positive cell ratio 26-50%; and 3, positive cell ratio >50%. The intensity score represents the staining intensity (score 0, no staining signal; score 1, weak positive signal; score 2, moderate positive signal; score 3, strong positive signal). Finally, a total expression score was given ranging from 0 to 12. According to the product of these two indicators scoring the results, they were divided into four levels: score 0-2 is considered as (-), score 3-4 as (+), score 5-7 as (+++) and score 8-9 is considered as (++++).
Western blot assay. MG-63 cells were harvested and extracted using lysis buffer (Tris-HCl, SDS, mercaptoethanol, glycerol). Cell extracts were boiled for 5 min in loading buffer and then equal amount of cell extracts was separated on 15% SDS-PAGE gels. Separated protein bands were transferred into polyvinylidene fluoride (PVDF) membranes and the membranes were blocked in 5% skim milk powder. The primary antibodies against COX-2, SUV, PCNA and CAS-3 were diluted according to the instructions of antibodies and incubated overnight at 4℃. Then, horseradish peroxidase-linked secondary antibodies were added at a dilution ratio of 1:1000, and incubated at room temperature for 2 h. The membranes were washed with PBS three times and the immunoreactive bands were visualized using ECL-PLUS/Kit according to the kit’s instruction. The relative protein level in different cell lines was normalized to GAPDH concentration. Three separate experiments were performed for each clone.

Cell proliferation assay. Cell proliferation was analyzed with the MTT assay. Briefly, cells infected with rAd5-SUV were incubated in 96-well-plates at a density of 1x10⁵ cells per well with DEME medium supplemented with 10% FBS. Cells were treated with 20 µl MTT dye at 0, 24, 48 and 72 h and then incubated with 150 µl of DMSO for 5 min. The color reaction was measured at 570 nm with enzyme immunoassay analyzer (Bio-Rad, USA). The proliferation activity was calculated for each clone.

Cell apoptosis analysis. To detect cell apoptosis, cells were trypsinized, washed with cold PBS and resuspended in binding buffer according to the instruction of the apoptosis kit. FITC-Annexin V and PI were added to the fixed cells for 20 min in darkness at room temperature. Then, Annexin V binding buffer was added to the mixture before the fluorescence was measured on FACsort flow cytometer. The cell apoptosis was analyzed using the CellQuest software (Becton-Dickinson, USA). Three separate experiments were performed for each clone.

In vivo tumor xenograft studies. Four mice were injected subcutaneously with 1x10⁸ MG-63 cells in 50 µl of PBS pre-mixed with an equal volume of matrigel matrix (Becton-Dickinson). Mice were monitored daily, and three out of four mice developed a subcutaneous tumor. When the tumor size reached approximately 5 mm in length, they were surgically removed, cut into 1-2 mm³ pieces, and re-seeded individually into 18 other mice. When tumor size reached ~5 mm in length, the mice were randomly assigned to MG-63, rAd5-GFP and rAd5-SUV groups. In rAd5-GFP and rAd5-SUV groups, 15 µl of adenovirus was injected into subcutaneous tumors using a multi-site injection format. Mice in the MG-63 group received 15 µl of PBS only. Injections were repeated on the third day after initial treatment. The tumor volume every three days was measured with a caliper, using the formula volume = (length x width)²/2.

Statistical analysis. The results of each experiment are shown as mean ± SD when applicable. Statistically significant difference in each assay was determined by SPSS version 11.5. Difference in each group was tested for significance using $\chi^2$ test and ANOVA analysis of variance. P<0.05 was considered significant.

Results

The expression of COX-2 and SUV in human OS. The expression of COX-2 and SUV in OS and OC was evaluated using IHC assays (x400). The results showed that the expression of COX-2 and SUV was mainly localized in the cytoplasm, and was, respectively, observed in 73.3 and 25.0% OS tissues and in 63.3 and 30.0% OC tissues, indicating their higher expression in OS compared with OC.

Table I. The expression of COX-2 and SUV in OS and OC tissues.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sample</th>
<th>n</th>
<th>+</th>
<th>−</th>
<th>Total</th>
<th>Positive rate (%)</th>
<th>$\chi^2$</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>COX-2</td>
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<td>22</td>
<td>30</td>
<td>73.3</td>
<td>11.06</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OC</td>
<td>15</td>
<td>5</td>
<td>20</td>
<td>25.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUV</td>
<td>OS</td>
<td>11</td>
<td>19</td>
<td>30</td>
<td>63.3</td>
<td>5.23</td>
<td>0.02</td>
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<td>OC</td>
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<td>6</td>
<td>20</td>
<td>30.0</td>
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$\chi^2$ test and ANOVA analysis of variance. P<0.05 was considered significant.
The relationship of COX-2 and SUV expression with the clinicopathologic features of OS. The relationship between the expression of COX-2 and SUV and clinicopathologic features was analyzed. As shown in Table II, no significant correlation was found between the expression of COX-2 and SUV with age and pathological grade and types of OS.

**Effects of NS-398 on mRNA expression of COX-2 and SUV in MG-63 cells.** To examine the effects of NS-398 on expression of COX-2 and SUV in MG-63 cells, MG-63 cells were treated with different concentrations of NS-398 (0, 50, 100 and 200 µmol/l), RT-PCR was performed at 48-h recovery to measure their mRNA expression levels. (C) The mRNA expression levels of COX-2 and SUV were significantly lower in NS-398 treated groups in a dose-dependent manner than the control group, suggesting that NS-398 inhibited the mRNA expression of COX-2 and SUV in MG-63 cells (*P<0.05; **P<0.01).

**Effects of NS-398 on proliferation and apoptosis of MG-63 cells.** (A) The proliferative activities of MG-63 cells treated with NS-398 were examined by MTT assay, and it was found that NS-398 could significantly reduce the proliferative activities of MG-63 cells in a dose- and time-dependent manner in comparison with the control group (***P<0.01). (B) The apoptotic index of MG-63 cells treated with NS-398 was examined by flow cytometric analysis. The results showed that the apoptosis index of MG-63 cells in NS-398 treated groups was markedly higher than the control group (*P<0.05; **P<0.01).

**Table II. The relationship of COX-2 and SUV expression with clinicopathological characteristics of OS.**

<table>
<thead>
<tr>
<th>Clinicopathologic factors</th>
<th>COX-2</th>
<th>SUV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Age &gt;60</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>&lt;60</td>
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<td>15</td>
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<td>Pathological grade I</td>
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<td>II</td>
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<td>8</td>
</tr>
<tr>
<td>Others</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 2. Effects of NS-398 on mRNA expression of COX-2 and SUV in MG-63 cells. (A and B) To examine the effects of NS-398 on expression of COX-2 and SUV in MG-63 cells, MG-63 cells were treated with different concentrations of NS-398 (0, 50, 100 and 200 µmol/l), RT-PCR was performed at 48-h recovery to measure their mRNA expression levels. (C) The mRNA expression levels of COX-2 and SUV were significantly lower in NS-398 treated groups in a dose-dependent manner than the control group, suggesting that NS-398 inhibited the mRNA expression of COX-2 and SUV in MG-63 cells (*P<0.05; **P<0.01).

Figure 3. Effects of NS-398 on proliferation and apoptosis of MG-63 cells. (A) The proliferative activities of MG-63 cells treated with NS-398 were examined by MTT assay, and it was found that NS-398 could significantly reduce the proliferative activities of MG-63 cells in a dose- and time-dependent manner in comparison with the control group (**P<0.01). (B) The apoptotic index of MG-63 cells treated with NS-398 was examined by flow cytometric analysis. The results showed that the apoptosis index of MG-63 cells in NS-398 treated groups was markedly higher than the control group (*P<0.05; **P<0.01).
COX-2 and SUV in MG-63 cells, MG-63 cells were treated with different concentrations of NS-398 (0, 50, 100 and 200 µmol/l). RT-PCR was performed at 48-h recovery to measure their mRNA expression levels. As shown in Fig. 2, the mRNA expression levels of COX-2 and SUV were significantly lower in NS-398 treated groups in a dose-dependent manner than the control group, suggesting that NS-398 inhibited the mRNA expression of COX-2 and SUV in MG-63 cells.

Effects of NS-398 on proliferation and apoptosis in MG-63 cells. The proliferative activities of MG-63 cells treated with NS-398 were examined by MTT assay, and it was found that NS-398 could significantly reduce the proliferative activities of MG-63 cells in a dose- and time-dependent manner in comparison with the control group (Fig. 3A). Also, the apoptotic index of MG-63 cells treated with NS-398 was examined by flow cytometric analysis. The results showed that the apoptotic index of MG-63 cells in NS-398 treated groups was markedly higher than the control group (Fig. 3B). Therefore, NS-398 inhibited the proliferation and induced apoptosis in MG-63 cells.

Effects of rAd5-SUV on expression of SUV, PCNA, and CAS-3 in MG-63 cells. In order to efficiently knockdown the expression of SUV in MG-63 OS cells, an adenovirus-mediated small hairpin RNA approach was used to construct the rAd5-SUV vector. In pilot studies, the transfection efficiency of rAd5-SUV (MOI=100) in MG-63 cells was >95.0% observed by fluorescence microscopy. Western blotting was performed at 48-h recovery to measure the protein expression levels of SUV, PCNA, and CAS-3 in MG-63 cells. The results demonstrated that the protein expression of SUV and PCNA was decreased, while that of CAS-3 was increased in rAd5-SUV group compared with the GFP group.

Effects of rAd5-SUV on proliferation and apoptosis of MG-63 cells. Deregulated cell proliferation is a hallmark of cancer (18). In order to test the effects of rAd5-SUV on OS cell proliferation and apoptosis, we investigated the proliferative activities and apoptotic index of MG-63 cells by MTT and flow cytometry.
analysis. As a result, it was indicated that knockdown of SUV could significantly reduce the proliferative activities of MG-63 cells compared with GFP group and CON group ("P<0.01). Also, cell nuclear fragmentation, apoptotic bodies and DNA ladder turned up in group rAd5-SUV compared with the group GFP, demonstrating the DNA fragmentation and increase of cell apoptosis induced by knockdown of SUV. Moreover, knockdown of SUV markedly increased the apoptotic index of MG-63 cells compared with GFP group and CON group indicated by flow cytometry ("P<0.01).
Cyclooxygenase-2 inhibited the proliferation and induce apoptosis in MG-63 cells.

**Effects of rAd5-SUV on xenograft tumor growth.** Our *in vitro* experiments demonstrated the inhibitory effects of knockdown of SUV on OS MG-63 cell proliferation. Therefore, it is necessary to further investigate the effect of knockdown of SUV on xenograft tumor growth *in vivo*. The mean volume of tumors in all experimental mice before treatment was 101.05±36.27 mm³. During the whole tumor growth period (Fig. 6A and B), the tumor growth activity was measured. Tumors treated with rAd5-SUV grew substantially slowly compared with the PBS and rAd5-GFP group. When the tumors were harvested, the average weight of tumors in group rAd5-SUV was significantly lower than PBS and rAd5-SUV group (Fig. 6C). This result *in vivo* indicated that knockdown of SUV could also inhibit OS cell growth.

**Discussion**

OS is the most frequent malignant bone tumor with a peak incidence in the second and third decade of life. SUV as a member of the inhibitor of apoptosis protein family is expressed both during normal fetal development and in human cancer. Importantly, it is a useful prognostic marker in OS and patients with OS exhibiting nuclear SUV expression could potentially benefit from stratification of neoadjuvant chemotherapy (19). Elevated SUV expression in OS correlates with histologic grade and mitotic index and a decreased disease-free interval. SUV attenuation in canine OS cells inhibits cell cycle progression, increased apoptosis, mitotic arrest and chemosensitivity, and cooperates with chemotherapy to significantly improve tumor control (20). Thus, SUV can be considered as an independent predictor of survival for OS patients (21,22). Coupled with the report that COX-2 expression does not correlate with outcome of OS (16), the relationship of COX-2 and SUV with OS need to be further evaluated. In our study, the expression of COX-2 and SUV was, respectively, observed in 73.3 and 63.3% OS tissues and in 25.0 and 30.0% OC tissues, indicating their higher expression in OS than in OC. Spearman rank correlation analysis showed their positive correlation. However, consistent with a previous study (17), our results showed no significant correlation between the expression of COX-2 and SUV with age and pathological grade and type of OS.

In addition, COX-2 inhibitors such as NS-398 and celecoxib have been shown to inhibit COX-2 expression, and produce an anti-proliferative and pro-apoptotic effect on different types of tumor cells (23,24). Meloxicam, the preferential COX-2 inhibitor, inhibits OS growth, invasiveness and metastasis by COX-2-dependent and -independent routes (25,26). RNAI-mediated knockdown of COX-2 inhibits the growth, invasion and migration of OS, and COX-2 signaling pathway may provide a novel therapeutic target for the treatment of human OS (27).

Some data indicate that selective inhibition of COX-2 exerts an effect on primary tumor growth in Ewing sarcoma (28). Furthermore, we investigated the effect of NS-398 on OS cell proliferation and apoptosis, and found that NS-398 significantly inhibited the proliferation and induce apoptosis in OS cells, enriching the anti-tumor evidence of COX-2 inhibitors. Also, the regulatory mechanisms of COX-2 inhibitors on OS are worth exploring. Celecoxib induces apoptosis in human OS cells via downregulation of PI3K/Akt, activating GSK-3β and inhibiting β-catenin-dependent signaling pathways (29,30). Differently, our study showed that NS-398 downregulated the mRNA expression of SUV in a dose-dependent manner in MG-63 cells, suggesting that MG-63 might inhibit the proliferation and induce apoptosis of MG-63 cells through downregulation of the SUV pathway.

SUV is very important in the development of OS and blockade of SUV markedly inhibits the proliferation and invasion of OS cells, partially reversing their malignant phenotype. Targeting SUV might be a promising option in the treatment of OS and downregulation of SUV is an effective strategy to improve the therapeutic effect of OS (31). Similarly, our study indicated that knockdown of SUV by adenovirus-mediated RNAI inhibited the proliferation, induced apoptosis, and slowed the growth of xenograft tumors in MG-63 cells, providing a strategy for the treatment of OS. PCNA is essential for the replication of deoxyribonucleic acid DNA and has been proved to be an important marker for tumor proliferation. SUV expression has been verified to correlate with PCNA and CAS-3 in OS (32,33). Moreover, we found that knockdown of SUV decreased the expression of PCNA and increased the expression of CAS-3 in MG-63 cells, suggesting that SUV might be involved in OS proliferation and apoptosis via regulation of PCNA and CAS-3 expression.

In conclusion, the expression of COX-2 and SUV is closely correlated with human OS, and NS-398 inhibition of COX-2 or knockdown of SUV by RNAI suppresses tumor proliferation and induces apoptosis in MG-63 cells, suggesting that COX-2 may be involved in OS cell growth and apoptosis through SUV-mediated regulation of PCNA and CAS-3 expression, and provide a potential therapeutic strategy for the treatment of cancer.

**References**