ASPP2 inhibits osteosarcoma cell metastasis by stabilizing the β-catenin/E-cadherin complex

Fu-jie Xie¹, #, Wei-le Liu¹, #, Yu-peng Zheng¹, Wei-cong Yin¹, Sheng-fa Li¹, *, Shao-wei Zheng¹, *, Chun-han Sun¹, *

Abstract

Objective To explore the effect and mechanism of apoptosis stimulating protein 2 of p53 (ASPP2) in osteosarcoma metastasis. Methods Quantitative polymerase chain reaction was performed to determine ASPP2 mRNA expression levels in eight samples of human osteosarcoma tissue and four osteosarcoma cell lines. ASPP2 protein levels were detected with western blot. Metastatic ability of SAOS2 cells was assessed using a transwell invasion assay. The β-catenin/E-cadherin interaction was analyzed by co-immunoprecipitation. Immunofluorescence was used to detect nuclear expression of ASPP2 protein in osteosarcoma cells. Results ASPP2 mRNA expression was significantly decreased in eight osteosarcoma tissue samples compared to normal bone tissue collected from the same patients. The levels of ASPP2 mRNA and protein were also significantly decreased in normal human osteoblast cells compared to OS187, SAOS2, HOS, and MG63 osteosarcoma cell lines. Upon ASPP2 overexpression in SAOS2 cells, metastatic ability of cells was reduced, the β-catenin/E-cadherin interaction was enhanced, and nuclear translocation of β-catenin was inhibited. Conclusion ASPP2 reduces the metastatic characteristics of SAOS2 osteosarcoma cells by stabilizing the β-catenin/E-cadherin complex to inhibit β-catenin nuclear translocation.
**Introduction**

Osteosarcoma is the most frequently occurring primary bone tumor affecting adolescents and young adults. The estimated incidence rate of osteosarcoma is 4 million people per year worldwide, with a peak incidence between 15 and 19 years [1]. Osteosarcoma tumors occur most commonly in the long bones and are prone to early metastasis [2]. Approximately 20% of osteosarcoma patients present with lung metastases at the initial diagnosis, and 40% of patients develop metastases at a later stage. Eighty percent of all osteosarcoma metastases arise in the lungs, most commonly in the periphery of the lungs, and exhibit resistance to conventional chemotherapy [3-8]. The 5-year survival rate for osteosarcoma patients with metastases is 20% compared to 65% for osteosarcoma patients with localized disease; most deaths associated with osteosarcoma are the result of metastatic disease [6, 9-12].

Apoptosis stimulating protein 2 of p53 (ASPP2) is a member of the family of p53-interacting proteins and is involved in a series of physiological and pathological responses including cell growth, apoptosis, injury, and cell migration [13, 14]. The p53 gene is mutated in 35%-40% of human tumors and the pathways that activate p53 are also disrupted in many other tumors[15, 16]. The p53 protein modulates cellular functions, such as gene transcription, DNA synthesis, DNA repair, cell cycle arrest, senescence, and apoptosis. Mutations of the gene can result in inhibition of protein function, and it is this dysfunction that is linked to tumor progression and genetic instability [17].

Previous studies have shown that ASPP2 can stabilize the β-catenin/E-cadherin complex and inhibit nuclear translocation of β-catenin, thereby inhibiting metastasis of tumor cells [18]. However, ASPP2 expression in osteosarcoma tumors and whether or not it plays a role in osteosarcoma metastasis remain unknown. This study aimed to investigate the relationship between ASPP2 expression and osteosarcoma cell metastasis, and to define the mechanism of ASPP2-mediated metastasis in osteosarcoma.

**Materials and Methods**

**Materials**

Osteosarcoma tumors were collected from a total of eight male patients with metastatic osteosarcoma at XX Hospital. Normal osteoblasts (HOSB) and osteosarcoma cells (OS187, SAOS2, HOS, and MG63) were donated by the Southern Hospital Orthopedic Laboratory. The following reagents were used: SYBR Green Real-time PCR Master Mix and Trizol (TaKaRa Company, Dalian of China, mouse anti-human monoclonal anti-ASPP2 (Abcam Inc Company, Colorado, United States), β-catenin and E-cadherin rabbit polyclonal antibody (CST Company, Boston, USA), human β-actin mouse monoclonal antibody (Santa Cruz, Santa Cruz County, USA), ASPP2 overexpression plasmids (synthesized by Hai Jima Biotechnology, Shanghai, China), and Lipofectamine® 2000 (Life Inc., New York, USA).
Collection and processing of human osteosarcoma surgical specimens

Human osteosarcoma carcinomas (5 cm in size) and adjacent normal bone tissue were collected. Fresh tissue was placed in a bowl that was pre-cooled with liquid nitrogen, an appropriate amount of Trizol was added, and the tissue was ground into a powder. Samples were stored at −80 °C.

Plasmid transfection

Synthetic plasmids were purchased from Shanghai Jiema Company. Approximately 2 × 10^5 SAOS2 cells were seeded into 24-well plates and cultured at 37 °C with 5% CO₂. When cells reached about 80% confluency, Lipofectamine® 2000 was used to transfect plasmids expressing ASPP2 or an empty vector control. Cell fluorescence was observed using a fluorescent microscope 72 h after transfection. Puromycin (0.2 µg/ml) was used to select for cells stably expressing ASPP2, and ASPP2 protein expression was detected with western blot.

Quantitative polymerase chain reaction (q-PCR) measurement of ASPP2 mRNA expression

Trizol was used to extract RNA from human osteosarcoma tissue or from cells cultured in six-well plates. RNA was reverse transcribed into cDNA using a TaKaRa reverse transcription kit, according to the manufacturer’s instructions. The reaction mixture consisted of 2 µl of cDNA, 10 µl of 2x SYBR, 6 µl of water, and 1 µl each of the upstream and downstream primers (Table 1). Reaction conditions were 95 °C for 5 min, 95 °C for 15 s, and 60 °C for 60 s, for 40 cycles. ASPP2 expression level was determined based on the cycle threshold (Ct) value (the number of cycles through which the fluorescent signal reaches the set threshold). GAPDH was used as an internal control.

Western blot detection of ASPP2 protein

First, cells were cultured in 60-mm dishes and protein was extracted. And then the test used 5% concentrated rubber 80 V regulator for 30 min, 10% of the separation of plastic pressure 110 V, 40 min, wet 120 mA constant current 50 min, 37 °C shaker 5% BSA closed 2 h. Membranes were washed with TBST for 5 min each wash, for a total of three washes. Goat anti-rabbit or goat anti-mouse anti-infrared secondary antibodies were incubated at 1:15000 at room temperature with shaking for 1.5 h. Membranes were imaged using an Odyssey V3.0 scanner (American LICOR company, New York, USA).

Transwell experiments

Cells stably expressing ASPP2 were harvested in serum-free DMEM (DMEM is a medium containing various amino acids and glucose.), resuspended at a density of 5 ×104 cells/ml, and 200 µl of the cell suspension was added to the upper portion of a Transwell chamber. In the lower chamber, 600 µl of DMEM containing 10% serum was added. After 12 h, the medium was removed, cells were fixed with 4% paraformaldehyde for 10 min, and stained with crystal violet.
for 2 h. Cells were photographed and observed under a microscope.

**Immunofluorescence**

Cells were seeded in a confocal dish and fixed with 4% paraformaldehyde for 10 min, harvested (β-catenin antibody, CST Company, Boston, USA) after 12 h. Then, cells were then washed with PBS (phosphate buffered solution) for 3 min each wash, for a total of three washes. Finally, fluorescence microscopy was used to observe the fluorescence of the cells.

**Co-immunoprecipitation (co-IP)**

Protein was extracted and incubated with β-catenin antibody beads at 4 °C for 4 h, washed three times with the appropriate amount of 2× loading buffer, and placed in a 100 °C water bath for 10 min. β-catenin and E-cadherin proteins were detected by Western blot.

**Statistical analysis**

The homogeneity of variance test was performed using the Levene method with one-way ANOVA and the χ² test, using SPSS 13.0 statistical software (Chicago, USA). The variance was equal and the difference between the two groups was statistically significant. Multiple comparisons were made using Least-Significant Difference P <0.05 was used to determine statistical significance.

**Results**

**ASPP2 expression in osteosarcoma tissue and cells**

Tumors and adjacent bone tissue were collected from eight osteosarcoma patients and ASPP2 mRNA expression levels were measured with qPCR. ASPP2 mRNA expression in osteosarcoma tissue (T) was significantly lower than that in para-cancerous bone tissue (N) (Fig. 1A). We also compared the expression levels of ASPP2 mRNA and protein in normal osteoblasts (HOSB) and osteosarcoma cell lines (OS187, SAOS2, HOS, and MG63). This analysis also revealed that ASPP2 mRNA and protein are expressed at significantly lower levels in osteosarcoma cells compared to normal HOSB cells (Fig. 1B and C).

<table>
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<th>Table 1. The sequence of q-PCR primer.</th>
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<tr>
<td>Primer</td>
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<tr>
<td>ASPP2</td>
<td>5′- TATCTAATCCTTACCGAAACC-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′- GTGGGCCGCTCTAGGCACCAA-3′</td>
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**ASPP2 overexpression inhibits SAOS2 cell invasion**

ASPP2 is a tumor suppressor gene, and its expression in osteosarcoma tissue and cells is significantly reduced (Fig. 1). We overexpressed ASPP2 in SAOS2 osteosarcoma cells, which have low endogenous expression of ASPP2 (Fig. 2A). Cell invasion assays showed a significant reduction in the invasive characteristics of SAOS2 cells upon overexpression of ASPP2 (Fig. 2B).

**ASPP2 inhibits nuclear translocation of β-catenin**

When complexed with E-cadherin, nuclear translocation of β-catenin is inhibited. We completed a co-IP assay to examine the interaction of E-cadherin and β-catenin in SAOS2 cells overexpressing ASPP2. We found that upon overexpression of ASPP2, the interaction between E-cadherin and β-catenin was increased significantly in SAOS2 cells (Fig. 3A). At the same time, ASPP2 overexpression inhibited nu-
clear translocation of β-catenin in SAOS2 cells (Fig. 3B).

Figure 1 | ASPP2 expression in osteosarcoma tissues and cell lines. (A) ASPP2 mRNA expression levels in human osteosarcoma tissues determined with qPCR (Quantitative real time polymerase chain reaction). (B) Expression of ASPP2 mRNA in normal osteoblast cells and osteosarcoma cell lines, determined with qPCR. (C–D) ASPP2 protein expression in normal osteoblast and osteosarcoma cell lines, determined with western blot (left) and quantified (right). T contrast with N; #P < 0.05.

Figure 2 | Effects of ASPP2 overexpression on SAOS2 cell invasion. (A–B) The expression of ASPP2 in SAOS2 cells was detected with western blot. (C–D) Transwell assay was used to detect the invasion of SAOS2 cells. *Compared with vector-NC, P < 0.05.

Figure 3 | Effect of ASPP2 overexpression on nuclear translocation of β-catenin. (A) The interaction between E-cadherin and β-catenin was detected with co-IP. (B) Nuclear translocation of β-catenin (red) was analyzed using immunofluorescence. DAPI (blue) was used as a nuclear stain.
**Discussion**

ASPP2 was first identified as a tumor suppressor and an activator of the p53 family [17, 19]. The p53 protein is one of the best known tumor suppressor proteins; it plays a key role in apoptosis and cell cycle arrest, and is frequently mutated in a wide range of human cancers [20]. The ASPP family is comprised of proteins with ankyrin repeats, an SH3 domain, and a prolinerich region. The family has three members: ASPP1, ASPP2, and inhibitory ASPP (iASPP) [13, 21, 22]. ASPP2 is the best characterized proapoptotic protein in the ASPP family [19]. ASPP2 expression is significantly decreased in lung cancer [23], breast cancer [24], gastric cancer [25], and other common tumors. Here we showed that ASSP2 mRNA expression is significantly lower in human osteosarcoma tissue compared to pericarcinomatous bone tissue. Furthermore, we showed that ASPP2 mRNA and protein expression is also significantly reduced in osteosarcoma cell lines compared to normal osteoblast cells. These findings suggest that loss of ASPP2 expression may be a contributing factor in the occurrence of osteosarcoma.

E-cadherin and β-catenin form a complex of the main cell adhesion protein structure, which can increase the connection between cells and reduce tumor cell metastasis [26, 27]. The E-cadherin/β-catenin complex can also stabilize β-catenin and prevent it from translocating to the nucleus, thereby reducing tumor metastasis [28, 29]. In addition to its involvement in the regulation of apoptosis, recent studies have found that ASPP2 can stabilize the β-catenin/E-cadherin complex in breast cancer cells to inhibit β-catenin nuclear translocation, thereby reducing metastasis of breast cancer cells [19, 30]. We overexpressed ASPP2 in the highly metastatic SAOS2 osteosarcoma cell line, which has low endogenous expression of ASPP2. We found that ASPP2 overexpression significantly decreased the metastatic capacity of SAOS2 cells. We further examined the effect of ASPP2 overexpression on the β-catenin/E-cadherin complex, and found that the β-catenin/E-cadherin interaction was enhanced while nuclear localization of β-catenin was reduced. Thus, ASPP2 acts to stabilize the β-catenin/E-cadherin complex in osteosarcoma cells, thereby reducing β-catenin nuclear translocation.

In summary, our findings demonstrate that low expression of ASPP2 in osteosarcoma is related to the high metastatic capacity of osteosarcoma cells. One possible mechanism by which low ASPP2 expression could drive metastasis is through reduced stabilization of the β-catenin/E-cadherin complex, which would lead to increased nuclear translocation of β-catenin and the promotion of osteosarcoma cell metastasis. It can be drawn, ASPP2 osteosarcoma cell metastasis has a certain inhibitory effect on the clinical ASPP2 expression for the treatment of osteosarcoma have a certain effect.

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Conflict of interest No potential conflicts of interest relevant to this article are reported.

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References


