Serum amyloid A promotes osteosarcoma invasion via upregulating αvβ3 integrin

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Received January 8, 2014; Accepted July 22, 2014

DOI: 10.3892/mmr.2014.2635

Abstract. Serum amyloid A (SAA) is regarded as an important acute phase protein involved in tumor progression and metastasis. However, at present there is no evidence of its involvement in osteosarcoma. The present study aimed to investigate the effect of SAA on the invasion of osteosarcoma cells. The effects of SAA on the migration and invasion of osteosarcoma cells were detected using scratch wound healing and transwell assays, respectively. The expression of $\alpha v\beta 3$ integrin was detected at the protein and mRNA levels in U2OS cells. Agonists, inhibitors or siRNA of formyl peptide receptor like-1 (FPRL-1), mitogen-activated protein kinases and $\alpha v\beta 3$ integrin were used to investigate the mechanism underlying the effects of SAA on the regulation of U2OS cell migration and invasion. The present study revealed that SAA promoted osteosarcoma cell migration and invasion. SAA upregulated the expression of $\alpha v\beta 3$ integrin in a concentration- and time-dependent manner. When inhibiting $\alpha v\beta 3$ integrin with its antagonist, the migration and invasion abilities of the U2OS cells were markedly inhibited. SAA-induced avß3 integrin production was significantly downregulated by inhibiting FPRL-1 with siRNA and inhibitors. The present study also found that extracellular signal-regulated kinase (ERK) 1/2, but not c-Jun N-terminal kinase or p38, was important in this process. These findings demonstrated that SAA regulated osteosarcoma cell migration and invasion via the FPRL-1/ERK/avß3 integrin pathway.

Introduction

Osteosarcoma, one of the most common types of malignant bone neoplasm, is characterized by a highly metastatic potential (1). Although multiple methods, including chemotherapy and radiotherapy, have been used and the principles of treatment have markedly changed in the past decades, the five-year survival rate of osteosarcoma remains at a very low level (2-4). Thus, it is important to further investigate the potential mechanisms underlying the pathogenesis of osteosarcoma.

Previous studies involving cancer development have suggested that chronic inflammation is a key contributor to carcinogenesis (5-7). Chronic inflammation may also have an elevated effect on carcinoma recurrence(8,9). Based on previous observations, inflammatory cytokines and pathways have been examined with respect to the pathogenesis of osteosarcoma (10). Serum amyloid A (SAA), an acute-phase protein, is synthesized and secreted predominantly by the liver. SAA is also expressed and secreted by other tissues and cells, including synovial tissue, artery tissue, adipocytes and tumor tissue. Under inflammatory conditions, the serum level of SAA can increase up to 1,000-fold. Previous studies have demonstrated that elevated SAA is associated with poor tumor prognosis in several types of cancer, including gastric cancer (11), renal cell carcinoma (12) and breast cancer (13). However, to the best of our knowledge, no study has investigated the association between SAA and the progression of osteosarcoma.

The integrins, a family of transmembrane receptor proteins, mediate the attachment of cells to its surroundings. The majority of integrins in mammals are composed of two distinct chains, the 18 α and 8 β subunits, which dimerize to form different complexes. Integrins have been considered to regulate a number of processes in tumor cells, including proliferation, apoptosis, adhesion, migration and invasion (14). Altered expression of $\alpha v\beta 3$ integrin is associated with accelerated development and increased metastatic potential of several types of cancer, including prostate cancer (15), breast cancer (16), ovary cancer (17) and particularly osteosarcoma (18). Previous studies have indicated that the expression and activation of $\alpha v\beta 3$ integrin are dependent on the phosphorylation of mitogen-activated protein kinases (MAPKs) (19,20), while SAA can induce the phosphorylation of the three MAPKs, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase 1/2 (ERK1/2) (21). Thus, the present study examined whether SAA promoted the progression of osteosarcoma via activation of $\alpha v\beta 3$ integrin.

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Key words: serum amyloid A, osteosarcoma, $\alpha\nu\beta3$ integrin, formyl peptide receptor like-1, extracellular signal-regulated kinase 1/2

Materials and methods

Reagents. Recombinant human apo-SAA1 was purchased from Peprotech (Rocky Hill, NJ, USA). The avß3 specific antibody inhibitor LM609 was purchased from Merck (Darmstadt, Germany). Pertussis toxin was purchased from Sigma (Shanghai, China). H2N-WRWWWW-CONH2 (WRW⁴) was purchased from Tocris Bioscience (Ellisville, MI, USA). Human formyl peptide receptor like-1 (FPRL-1) siRNA was obtained from Santa Cruz Biotechnology, Inc. (cat no. sc-40123; Santa Cruz, CA, USA). The polyclonal rabbit anti-human antibodies for FPRL-1 and β3 integrin were purchased from Abcam (Cambridge, MA, USA). The polyclonal rabbit anti-human antibodies for total-ERK1/2, p-ERK1/2, total-JNK1/2 p-JNK1/2, total-p38 and p-p38 were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). The selective JNK inhibitor SP600125, selective ERK1/2 inhibitor PD98059 and selective P38 inhibitor SB203580 were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Monoclonal mouse anti-GAPDH was purchased from Bioworld Technology (Minneapolis, MN, USA). The study was approved by the Ethics Committee of The Second Hospital of Shandong University (Jinan, China).

Cell culture. U2OS cells were obtained from the American Type Culture Collection (Manassas, VA, USA). U2OS cells were cultured in Dulbecco's modified Eagles medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone) at 37°C in a humidified atmosphere of 5% CO_2 .

FPRL-1 siRNA and its transfection. FPRL-1 siRNA sequences were obtained from Santa Cruz Biotechnology, Inc. (cat no. sc-40123). Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) was used in the presence of 100 nM FPRL-1 siRNA and siRNA/lipofectamine 2000 particles were added to the U2OS cells for 48 h. The cells were then collected for western blot analysis and quantitative polymerase chain reaction (qPCR) detection to determine the effect of the siRNA sequences.

qPCR. Total RNA was extracted from the osteosarcoma cells using TRIzol reagent (Invitrogen Life Technologies), which was then measured using an UV spectrophotometer (Bio-Rad, Beijing, China) to determine the concentration. Total RNA $(1 \ \mu g)$ was reverse-transcribed into cDNA using oligo (dT) primers and the reverse transcription system (Fermentas, St. Leon-Rot, Germany). qPCR analysis was performed using a LightCycler (Roche Diagnostics, Mannheim, Germany) and SYBR Green I kit (Takara Bio, Inc., Kyoto, Japan), according to the manufacturer's instructions. The primer sequences used for qPCR were as follows: FPRL-1, forward 5'-CACGGCCACATTACCATTCCT-3' and reverse 5'-AGCGGTCCAGTGCAATGAAA-3' (22) and avß3 integrin, forward 5'-GCTTCAAGGACAGCCTGATCG-3' and reverse 5'-CTTTATACAGTGGGTTGTTGGCTG-3' (23). 18s was selected as the reference gene, forward 5'-CTTAGTTGG TGGAGCGATTTG-3' and reverse 5'-GCTGAACGCCACT TGTCC-3'. Melting curves were assessed to confirm the specificity of the products generated for each set of primers. The $ddC_{\scriptscriptstyle T}$ comparative method was then used to normalize the relative levels of gene expression.

Western blot analysis. Following stimulation with SAA or other reagents, U2OS cells were collected and lysed with lysis buffer containing protein inhibitors (Beyotime Institute of Biotechnology) for 20 min. The extracted protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology). Different molecular weights of proteins were separated via a 10% SDS-polyacrylamide gel in a running buffer (Beyotime Institute of Biotechnology) and the proteins were transferred onto nitrocellulose membranes (Merck KGaA, Darmstadt, Germany. The protein bands were then inhibited using 5% milk for 2 h and incubated with the following specific primary antibodies: Rabbit anti-human polyclonal antibody for FPRL-1 (Abcam, Cambridge, MA), rabbit anti-human polyclonal antibody for ß3 integrin was from (Abcam) and rabbit anti-human polyclonal antibodies for t-JNK, p-JNK, t-ERK, p-ERK, t-p38, p-p38 (Cell Signaling Technology Inc.) at 4°C overnight. This was followed by incubation with the secondary antibody is goat anti-rabbit antibody (ZSGB-Bio, Beijing, China), goat anti-rabbit antibody (ZSGB-Bio) and goat anti-rabbit antibody (ZSGB-Bio) for another 2 h at room temperature. Finally, an electrochemiluminescence detection system (Millipore, Billerica, MA, USA) was used to visualize the antigen-antibody complexes bands.

Scratch wound healing assay (migration assay). A scratch wound healing assay was used to evaluate the migration ability of the U2OS cells. Briefly, the U2OS cells ($1x10^6$ /well) were seeded in six-well plates cultured with DMEM supplemented with 10% FBS. When reaching confluency, straight scratches of the same width were made in the monolayer of the U2OS cells with a pipette tip. To assess the effects of SAA on the migration of U2OS cells, $10 \mu g$ /ml SAA was added to the plates and the same volume of physiological saline was also added as a control. Following culture for 24 h, the wound healing areas were measured under a microscope (IX71; Olympus, Tokyo, Japan) and analyzed.

Transwell assay (invasion assay). A transwell assay was used to evaluate the effect of SAA on the invasive ability of U2OS cells. The U2OS cells were cultured in modified Boyden chambers, with 8-µm pore filter inserts, in 24-well plates (Corning Costar, Cambridge, MA, USA). The pore inserts were pre-coated with Matrigel (BD Biosciences, Bedford, MA, USA) overnight and washed in phosphate-buffered saline. The media in the upper chamber consisted of DMEM with 1% FBS and the lower chamber consisted of DMEM with 10% FBS. Cells (1x10⁵ cells/ well) were seeded in the upper chamber. After 24 h of incubation, the cells remaining on the upper surface of the filters were removed using a cotton wool swab, while the cells invading to the lower surface were fixed with methanol for 30 min followed by hexamethylpararosaniline staining (Sigma). Six random high-power fields (magnification, x200) of each sample were selected and counted under a microscope (IX71; Olympus) to evaluate the average number of invasive cells.

Statistical analysis. Data were analyzed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Continuous data are expressed



Figure 1. SAA induces the migration and invasion of U2OS cells. The effects of SAA on the migration and invasion of U2OS cells were assessed by wound healing assay and Transwell assay, respectively. U2OS cells were treated with 10 μ g/ml SAA for 24 h. (A) Healing over the scratch in the SAA group increased markedly compared with the control group. (B) Cells invading to the underside surface of the Transwell filter were stained with hexamethylpararosaniline and the invasive cell numbers indicated U2OS cell invasion ability. *P<0.05, compared with the control group. Data are expressed as the mean \pm standard error of the mean from three independent experiments in duplicate. SAA, serum amyloid A.

as the mean \pm standard error of the mean. Differences among the groups were calculated using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

SAA induces the migration and invasion of U2OS cells. The effect of SAA on the migration of U2OS cells was assessed using a wound healing assay. U2OS cells were treated with SAA at a concentration of $10 \,\mu$ g/ml for 24 h in the SAA group, while an equal volume of physiological saline was used in the control group. As shown in Fig. 1A, the results of the wound healing assay demonstrated that $10 \,\mu$ g/ml SAA had a significant effect on the healing over the scratch compared with the control group (P<0.05), indicating that SAA induced migration of the U2OS cells.

The effect of SAA on the invasion of U2OS cells was assessed using Transwell assays. U2OS cells were also treated with SAA at a concentration of 10 μ g/ml or an equal volume of physiological saline for 24 h. As shown in Fig. 1B, the result of the Transwell assays revealed that 10 μ g/ml SAA significantly promoted the invasive activity of U2OS cells compared with the control group. The above findings suggested that SAA induced the migration and invasion of U2OS cells.

SAA upregulates the expression of $\alpha\nu\beta\beta$ integrin at the protein and mRNA levels. To investigate the effect of SAA on the expression of $\alpha\nu\beta\beta$ integrin, U2OS cells were stimulated with SAA at different concentrations (0, 0.1, 1 and 10 µg/ml) for 24 h. As shown in Fig. 2A, following stimulation with SAA at a concentration of 0.1 µg/ml, the relative expression of $\alpha\nu\beta\beta$ integrin did not change significantly. However, when the stimulating concentrations were elevated to 1 and 10 μ g/ml, the expression of $\alpha\nu\beta3$ integrin was significantly increased compared with the control group, and reached its maximal activity at 10 μ g/ml. The cells were then stimulated with 10 μ g/ml SAA for different time periods (0, 3, 6, 12 and 24 h). Following stimulation with 10 μ g/ml SAA for 3 h, the relative expression of $\alpha\nu\beta3$ integrin marginally increased (Fig. 2B). However, when the stimulating time period was extended to 6, 12 and 24 h, the relative expression of $\alpha\nu\beta3$ integrin was significantly increased compared with the control group.

In addition, identical stimulations were performed and qPCR was used to examine the mRNA levels of $\alpha\nu\beta3$ integrin. As shown in Fig. 2C, the relative mRNA expression of $\alpha\nu\beta3$ integrin was significantly increased following stimulation with SAA at the concentrations of 1 and 10 μ g/ml. In addition, it also increased gradually compared with the control group following stimulation with 10 g/ml SAA for 1, 3, 6 and 12 h (Fig. 2D), similar to the protein level. A similar increasing tendency was observed at the protein and mRNA levels and the transcriptional activation appeared ahead of protein synthesis, suggesting that this transcriptional activation was required for $\alpha\nu\beta3$ integrin protein synthesis. These data suggested that SAA induced the production of $\alpha\nu\beta3$ integrin in a concentration- and time-dependent manner at the protein and mRNA levels.

Treatment with LM609 eradicates SAA-induced cell migration and invasion. To determine the effect of $\alpha\nu\beta3$ integrin on the invasiveness and migration of osteosarcoma cells, the U2OS cells were pre-incubated with LM609, a specific inhibitor of $\alpha\nu\beta3$ integrin, and then subjected to migration and invasion assays. As shown in Fig. 3A, no difference was identified



Figure 2. SAA upregulates the expression of $\alpha\nu\beta3$ integrin. (A and C) U2OS cells were stimulated with SAA of varying concentrations (0,0.1, 1 and $10\,\mu$ g/ml) for 24 h. The levels of $\alpha\nu\beta3$ integrin expression were determined by western blot analysis and qPCR. (B and D) U2OS cells were stimulated with $10\,\mu$ g/ml SAA for different time periods (0, 3, 6, 12 and 24 h). The levels of $\alpha\nu\beta3$ integrin expression were also determined by western blot analysis and qPCR. *P<0.05 and **P<0.01 compared with the control group. Data are expressed as the mean ± standard error of the mean from three independent experiments in duplicate. SAA, serum amyloid A; qPCR, quantitative polymerase chain reaction.



Figure 3. Treatment with LM609 eradicates SAA-induced cell migration and invasion. U2OS cells were treated with $10 \mu g/ml$ SAA for 24 h in the control group, pre-treated with $20 \mu g/ml$ LM609 for 2 h prior to the addition of SAA in the LM609 + SAA group and treated with $20 \mu g/ml$ LM609 only for 2 h in the LM609 group. (A) No difference was observed in the healing areas among the three groups. (B) No difference was observed in the number of invasive cells among the three groups. Data are expressed as the mean ± standard error of the mean from three independent experiments in duplicate. SAA, serum amyloid A.

among the control, LM609 and the LM609 + SAA groups, which indicated that pre-incubation with 20 μ g/ml LM609 for 2 h significantly eradicated SAA-induced increases in U2OS cell migration. Similar results were observed when the transwell assay was conducted, which indicated that

SAA-induced increases in U2OS cell invasion were also eradicated following inhibition of the $\alpha\nu\beta3$ integrin pathway with LM609 (Fig. 3B). These results indicated that $\alpha\nu\beta3$ integrin was required for SAA-induced U2OS cell migration and invasion.



Figure 4. ERK1/2, but not JNK or p38, mediates SAA-induced production of $\alpha\nu\beta3$ integrin. (A-C) U2OS cells were stimulated with $10 \mu g/ml$ SAA for varying time periods (0, 5, 15 and 30 min, 1 h and 2 h). Western blot analysis revealed that SAA upregulated the expression of (A) p-JNK, (B) p-p38 and (C) p-ERK1/2. *P<0.05 and **P<0.01 compared with the control group. (D) SP600125 (50 μ mol/1), SB203580 (10 μ mol/1) and PD98059 (20 μ mol/1) were added into U2OS cells for 1 h prior to SAA treatment for 24 h. Western blot analysis revealed that only PD98059 inhibited SAA-induced production of $\alpha\nu\beta3$ integrin. *P<0.05 and **P<0.01 compared with the control group. #P<0.05, compared with the SAA group. Data are expressed as the mean ± standard error of the mean from three independent experiments in duplicate. SAA, serum amyloid A; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase.

ERK1/2 mediates SAA-induced $\alpha \nu \beta 3$ integrin production. Previous studies have demonstrated that the MAPKs p38 and ERK1/2, are important in the expression and activation of $\alpha v\beta 3$ integrin (17,18). As a result, the present study aimed to determine whether SAA induced the production of $\alpha v\beta 3$ integrin in U2OS cells via the phosphorylation of the three members of the MAPK family: JNK, p38 and ERK1/2. To determine the association between MAPKs and the production of $\alpha v\beta 3$ integrin production, the relative expression of phospho-JNK, phospho-p38 and phospho-ERK1/2 was investigated following stimulation of U2OS cells with 10 mg/ml SAA for 5, 15, 30, 60 and 120 min. As shown in Fig. 4A-C, the relative expression of the three members were all significantly upregulated compared with the control group. The activity of the three members rapidly increased after 5-30 min stimulation and gradually decreased after 1-2 h stimulation. However, when the JNK, p38 and ERK signaling pathways were inhibited by their respective inhibitors, SP600125 (50 μ mol/l), SB203580 (10 µmol/l) and PD98059 (20 µmol/l) for 1 h prior to SAA treatment for another 24 h, only PD98059, the ERK1/2 specific inhibitor, markedly inhibited SAA-induced $\alpha v\beta 3$ integrin production. The results indicated that all three members of the MAPK family were activated by SAA, but only the ERK1/2 signaling pathway was involved in the reaction in U2OS cells.

FPRL-1 is involved in SAA-induced production of $\alpha\nu\beta3$ integrin. As FPRL-1 has been considered the most important receptor of SAA in the cellular membrand. The present study aimed to investigate the involvement of FPRL-1 in SAA-induced expression of $\alpha v\beta 3$ integrin. Initially, the FPRL-1 gene was silenced by human FPRL-1 siRNA, which was designed and used to treat U2OS cells for 48 h. Its effect was carefully examined via qPCR and western blot analysis. The results demonstrated that FPRL-1 siRNA effectively downregulated the expression of FPRL-1 siRNA at the protein and mRNA levels (Fig. 5A and B). In addition, pertussis toxin (an antagonist of the G protein-coupled receptor) and WRW⁴ (a novel specific inhibitor for FPRL-1) were also selected to determine whether the SAA-induced production of $\alpha v\beta 3$ integrin in U2OS cells was mediated by FPRL-1. The data indicated that the SAA-induced secretion of $\alpha v\beta 3$ integrin was significantly inhibited by the three reagents (Fig. 5C and D), which supported the hypothesis that FPRL-1 is required for SAA-induced production of $\alpha v\beta 3$ integrin.

Discussion

Several new findings have been demonstrated in the present study. SAA was able to induce U2OS cell migration and invasion and exerted its effect on U2OS cells via $\alpha\nu\beta3$ integrin.



Figure 5. SAA induces $\alpha\nu\beta3$ integrin production via FPRL-1. (A and B) Following pre-incubation with the siRNA sequences of FPRL-1 (100 nM) for 48 h, U2OS cells were harvested to investigate the effect of the siRNA sequences by western blot analysis and qPCR. (C andD) U2OS cells were pre-incubated with pertussis toxin (500 ng/ml) or WRW⁴ (30 μ M) for 1 h or human FPRL-1 siRNA (100 nM) for 48 h and then stimulated with 10 μ g/ml SAA for another 24 h. Levels of SAA-induced $\alpha\nu\beta3$ integrin production were determined by western blot analysis and qPCR. **P<0.01 compared with the control group. #P<0.05, compared with the SAA group. Data are expressed as the mean ± standard error of the mean from three independent experiments in duplicate. SAA, serum amyloid A; FPRL-1, formyl peptide receptor like-1; WRW⁴, H2N-WRWWW-CONH2; qPCR, quantitative polymerase chain reaction.

In addition, SAA induced the production of $\alpha\nu\beta3$ integrin in a concentration- and time-dependent manner and it induced $\alpha\nu\beta3$ integrin through the FPRL-1/ERK1/2 signaling pathway.

SAA is mainly produced and secreted by hepatocytes (24), although other types of cells also have the same ability. Following secretion into the serum, SAA rapidly binds to high-density lipoprotein (235). In healthy individuals, only a low level of SAA is present in the serum (26), while a marked elevation of SAA can be found in the serum of patients with neoplastic diseases, including renal (12) and breast cancer (13). To the best of our knowledge, the present study is the first to focus on the association between SAA and osteosarcoma. In order to determine the effect of SAA on osteosarcoma, U2OS cells were cultured and stimulated by SAA. The results demonstrated that SAA induced the migration and invasion of U2OS cells *in vitro*.

Integrins are implicated in the regulation of a number of cellular processes during tumor progression, including adhesion, invasion and differentiation (27). $\alpha\nu\beta3$ integrin is considered to be highly expressed in the lesion of osteosarcoma and has the specific ability to promote the migration and invasion of osteosarcoma cells, suggesting that $\alpha\nu\beta3$ integrin contributes to the metastatic process of osteosarcoma (28). In the present study, SAA increased the expression of $\alpha\nu\beta3$ integrin at the protein and mRNA levels. The specific inhibitor of $\alpha\nu\beta3$ integrin, LM609, was used and was found to inhibit SAA-induced U2OS cell migration and invasion, indicating the possible involvement of $\alpha\nu\beta3$ integrin in the course of SAA-induced U2OS cell migration and invasion.

FPRL-1 is known to be the most important receptor of SAA, thus it is necessary to investigate the effect of FPRL-1 on the SAA-induced expression of $\alpha\nu\beta3$ integrin. FPRL-1 siRNA sequences, as well as pertussis toxin and WRW⁴, which inhibit binding of SAA to FPRL-1 were all used to inhibit SAA at different levels and the results confirmed that the expression of $\alpha\nu\beta3$ integrin required the activation of FPRL-1.

MAPK signal transduction pathways, including JNK, p38 and ERK1/2, are considered to be important in the regulation of inflammation, cellular proliferation and tumor metastasis. SAA has been reported to induce the expression of various cytokines, including interleukin (IL)-8, tumor necrosis factor- α , IL-10 and IL-12 (29), the majority of which involve MAPK signaling pathways. The present study demonstrated that SAA induced the phosphorylation of MAPKs after 5 min stimulation, which was maintained for 15 and 30 min, in accordance with a study by Li *et al* (21). However, the expression of $\alpha\nu\beta$ 3 integrin was also implicated in the effects of MAPK. Pechkovsky *et al* revealed that tumor growth factor- β induces the expression of $\alpha\nu\beta3$ integrin via the p38MAPK pathway (30), while Kurihara *et al* verified that phorbol myristate acetate induces the expression of $\alpha\nu\beta3$ integrin via the ERK1/2 pathway (19). Using pharmacological inhibitors, the present study demonstrated that although SAA activated the JNK, p38 and ERK1/2 pathways, only ERK1/2 was involved in SAA-induced expression of $\alpha\nu\beta3$ integrin. These data suggested that ERK1/2, but not JNK or p38, is important in the regulation of $\alpha\nu\beta3$ integrin expression in U2OS cells.

In conclusion, to the best of our knowledge, the present study for the first time provides evidence that the SAA/FPRL-1/ERK1/2/ $\alpha\nu\beta3$ integrin pathway may be important in the migration and invasion of osteosarcoma cells. According to these results, inhibiting SAA may be a potential way to restrain the progression of osteosarcoma via inhibiting $\alpha\nu\beta3$ integrin.

Acknowledgements

This study was supported by the Natural Science Foundation of Shandong Province (grant no. 2012ZRE27087).

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