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Gas6/Axl mediates tumor cell apoptosis, migration and invasion and predicts the clinical outcome of osteosarcoma patients ☆

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ABSTRACT

Dysregulation of the receptor tyrosine kinase Axl and its ligand Gas6 has been shown to promote multiple tumorigenic processes, as well as to correlate with worse prognosis in many different tumor types. However, studies of Axl expression and function in osteosarcoma have rarely been reported. In this study, we report that activated Axl is highly expressed in osteosarcoma cells, and this expression is significantly correlated with the recurrence and lung metastasis of osteosarcoma patients. High expression of activated Axl was an independent predictor for worse prognosis in osteosarcoma. Additionally, we confirmed a strong positive correlation between P-Axl and MMP-9 expression in those osteosarcoma patients. In osteosarcoma cell lines MG63 and U2OS, 200 ng/ml rhGas6 could cause obvious increase of P-Axl expression within 30 min, consistent with the expression of P-AKT. In both of the cell lines, Axl activated by rhGas6 could protect the tumor cells from apoptosis caused by serum starvation, and promote tumor cells' migration and invasion *in vitro*. Together with previous data, these studies suggest that activated Axl participate in the progression of osteosarcoma by resisting tumor cells apoptosis and promoting their migration and invasion, which may be linked to the expression of MMP-9. In the mechanism, AKT signaling pathway may contribute to the function of P-Axl in osteosarcoma rather than ERK pathway.

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1. Introduction

Osteosarcoma (OS) is the most common primary malignant tumor arising from bone in children and adolescents. Recent finding suggest that most patients with localized OS have micrometastases at presentation [1].

Receptor tyrosine kinases (RTKs) regulate important cellular processes in tumor progression. Axl, along with Tyro3 and Mer, is a member of the TAM family of RTKs and was first identified as a transforming gene in chronic myeloid leukemia [2]. The ligand for Axl, Growth arrest-specific 6 (Gas6), is a vitamin K-dependent protein that binds Axl with high affinity [3]. Axl activation and signaling have been implicated in multiple cellular responses, including cell survival, proliferation, migration, and adhesion [4]. Additionally, Axl has been shown to regulate vascular smooth muscle homeostasis, endothelial cell migration and vascular network formation [5].

Overexpression of the Axl receptor has been identified in multiple malignancies and is observed in both the primary tumor and metastatic lesions [6]. Axl acts as a transforming gene, and the Gas6/Axl signaling system regulates processes fundamental for both angiogenesis and tumorigenesis [5]. Furthermore, in multiple tumor types, increased levels of Axl protein has been observed at higher levels in metastases than in primary tumors, and a higher level of Axl protein expression is associated with a poor clinical outcome [7–11].

As one of the major matrix metalloproteinases (MMPs), MMP-9 activity increases with the degree of malignancy. Multiple signaling transduction pathways are involved in the regulation of MMP-9 production in human cancer cells, including the ERK and AKT pathway [12]. The signaling molecules and the phosphorylation of cytoplasmic substrates activated by Axl in various cell types include phosphatidylinositol 3-kinase (PI3-K), AKT, S6K, Src, ERK, p38 MAPK and NF-kB [13]. However, the functional relevance of Axl to the regulation of MMP-9 gene expression remains unclear.

Few studies have explored the relationship between the expression of Axl and tumor progression in osteosarcoma. In highly metastatic osteosarcoma sublines, Axl is one of the five genes

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(AXL, TGFA, COLL7A1, WNT5A and MKK6) associated with tumor cell motility and/or invasiveness by cDNA microarray [14]. To date, no clinically relative analysis has explored the relationship between osteosarcoma and Axl protein expression. This paper focuses on the relationship between Axl expression and patient prognosis with the aim of identifying the mechanism of Axl action in osteosarcoma cells.

2. Materials and methods

2.1. Patients and tissues

Paraffin-embedded punctured biopsy samples were collected individually from 62 patients who presented to the 1st Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) with primary non-metastatic osteosarcoma between 1999 and 2008. This population included 43 males and 19 females from 8 to 58 years old; the average patient age was 16.32 years. The patients were followed for 12 months to 124 months, with an average follow-up of 75 months. Sixteen cases recurred or developed lung metastases, and 28 patients died of the disease. Fresh samples were collected and immediately frozen at −80 °C. Twenty-one cases of osteofibrous dysplasia were used as a benign lesion group.

2.2. Antibodies and other reagents

Antibodies against human phosphorylated Axl and recombinant human Gas6 were obtained from RD Systems (Minneapolis, MN, USA). Antibodies against phosphorylated AKT (Ser 473), phosphorylated p38 MAPK (Thr180/Tyr182), and GAPDH were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against human MMP-9 and HRP-conjugated secondary antibodies (Envision) were obtained from DAKO, Denmark.

2.3. Immunohistochemical analysis

An Envision two-step assay was used for the immunohistochemistry stain for P-Axl and MMP-9. Staining results were scored semiquantitatively based on the combined percentage [five-tiered algorithm for positive cells (0: 0%; 1: <25%; 2: 25%–50%; 3: 56%–75%; 4: >75%)] and intensity of cytoplasmic and nuclear staining [four-tiered system (0: negative; 1: weak; 2: moderate; 3: strong)], and scores were tabulated as the expression index [(percentage positive) × (intensity)]. The index scores of three pathologists were averaged to obtain the final expression index. For P-Axl grading, high expression was defined as a score of 2 or more; scores was less than 2 were defined as low expression. For MMP-9 grading, high expression was defined as a score of 3 or greater; lower scored denoted low expression.

2.4. Cell culture and immunohistochemistry

Osteosarcoma cell lines MG63 and U2OS were obtained from ATCC. All cells were regularly cultured and prepared as cell slides. Cell slides were subjected to immunohistochemistry staining for P-Axl.

2.5. Cell viability assay

Cells were seeded in 96-well plates and cultured for 12 h. After 12 h, the full medium was changed to serum free medium for 6 h for MG63 cells or 12 h for U2OS cells. Cells were then incubated with medium plus 0, 100, 200, or 400 ng/ml rhGas6. After treatment, cell viability was assessed using MTT as described previously [15]. This experiment were repeated three times separately.

2.6. In vitro wound healing assays

Cell wounding studies were performed using methods similar to those previously described [16]. A slash was created in near-confluent cell cultures using the tip of a P-1000 pipetman after 6 and 12 h of serum-free incubation for MG63 and U2OS cells, respectively. The medium were changed to full medium plus 0, 100, 200 or 400 ng/ml rhGas6. The plates were then incubated at 37 °C, and pictures were taken at 0, 24, 48 and 72 h using a Nikon Eclipse TE2000-5 microscope. This experiment were repeated three times separately.

2.7. Cell migration and invasion assays

For migration and invasion assays, Transwell champers with 8-μm pore size (Corning Inc., NY, USA) were used without or with the reconstituted Matrigel matrix (BD Bioscience, USA). MG63 (2×10^5/ml) and U2OS (4×10^5/ml) cell suspensions in serum-free media were seeded in the upper champers. After 12 h, rhGas6 was added to the upper chamber at a concentration of 200 ng/ml for MG63 and 400 ng/ml for U2OS. After fixation and staining, the invading cells were counted. Experiments were repeated at least three times separately [17].

2.8. Cell cycle analysis

Cell cycle assays were performed using a cell DNA content detecting kit (KeyGen, China). MG63 and U2OS cells were starved with serum free media 6 h or 12 h, respectively. rhGas6 was added to the samples at 0, 100, 200 or 400 ng/ml at 37 °C for 48 h. The cells were collected and fixed. After staining with PI, the cell cycle was analyzed in a flow cytometer using an argon laser (Beckman Coulter, USA) at a wavelength of 488 nm.

2.9. Apoptosis detection (Hoechst 33258 staining)

MG63 and U2OS cells were pretreated with 0, 100, 200 or 400 ng/ml rhGas6 for 48 h. Cells were subsequently stained with 1 μg/ml Hoechst 33258 for 10 min at room temperature in the dark, and photos were taken using a fluorescence microscope (Leica, Germany). This experiment were repeated three times separately.

2.10. Western Blot

MG63 and U2OS cells were stimulated for 30 and 15 min, respectively, with 0, 100, 200 or 400 ng/ml rhGas6. Alternatively, cells were treated with 200 ng/ml rhGas6 for 0 to 30 min. Total proteins was isolated, and the protein concentration was measured. Western Blotting was performed as described previously [18]. Primary antibodies against P-AXL, P-AKT and P-ERK1/2 were used; GAPDH was used as a loading control. The hybridization signals were detected using Femto Maximum Sensitivity chemiluminescent substrate from Thermo Scientific.

2.11. Statistical analysis

Disease-free survival (DFS) was calculated as time from the date of diagnosis to the date of the first local recurrence or metastatic failure after surgery or to the date of 1st follow-up in patients without recurrence or metastasis. Overall survival (OS) was calculated as the time from the date of diagnosis to the date of death or the date of last follow-up if the patient was still alive. Survival rate was calculated using the Kaplan–Meier method. Univariate and multivariate survival analyses were performed to test the association of clinicopathological features with DFS and OS, incorporating log-rank testing and Cox proportional hazard regression models.

Correlations between Axl and clinicopathological features were examined by chi-square or ANOVA testing. Pearson analyses were used to test correlations between P-Axl and MMP-9 expression in osteosarcoma tissues. Statistical analyses were conducted using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) with a 2-sided significance level of $P < 0.05$.

3. Result

3.1. P-Axl is overexpressed and translocated to the nucleus in osteosarcoma cells and promotes tumor invasion and lung metastasis

P-Axl expression was detected in the nucleus and cytoplasm in cultured osteosarcoma cell lines (Fig. 1A). Western Blot analysis revealed high P-Axl expression in 3 cases of fresh osteosarcoma tissues compared with benign osteofibrous dysplasia (Fig. 1B). The expression of P-Axl in osteosarcoma tissues was consistent with the results in tumor cell lines. Next, we examined the correlation between P-Axl expression and clinicopathological characteristics. Out of 62 osteosarcoma cases, P-Axl was highly expressed in 27 cases, and positive expression was detected in both the nucleus and cytoplasm of tumor cells. P-Axl exhibited a high expression rate of 43.5%, and the average score as 3.15 (according to the calculating method in Section 2). Only 3 cases were positive out of the 21 cases of osteofibrous dysplasia; the positive expression rate was only 14.3%, with an average score of 0.62. There were significant differences between the tumor group and the benign group ($P < 0.05$) (Fig. 1C).

As shown in Table 1, P-Axl is positively correlated with the clinicopathological features of osteosarcoma. The median follow-up time was 87 months (range: 8–144 months). Among the 62 patients, 16 cases developed recurrence or lung metastasis. Twelve of the 16 cases exhibited high P-Axl expression. The difference in the level of P-Axl expression between the high expression group and the low expression group was significant ($P < 0.05$), indicating that P-Axl was positively correlated with tumor recurrence or lung metastasis ($P < 0.05$). However, P-Axl expression exhibited no significant relationship with other clinical features, including surgery type, gender, age or site and size of tumor.

3.2. P-Axl acts as an independent predictor of worse prognosis and is correlated with MMP-9 expression in osteosarcoma patients

To elucidate the possible relationship between P-Axl expression and patient survival, we evaluated the correlation between P-Axl and disease-free survival (DFS) and overall survival (OS) in the cases with follow-up data. The 5-year survival rate of patients whose samples represented low P-Axl expression was 65.7% (23/35), and the survival rate significantly decreased to 40.7% (11/27) in the high-expression group ($P < 0.05$). Furthermore, the DFS

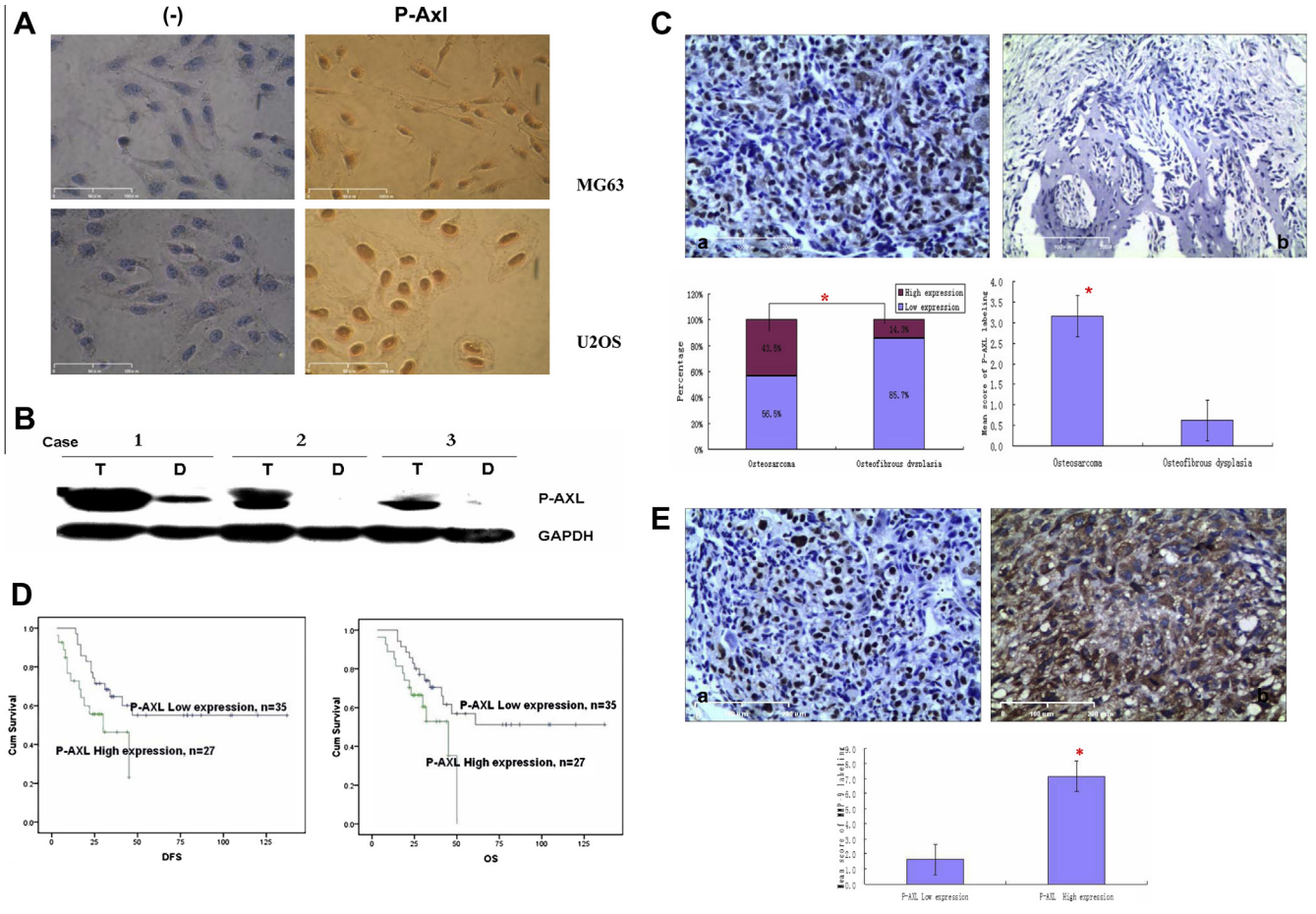


Fig. 1. The expression of P-Axl in osteosarcoma and the correlationship between P-Axl and clinicopathological characteristics. (A) Both the nucleus and cytoplasm were positive for P-Axl in the cultured osteosarcoma cell lines. (B) P-Axl was highly expressed in 3 pairs of osteosarcoma and benign osteofibrous dysplasia by Western Blot assay. (T, tumor; D, osteofibrous dysplasia). (C) Immunohistochemistry staining on osteosarcoma tissues. In osteosarcoma (a) and osteofibrous dysplasia (b) the positive signals were located in the tumor cell nucleus and cytoplasm. The expression intensity of P-Axl was significantly higher in osteosarcoma than in osteofibrous dysplasia ($P < 0.05$). (D) In the Axl highly expressed group, DFS and OS was significantly lower than in the P-Axl lowly expressed group ($P < 0.05$). (E) Expression of P-Axl (a) and MMP-9 (b). MMP-9 expression was obviously higher in the P-Axl highly expressed group than in the lowly expressed group ($P < 0.05$).

Table 1
Correlation between P-AXL and clinicopathologic characteristics.

Clinicopathologic characteristics	P-Axl low expression ($n = 35$)	P-Axl high expression ($n = 27$)	P
Gender			
Male	23	20	0.479
Female	12	7	
Age			
<18	22	14	0.384
⩾18	13	13	
Primary location			
Humerus	3	5	0.22
Radius	0	1	
Femur	19	8	
Tibia	10	8	
Fibula	3	4	
Others	0	1	
Histological type			
Osteoblastic	19	16	0.61
Chondroblastic	8	4	
Fibroblastic	5	6	
Dilated blood vessels	1	1	
Others	2	0	
Tumor size			
<6 cm	21	11	0.132
⩾6 cm	14	16	
Surgery type			
Limb sparing	20	18	0.445
Amputation	15	9	
Local recurrence/lung metastasis			
Yes	4	12	<0.05
No	31	15	
Survival status			
Alive	23	11	<0.05
Dead	12	16	

was 85.43 ± 10.21 months in the low P-Axl expression group, compared with 28.82 ± 3.55 months in the highly expressed P-Axl group. In addition, the OS was 86.42 ± 10.17 months in the low expression group, compared with 34.61 ± 3.44 months in the high expression group (Fig. 1D). These results demonstrate that P-Axl expression predicts significant differences in DFS and OS, as determined by the survival rates of the two P-Axl expression groups ($P < 0.05$).

By univariate Cox regression analysis (Table 2), the expression of P-Axl in osteosarcoma was correlated with patient prognosis. Increased P-Axl expression was correlated with an increased relative risk (*RR*) and a decreased DFS and OS. Other predictors of poor prognosis included the tumor size (⩾6 cm), amputation, local recurrence or lung metastasis. The multivariate Cox regression model for clinicopathological diagnoses demonstrated that high P-Axl expression in osteosarcoma tumors was an independent factor predicting DFS ($P = 0.035$) and OS ($P = 0.042$) (Table 3).

In the osteosarcoma tissues, we observed that the average score of MMP-9 in the high P-Axl expression group was significantly higher than that of the low P-Axl expression group, demonstrating a strong positive correlation between P-Axl and MMP-9 expression ($r = 0.356$, $P < 0.05$) (Fig. 1E).

3.3. Activated Axl protect osteosarcoma cell lines from apoptosis in vitro with no effect on viability and cell cycle

To determine whether Axl kinase activity regulates osteosarcoma viability, Axl kinase activity was stimulated using its ligand, Gas6. These studies were performed in two osteosarcoma cell lines, MG63 and U2OS. Western Blot analysis revealed that the expression of phosphorylated Axl increased in a dose- and time-dependent manner (Fig. 2A and B). There was no effect of Gas6 stimulation on the proliferation of the two cell lines by MTT assay (Supplementary Fig. S1) or on cell cycle arrest in either of the two cells (Supplementary Fig. S2). Hoechst 33258 staining revealed that cells stimulated with 200 ng/ml Gas6 were protected from serum starvation-induced apoptosis, the apoptosis inhibitory rate of the tumor cells dicreased dramatically with the protection of 200 ng/ml and 400 ng/ml rhGas6 (Fig. 2C).

3.4. Activated Axl promotes osteosarcoma cell migration and invasion in vitro

Osteosarcoma clinical spread is characterized by invasive growth and lung metastasis. Therefore, we evaluated the role of Axl in osteosarcoma migration and invasion. Wound-healing assays in Axl-phosphorylated MG63 and U2OS cells demonstrated that Gas6 stimulation resulted in a marked increase in wound closure at 24–48 h (Fig. 3A and B). Compared to the control, the cells with the stimulation of 200 ng/ml to 400 ng/ml rhGas6 have obviously higher ability to migrate. Transwell chamber assay also confirmed that Axl activated by at least 200 ng/ml rhGas6 could promote cells migration and invasion *in vitro* (Fig. 4A and B).

3.5. The AKT signaling pathway is involved in Axl phosphorylation by Gas6 in osteosarcoma cells

Ligand-mediated Axl modulation was evaluated by suppressing endogenous GAS6 in osteosarcoma cell lines and by treating the cell lines with exogenous GAS6. In both of the osteosarcoma cell lines, Western Blot analysis revealed that P-Axl was overexpressed in response to rhGas6 stimulation, whereas P-Axl expression was nearly undetectable in the absence of Gas6 treatment. Notably, the highest P-AKT expression was observed in MG63 after treatment with 100 ng/ml Gas6 and in U2OS after 200 ng/ml Gas6 stimulation. The trend in P-AKT expression was consistent with the P-Axl expression in both of the two OS cells, whereas the expression of phosphorylated ERK exhibited no obvious changes(Supplementary Fig. S3A). After stimulation with 200 ng/ml rhGas6, P-AKT expression increased in both of the OS cells within 30 min, but no change was observed in P-ERK expression (Supplementary Fig. S3B).

4. Discussion

Numerous studies have demonstrated that the TAM RTK family members, notably Axl, play an important role in tumor development and cancer progression due to their involvement in tumor cell survival and growth, increased migration and angiogenesis [19]. Nevertheless, little is known concerning the role of Axl in osteosarcoma. Nakano et al. [14] have reported that the AXL gene was one of the five genes associated with osteosarcoma cell motility and/or invasiveness, but the role of activated Axl protein and its clinicopathological relationship to osteosarcoma has yet to be elucidated.

In this study, we demonstrate that activated Axl (P-Axl) is significantly increased in osteosarcoma patient samples, and the expression level of P-Axl is statistically correlated to tumor recurrence and lung metastasis. Furthermore, we also demonstrate that the level of P-Axl expression in osteosarcoma is an independent factor that correlates with patient prognosis. All these studies prove that activated Axl participate in the progression of osteosarcoma. Thus, P-Axl is an useful predictor of patient prognosis and justifies further investigation of Axl as a potential target for cytokine therapy.

Table 2
Univariate Cox regression analysis of potential prognostic factors for 62 patients with osteosarcoma.

Clinicopathologic characteristics	DFS		OS	
	Hazard ratio (95% *CI*)	*P*	Hazard ratio (95% *CI*)	*P*
Gender				
Male	1.000		1.000	
Female	0.586 (0.245, 1.399)	0.228	0.563 (0.235, 1.346)	0.196
Age				
<18	1.000		1.000	
⩾18	0.784 (0.359, 1.713)	0.542	0.801 (0.366, 1.751)	0.578
Primary location				
Humerus	1.000		1.000	
Radius	1.451 (0.161, 13.09)	0.740	2.092 (0.230, 18.998)	0.512
Femur	0.723 (0.233, 2.245)	0.575	0.736 (0.236, 2.289)	0.596
Tibia	0.433 (0.122, 1.54)	0.196	0.489 (0.137, 1.743)	0.270
Fibula	0.584 (0.130, 2.267)	0.484	0.598 (0.133, 2.687)	0.503
Others	2.422 (0.263, 22.26)	0.434	4.374 (0.461, 41.52)	0.199
Histological type				
Osteoblastic	1.000		1.000	
Chondroblastic	0.654 (0.237, 1.964)	0.530	0.448 (0.129, 0.952)	0.125
Fibroblastic	0.875 (0, 437, 2.054)	0.823	0.761 (0.301, 1.821)	0.281
Dilated blood vessels	1.263 (0.563, 1.753)	0.174	1.386 (0.453, 2.271)	0.852
Others	0.531 (0.128, 1.274)	0.224	0.402 (0.102, 0.742)	0.406
Tumor size				
<6 cm	1.000		1.000	
⩾6 cm	1.873 (0.857, 4.093)	0.116	2.730 (1.202, 6.201)	0.016
Surgery type				
Limb sparing	1.000		1.000	
Amputation	2.360 (1.010, 5.058)	0.027	2.527 (1.179, 5.416)	0.017
Local recurrence/lung metastasis				
Yes	1.000		1.000	
No	0.155 (0.064, 0.376)	<0.05	0.397 (0.181, 0.872)	0.021
P-Axl expression				
Low	1.000		1.000	
High	2.226 (1.017, 4.871)	<0.05	2.184 (0.983, 4.850)	<0.05

Table 3
Multivariate Cox regression analysis of potential prognostic factors for 62 patients with osteosarcoma.

Clinicopathologic characteristics	DFS		OS	
	Hazard ratio (95% *CI*)	*P*	Hazard ratio (95% *CI*)	*P*
Tumor size				
<6 cm	–	–	1.000	
⩾6 cm			3.191 (1.366, 7.455)	0.007
Surgery type				
Limb sparing	1.000		1.000	
Amputation	2.206 (1.026, 4.743)	0.043	2.474 (1.153, 5.307)	0.020
Local recurrence/lung metastasis				
Yes	1.000		1.000	
No	0.163 (0.067, 0.396)	<0.05	0.316 (0.140, 0.712)	0.005
P-AXL expression				
Low	1.000		1.000	
High	1.867 (0.800, 4.357)	0.035	1.385 (0.539, 3.641)	0.042

Although RTKs, such as Axl, are typically located on cell membranes, we detect P-Axl localization in both the nucleus and cytoplasm in osteosarcoma cells and tissue. Nuclear translocalization of several cell surface RTKs has been previously reported, including EGFR, HER-2 and HER-3 receptors, TrkA,B/NGFR, FGFR, VEGF receptor 2 (VEGFR-2) and type I TGF-b receptor [20]. Further, nuclear import of cell-surface receptors has been shown to occur in ligand-dependent and -independent manners. Nuclear EGFR, HER-2, HER-4 and FGFR possess the intrinsic ability to enhance gene transcription. In addition, nuclear RTKs may have other functions, such as DNA damage/repair [20]. Our results suggest that in osteosarcoma, as EGFR in breast cancer, Axl translocate to the nucleus through a ligand-dependent manner, and this translocation may contribute to tumor progression.

Activation of Axl can enhance cell proliferation in mesothelioma cells [21]. However, in the osteosarcoma cell lines MG63 and U2OS, we did not observed any effects of Axl activation on cellular proliferation as measured by MTT assay, nor did we observe any changes in the cell cycle. However, we found that when we cultured cells in a serum-free environment, the introduction of rhGas6 was able to protect cells from apoptosis that is otherwise normally observed. Consistent with our results, in squamous cell carcinoma, Gas6 stimulation of Axl has no effect on cell proliferation but does enhance the ability to resist apoptosis [22]. This evidence suggests

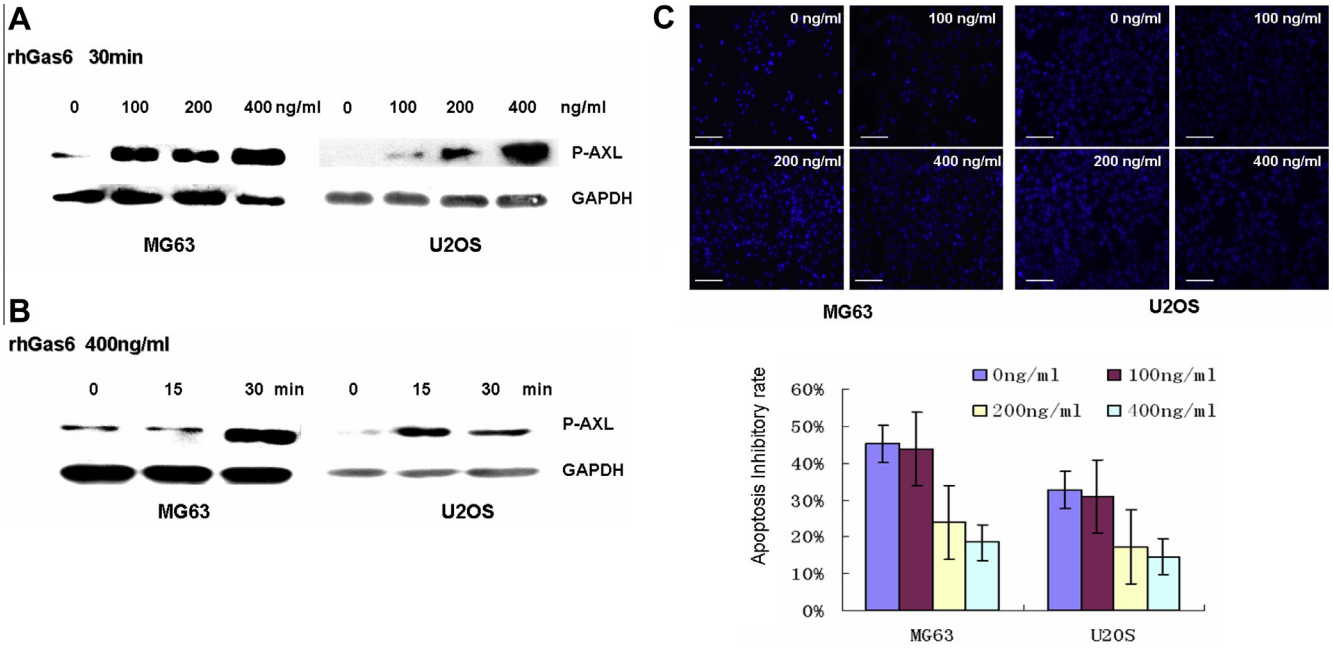
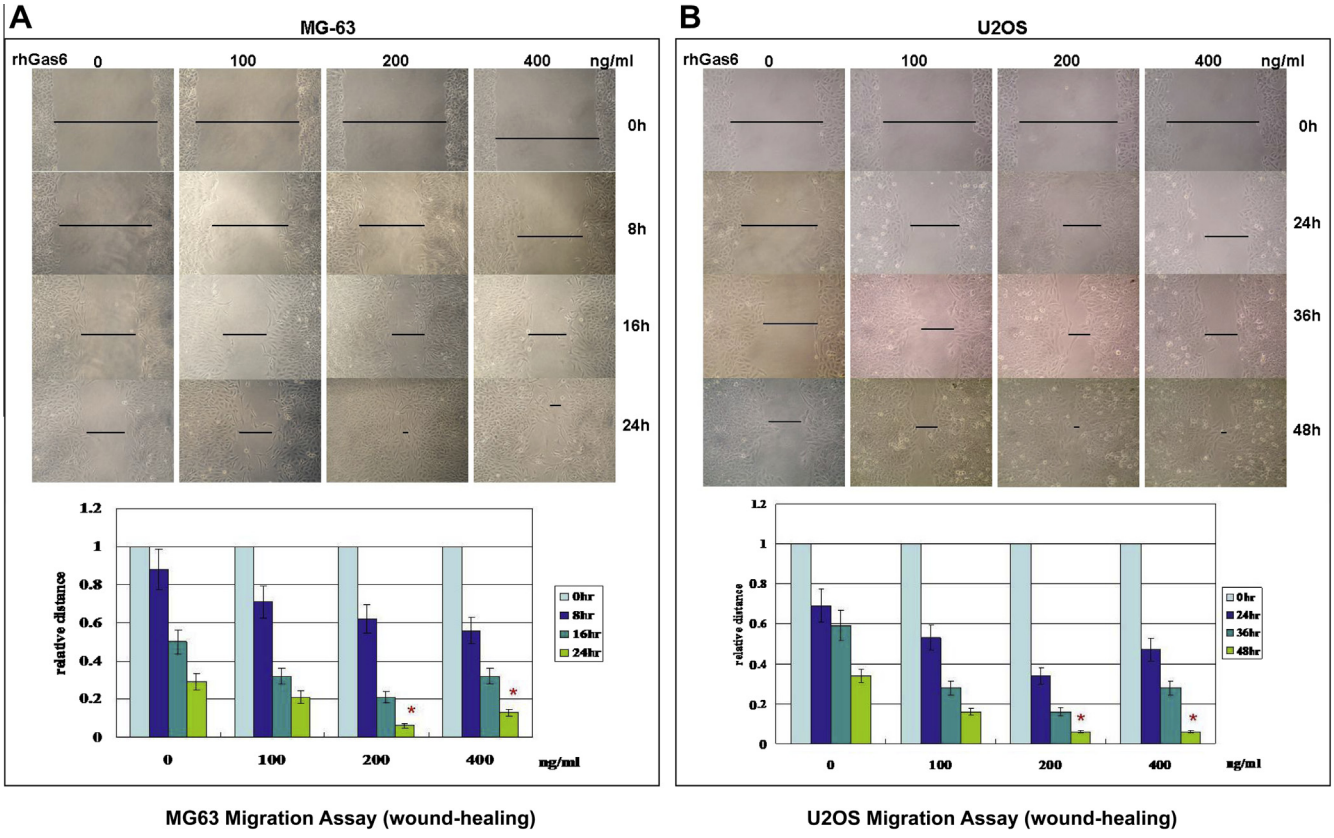


Fig. 2. P-Axl expression and function in osteosarcoma cells stimulated with rhGas6. (A) P-Axl expression increased obviously with 100 ng/ml rhGas6 in MG63 and 200 ng/ml in U2OS within 30 min. Within the concentration of 400 ng/ml rhGas6, P-Axl expression increased in a dose dependent manner. (B) In MG63 and U2OS cell lines stimulated with 400 ng/ml rhGas6, P-Axl increased obviously in 30 min in MG63 and increased obviously in 30 min in U2OS, which changed in a time-dependent manner within 30 min. (C) In both MG63 and U2OS, 200 ng/ml rhGas6 resisted obviously the apoptosis of the tumor cells caused by serum starvation. Especially for U2OS, there were a few apoptotic cells with the protection of 400 ng/ml rhGas6 (the size bar represent 500 μm).



MG63 Migration Assay (wound-healing) **U2OS Migration Assay (wound-healing)**

Fig. 3. Osteosarcoma cell lines MG63 and U2OS migration assay *in vitro*. (A) In MG63 cells, wound healing assays revealed that 200 ng/ml rhGas6 for 24 h can promote wound closure. Compared to the control, the cells with the stimulation of 200 ng/ml to 400 ng/ml rhGas6 have obviously higher ability to migrate. (B) In U2OS cells, both 200 ng/ml and 400 ng/ml rhGas6 promoted wound closure within 48 h. As the same as MG63, 200 ng/ml rhGas6 also promote the ability to migrate in U2OS.

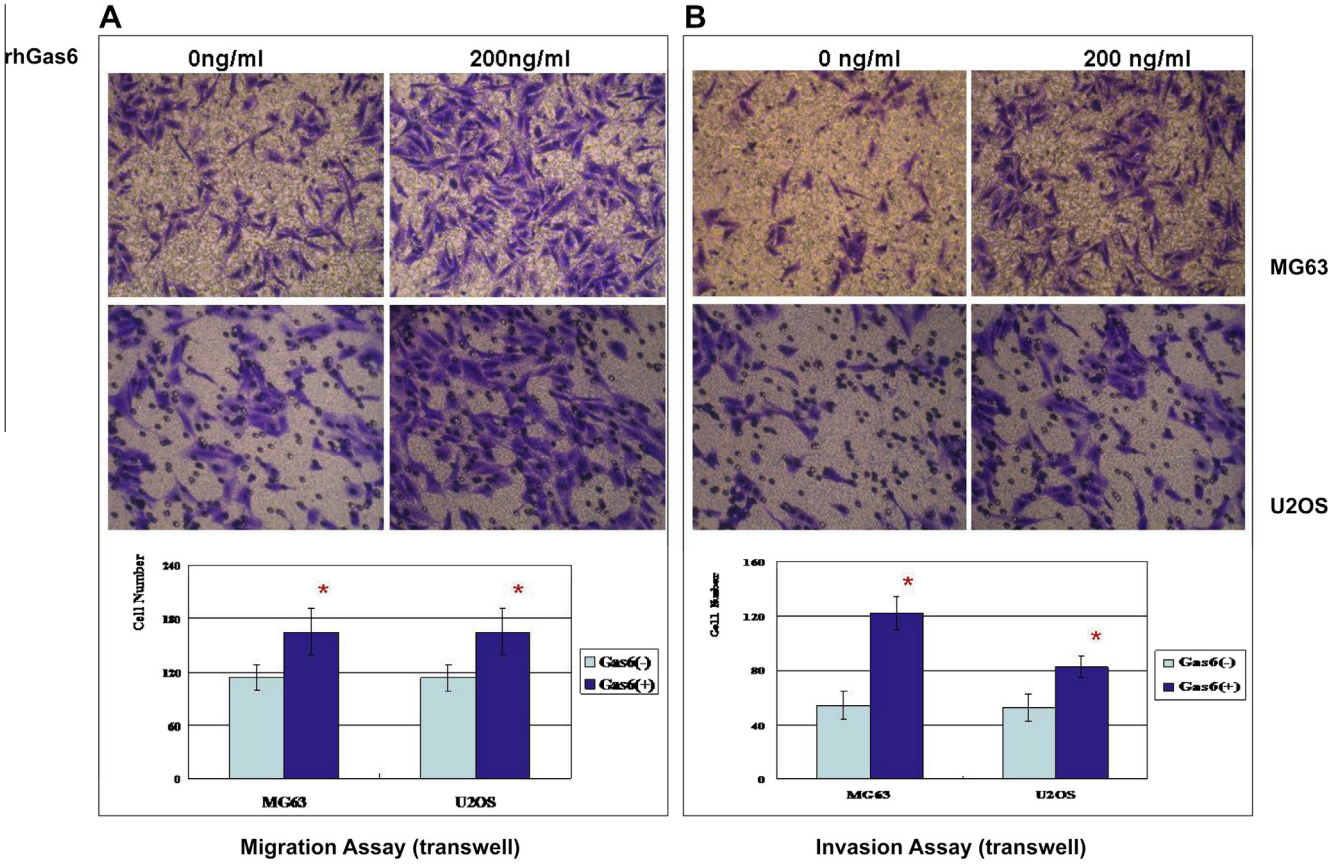


Fig. 4. Osteosarcoma cell lines MG63 and U2OS invasion assay *in vitro*. Transwell chamber assay also revealed that 200 ng/ml rhGas6 promote significantly the ability *of* migration (A) and invasion (B) *in vitro* in both the two cell lines.

that Axl promotes osteosarcoma proliferation by protecting tumor cells from apoptosis.

In some cancer cells, Axl mediate invasive signaling and enhance the expression of MMP-9 [23]. We confirmed that activation of Axl enhanced osteosarcoma cellular migration and invasion *in vitro*, and the expression of P-Axl and MMP-9 was significantly correlated in osteosarcoma patients. It indicate that in osteosarcoma P-Axl may mediate the tumor progression either by promoting tumor cells migration and invasion, which is linked to the expression of MMP-9, or by protecting tumor cells from apoptosis. However, its detailed role in the mechanism is still not completely understood.

In some cancer cell lines as MCF-7 and MDA-MB-415 etc., P-Axl has been proved to regulate MMP-9 promoter activity by MAP kinase kinase (MEK)/ERK signaling pathway [23]. While, in cutaneous SCC Axl controls the relative balance between AKT and PTEN activities to modulate the apoptotic response [22]. In our study, we further confirm that the expression of P-AKT protein increased with the concentration increasing of rhGas6, which was in consistence with the change of P-Axl. It throws light on the theory that P-Axl may mediate the migration and invasion with MMP-9 and inhibit tumor apoptosis by AKT signaling pathway in osteosarcoma cells. Further investigation is needed to know well the pathway and find more potential target for cytokine therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.019.

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