SerpinE2 promotes multiple cell proliferation and drug resistance in osteosarcoma

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Abstract. SerpinE2 is a member of the Serpins family, which could inhibit serine protease and promote tumor progression, particularly in tumor metastasis. However, at present, its role in the progression of osteosarcoma has not been determined. The present study analyzed the expression profiles of SerpinE2 in cancer tissues, including tissues from osteosarcoma of different stages. Higher expression of SerpinE2 was shown in osteosarcoma tissues, particularly in tissue from patients with metastasis and a tumor-node-metastasis stage II-III. Following chemotherapy, the SerpinE2 expression levels were shown to be higher than those at diagnosis. Cell proliferation and colony formation were increased after transfection with SerpinE2 over-expression vector. Additionally, drug resistance to bortezomib and doxorubicin treatment following SerpinE2 transfection was analyzed. MG-63 and SAOS-2 cells showed less sensitivity following transfection with SerpinE2. The cell cycle-related genes, cyclin-dependent kinase (CDK)4 and cyclin D1 were positively correlated with SerpinE2 expression in patient-derived tissue and in osteosarcoma cells. Finally, the high expression of SerpinE2 contributes to poor survival rates in patients with osteosarcoma. In conclusion, high expression of SerpinE2 in osteosarcoma stimulates cell proliferation, promotes drug-resistance, and results in poor survival by regulating CDK4 and cyclin D1. Thus, SerpinE2 could be a potential target for treatment of patients with osteosarcoma.

Introduction

Osteosarcoma is one of the most fatal tumors worldwide (1). The most common treatments for osteosarcoma are radiotherapy, chemotherapy and surgical resection (2,3). However, the side effects and cure rate remain unsatisfactory. Understanding the molecular mechanisms of oncogenes and tumor suppressor genes that are involved in cancer progression may provide novel targets and biomarkers for diagnosis and therapy of osteosarcoma.

Serpins are a group of proteins, which are able to inhibit proteases (4). Certain members of the Serpin family, including SerpinI1, SerpinB2, SerpinE2 and SerpinD1, have been demonstrated to participate in tumor progression, particularly in tumor metastasis (4-8). These proteins are involved in sustaining cancer cells in brain metastasis (5). Serpins also exhibit a role as anti-plasminogen activators, which act as a shield for cancer from deleterious signals of astrocytes (9,10). Among these proteins, SerpinE2 is an extracellular plasminogen activator inhibitor and regulates broad key factors of tumor progression (11-13). In addition, SerpinE2 was found to be increased in pancreatic tumors (14), breast tumors (15), colorectal tumors (16) and oral squamous carcinomas (17). SerpinE2 also promotes tumor metastatic activity (14,18). Thus, it was hypothesized that SerpinE2 mediates cell growth and invasion of osteosarcoma. However, the role of SerpinE2 in osteosarcoma has not previously been reported and its molecular mechanisms are poorly understood in osteosarcoma progression.

In the present study, the expression of SerpinE2 in different osteosarcoma tissues was investigated. The cell proliferation and drug resistance following SerpinE2 transfection were determined. Moreover, the alteration of the cell cycle following SerpinE2 over-expression and its mechanism was discussed. Finally, the correlation between SerpinE2 expression and overall survival rates were also investigated.

Materials and methods

Patients. Clinical osteosarcoma and tumor-adjacent normal samples from 80 patients with osteosarcoma were obtained from the Department of Orthopedics, The Second Xiangya Hospital, Central South University (Changsha, China). Informed consent was obtained from all patients, and the study was approved by the ethics committee of the Second Xiangya Hospital. The patients had not received any treatment prior to tissue collection. The tissue histology classification was demonstrated using the World Health Organization classification (19). The characteristics of the patients are shown in Table I. After tissue collection, 20 patients with T1/T2 stage osteosarcoma were treated with cis-platinum (30 mg once daily; Sigma-Aldrich, St. Louis, MO, USA) and the samples of osteosarcoma and the tissues

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were collected at all three stages prior to the second and fourth chemotherapeutic treatment.

Cell lines. MG-63 and SAOS-2 osteosarcoma cell lines (The Second Xiangya Hospital, Central South University) were cultured at 37˚C in an incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.) and penicillin/streptomycin (100 μg/ml, Sigma-Aldrich).

Reagents. Antibodies against SerpinE2 (rabbit polyclonal; cat. no. ab75348), CyclinD1 (rabbit monoclonal; cat. no. ab134175), CDK4 (rabbit monoclonal; cat. no. 108357) and GAPDH (rabbit polyclonal; cat. no. ab9485) were purchased from Abcam (Cambridge, UK), and anti-Annexin V-APC was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Bortezomib was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada) and doxorubicin was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.) was used to extract the total RNAs (2 μg). RT was performed using a PrimeScript RT Reagent Kit with a gDNA Eraser (Takara Bio, Inc., Dailan, China) qPCR was performed on an ABI 7500 Real Time PCR system (Thermo Fisher Scientific, Inc.) using SYBR premix Ex Taq II (Takara Bio Inc.). The primers were designed by Primer Express version 3.0 software (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the sequences were as follows: Forward: 5'-TCTCATTGC AAGATCATCGCC-3' and reverse: 5'-CCCCATGAATAACAC AGCACC-3' for SerpinE2; forward: 5'-CCTAGTACTGCA ATTCGGGAAATT-3' and reverse: 5'-CCTGGAATCCTGCAT AAGCAC-3' for cyclin-dependent kinase (CDK)1; forward: 5'-CCAGGAGTTACTTCTATGCCTGA-3' and reverse: 5'-AATCCGCTTGTTAGGGTCGTA-3' for CDK2; forward: 5'-CACAGTTCGTGAGGTGGCTTTA-3' and reverse: 5'-TGT CCTTAGGTCCTGGTCTACATG-3' for CDK4; forward: 5'-TGCACAGTGTCACGAACAGA-3' and reverse: 5'-ACC TCGGAGAAGCTGAAACA-3' for CDK6; forward: 5'-CTC TTAACCGCGATCCTCCAG-3' and reverse: 5'-CAATAA AAGATCCAGGGTACATGATTG-3' for Cyclin A; forward: 5'-AAAGGCGTAACTCGAATGGA-3' and reverse: 5'-CCG ACCTTTTATTGAAGAGCA-3' for Cyclin B; forward: 5'-TCG CTGGAGCCCGTGAA-3' and reverse: 5'-CCGCCTCTGGCA TTTTGG-3' for Cyclin D1; forward: 5'-ATACAG ACCCAC AGAGACAG-3' and reverse: 5'-TGCCATCCACAGAAATAC TT-3' for Cyclin E; and forward: 5'-CGCTCTCTGCTCCTC CTGTT-3' and reverse: 5'-CCATGGTGTCTGAGCGATGT-3' for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The reaction conditions were as follows: 1 cycle at 95˚C for 10 sec, 40 cycles at 95˚C for 10 sec and 60˚C 30 sec. The relative expression levels were normalized to GAPDH expression using the $2^{-\Delta\Delta Cq}$ method (20).

Cell transfection. The DNA fragment of SerpinE2 was obtained from Invitrogen, Thermo Fisher Scientific, Inc. and cloned into the pEF-BOS-EX expression vector, as previously described (21). The vectors were then transfected into MG-63 and SAOS-2 cells using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc.). The wild-type controls of MG-63 and SAOS-2 cells were not transfected with vectors. Briefly, 1 μg SerpinE2 over-expressing (OE) vector was added to cells in the OE groups. There were five replicates for each group. After 24 h transfection according to the manufacturer's instructions, the cells were used for the following experiments.

Table I. Patient characteristics.

Variable	Number
Age (years)	
≤50	43
>50	37
Gender	
Male	48
Female	32
T stage	
T1/T2	39
T3/T4	41
Histologic grade	
I	30
II	36
III	14
Metastasis	
No (M0)	36
Yes (M1)	44
Tumor size (cm)	
≤5	29
>5	51

Cell proliferation. The cells were counted using a hemocytometer (cat. no. Z359629; Sigma-Aldrich) under an inverted microscope (Axiovert 200 M; Zeiss, Thornwood, NY, USA). The dead cells were determined by 0.4% Trypan Blue (Sigma-Aldrich, St. Louis, MO, USA) staining. A minimum of 100 cells were counted.

Clonogenicity assay. Clonogenic growth was assayed by seeding $1x10^3$ cells in 0.5 ml DMEM containing 10% FBS and 0.33% agar. The cells were cultured for 1 week, the medium was removed and the cells were fixed in 75% methanol and 25% acetic acid for 15 min. The cells were then stained using 0.5% crystal violet (Shanghai Sangon Biological Engineering Technology Co., Ltd., Shanghai, China) in methanol for 20 min. Then the colonies were counted. The clonogenicity was indicated as % colonies relative to MG-63 WT cells. The clonogenic analysis was performed in triplicate.

Flow cytometry. To analyze cell differentiation, the cells ($1x10^5$ cells) were collected using TrypLE (Invitrogen, Thermo Fisher Scientific, Inc.) with 100 U/ml DNase (Invitrogen, Thermo Fisher Scientific, Inc.). The cells were fixed in cold methanol, blocked using 4% FBS and washed with phosphate-buffered saline with Tween 20 (EMD Millipore, Billerica, MA, USA) and

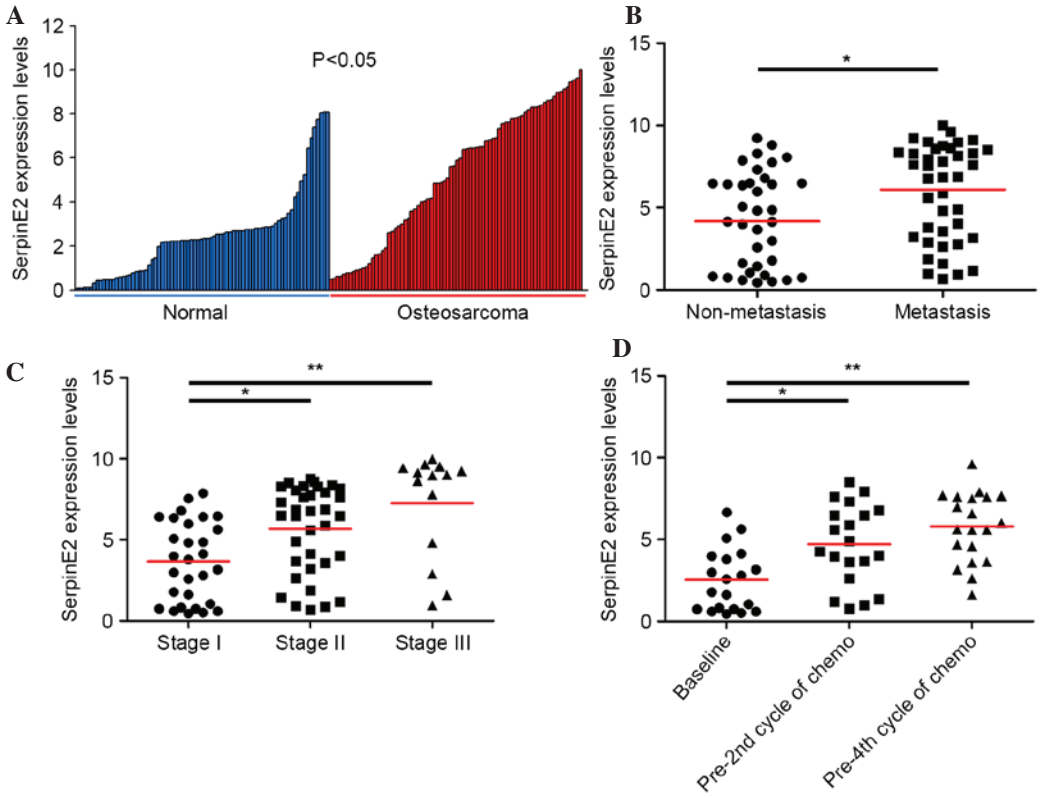

Figure 1. SerpinE2 expression increases in osteosarcoma. (A) SerpinE2 expression in tumor-adjacent normal and osteosarcoma tissues (n=80). (B) Expression of SerpinE2 in tissues that had metastasized compared with non-metastasis tissues. (C) SerpinE2 level changes in tumor-node-metastasis stage I, II and III. (D) SerpinE2 expression in patient samples (n=20) collected at diagnosis, pre-2nd cycle of chemotherapy and pre-4th cycle of chemotherapy. *P<0.05 and **P<0.01.

then incubated with Annexin V-APC antibody (cat. no. A35110; Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Finally, the cells were stained with propidium iodide (Invitrogen; Thermo Fisher Scientific, Inc.) and were analyzed using an FC500 machine and CXP version 2.1 software (both purchased from Beckman Coulter, Pasadena, CA, USA).

Effects of SerpinE2 on drug resistance in osteosarcoma. The cells were treated with bortezomib (5 nM) and doxorubicin (50 nM) as pervious reported (21). After 48 h treatment, the cells were analyzed by a clonogenicity assay.

Western blotting. The proteins were extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Wuhan, China). After determining the protein concentration using a Bradford assay method (Beyotime Institute of Biotechnology), 15 μg protein for each sample was electrophoresed using 15% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies (dilution SerpinE2 1:1,000; Cyclin D1 1:500; CDK4 1:1,000; GAPDH 1:2,000) overnight at 4˚C. After three (5 min/time) washes in phosphate-buffered saline with Tween-20, the secondary antibodies (dilution 1:200) were incubated with the membrane for 30 min at room temperature. Finally, the protein signals were visualized with an enhanced chemiluminescent detection system (EMD).

Statistical analysis. The survival data of osteosarcoma patients were indicated using a Kaplan-Meier curve and analyzed using log-rank test by SPSS v 17.0 (SPSS Inc, Chicago, IL, USA) using the data of the 80 patients. The difference between paired groups was analyzed by paired t-test and the differences among multiple groups (>2) was analyzed by one-way analysis of variance followed by Tukey's test. Gene expression correlations were analyzed using the Spearman's rank correlation coefficient test. P<0.05 was considered to indicate a statistically significant difference.

Results

Increased expression of SerpinE2 in osteosarcoma. Significantly higher expression of SerpinE2 was found in osteosarcoma tissues compared with the tumor-adjacent normal tissues from 80 patients (Fig. 1A). In addition, higher SerpinE2 expression in tissues that had metastasized compared with non-metastatic tissues (Fig. 1B). Notably, SerpinE2 expression in tumor-node-metastasis stage II-III was markedly higher than expression in stage I (Fig. 1C). In addition, the levels of SerpinE2 increased substantially at the three serial stages at diagnosis, prior to the second and fourth chemotherapeutic treatment (Fig. 1D).

SerpinE2 increases cell proliferation of osteosarcoma. To demonstrate the effects of SerpinE2 on osteosarcoma cells, MG-63 and SAOS-2 cell lines were transfected with SerpinE2 OE vectors. The increased expression of SerpinE2

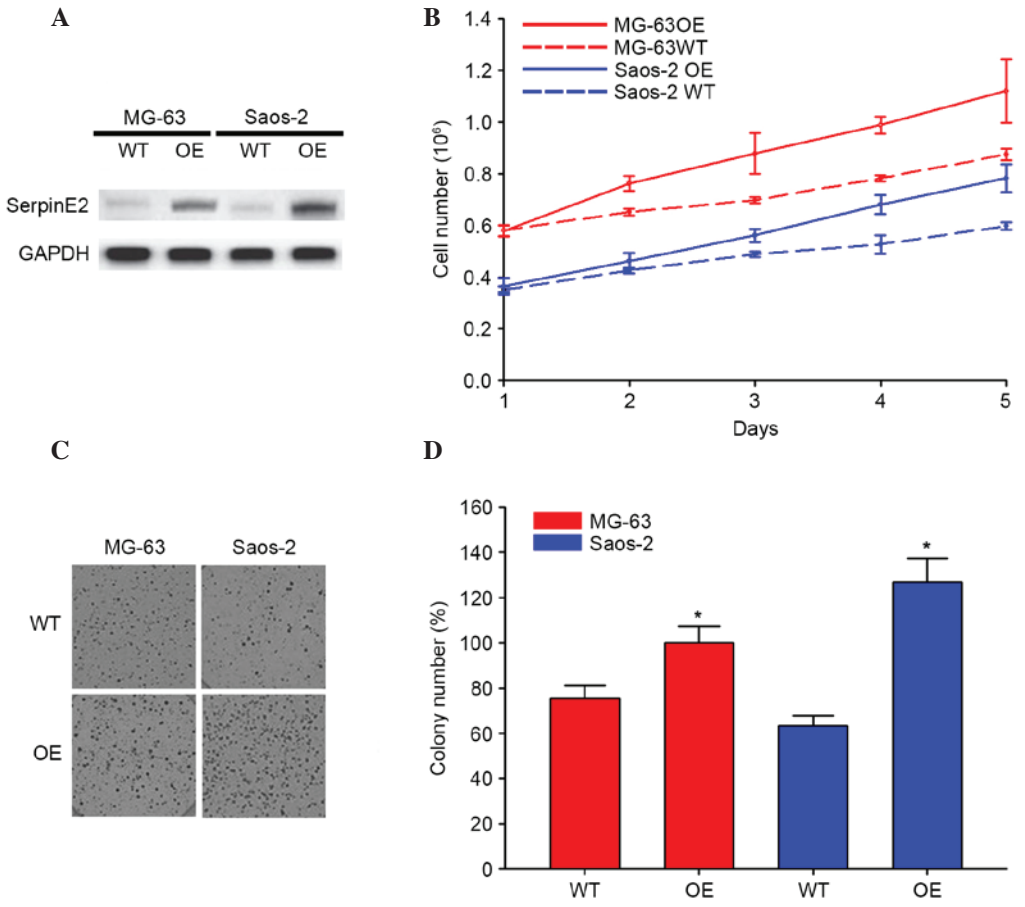

Figure 2. SerpinE2 promotes proliferation of osteosarcoma cells and drug resistance. (A) Western blotting assay of SerpinE2 on transfected (OE) and untransfected (WT) cells. (B) Growth curve of OE and WT cells. (C) The clonogenic capacity of SerpinE2-OE and WT cells (magnification x40). (D) Colony numbers of SerpinE2-OE and WT cells. N=5 for each group; *P<0.05, compared with WT. WT, wild type; OE, cells over-expressing SerpinE2; GAPDH, glyceraldehyde 3-phsophate dehydrogenase.

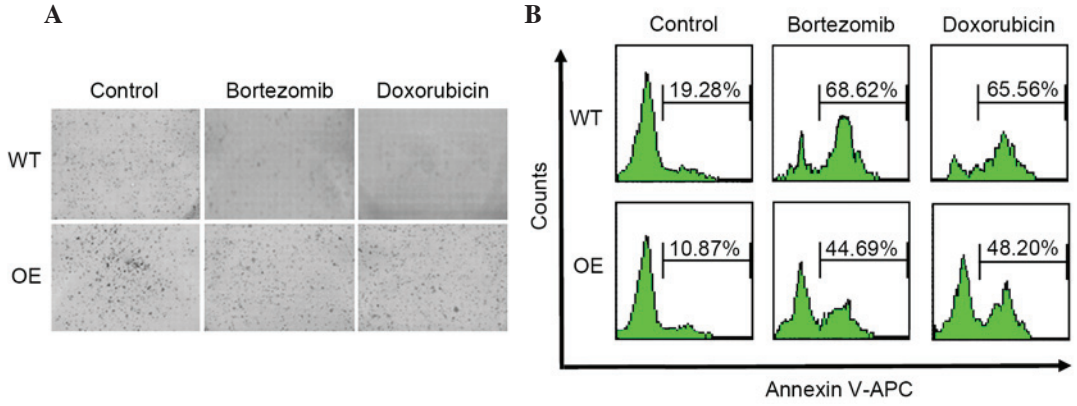

Figure 3. Cell activities of SerpinE2-OE and WT cells after treatment of bortezomib and doxorubicin. (A) The clonogenic capacity of SerpinE2-OE and WT cells after treatment of bortezomib and doxorubicin, respectively (magnification x40). (B) Cell apoptosis was compared between SerpinE2-OE and WT cells treated with bortezomib and doxorubicin by flow cytometry. WT, wild type; OE, cells over-expressing SerpinE2.

in the OE cells compared with untransfected control cells (WT) were verified by western blotting (Fig. 2A). The growth of WT and OE cells was evaluated for 5 days and the results showed significantly increased cell growth of OE cells compared with WT cells (Fig. 2B). The colony formation of MG-63 and SAOS-2 cells was also significantly promoted by over-expression of SerpinE2 (Fig. 2C and D).

SerpinE2 promotes drug resistance in osteosarcoma. Colony formation assays were used to investigate the possibility that SerpinE2 promotes drug resistance in osteosarcoma. There were more colonies of SerpinE2 MG-63-OE cells generated following bortezomib (5 nM) and doxorubicin (50 nM) treatment compared with those of MG-63-WT cells (Fig. 3A). Annexin V as a marker of apoptotic cell death was detected by

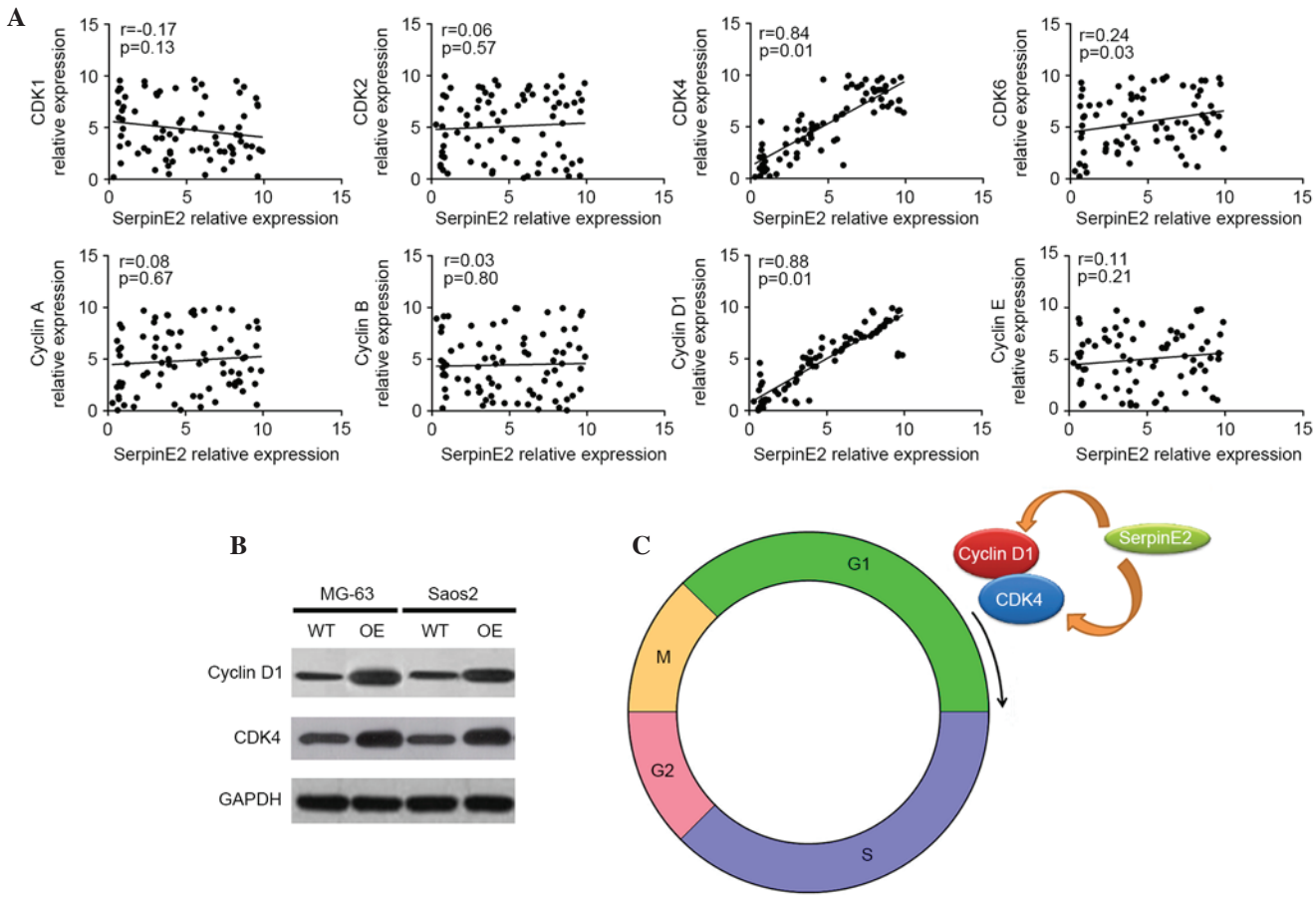

Figure 4. Increased SerpinE2 activates CDK4 and cyclin D1 in osteosarcoma cells. (A) Scatter plots showed the positive correlation between SerpinE2 and CDK4 as well as cyclin D1 (n=80). (B) Western blotting demonstrated the protein expression of CDK4 and cyclin D1 expression in SerpinE2 OE and WT osteosarcoma cells. (C) An illustration of the SerpinE2, CDK4 and cyclin D1 interaction model. WT, wild type; OE, cells over-expressing SerpinE2; CDK, cyclin-dependent kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

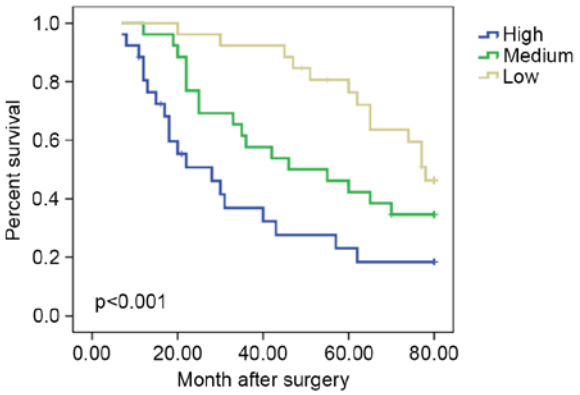

Figure 5. Patients with high expression of SerpinE2 (cut off: 30%) had significantly worse overall survival rates compared with those who had cancers with low and medium SerpinE2 expression.

flow cytometry, which revealed that treatment of cells for 48 h with bortezomib (5 nM) and doxorubicin (50 nM) contributed to fewer apoptotic MG-63-OE cells compared with MG-63-WT cells (Fig. 3B).

SerpinE2 is positively correlated with CDK4 and cyclin D1. To detect the mechanism of SerpinE2 promoting cell proliferation in osteosarcoma, the cell cycle-related genes, CDK1, CDK2, CDK4, CDK6, cyclin A, cyclin B, cyclin D1 and cyclin E were analyzed after transfection with SerpinE2 (Fig. 4A). Only CDK4, CDK6 and cyclin D1 were significantly correlated with SerpinE2 expression (P<0.05). According to the correlation coefficients, CDK4 (r=0.84) and cyclin D1 (r=0.88) were positively correlated with SerpinE2 expression, while no correlation was observed between CDK6 and SerpinE2 (r=0.24). In the cell lines, the protein levels also suggested the correlation between CDK4 and SerpinE2 as well as cyclin D1 and SerpinE2 expression (Fig. 4B). Notably, CDK4 and cyclin D1 mediate the development of tumors by driving cell-cycle progression through G1, which leads to cell proliferation (Fig. 4C). Thus, SerpinE2 can promote cell proliferation of osteosarcoma cells via promoting CDK4 and cyclin D1 expression (Fig. 4C).

Correlation between SerpinE2 expression and survival rates. The expression of SerpinE2 was determined by RT-qPCR. To confirm the correlation between SerpinE2 expression and survival rates, it was demonstrated that patients with osteosarcoma with high SerpinE2 expression [cut off: 30%, high (>30%) vs. low (<30%)] had the worst outcome compared with the patients with low SerpinE2 expression and medium SerpinE2 expression (P<0.05), indicating that patients with high SerpinE2 expression had poor overall survival (Fig. 5).

Discussion

Osteosarcoma, as one of the most dangerous malignant bone tumors, shows high propensity for invasion and proliferation. Recent studies have reported that SerpinE2 is highly expressed in different tumor tissues (12-14,16). The over-expression of SerpinE2 can promote tumor progression. A previous study suggested that SerpinE2 is upregulated by RAS, BRAF and MEK1, which results in oncogenesis of intestinal epithelial cells (16). Thus, SerpinE2 may be a potential target for cancer treatment. The present study demonstrated that SerpinE2 increased the proliferation and invasion of osteosarcoma cells and discussed the mechanism underlying these effects.

The expression levels of SerpinE2 were upregulated in high-grade osteosarcoma cells, which exhibited a similar expression profile to other cancer types. This evidence strongly suggested that SerpinE2 could be regarded as an oncogene as previously suggested (22). In addition, by tracking 20 patients following chemotherapy, SerpinE2 expression profiles during the therapy were analyzed. Prior to the second and fourth chemotherapeutic treatment, the expression of SerpinE2 increased significantly compared with the samples at the beginning of treatment. The higher expression of SerpinE2 was observed in the present study, and this is in accordance with previous studies (23-25). These results showed that SerpinE2 may lead to drug resistance and promote cell proliferation.

Next, the effects of SerpinE2 on osteosarcoma cell lines were assayed. Over-expression of SerpinE2 induced proliferation and increased clonogenic formation of MG-63 and SAOS-2 cells confirming the hypothesis that SerpinE2 promotes the proliferation of osteosarcoma cells. Similar results were found in pancreatic cancer (14), testicular cancer (11) and medulloblastoma (26). In addition, the association between the drug resistance and the expression of SerpinE2 was investigated. Following treatment with bortezomib and doxorubicin, drug resistance was shown. MG-63 cells were shown to be less sensitive to bortezomib and doxorubicin following transfection with SerpinE2. To the best of our knowledge, this is the first study to demonstrate that SerpinE2 contributes to drug resistance in osteosarcoma. Vaillant *et al* (27) indicated that Serpine2 is required for drug resistance.

Currently there is no information regarding the association between SerpinE2 and the cell cycle. Herein, by screening 8 cell cycle-related genes from the 80 patients, it was demonstrated that SerpinE2 expression was positively correlated with cyclin D1 (R=0.88) and CDK4 (R=0.84), respectively. Notably, CDK4 and cyclin D1 mediate the development of tumors by driving cell-cycle progression through G1, which leads to cell proliferation. Therefore, it was proposed that SerpinE2 promotes osteosarcoma by inducing the expression of cyclin D1-CDK4 kinase complexes. These results were further demonstrated by western blot analysis, in which increased SerpinE2 expression upregulated CDK4 and cyclin D1 in cells. Thus, it was suggested that increased SerpinE2 expression in osteosarcoma induces cell proliferation by activating cyclin D1/CDK4 expression resulting in poor survival of patients with osteosarcoma.

In conclusion, the present study demonstrated that increased SerpinE2 expression demonstrates drug-resistance, promotes osteosarcoma cell proliferation and contributes to poor survival in osteosarcoma patients. Thus, targeting SerpinE2 may be a potential therapeutic strategy for patients with osteosarcoma.

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