RESEARCH COMMUNICATION

Knockdown of S100A4 Decreases Tumorigenesis and Metastasis in Osteosarcoma Cells by Repression of Matrix Metalloproteinase-9

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Abstract

Osteosarcoma (OS), the most frequent bone tumor in children and adolescents, is highly malignant. Metastases are the major cause of death, and patients with relapse have a poor prognosis. Given the associations of S100A4 with OS and tumor metastasis, we explored its potential roles in OS metastasis. Among 32 OS (16 metastatic and 16 non- metastatic) specimens examined, we found a significant increase of S100A4 mRNA in metastatic tissues, and more importantly, expression of S100A4 and MMP-9 to be strongly correlated in patients who had lymph node or distant metastasis. We observed that siRNA mediated suppression of the S100A4 gene significantly reduced the proliferative and invasive capability of highly invasive OS cells, with a reduced rate of tumor growth and metastasis under *in vivo* conditions. Matrix metalloproteinase 9 (MMP-9) proved highly responsive to S100A4 gene suppression, demonstrating significant reduction in proteolytic activity, while overexpression of S100A4 increased the expression and proteolytic activity of MMP-9. Links of S100A4 with cell motility were confirmed by depletion which resulted in reduced cell migration. Moreover, loss of cell metastatic potential was completely rescued by overexpression of MMP-9. Collectively, our findings indicate that S100A4 contributes to OS metastasis by stimulating MMP-9 expression, suggesting potential as a novel diagnostic biomarker for OS progression as well as a therapeutic target.

Keywords: Osteosarcoma - metastasis - S100A4 - MMP-9 expression - cell migration

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Introduction

Over 80% of patients with osteosarcoma treated with excision alone develop pulmonary metastases, suggesting that the majority of patients with this disease harbor "micrometastases" at diagnosis. There are no histologic or molecular variables which can predict the presence or absence of micrometastasis, and no therapeutic target in OS (Bjørnland et al., 2005).Thus, the identification of new predictive biomarkers, especially those that are indicative of invasiveness of the disease, which could serve as targets for establishing effectiveness of therapeutic and chemopreventive interventions, will improve clinical management of OS.S100A4 (also known as Mts1, metastasin, p9Ka, pEL98, CAPL, calvasculin, Fsp-1, placental calcium-binding protein) belongs to the family of EF-hand calcium-binding proteins, whose expression is elevated in a number of pathological conditions (Ismail et al., 2008; Mencia et al., 2010; Huang et al., 2011; Lo et al., 2011). Although it is well documented that S100A4 is expressed in cancer cells and contributes to tumor cell motility and metastatic progression, the exact underlying mechanisms remain elusive (Berge et al., 2010; Tarabykina et al., 2010). Matrix metalloproteinases (MMPs) are a class of matrix- and basement membrane-degrading enzymes whose expression is associated with tumor cell invasive and metastatic behavior. One of these enzymes, MMP-9 is expressed in developing and remodeling bone and in osteosarcoma cell lines. It has reported that MMP-9 expression was associated with the micrometastatic behavior of osteosarcoma (Himelstein et al., 1998; Kido et al., 1999).

In this study, we investigated the expression of S100A4 and matrix metalloproteinase (MMP)-9 in OS patients and the potential functional relationship in tumor metastasis.

Materials and Methods

Tissue sample collection

A total of 32 cryostat sections of frozen tissue of osteosarcoma (OS) cases were enrolled in this study: 16 with lymph node or distant metastasis and 16 without metastasis. These patients did not receive any preoperative adjuvant radiation or chemotherapy.

Real-time quantitative RT-PCR for archival material

Total RNA from OS specimens containing at least 80% of tumour cells was extracted from cryostat sections

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of frozen tissue.The RNA was reverse-transcribed in a final volume of 20 µl using Superscript II reverse transcriptase(Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions, with the following conditions:1 mmol/l dNTPs, 40 U of RNase inhibitor, and 10 µl of RNA solution. The reactions were performed at 42˚C for 45 min, followed by inactivation of the enzyme at 70˚C for 15 min. The cDNA was stored at –20˚C.Real-time RT-PCR analyses of mRNAs for S100A4 and MMP-9 were performed using the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Inc, Foster City, CA, USA). Intronspanning primers and probes for the TaqMan system were designed using the Primer Express software (Perkin Elmer, Foster City, CA, USA). Probes were purchased from Shanghai Applied Biosystems.Real-time RT-PCR was performed with the TaqMan Universal MasterMix (PE, Applied Biosystems) using 5 µl of diluted cDNA, 300 nmol/l of the probe, and 300 nmol/l of the primers in a 30 µl final reaction mixture. After 2 min incubation at 50˚C to allow for uracil N-glycosylase (UNG) cleavage, AmpliTaq Gold was activated by incubation for 10 min at 95˚C. Each of the 40 PCR cycles consisted of 15 s of denaturation at 95 ˚C and hybridization of the probe and primers for 1 min at 60˚C. The products of Q-PCR were verified on agarose gels.

Cell culture

The human osteosarcoma cell line MG-63 and SAOS-2 was obtained from the American Type Culture Collection (Manassas, VA, U.S.) and was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 U/mL) at 37°C in an atmosphere of 5% CO2.

Silencing of S100A4

siRNAs were commercially purchased from Qiagen (Valencia,CA).The sequence of selected regions to be targeted by siRNAs was 5'-AACGAGGTGGACTTCCAAGAG-3' for S100A4, and 5'-AATTCTCCGAACGTGTCTCGT-3'for a nonsilencing siRNA (control).Two siRNA sequences were cloned into siRNA expression vector pRNA6.1 using BamHI and HindIII sites. MG-63 and SAOS-2 cells were transfected with the siRNA plasmids in the presence of Lipofectamine. Stable transfectants were selected with 2.0 mg/ml G418

S100A4 cDNA and MMP-9 cDNA Plasmid Construction and Transfection.

S100A4-Luciferase reporters were prepared based on plasmid pGL3-Basic (Promega, Madison, WI, USA). The promoter fragments were generated by PCR amplification of human genomic DNA. The PCR products were then cloned into pGL3-Basic using KpnI and BglII sites. The full-length ORF of human S100A4(MMP-9) was amplified by PCR from the cDNA of an OS tumor tissue. The PCR product was then cloned into pBabe retroviral vector using BglII and SalI sites. The constructs were confirmed by DNA sequencing and restriction enzyme digestion.For transfection studies, MG-63 and SAOS-2 cells were plated at a density of 1 × 106 cells per well in six-well plates and incubated for 24 h in complete medium. The cells were then transfected with S100A4 cDNA(MMP-9) construct by using an Lipofectamine transfection kit for 48 h. For controls, the same amount of empty vector was also transfected.

RT-PCR and Real-Time RT-PCR Analysis of S100A4 and MMP-9 in MG-63 and SAOS-2 cells

PCRs were carried out by using forward and reverse primer combinations for S100A4 (forward 5'-TCAGAACTAAAGGAGCTGCTGACC-3', reverse 5'-TTTCTTCCTGGGCTGCTTATCTGG-3'), MMP-9 (forward 5'-TACCACCTCGAACTTTGACAGCGA-3', reverse 5'-AAAGGCACAGTAGTGGCCGTAGAA-3') and GAPDH (forward 5'-AATCCCATC-ACCATCTTCCAGGAG-3', reverse 5'-GCATTGCTGAT-GATCTTGAGGCTG-3'). The cDNA was amplified with an initial denaturation at 94°C for 3 min followed by the sequential cycles of denaturation at 94°C for 45 sec, annealing at 52°C for 1 min, and extension at 72°C for 1 min for 30 cycles, with final extension at 72°C for 5 min. Real-Time RT-PCR in MG-63 and SAOS-2 cells was performed according to the same instructions detailed above.

Western blot analysis

Whole-cell proteins in different groups were isolated and the lysates were centrifuged and the supernatant collected and stored at -70˚C. SDS-PAGE and Western blotting was performed according to the manufacturer's instructions. Primary antibodies were as follows: anti-S100A4 (1:200 dilution) and anti-MMP-9(1:200 dilution; all from Santa Cruz Biotechnology).The membrane was probed using horseradish peroxidase–conjugated immunoglobulins (DAKO).Western blot images were captured using ImageQuant software (Amersham Pharmacia Biotech).

Zymography for gelatinase

Zymographic analysis of gelatinase activity in secreted medium was done in 10% polyacrylamide gels containing 0.1% gelatin. Cells were cultured in RPMI 1640 containing 10% FBS. After 2 hours, cells were washed extensively and changed to serum-free RPMI 1640. After an overnight incubation, media were collected and mixed with sample buffer for electrophoresis.

After electrophoresis, the SDS was removed from the gel by incubating in 2.5% (v/v) Triton X-100 for 30 minutes. The gels were then incubated at 37˚C overnight in development buffer [50 mmol/L Tris-HCl (pH 7.6), containing 0.2 mol/L NaCl, 5 mmol/L CaCl2] and stained with 40% methanol/10% glacial acetic acid containing 0.5% (w/v) Coomassie brilliant blue G-250 for 20 minutes.

Colony formation assay

Stable transfectants MG-63 and SAOS-2 cells (5,000 cells per well) in 20% FCS/0.7% agarose (top layer) were plated and incubated at 37°C for 14 days. After incubation, plates were stained with 0.005% crystal violet for more than 2 h, and colonies were counted in two colony grids

per well under a stereomicroscope.

Transwell migration assay

Cells (1×10^4) were seeded in 8-mm porous transwell chambers in DMEM without serum and incubated in 24-well-plates with 10% FBS supplemented DMEM for 24 h. Transmigration cells were stained with 0.1% crystal violet and evaluated using microscopy

Tumorigenicity studies in nude mice

After growth to subconfluency, transfected (S100A4 siRNA or mock siRNA) and nontransfected cells were trypsinized and harvested. A total of 1×10^6 cells were suspended in 50 µl of medium and 50 µl of BPS.For s.c. tumor growth, the cell suspension was inoculated s.c. into the right flank on 4-6-week-old male nude mice by using a 27-gauge needle. Every week, tumor volume was measured for 5 weeks. For matastasis assay, MG-63 and SAOS-2 cells stably transfected with S100A4 siRNA were injected into the pancreas under the envelope near spleen of nude mice (n = 6 for each variant). 35 days latter,the mice were killed following the operation. The number of the seeded tumor in the liver and lung is used for assessment of metastases.

Statistical assessment.

All statistical analyses were performed using the SPSS13.0 software. The results were presented as means ± SD of three replicate assays. Differences between different groups were assessed using ANOVA or Dunnett t-test. A P value of <0.05 was considered to indicate statistical significance.

Results

Increased expression of S100A4 mRNA and MMP-9 is associated with lymph node and distant metastasis in OS

Real-time RT-PCR is a sensitive and fast technique for detecting relative mRNA transcription levels and seemed suitable for the analysis of our samples. Moreover, we have tried several commercially available antibodies against human S100A4 in western blot analysis but could not identify an apparently mono-specific antibody useful for immunohistochemistry (data not shown); we therefore analysed S100A4 mRNA instead of protein expression. In the present study,we found that levels of transcripts of S100A4 mRNA and MMP-9 mRNA were increased in metastasis (M) tumor samples in comparison to non-metastasis tumor(N) samples(expressed as transcript copy number per 50 ug of messenger RNA and standardized with β-actin; Figure 1A). Transcript copy numbers for S100A4 were 69.4 ±8.2 for M tumor and 19.4 ±4.8 for N tumor(P=0.015); for MMP-9, they were 51.5 ±7.2 for M tumor and 18.7 ±4.53 for N tumor (P = 0.042); A significant relationship (p = 0.027) was observed between S100A4 mRNA and MMP-9 expression. The products of Q-PCR were verified on agarose gels (Figure 1B). These results indicate that OS metastasis is associated with significantly increased expression of S100A4 and MMP-9, and interestingly, these two proteins seem to coexpress in OS.

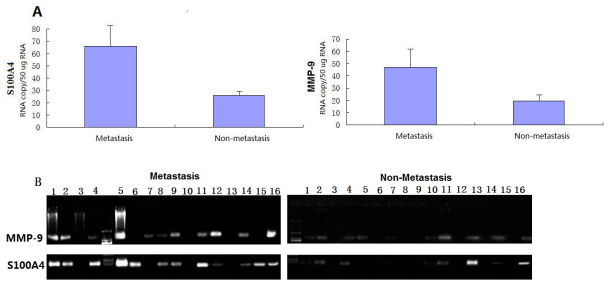

Figure 1. S100A4 and MMP-9 mRNA Analysis. A, Levels of transcripts of S100A4 and MMP-9 in M tumor samples in comparison to N tumor (expressed as transcript copynumber per 50ug of messenger RNA and standardized withβ-actin). B, The products of Q-PCR were verified on agarose gels

S100A4 expression is associated with the levels of MMP-9 protein and mRNA in MG-63 and SAOS-2 cells

The coexpression of S100A4 and MMP-9 in OS suggests a potential interaction of these two proteins, which we studied by examining the MG-63 and SAOS-2 cells stably expressing S100A4 siRNA. By Western blot and PCR analysis, cells transfected with S100A4 siRNA displayed a significantly reduction in the expression of S100A4 protein and mRNA (Figure 2A-J). Consistent

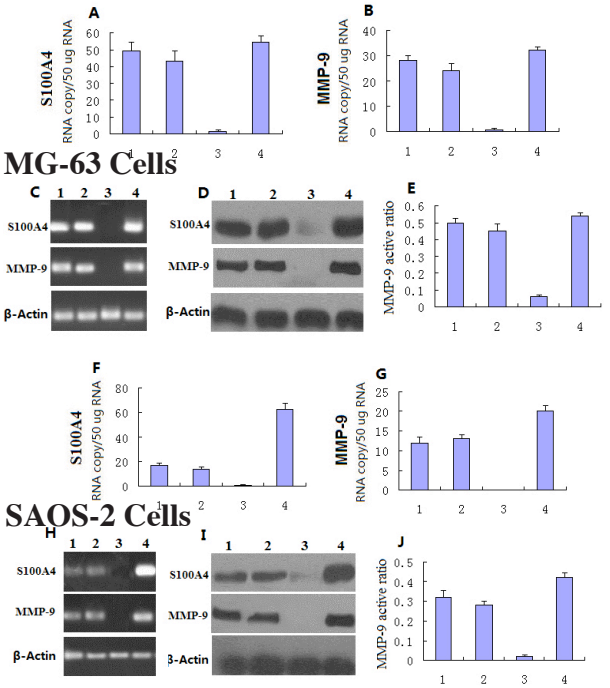

Figure 2. Effect of S100A4 on the MMP-9 Expression Level and Gelatinolytic Activity in MG-63 and SAOS-2 Cell Lines. A,B,F and G, Real-time PCR evaluates mRNA level of S100A4 on the MMP-9 in MG-63 and SAOS-2 cell lines transfected with S100A4 siRNA or/and S100A4 c DNA. Columns, mean of three separate experiments; bars, s.d.C and H,Representative images showing the expression of S100A4 and MMP-9 mRNA as determined by RT-PCR analysis. D and I, Representative images showing the expression of S100A4 and MMP-9 protein as determined by western blot analysis.
E and J,Representative image showing gelatinolytic activity of MMP-9 in cells. Densitometric measurements of the bands in RT-PCR and western blot analysis were performed by using the digitizing software.All experiments were repeated three times with similar results. (1, Control; 2, Mock siRNA; 3, S100A4 siRNA; 4, S100A4 siRNA+S100A4 cDNA)

with these data,Western blot and PCR analysis of the transfectants showed significantly reduced MMP-9 protein and mRNA in MG-63 and SAOS-2 cells that stably expressed S100A4 siRNA(Figure 2A-J). Zymography assay indicated lower activity of MMP-9 in MG-63 and SAOS-2 cells that stably expressed S100A4 siRNA than control and mock siRNA cells (Figure 2.E and J).These results demonstrate that knockdown of S100A4 is associated with decreased MMP-9 expression and activity in MG-63 and SAOS-2 cells.

We also found when the MG-63 and SAOS-2 cells stably expressed S100A4 siRNA were transfected with S100A4 cDNA for 48 h, S100A4 and MMP-9 protein and mRNA were all increased (Figure 2A-J) .These results demonstrate that S100A4 overexpression is associated with increased MMP-9 expression and activity in MG-63 and SAOS-2 cell lines. (1, Control; 2,Mock siRNA; 3, S100A4 siRNA; 4, S100A4 siRNA+S100A4 cDNA).

S100A4 gene suppression or overexpression on invasive capability of MG-63 and SAOS-2 Cells.

The S100A4 gene is reported to confer invasive characteristics to various cancer cells;In the present study, we analyzed the effect of S100A4 gene suppression on the invasive capability of MG-63 and SAOS-2 Cells by Transwell migration assay.As shown in Figure 3 A, suppression of the S100A4 caused a over 50 % reduction (P<0.05) in the number of cells that traversed the membrane versus nonsilencing control in MG-63 cells,and 60 % reduction (P<0.05)in SAOS-2 Cells Figure 3B.When the Stable transfectants(S100A4 siRNA)were transfected with S100A4 cDNA to restore the expression of S100A4 in the stable transfectants cells,only to found significant invasion ability in MG-63 and SAOS-2 cells was restored (Figure 3 A-B).These data suggest that the S100A4 gene controls the motility and invasion of cancerous cells during the metastasis of human MG-63 and SAOS-2 cells.

Transmigration cells of control,mock siRNA,S100A4 siRNA and S100A4 siRNA+S100A4 cDNA cells was calculated from three independent experiments. The indicated cells (1×10^4) were seeded on 8-mm porous transwell chambers. After 24 h of plating, transmigration cells were fixed and stained with crystal violet. Transmigration cells were counted for each of the indicated cells. Columns, mean number of cells obtained in three independent experiments; bars, s.d.; *P<0.05.

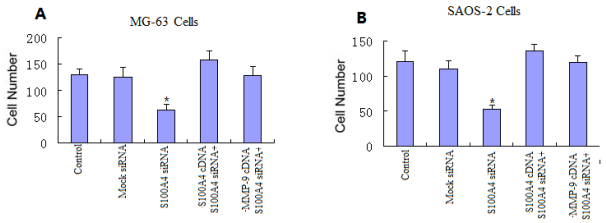

Figure 3. S100A4 gene suppression or overexpression on Invasive Capability in MG-63 and SAOS-2 Cells

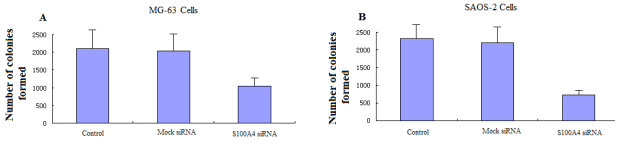

Figure 4. Soft Agar Colony Formation Analysis

Knockdown of S100A4 inhibited metastasis in osteosarcoma cells by repression of matrix metalloproteinase-9

Tumor metastasis consists of numerous consequent processes,which include migration, homotypic and heterotypic cell–cell adhesion, cell–matrix interaction, invasion into surrounding tissues, release from the primary tumor, intravasation, adhesion to vascular walls, extravasation and formation of new foci (Chen et al., 2008; Hoon et al., 2011).Compiled studies have implicated MMPs in cell metastatic potential in physiological and pathological processes through the basis for the mechanism of ECM degradation (Hoon et al., 2011). It has demonstrated above that knockdown of S100A4 is associated with decreased MMP-9 expression and activity in MG-63 and SAOS-2 cells,and knockdown of S100A4 inhibited invasion of MG-63 and SAOS-2 cells. In the present study,we found when the stable transfectants(S100A4 siRNA) were transfected with MMP-9 cDNA to restore the expression of MMP-9 in the stable transfected MG-63 and SAOS-2 cells,only to found significant invasion ability in MG-63 and SAOS-2 cells was shown(Fig.3 A-B).These data suggest that knockdown of S100A4 inhibited invasion of MG-63 and SAOS-2 Cells by downregulation of MMP-9.

Knockdown of S100A4 Gene inhibits Colony Formation in MG-63 and SAOS-2 Cells

To investigate the effect of S100A4 gene suppression on the growth of MG-63 and SAOS-2 Cells, we performed a soft agar colony-formation assay. As shown in Figure 4 A –B, S100A4 siRNA transfected cells formed a significantly reduced (P<0.01) number of colonies compared with control and mock siRNA-treated cells, suggesting that S100A4 might have growth-promoting effects on MG-63 and SAOS-2 cells and that suppression of S100A4 gene reduces the proliferative property of OS cells.

Knockdown of S100A4 Gene on Tumorigenicity and metastasis in Nude Mice

Because suppression of the S100A4 gene reduced OS cell invasion and colony-formation in vitro, we next examined whether these in vitro data have in vivo relevance. To accomplish this goal, MG-63 and SAOS-2 cells stably transfected with S100A4 siRNA were injected

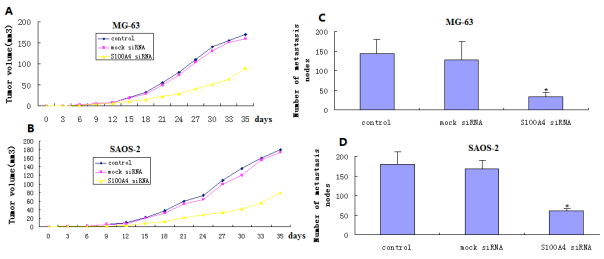

Figure 5. Knockdown of S100A4 inhibits the growth and Metastasis of xenograft OS tumors. A, the growth bar of MG-63 tumors as described in Materials and Methods (n = 6 per group); B, the growth bar of SAOS-2 tumors; C, The metastasis nodes in MG-63 tumors; D, The metastasis nodes in SAOS-2 tumors. Compared to control or mock siRNA groups,*P < 0.01

into the flanks of 4- to 6-week-old female nude mice (1×10^6/100 μL serum-free culture medium).Tumor growth was monitored daily for 35 days. We found mock siRNA did not have any effect on tumor growth compared with controls alone. In contrast, S100A4 siRNA transfected xenograft tumors grew much slower, with at least 50%-60% growth suppression compared with controls and mock siRNA (respectively, $P < 0.01$; Figure 5 A and B).

For metastasis assay, three weeks after the graft, the animals were sacrificed and autopsy was carried out to remove organs.In S100A4 siRNA transfected MG-63 and SAOS-2 cells,fewer metastasis nodes (total nodes in 6 mice)were found than that of controls and mock siRNA ($P < 0.01$; Figure 5 C and D).

Discussion

Osteosarcoma is the most common bone tumor seen in the pediatric and adolescent age group. Survival rates in osteosarcoma have improved considerably from 20 to 65% since the 1980s with the advent of multiagent chemotherapy. Further improvement in survival has not been achieved owing to lack of well-validated prognostic markers and better therapeutic agents. Markers involved with angiogenesis, cell adhesion, apoptosis and cell cycle have been shown recently to play an important role in osteosarcoma growth, differentiation and metastasis (Bakhshi et al.,2010).

MMP-9 has a key role in extracellular matrix degradation and tumor metastasis(Bjørnland et al.,2005;Cho et al.,2007;Kushlinsky et al.,2010) and its overexpression was reported in OS(Himelstein et al.,1998; Kido et al.,1999; Ferrari et al.,2004).The expression of S100A4 in tumors may function as an prometestasis molecule that inhibits cell–cell aggregation (Tarabykina et al., 2007; Ismail et al., 2008; Berge et al., 2010; Mencía et al., 2010; Huang et al., 2011). In the present study, we demonstrate co-overexpression of S100A4 and MMP-9 in OS patients, especially in patients with metastasis. Significant statistical correlation between overexpression of these two genes was found.The data demonstrated trends toward decreased metastasis in patients with overexpression of either one gene or both of them.However, the mechanism in which manner S100A4 promotes OS metastasis remains unknown. It has reported S100A4 promoted metastasis through NF-Kb-dependent expression and secretion of osteopontin in a selection of osteosarcoma cell lines (Berge et al., 2010). Ma(2001) has reported S100A4 promoted the proliferation, invasion and metastasis of osteosarcoma cells by regulating the expression of other proteins that are crucial in modulating cell-ECM adhesion and facilitating ECM degradation.

In this study, we show that overexpression of the S100A4 gene increases the invasiveness of OS cells and that its suppression reverses this effect. We also show that suppression of S100A4 gene reduces the growth and proliferative potential of human OS cells. These data provide evidence that the S100A4 gene may be associated with proliferation, invasion, and metastatic spread of human OS.

Degradation of ECM is required by tumor cells to invade distant tissues (Yang et al., 2001; Matsuyama et al., 2002), and recently metastatic effects of S100A4 have been linked with ECM destruction in some cancer types (Chen et al., 2008).

MMPs are known to degrade the ECM by proteolysis, and a positive correlation has been shown to exist between MMP-9 expression levels in OS patients (Himelstein et al., 1998; Kido et al., 1999). In the present study, we observed that S100A4 gene suppression significantly decreased the expression of MMP-9 and that overexpression of the S100A4 gene significantly increased MMP-9 expression. We also observed that suppression of the S100A4 gene caused a decrease in the proteolytic activity of MMP-9 protein, whereas overexpression of S100A4 caused a reverse effect, suggesting that transcriptional activation of MMP-9 is regulated by the S100A4 gene in OS cells.

In Vivo,we found S100A4-siRNA-transfected OS cells displayed a significantly slower tumor growth.Based on our observations that S100A4-siRNA-transfected cells proliferated more slowly under in vitro and in vivo conditions, we suggest that suppression of the S100A4 gene may have resulted in the loss of its proliferative property. We also found S100A4-siRNA-transfected OS cells displayed a significantly fewer metastasis nodes in vivo,this was indicated suppression of the S100A4 gene may have resulted in the suppression of its invasion property.These data strengthen our notion that the S100A4 gene plays a role during the progression of human OS.

In summary, we demonstrated that the S100A4 gene controls proliferation, invasion, and tumorigenicity of human OS cells through the transcriptional regulation of MMP-9 gene. We suggest that control of proliferation, invasion, and metastasis through suppression of the S100A4 gene may contribute to a novel therapeutic approach against OS. This approach could be realized through development of specific S100A4 inhibitors or use of a gene therapy approach.

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