

ORIGINAL ARTICLE

Elevated expression of matrix metalloproteinase-3 in human osteosarcoma and its association with tumor metastasis

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Summary

Purpose: Matrix metalloproteinase-3 (MMP-3) is one of the several MMPs that is associated with malignant tumors of breast, colon, cervix and lung, where its expression has been correlated with tumor invasion and metastasis. However, the role of MMP-3 in metastasis of osteosarcoma has not yet been explored.

Methods: MMP-3 expression in 15 primary and metastatic osteosarcomas with case-matched adjacent non-tumor tissue was assessed by immunohistochemistry and quantitative RT-PCR. Further, MMP-3 mRNA and protein levels were also determined in osteoblast and osteosarcoma cell lines. Additionally, migration and invasion assays were performed in MMP-3 knockdown cells.

Results: MMP-3 was expressed in 86.6% (13/15) of the

osteosarcoma patients and its expression was significantly higher in metastatic tumors as compared to the primary osteosarcoma tumor tissues. Furthermore, osteosarcoma cell lines showed higher MMP-3 expression as compared to osteoblast cell lines. siRNA-mediated MMP-3 knockdown in osteosarcoma cell lines significantly inhibited their migration and invasion properties.

Conclusion: Our results demonstrated that MMP-3 expression is deregulated in osteosarcomas and this potentially contributes to metastasis and might be a promising marker for the prognosis and therapy of metastatic osteosarcoma.

Key words: EMT, invasion, metastasis, MMP-3, osteosarcoma.

Introduction

Osteosarcoma is the most common histological type of primary malignant bone cancer and is the 8th most common form of malignancy in children and young adults [1,2]. Additionally, 5-year overall survival rate of patients with localized osteosarcoma has reached a plateau of 60–70% through combination of chemotherapeutics and surgery [3]. Osteosarcoma has high propensity to local invasion and early metastasis and, as a result, 5-year overall survival rates have been reported to

be around 20–30% and 5-year event free-survival about 20% [4]. Although 80% of the patients are diagnosed with metastasis or micro-metastasis at the initial stage, only 20% of the patients have clinically detectable disease since most patients suffer from micro-metastasis [5,6]. Therefore, it is critical to know the genes involved in the progression of osteosarcoma, giving rise to a metastatic state [7]. Due to lack of metastatic markers that could potentially differentiate a primary from a

metastatic bone tumor, nearly all patients receive combination treatment with chemotherapy and surgical resection. Hence, there is a clinical demand to identify genetic markers that can precisely predict the presence or absence of metastasis in order to develop better and more effective treatment protocols.

Extracellular matrix (ECM)-degrading MMPs play an important role in cancer progression and are upregulated in almost every cancer type. Their expression, therefore, has been linked to tumor proliferation, angiogenesis, invasion, and metastasis [8-10]. During metastasis, MMPs have also been shown to activate invasive characteristics in cancer cells, thereby inducing the phenomenon of epithelial to mesenchymal transition (EMT), a programmed phenotypic transformation that is also instrumental in developmental as well as wound healing processes. More specifically, MMP-3, also known as stromelysin-1, is one of the several MMPs that regulate angiogenesis, invasion and metastasis. As an example, MMP-3 expression in mammary gland and the lung of transgenic mice is responsible for an invasive carcinoma phenotype as a consequence of EMT and has also been linked to tumor growth and metastasis in human cancers of the colon and cervix [8,11-14]. Although MMP-3 has been reported to be associated with several types of cancer, its expression levels in patients with osteosarcoma have not yet been examined.

Therefore, the aim of this study was to determine the expression levels of MMP-3 in tumor specimens from osteosarcoma patients as well as adjacent non-tumor tissues. Additionally, MMP-3 expression in osteoblast and osteosarcoma tumor cell lines was examined followed by assessing its role in migration and invasion in MMP-3 knock-down cells. To the best of our knowledge, this is the first report that directly correlates MMP-3 expression to the metastatic potential of tumor cells. Taken together, this report suggests the application of MMP-3 as a novel prognostic and invasive marker, as well as a potential target for alternative chemotherapy-based approaches in patients with metastatic osteosarcoma.

Methods

This study was approved by the First Affiliated Hospital of Zhejiang Chinese Medical University Ethics Review Committee, and a written informed consent was obtained from each patient.

Human osteoblast and osteosarcoma cell lines

Human osteoblast HOB-c cell line was purchased from Fisher Scientific (Pittsburgh, PA), and NHOst osteoblast cell line was procured from Lonza (Walkersville, MD). These cell lines were maintained in osteoblast growth medium with supplement mix (Fisher Scientific). Human osteosarcoma cell lines (MG-63, Saos-2 and TE85) were procured from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM or MEM medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. All cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated.

Tumor samples from patients

Human osteosarcoma samples (N=15) along with adjacent non-tumor tissues were collected by biopsy or surgical resection from patients admitted to the First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou City, Zhejiang Province, China. One-half of each tumor sample was frozen (for RT-PCR studies) and the other half was fixed for immunohistochemistry. All the patients used in this study did not receive any pre-operative chemotherapy or radiotherapy prior to surgery.

Immunohistochemistry

Formalin-fixed tumor samples were stained for MMP-3. For this, the samples were paraffin-embedded and sectioned into 4-µm slices. Antigen retrieval procedure was performed for 30 min in 10 mM citrate buffer (pH 6.0), immediately prior to deparaffinization. The slides were then incubated with 3% H₂O₂ for 20 min in order to quench endogenous peroxidase activity. Following this, the sections were incubated overnight at 4°C with primary antibody against MMP-3 (rabbit monoclonal anti-human, EP1186Y, ab52915 1:50 dilution; Abcam, Burlingame, CA, USA) in a humidified chamber. The next day, the sections were incubated with biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, 1:200 dilution) for 1 h at room temperature. For detection, peroxidase-based Elite ABC (avidin/biotin complex) kit (Vector Laboratories) was used together with the substrate, 3,3'-diaminobenzidine, and color development was monitored under the microscope. The slides were briefly counterstained with hematoxylin, dehydrated, cleared, and mounted in Permount medium (Fisher Scientific).

Evaluation of immunohistochemical staining

The intensity of MMP-3 immunostaining of the tumor tissues was evaluated by two pathologists with no prior knowledge of the patients' clinical information. MMP-3 expression scores were given separately and samples that were strongly stained, i.e. in which the proportion of positive cells was above 30% were

considered as strongly positive (+++); cases where the percentage of stained cells was between 20-30% were considered as moderately positive (++); samples that were weakly stained, i.e. in which the percentage of stained cells was below 10% were considered as weakly positive (+). All detection results were evaluated via a double-blind trial by the researchers.

Immunoblotting analysis

Total protein was extracted from the cells in culture using a lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 1 mM PMSF with protease inhibitor cocktail. The protein concentration of the supernatant was determined using the Bio-Rad assay. Following this, samples (standardized to equal amounts of protein) were separated using 10% SDS-PAGE and then transferred onto a nitrocellulose membrane. The membranes were incubated with primary antibodies (MMP-3, mouse monoclonal antihuman, 1:1000 dilution, SC-21732, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA); GAPDH (mouse monoclonal anti-human, 1:1000 dilution, sc-47724, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) overnight at 4°C, followed by incubation with alkaline phosphatase conjugated goat anti-mouse secondary IgG antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) for 2 hrs at room temperature. The blots were then developed using a chemiluminescence reagent.

siRNA transfection

Transfection of MMP-3-specific siRNA (siMMP-3) or nonspecific siRNA (siCtrl) was carried out using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 3×10^5 cells/well were seeded on a 6-well plate. Following an overnight period of attachment and acclimatization, cells were transfected with 1.5 µg of siRNA and Lipofectamine (Life Technologies, Grand Island, NY) and medium change was performed after 6 hrs of transfection. After 2 days of transfection, cells were collected and MMP-3 knockdown was verified by immunoblotting (IB).

qRT-PCR analysis

Total RNA was extracted from tumor tissues using TRIzol reagent. cDNA was synthesized from 2 µg of total RNA using Oligo(dT)12-18 primer and Superscript II reverse transcriptase. TaqMan probes (Invitrogen) were used to measure human MMP-3 (assay ID number Hs00968305_m1) and GAPDH (assay ID number Hs02758991_g1) mRNA expression. PCR was carried out in a 20 µl mixture containing 150 ng cDNA, 10 µl TaqMan 2X universal PCR master mix and 1 µl probe and the samples were run on the ABI Prism 7900 Fast Real-time PCR system for each gene and each sample in triplicate. The following amplification parameters were used: 95 °C for 10 min, 45 cycles of a 15-s dena-

turing at 95 °C, and 1 min annealing at 60 °C. SDS 2.1 Software (ABI) was used to calculate MMP-3 mRNA expression levels, normalized to an endogenous control gene (GAPDH), and relative to either normal tissue or scrambled controls.

Migration and invasion assays

Cell migration and invasion assays were performed using the transwell system (from BD Bioscience, San Jose, CA) following the manufacturer's instructions. Migration and invasion assays were conducted as previously described [14]. Briefly, cells suspended in serum free media were added to each transwell insert and the medium in the lower chamber contained 10% fetal calf serum (FCS). Following incubation for 18 hrs at 37 °C, cells attached to the insert were removed using cotton swabs and the cells that migrated across the insert and attached to the surface were fixed with 4% paraformaldehyde for 10 min and subsequently stained with 0.5% crystal violet. Stained samples were then dissolved in 10% acetic acid and migrated cells were quantitatively assessed at 560 nm. Transwell cell invasion assays were performed in the same way, except that the transwell inserts were pre-coated with growth factor-reduced Matrigel (BD Biosciences, San Jose, CA).

Statistics

All quantitative results in this study were expressed as mean±standard deviation. Statistical analysis was performed using Student's t-test and one-way analysis of variance (ANOVA) using the SPSS 17.0 software package. A p value <0.05 was considered to be statistically significant.

Results

MMP-3 is strongly expressed in osteosarcoma tissues

To assess MMP-3 expression in human osteosarcoma tissue, tumors obtained following sur-

Table 1. Clinical features of osteosarcoma patients

Clinical features	N (%)
Age, years	
≤25	3 (20.0)
>25	12 (80.0)
Gender	
Male	10 (66.6)
Female	5 (33.3)
Stage	
Primary	4 (26.6)
Metastatic	11 (73.4)
MMP-3 expression	
Weak	2 (13.3)
Moderate	4 (26.6)
Strong	9 (60.0)

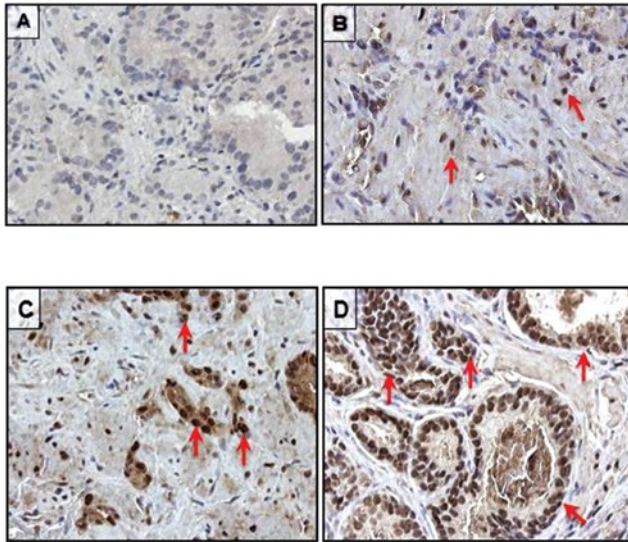


Figure 1. Visualization of MMP-3 expression in osteosarcoma and adjacent non-tumor tissues. Representative micrographs depicting immunohistochemistry for MMP-3 in (A) adjacent non-tumor tissues and (B-D) in osteosarcoma tissues showing (B) weak positive, (C) moderate positive, and (D) strong positive expression. Tumor cells exhibited enhanced expression of MMP-3 (stained brown and denoted by red arrows), while the adjacent non-tumor tissue stained negative for MMP-3. All sections were counterstained with hematoxylin (magnification x400).

gical resection or during autopsy were examined histologically. The clinical and pathological features of 15 osteosarcoma samples are presented in Table 1. Immunostaining for MMP-3 (Figure 1) revealed that it was highly expressed among all osteosarcoma patients, with a median of 85% tumor cells stained positively. Based on the area of positive immunoreactivity, a final overall score

(weak/moderate/strong) was established as described in the Methods section. According to the staining intensity, there were 2 (13.1%) cases with weak MMP-3 expression, 4 (26.6%) with moderate MMP-3 expression, and 9 (60%) with strong MMP-3 expression. Immunostaining results also revealed that MMP-3 was mainly localized in the membrane and cytoplasm of tumor cells. The case-matched adjacent non-tumor tissues were essentially negative for MMP-3 staining. Statistical analysis showed that MMP-3 expression levels were significantly higher in metastatic tumors than in primary osteosarcoma tissues (metastatic vs primary, $p < 0.0001$).

Quantitative MMP-3 expression in osteosarcoma tumor samples

To confirm immunohistochemical staining for MMP-3, qRT-PCR was used to analyze MMP-3 mRNA expression in 15 tumor samples as well as in the adjacent non-tumor tissues. As shown in Figure 2, the MMP-3 mRNA expression levels were upregulated ($p < 0.001$) in 4 samples (sample # 1, 3, 10 and 15) as compared to matched normal tissues. Additionally, 9 metastatic samples had 6-fold greater MMP-3 expression as compared to primary tumors and the corresponding normal tissues ($p < 0.0001$). However, in 2 samples (sample # 8 and 15), the levels of MMP-3 expression were not significantly different when compared to normal tissues. These findings corroborated with immunostaining data, thereby suggesting that MMP-3 expression might be functionally impor-

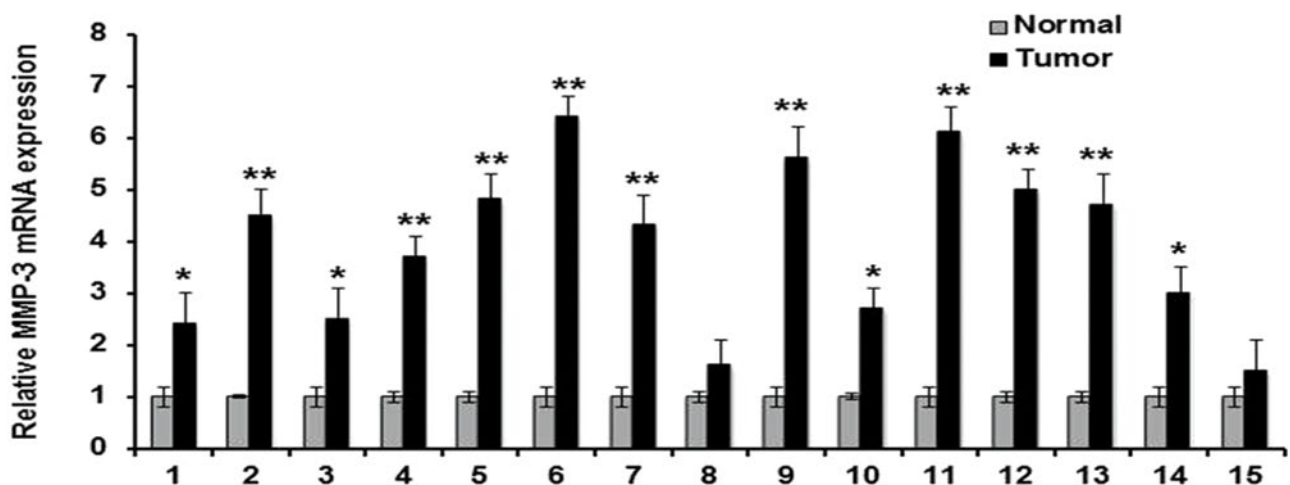


Figure 2. MMP-3 mRNA expression in osteosarcoma and adjacent non-tumor tissues. MMP-3 mRNA levels in tumor samples as determined by qRT-PCR normalized to GAPDH and expressed as relative to corresponding non-tumor tissue. Statistical analysis was performed using Student's t-test and * $p < 0.001$, ** $p < 0.0001$ indicate significance between osteosarcoma tissue and its corresponding non-tumor tissue.

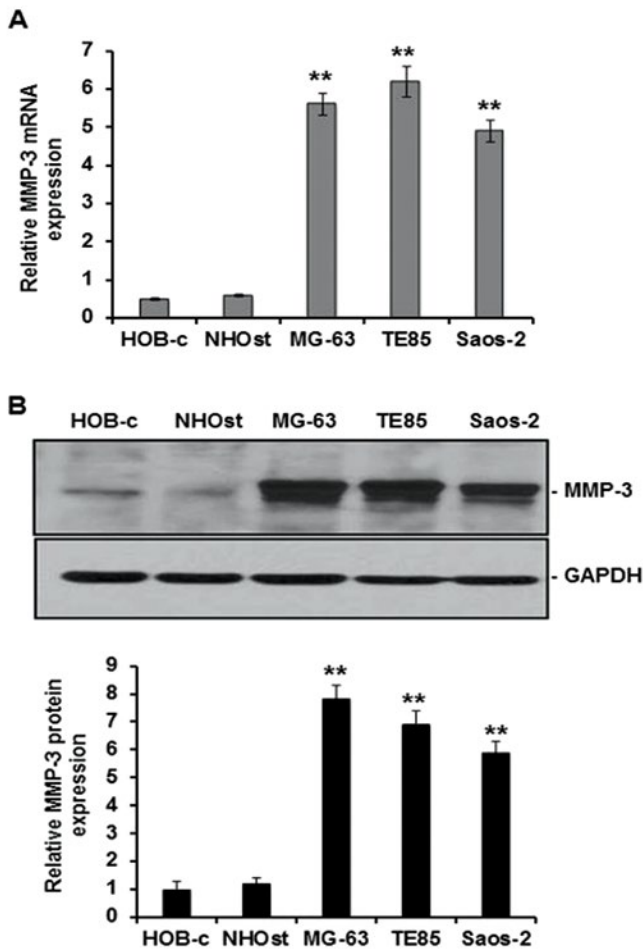


Figure 3. MMP-3 expression in osteoblast and osteosarcoma cell lines. **(A)** MMP-3 mRNA levels in osteoblast (HOB-c and NHOst) and osteosarcoma (MG-63, TE85 and Saos-2) cell lines, as determined by qRT-PCR. **(B)** Immunoblot analysis of MMP-3 protein expression in osteoblasts and osteosarcoma cells. Protein signals were quantified by ChemiDoc and normalized to cognate GAPDH signals, and presented as relative expression. Statistical analysis was performed using Student's t-test; ** $p < 0.0001$ indicates significant difference between osteosarcoma and osteoblast cell lines ($N=3$).

tant in tumor progression and metastasis in osteosarcoma.

MMP-3 is overexpressed in osteosarcoma cell lines

In the next set of experiments, the *in vitro* expression levels of MMP-3 in osteosarcoma cells was determined. For this, mRNA and protein levels of MMP-3 in osteoblast and osteosarcoma cell lines were examined by qRT-PCR and Western blotting, respectively. As shown in Figure 3, the mRNA (A) and protein (B) levels of MMP-3 were modest in HOB-c and NHOst osteoblast cell lines. However, MG-63, TE85 and Saos-2 cell lines exhibited significantly higher expression of MMP-3 as compared to osteoblast cells. Difference in the levels of MMP-3 expression in tumor cell lines

as compared to osteoblast cells indicated endogenous overexpression of MMP-3 in tumor specimens (Figure 2).

MMP-3 knockdown inhibits the migration and invasion ability of osteosarcoma cells

To further investigate the functional role of MMP-3 in osteosarcoma, MMP-3 knockdown was performed in MG-63 and TE85 cells that were shown to express high levels of MMP-3 (Figure 3). Western blot demonstrated the knockdown to be specific and efficient (Figure 4A). Quantitatively, MMP-3 siRNA (siMMP-3) resulted in knockdown efficiencies ranging from 3.5 to 4-fold against siRNA control (siCtrl). To further evaluate the effect of MMP-3 knockdown on migration and invasive properties of MG-63 and TE85 cells, transwell migration and invasion assays were conducted. As shown in Figure 4B, MMP-3 knockdown led to significant reduction in migration rates of ME-63 and TE85 cells as compared to the siCtrl cells at the end of 18 hrs ($p=0.0009$ and 0.0003 , respectively). Quantitatively, fewer MG-63 and TE85 cells with siMMP-3 penetrated the Matrigel-coated membrane when compared with siCtrl cells ($p=0.0003$) (Figure 4C). Taken together, the results of this study suggest a potential role of MMP-3 on migratory and invasive properties of MG-63 and TE85 cells.

Discussion

Osteosarcoma is the most common type of bone cancer in children and adults with increased propensity for invasion and metastasis. During the last two decades, no drastic change in overall prognosis of osteosarcoma has been seen [16,17]. Although osteosarcoma commences in a specific bone, it can potentially migrate and invade to secondary sites in the body, such as lungs and other bones, which is the most common cause of osteosarcoma-related deaths. Therefore, a clear understanding of factors responsible for osteosarcoma metastasis is of critical importance. The aim of this study was to examine and characterize a potential marker, MMP-3, which may play a crucial role in osteosarcoma patients. Immunohistochemistry results showed that MMP-3 is highly expressed in metastatic osteosarcoma in comparison to primary osteosarcoma tissues, with a correlation between the levels of MMP-3 expression (Figure 2) to the metastatic status in the clinical setting.

The current model pertaining to metastasis

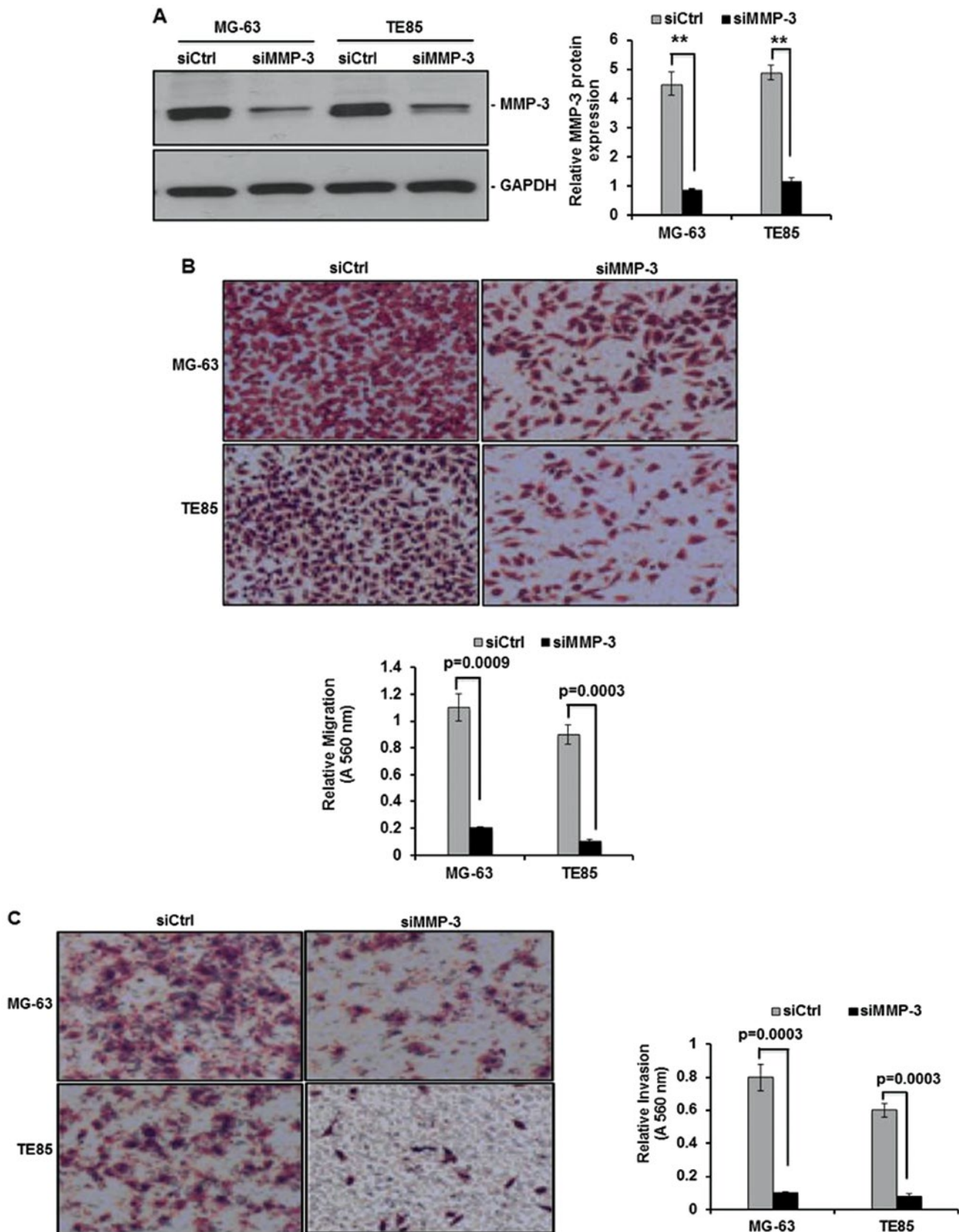


Figure 4. Effect of MMP-3 knockdown on migration and invasion of osteosarcoma cell lines. **(A)** Efficiency of MMP-3 knockdown in MG-63 and TE85 cells determined by Western blot analysis; samples were treated with MMP-3-specific siRNA (siMMP-3) or nonspecific siRNA (siCtrl). Protein signals were quantified as in Figure 3 and presented as bar graph. **(B)** Migration and **(C)** invasive ability of MMP-3 knockdown cells as assessed by crystal violet staining of migrated/invaded cells. Migration/invasion was quantified at $\lambda 560$ and presented as bar graph. Statistical analysis was performed with Student's t-test; ** $p < 0.0001$ indicates significance between cells treated with siMMP-3 and siControl (siCtrl) (N=3).

suggests that clonal expansion of mutated cells can cause tumor progression, potentially facilitating metastasis [18]. It has been reported that in some patients, most of tumor cells in the primary tumor can exhibit changes that promote metastasis [18,19]. In addition, there could be a small proportion of cells in the primary tumor that have inherited further alteration to facilitate invasion and metastasis [19,20]. In this study, the metastatic advantage of osteosarcoma cells was potentially due to their capability to express MMP-3. We hypothesize that metastasizing tumor cells could possibly be derived from metastatic clonal expansion of rare MMP-3 expressing cells from the primary lesion resulting in a subset of cells with enhanced expression of MMP-3.

Previously, MMP-3 has been shown to induce EMT in mammary epithelial cells [21], and has also shown to play a significant role in the growth and/or metastatic transformation of breast cancer, hepatocellular carcinoma, prostate cancer, gliomas, and chondrosarcoma [14,22-26]. Moreover, overexpression of MMP-3 promoted the development of premalignant and malignant mammary lesions in transgenic mice [8]. Furthermore, elevated levels of MMP-3 in serum from gastric cancer patients suggest its application as a tumor marker [27-29].

Our results demonstrated higher MMP-3 expression in osteosarcoma cell lines, MG-63 and TE85 cells, that are associated with higher metastatic potential compared to Saos-2 cells (Figure 3 A,B). Also, silencing MMP-3 expression significantly inhibited the migration and invasion of MG-63 and TE85 cells (Figure 4 B,C), thereby indi-

cating that MMP-3 plays a significant role in promoting the malignant phenotype in osteosarcoma cells, and could be a potential therapeutic target.

One of the most common mechanisms for tumor invasion and metastasis is EMT, although EMT-independent mechanisms of tumor cell metastasis have also been reported [29]. A foremost step in this process is the detachment of malignant cells from the primary site of invasion followed by ECM degradation [31]. Interestingly, MMP-3 has been reported to induce EMT *in vitro* [21], thereby suggesting that a sustained increase in postoperative MMP-3 might encourage micro-metastasis or the development of neo metastasis from circulating tumor cells.

Taken together, MMP-3 expression in osteosarcoma patients was found upregulated during tumor progression from primary to metastatic osteosarcoma. Thus, our findings suggest that MMP-3 might be a potential clinical marker as well as a therapeutic target for the prevention of osteosarcoma-based metastasis. However, further studies are required to investigate the molecular mechanisms of EMT regulation by MMP-3 in osteosarcoma patients.

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Conflict of interests

The authors declare no conflict of interests.

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