Int J Clin Exp Pathol 2019;12(10):3752-3760
www.ijcep.com /ISSN:1936-2625/IJCEP0100191

Original Article

Matrilin-2 is a novel prognostic marker in osteosarcoma

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Received July 23, 2019; Accepted September 23, 2019; Epub October 1, 2019; Published October 15, 2019

Abstract: Objective: Osteosarcoma is the most malignant bone tumor in adolescents. Molecules that are significantly associated with prognosis of osteosarcoma remain to be elucidated. In this study, we aimed to explore the prognostic role of Matrilin-2 (MATN2) in osteosarcoma. Methods: By in silico analysis, we used GSE42352, GSE33382, and GSE21257 to explore the differential expression in osteosarcoma patients with different tumor status. Tissue and serum levels of MATN2 in 56 osteosarcoma patients were verified by RT-PCR and ELISA kits, respectively. Small interfering RNA was used to interfere with the expression of MATN2 in U-2OS and MG-63. CCK-8 and transwell assay were used to detect cell proliferation and migration, respectively. Western blot was used to detect the expression of invasion-related proteins. Results: MATN2 was overexpressed both in osteosarcoma cells and tissues when compared to normal controls. Both tissue and serum level of MATN2 correlated with tumor Enneking stage and metastasis status in osteosarcoma patients according to GEO datasets and validation cohort. Further Kaplan-Meier analysis revealed that MATN2 was a novel prognostic marker in patients with osteosarcoma. Knockdown of MATN2 by siRNA significantly reduced the proliferation and invasion of osteosarcoma cells in vivo. Furthermore, inhibition of MATN2 in U-2OS and MG-63 cells dramatically reduced the expression of mmp2, vimentin and snail1. Conclusion: Elevated MATN2 in tissues and serum significantly associated with both tumor malignancy and poor outcome, which indicates MATN2 could be a prognostic biomarker in patients with osteosarcoma.

Keywords: MATN2, osteosarcoma, biomarker, prognosis, invasion

Introduction

Osteosarcoma (OS) is the most common malignant tumor of bone tissue, and commonly occurs in adolescents. The incidence rate in patients aged 0-24 years is between 2.0% and 7.6% depending on the region [1, 2]. The 5-year survival rate for patients with OS is 60%-70%. About 8.9% of all child and adolescent deaths were caused by bone tumors. Although surgical treatment combined with chemotherapy could improve the survival rate of OS patients, the outcome was still unsatisfactory, especially in patients with clinical metastasis at the time of initial examination. OS often metastasizes to the lungs, which is the leading cause of death in OS patients [3]. It was reported that about 20% of osteosarcoma patients presented with clinical metastasis to the lungs at the time of initial examination and the 5-year survival rate was less than 20% in these patients [4]. New evidence in the field of epigenetics research indicated that osteosarcoma is caused by genetic alteration and genetic accumulation. The malignant progression of tumors depends on the regulation of genes, so it is particularly important to find novel markers that are significantly associated with the malignant phenotype and clinical prognosis of osteosarcoma [5]. Therefore, screening a new biomarker for early diagnosis of OS and predicting disease progression may help improve the clinical outcome of OS patients.

Previous studies found that many markers, such as serum alkaline phosphatase (ALP), CA125, and noncoding RNAs, were closely associated with clinical prognosis of OS patients and could be used for early diagnosis and outcome prediction of OS [6, 7]. In particular, ALP was a crucial biomarker in outcome prediction of OS [8]. But it was sometimes unreliable due to the fact that it could be elevated during non-neoplastic destruction of bone. Besides, the serum concentration of ALP presented no significance difference between metastatic and non-metastatic patients and the sensitivity and specificity of ALP on metastasis was also unfavorable [9, 10]. Therefore it is urgently needed to identify novel biomarkers for early prediction of outcome for OS patients.

MATN2 is an extracellular matrix protein which is involved in the formation of filamentous networks during the extracellular matrices of various tissues [11]. MATN2 can be released into peripheral blood during pathological processes [12]. Previous studies found that the expression of MATN2 was significantly altered during cancer progression. Expression of MATN2 was elevated in hepatocellular carcinoma (HCC) and melanoma, while decreased in human endometrium and cervical cancer [13, 14]. It was found that MATN2 could act as either an oncogene that promoted cancer cell proliferation and invasion, or a tumor suppressor that inhibited tumor formation in vivo [15, 16]. A previous study revealed that both developmental bone and adult bone expressed MATN2 and MATN2 was significantly elevated in the early stage of increased osteoarthritis [17]. While the expression of MATN2 in OS tissue and its role in OS prognosis prediction are still unknown. Whether MATN2 or its secreted form can be used as a prognostic marker in patients with OS remain unclear. Therefore, we performed this study to investigate the level of MATN2 both in tissues and peripheral blood and tried to find an association between secreted MATN2 (sMATN2) and clinical parameters, as well as patients' prognosis.

Materials and methods

Patients and serum samples

This study was carried out on 56 osteosarcoma frozen tissues that were collected from the Department of Orthopedics, the Third People's Hospital of Shenzhen. Another 24 frozen benign bone tumors tissues were also collected. Blood samples of patients with malignant and benign tumors were all obtained on admission. No patients received any chemo- or radiotherapy before surgery. This study received approval of the Ethics Committee of the third people's hospital of Shenzhen. All patients included in this study signed informed consent.

Measurement of serum markers

2 ml of blood samples were collected. All samples were immediately centrifuged at 2,500 rpm for 5 min at 4°C and the supernatant fluid was stored at -80°C for future assayed. ELISA kits (MATN2 (SEE149Mu), ALP (SEB472Hu), Wuhan USCN Business Co., Ltd)) were used to detect serum level of MATN2 and ALP. All operations were performed according to the manufacturer's instruction.

Cell lines and cell transfection

U-2OS and MG-63 cells were purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured with RIPM 1640 supplemented with 10% fetal bovine serum (Gibco, USA) in cell culture incubator. siRNAs was purchased from RIBOBIO (Guangzhou, China). Transfection of siRNA using lip2000 was performed according to the instructions.

Bioinformatics analysis

Normalized gene-level RNAseq and clinical data of GSE42352, GSE33382 and GSE21257 were downloaded from GEO database (https://www.ncbi.nlm.nih. gov/geo/).

RNA isolation and RT-PCR

Total RNA was extracted from osteosarcoma tissues by using TRIzol regent (Invitrogen). PrimeScript RT reagent kit with gDNA Eraser (Takara, Tokyo, Japan) was used to prepare for cDNA and SYBR Green II Mixture (TaKaRa) was used for real-time PCR. All operations were performed according to the manufacturer's protocol. The specific primer pairs were as follows: GAPDH primer (forward primer, 5'-ATCGTGACTTACATAGGGGTGA-3'; reverse primer, 5'-AGTTTCCCACACGCGCCAT-3'); MATN2 primer (forward primer, 5'-TGAATCGACTGAGGGACAGGAGCA-3'; reverse primer, 5'-ATGTTGCAAGTGTACAGGTTGGA-3').

Cell Counting Kit-8 assay and transwell assay

We used Cell Counting Kit-8 to measure the proliferative ability of cells. 5,000 transfected cells were seeded into 96-well plates and the proliferative rates of the cells at 0 h, 24 h and 48 h were calculated, respectively. Transwell assay was used to detect the invasive ability of cells. 20,000 cells were placed on the top of matrigel and cultured with serum-free medium. We added 600 μl of complete medium under the matrigel. Cells were cultured for 48 hours in a cell culture incubator. Then cells were washed with PBS, fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. Photographs were taken using an inverted microscope, and six high-power fields were randomly selected for cell counting.

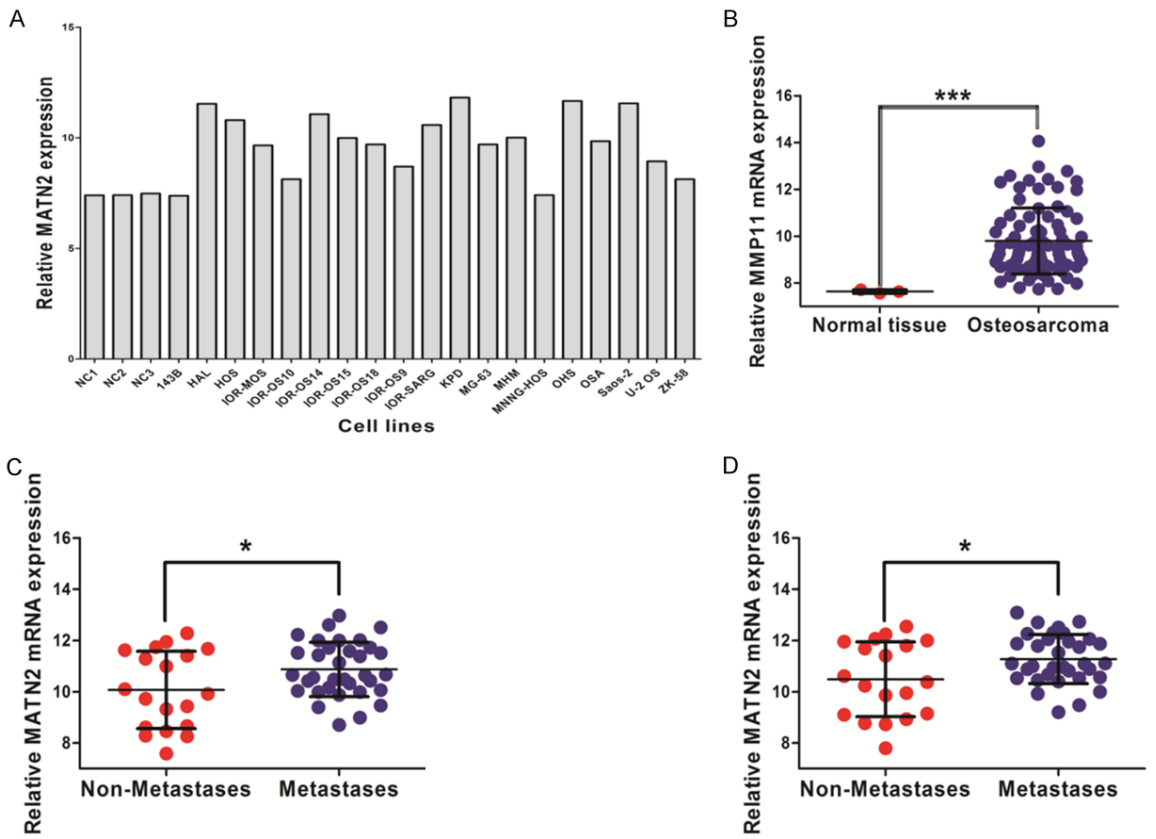

Figure 1. In silico analysis of MATN2 expression in osteosarcoma cells and tissues. A: MATN2 expression in 3 control cell lines and 19 osteosarcoma cell lines according to GSE42352. B: Comparison MATN2 expression between normal tissues and osteosarcoma tissues according to GSE33382. C, D: Differential expression of MATN2 between metastatic and non-metastatic groups in GSE33382 and GSE21257. *, $P < 0.05$, ***, $P < 0.001$.

Western blot

RIPA cell lysate was used to extract total cellular protein. The BCA protein concentration assay kit performed protein quantification. We added equal protein amounts on SDS-PAGE and then transferred them to a PVDF membrane. After washing three times with TBST, membrane was blocked with 5% skim milk for 1 hour at room temperature. Then, membrane was incubated with primary antibody (overnight) and secondary antibody for 1 hour, respectively, and finally scanned with Oddesey.

Statistical analysis

Continuous variables were presented by Mean ± standard deviation and median was used for categorical variables. t test was used for comparisons between two groups and one-way ANOVA analysis for three or more groups comparisons. Box-plots were used to compare levels of MATN2 between different groups. Kaplan-Meier survival analysis was performed to explore the relationship between MATN2 and prognosis. Univariate and multivariate regression analysis were used to analysis the independent risk factors of poor outcome. Picture production was done by Graphpad 5.0. Statistical analyses were conducted using SPSS 21.

Results

In silico analysis of MATN2 expression in osteosarcoma

First, we used GSE42352 to analyze the relative expression of MATN2 in osteosarcoma cells and the results showed that MATN2 was elevated in osteosarcoma cells compared with osteoblast cells (**Figure 1A**). GSE33382 contained 84 high grade osteosarcoma and 3 osteoblast cells. We found that patients with osteosarcoma had higher level of MATN2 than normal controls (**Figure 1B**). It was mentioned before that about 20% of osteosarcoma patients presented with clinical metastasis to the

Table 1. Baseline information of 56 OS patients included

Variables	
Age (years)	19.28 ± 10.23
Sex	
Female	35
Male	21
Tumor size (cm)	
> 8 cm	15
≤ 8 cm	41
Location	
Femur/Tibia	39
Other	17
Lung metastasis at diagnosis	
Yes	19
No	37
Enneking stage	
I-IIA	29
IIB-III	27

lungs at the time of initial examination, and the 5-year survival rate was less than 20% in these patients. We analyzed the data in GSE33382 and GSE21257 and found that MATN2 was elevated in OS patients with metastases presented at diagnosis when compared with those with no metastases at diagnosis (**Figure 1C**, **1D**).

MATN2 expression in osteosarcoma validation cohort

In order to verify our findings, we used RT-PCR detect the expression of MATN2 in 56 OS tissues and 24 benign bone tumor tissues. The clinical data of OS patients included in this study are shown in **Table 1**. The results showed that MATN2 was higher in patients with OS than in patients with benign tumors (**Figure 2A**). A non-invasive serum marker is important in predicting outcome of patients with OS. MATN2 is an extracellular matrix protein that is released into peripheral blood during pathologic processes. We found that the serum level of MATN2 was significantly correlated with its expression in osteosarcoma tissues (**Figure 2B**). Moreover, the results of ELISA showed that serum MATN2 was increased in patients with OS, compared to patients with benign bone tumors (**Figure 2C**).

MATN2 correlation with tumor malignancy

Among 56 OS patients, 19 patients presented with lung metastasis and 37 patients had no clinical metastasis at diagnosis. The results showed that either expression of MATN2 in tissues or in blood was higher in the metastasis group than the non-metastasis group (**Figure 3A**, **3B**). Besides, the mRNA level in tissues and secreted level of MATN2 in peripheral blood of osteosarcoma patients with advanced Enneking stage were significantly higher than patients with early stage (**Figure 3C**, **3D**). ALP has been identified and reported as a prognostic serum markers in OS. Our results revealed that the serum level of MATN2 was positively correlated with ALP in OS patients ($r=0.706$, $P < 0.001$, **Figure 3E**).

MATN2 association with prognosis of OS patients

To further investigate the prognostic role of MATN2 in OS, we used a Kaplan-Meier method to estimate the effect of MATN2 on overall survival (OS) and disease-free survival (DFS). The results showed that patients with higher serum MATN2 level had shorter OS time and DFS than patients with lower serum MATN2 (**Figure 4A**, **4B**). Moreover, results of Cox regression analysis revealed that higher serum MATN2 level was an independent risk factor of overall survival in patients with OS (**Table 2**).

MATN2 promoted proliferation and invasion of osteosarcoma cells

We used small interfering RNAs to inhibit the expression of MATN2 in U-2OS and MG-63 cells, and the results showed that specific siRNA dramatically reduced the MATN2 expression in osteosarcoma cells. Inhibition of MATN2 reduced the proliferation of U-2OS and MG63 cells detected by CCK-8 assay (**Figure 5A**, **5B**). Next, transwell assay revealed that knockdown MATN2 could significantly reduce the invasion of U-2OS and MG-63 cells (**Figure 5C**, **5D**).

MATN2 increased invasion-related marker of osteosarcoma cells

Invasion-related markers, such as vimentin, snail1, and mmp2, are crucial for invasion of cancer cells. We used the GSE33382 dataset to explore the correlation between MATN2 and invasion-related markers. Results showed that MATN2 expression positively correlated with mmp2, snail1, and Twist1 expression (**Figure 6A**). Furthermore, we performed WB to verify

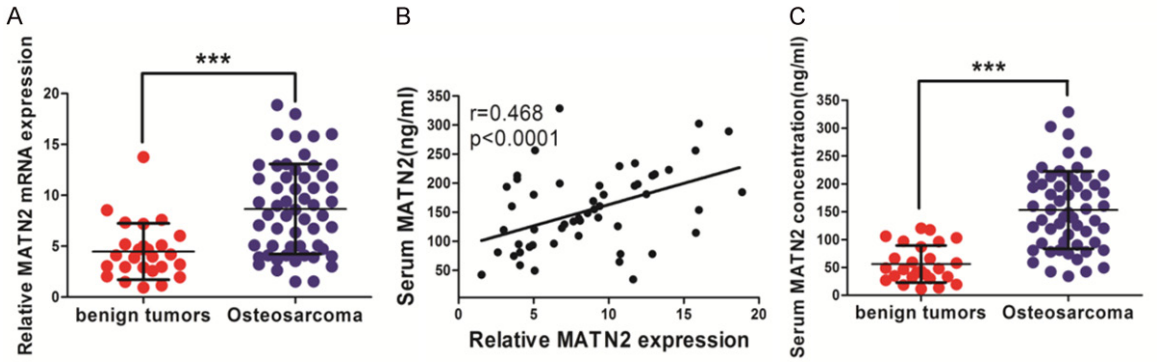

Figure 2. MATN2 expression in osteosarcoma validation cohort. A: RT-PCR was performed to detect mRNA level of MATN2 in 56 osteosarcoma tissues and 24 benign bone tumor tissues; B: Correlation between serum MATN2 level and MATN2 mRNA expression in osteosarcoma tissues. C: Serum level of MATN2 in osteosarcoma and benign tumors. ***, P < 0.001.

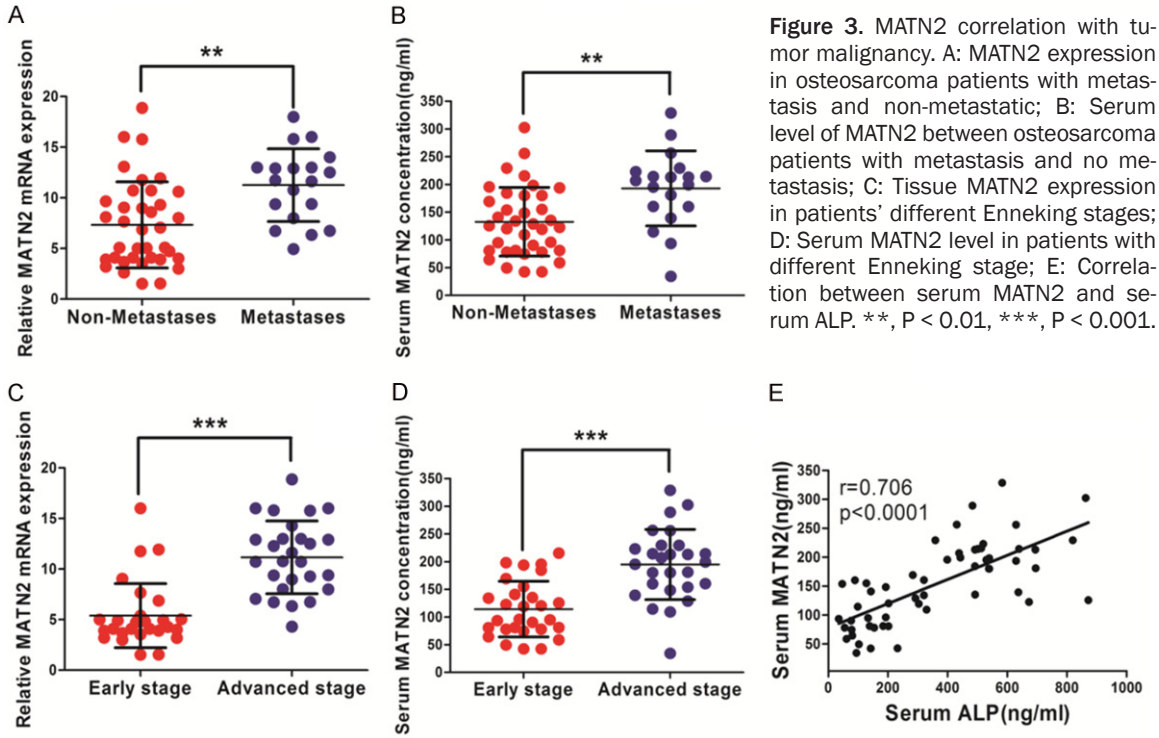

Figure 3. MATN2 correlation with tumor malignancy. A: MATN2 expression in osteosarcoma patients with metastasis and non-metastatic; B: Serum level of MATN2 between osteosarcoma patients with metastasis and no metastasis; C: Tissue MATN2 expression in patients' different Enneking stages; D: Serum MATN2 level in patients with different Enneking stage; E: Correlation between serum MATN2 and serum ALP. **, P < 0.01, ***, P < 0.001.

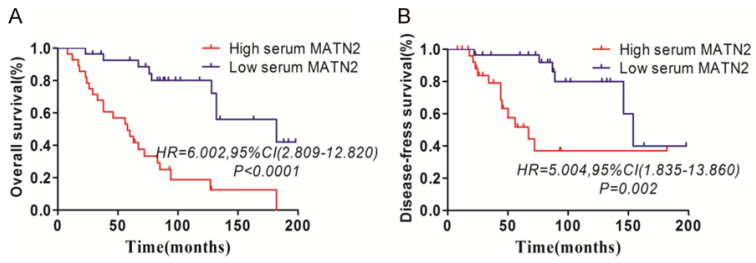

Figure 4. Prognostic role of MATN2 in osteosarcoma patients. Expression of MATN2 in osteosarcoma was divided into two groups and Kaplan-Meier analysis was used to estimate the effect of MTAN2 on OS and DFS (A, B). HR, hazard ratio.

our findings and we found that knockdown of MATN2 in osteosarcoma reduced the expression of mmp2 and snail1 (**Figure 6B**, **6C**).

Discussion

In this study, we found that MATN2 was significantly elevated in human osteosarcoma tissues both through in silico analysis and in our cohort vali-

Table 2. Univariate and multivariate Cox regression analysis of prognostic parameters in validation cohort

	Univariate Cox regression		Multivariate Cox regression	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (≥ 16 y vs < 16 y)	0.781 (0.462-1.357)	0.621	-	-
Gender (Female vs male)	0.463 (0.163-0.939)	0.48	-	-
Location (Femur/Tibia vs Other)	1.829 (1.293-4.254)	0.081	1.283 (1.024-3.216)	0.712
Tumor size (> 8 cm vs ≤ 8 cm)	1.192 (1.634-3.342)	0.176	-	-
Lung metastasis at diagnosis (Yes vs No)	3.223 (1.382-6.483)	0.017	4.018 (2.65-11.393)	< 0.001
Enneking stage (IIB-III vs I-IIA)	2.206 (1.237-8.983)	< 0.001	2.740 (1.819-6.392)	0.003
Serum ALP level (High vs Low)	3.218 (1.901-4.293)	0.028	2.102 (1.293-4.203)	0.068
Serum MATN2 level (High vs Low)	2.75 (1.531-4.320)	< 0.001	2.19 (1.174-3.021)	0.002

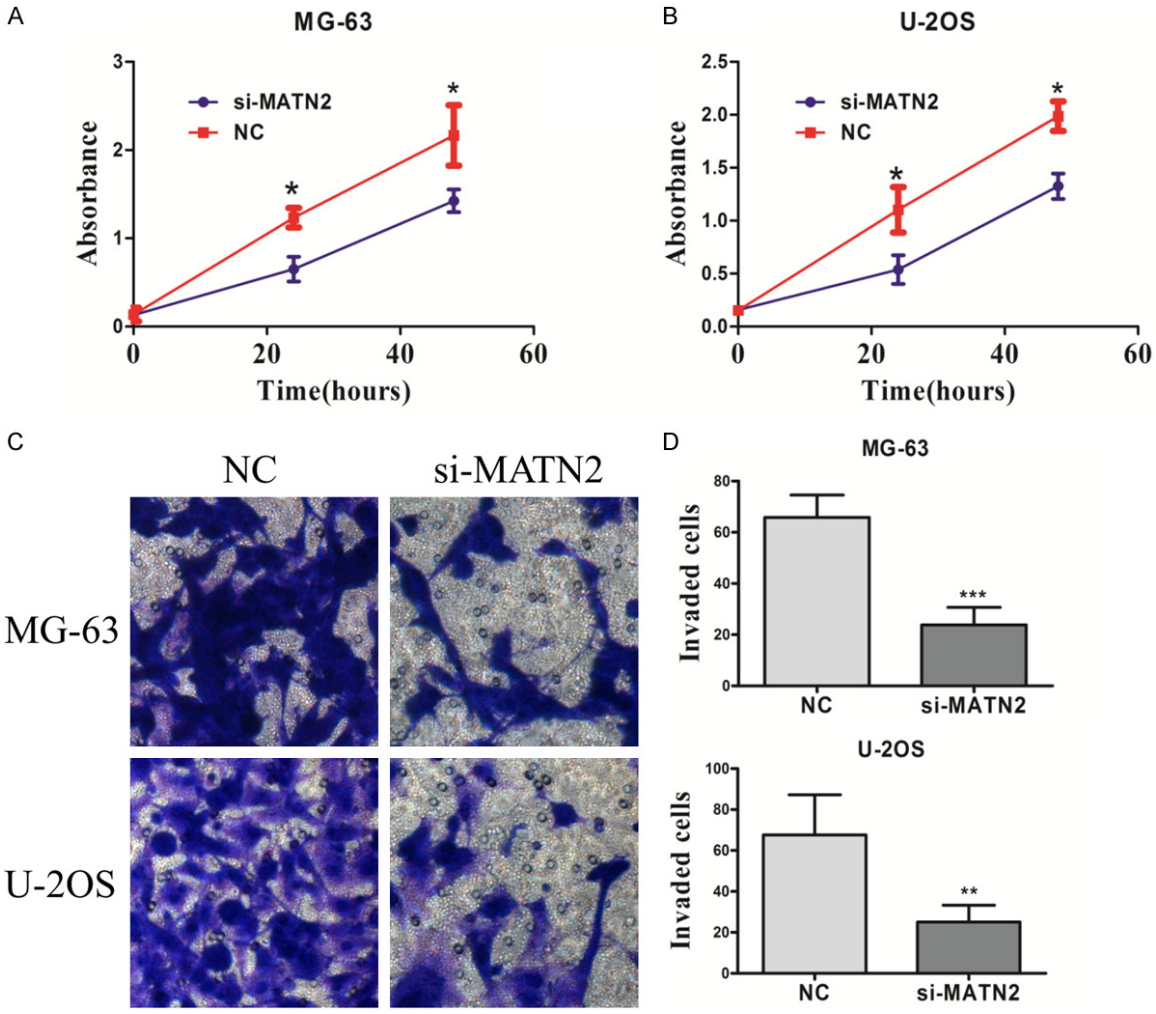

Figure 5. MATN2 promoted proliferation and invasion of osteosarcoma cells. A, B: CCK-8 assay was used to detect the proliferation of osteosarcoma cells; C, D: Invasive ability of osteosarcoma cells was measured by transwell assays. NC: normal control, *, P < 0.05, **, P < 0.01, ***, P < 0.001.

dation. Patients presenting with clinical metastasis had a higher level of MATN2 both in tissue and peripheral blood when compared to patients with no metastasis at diagnosis. Serum biomarkers were important in predicting the outcome of patients with osteosarcoma. We

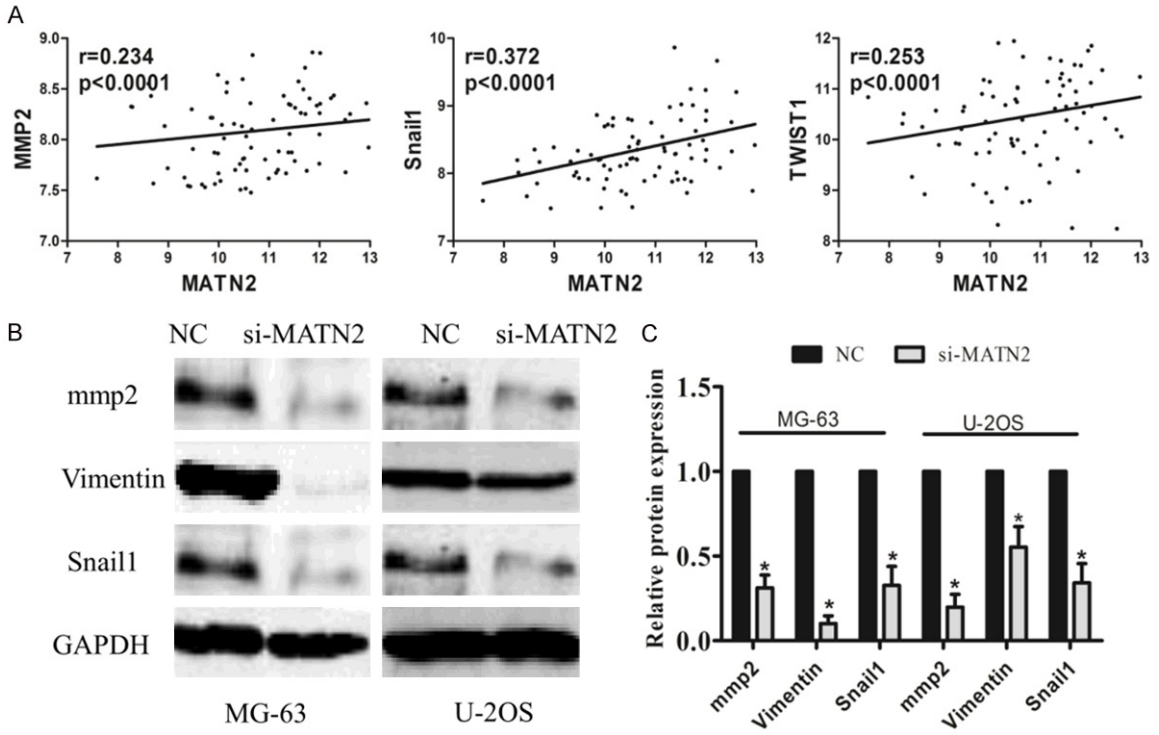

Figure 6. MATN2 altered invasion-related markers of osteosarcoma cells. A: Correlation between MATN2 and mmp2, snail1, and twist1 based on GSE33382 dataset; B, C: Inhibition of MATN2 in osteosarcoma cells altered the invasion-related markers. NC: normal control, *, $P < 0.05$.

found that the serum level of MATN2 was significantly correlated with its expression in osteosarcoma tissues. Also, both tissue expression and serum expression of MATN2 significantly correlated with the Enneking stage. Further Kaplan-Meier method and Cox regression analysis revealed that MATN2 was a prognostic marker and an independent risk factor for overall survival in osteosarcoma patients. Inhibition of MATN2 reduced the proliferation and invasion of U-2OS and MG-63 cells detected by CCK-8 and transwell assay, respectively. Furthermore, knockdown of MATN2 in osteosarcoma reduced the expression of mmp2 and snail1 in vivo. Consequently, MATN2 could be used as a novel biomarker in patients with OS.

MATN2 consists of two von Willebrand factor A-like domains, a unique sequence, 10 epidermal growth factor-like modules, and a curly spiral domain [18]. MATN2 has been found to play an important role in promoting the progression of inflammation [19, 20] but the role of MATN2 in tumors remains controversial. In order to explore the role of ECM in cell resistance, Radosław et al. used resistant variants of the W1 cell generated by different drugs, such as methotrexate, cisplatin, doxorubicin, vincristine-, topotecan, and paclitaxel. The results showed that MATN2 was had increased expression (> 20 fold) by studying the gene expression profile of ovarian cancer drug-resistant cells [21]. But other studies presented different results. RT-PCR was used to detect the expression of MATN2 in 10 normal liver samples and 35 hepatocellular carcinomas (HCC) and the results showed that there was no difference in MATN2 level between HCC and normal tissues [22]. Interestingly, Alexandra et al. used a MATN2-/- mouse to explore the oncologic role of MANT2 in HCC and they found that knockout of MATN2 promotes tumor progression by regulating ERK1/2 and GSK3β signaling [23]. In this study, we used bioinformatics and a cohort of OS tissues to explore the potential role of MATN2 in OS. We found that patients with OS had higher MATN2 expression than those with benign bone tumors. Further analysis also demonstrated that the expression of MATN2 was closely associated with metastatic status and the Enneking stage of OS. These results sug-

gested that MATN2 might be an important oncogene in promoting the progression of OS.

Our study revealed that MATN2 could inhibit the proliferation and invasion of osteosarcoma cells and knockdown of MATN2 dramatically reduced the expression of mmp2, vimentin, and snail1. There have been few studies focused on MATN2 in extracellular matrix remodeling. Extracellular matrix (ECM) is an important factor affecting chemotherapy resistance. A previous study revealed that MATN2 could interacted with multiple extracellular components, such as fibrillar collagens, fibronectin and laminin-nidogen-1 [24]. Korpos et al. reported that MATN2 expression in myoblasts is essential for TGF/BMP7/Smad signaling transduction, and enhanced MATN2 expression promoted cell migration and wound healing [25]. Also, MATN2 was reported to be an crucial role in mediating the invasion of HaCaT cells. However, in another study, the results showed that MATN2 had no significant effect on promoting cell migration and invasion. MATN2 may play a different role in different cells, especially for normal cells and tumor cells. This might because osteosarcoma cells have different gene regulation networks from normal cells.

MATN2 had been suggested to function as a biomarker in several cancers. Previous studies have investigated various genes by analyzing gene chips, and found that MATN2 was a biomarker that can improve the diagnosis of thyroid cancer [26]. Furthermore, Sharma et al. found that MATN2 was overexpressed in pilocytic astrocytoma (PA) and the expression of MATN2 was significantly associated with an aggressive clinical phenotype. This indicated MATN2 might be a novel biomarker for discriminating the diagnosis between indolent and aggressive PA [27]. MATN2 is a secreted protein and it was only detected in burn patients, wherein burn patients had a higher serum level of MATN2 than healthy controls [28].

Still, secreted MATN2 had not been investigated in tumor. Serum biomarkers were important in predicting outcome of patients with OS. MicroRNAs, cytokines, and several extracellular proteins had been proven to be important biomarkers in predicting prognosis of OS patients. Among these markers, ALP was a crucial biomarker in outcome prediction of OS. ALP may be elevated during non-neoplastic destruction of bone. Besides, the serum concentration of ALP presented no significance difference between metastatic and non-metastatic patients. In our study, we found that both tissue and serum MATN2 expression were significantly associated with metastatic status and Enneking stage of OS. Our results revealed that patients with higher MATN2 expression had shorter overall survival times than those with lower MATN2 expression. These results indicated that MATN2 level was a novel biomarker in predicting outcome of patients with OS.

Disclosure of conflict of interest

None.

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