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RESEARCH ARTICLE

EMMPRIN co-expressed with matrix metalloproteinases predicts poor prognosis in patients with osteosarcoma

Naohisa Futamura · Yoshihiro Nishida · Hiroshi Urakawa · Eiji Kozawa · Kunihiro Ikuta · Shunsuke Hamada · Naoki Ishiguro

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Abstract Several studies have focused on the relationships between the expression of extracellular matrix metalloproteinase inducer (EMMPRIN) and the prognosis of patients with malignant tumors. However, few of these have investigated the expression of EMMPRIN in osteosarcoma. We examined expression levels of EMMPRIN immunohistochemically in 53 cases of high-grade osteosarcoma of the extremities and analyzed the correlation of its expression with patient prognosis. The correlation between matrix metalloproteinases (MMPs) and EMMPRIN expression and the prognostic value of co-expression were also analyzed. Staining positivity for EMMPRIN was negative in 7 cases, low in 17, moderate in 19, and strong in 10. The overall and disease-free survivals (OS and DFS) in patients with higher EMMPRIN expression (strong-moderate) were significantly lower than those in the lower (weak-negative) group (0.037 and 0.024, respectively). In multivariate analysis, age (P=0.004), location (P=0.046), and EMMPRIN expression (P=0.038) were significant prognostic factors for overall survival. EMMPRIN expression (P=0.024) was also a significant prognostic factor for disease-free survival. Co-expression analyses of EMMPRIN and MMPs revealed that strong co-expression of EMMPRIN and membrane-type 1 (MT1)-MMP had a poor prognostic value (P=0.056 for DFS, P=0.006 for OS). EMMPRIN expression and co-expression with MMPs well predict the prognosis of patients with extremity osteosarcoma, making EMMPRIN a possible therapeutic target in these patients.

Keywords EMMPRIN · Osteosarcoma · Matrix metalloproteinase · Prognosis

N. Futamura · Y. Nishida (✉) · E. Kozawa · K. Ikuta · S. Hamada · N. Ishiguro
Department of Orthopaedic Surgery, Nagoya University Graduate School and School of Medicine, 65-Tsurumai, Showa, Nagoya, Aichi 466-8550, Japan
e-mail: ynishida@med.nagoya-u.ac.jp

H. Urakawa
Department of Clinical Oncology and Chemotherapy, Nagoya University Graduate School and School of Medicine, 65-Tsurumai, Showa, Nagoya 466-8550, Japan

Introduction

Osteosarcoma is the most common malignant bone tumor in children and adolescents. Advances in diagnostic techniques, introduction of adjuvant and neoadjuvant chemotherapy, and adequate wide tumor excision have significantly improved the prognosis of these patients. Nevertheless, recurrence occurs in 30 to 40 % of patients with osteosarcoma and 70 % of patients with recurrence die despite second-line treatment [1–3]. Determination of sensitive and specific prognostic factors in patients with osteosarcoma is urgently needed to identify patients at high risk for relapse, while the determined factor(s) might also be suitable as therapeutic targets in the patients. Alteration of the chemotherapy protocol based on risk factors would be important to avoid unnecessary side effects and their substantial economic and social consequences. Because chemotherapy is a requisite treatment for osteosarcoma, to predict the upcoming clinical course of patients after conventional neoadjuvant and adjuvant chemotherapy would facilitate adjusting the treatment regimen. A reliable risk factor would also allow patients with low risk to avoid onerous and unnecessary chemotherapy. Although several clinical prognostic factors such as tumor size, location, age, stage, response to chemotherapy, metastasis, and surgical margin have been proposed [4–7], factors based on the biological profile seem to better reflect the malignancy of tumors. Several reports have revealed that molecules expressed in osteosarcoma such as COX-2 and matrix metalloproteinases (MMPs) are significant prognostic factors [8, 9].

Extracellular matrix metalloproteinase inducer (EMMPRIN/CD147) is a transmembrane glycoprotein that was first identified as a surface protein on tumor cells and acts to stimulate MMP expression [10, 11]. MMPs play significant roles in vascular remodeling and regulate degradation of the extracellular matrix (ECM) [12, 13]. Thus, tumor cells can invade lymphatic tissue, blood vessels, and adjacent organs through the expression of MMPs. Experimental studies have elucidated the possible crucial roles of EMMPRIN in malignant tumors. Effects of EMMPRIN on invasiveness and angiogenesis in malignant tumor cells have been reported in several cancers such as mammary carcinoma and head and neck carcinoma [14, 15].

Clinically, several studies have reported a relationship between expression of EMMPRIN and chemoresistance, metastatic activity, and prognosis of patients with malignant melanoma, lymphoma, adenoid cystic carcinoma, and colorectal cancer [16–19]. Two previous studies investigated the expression of EMMPRIN in osteosarcoma and analyzed the correlation of the expression and patient prognosis [20, 21]. However, these studies included patients with distant metastasis at presentation (American Joint Committee on Cancer (AJCC) stage IV), precluding any exact proof of the usefulness of EMMPRIN as a "prognostic factor." Moreover, given that location such as the pelvis significantly affects the patient prognosis, study of an identical cohort would be helpful in identifying significant prognostic factors. Furthermore, these studies did not investigate the relationship between EMMPRIN and MMPs, which seems to be crucial for EMMPRIN function.

In this study, we determined the expression of EMMPRIN in high-grade osteosarcoma of the extremities without metastasis at presentation and analyzed the correlation of its expression with patient prognosis in addition to other clinicopathological variables. In addition, the prognostic significance of co-expression of EMMPRIN with that of membrane-type 1 (MT1)-MMP, MMP-2, and MMP-9 was also analyzed.

Materials and methods

Cell culture

The human osteosarcoma cell lines, HOS, MG63, and Saos2; human mammary carcinoma cell line, MDA-MB-231; and human fibroblast cell line, WI-38, were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured as monolayers in Dulbecco's modified eagle's medium (DMEM), supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere with 5 % CO_2.

Patients and tissue samples

From 2000 to 2009, a total of 90 patients were diagnosed with osteosarcoma and treated in our institutions. To evaluate whether EMMPRIN is of prognostic value, patients with low-intermediate-grade osteosarcoma, distant metastasis at referral, or prognostically unfavorable locations such as the pelvis were excluded. There were 53 cases of high-grade osteosarcoma arising in an extremity without distant metastasis at presentation. Tumor tissue samples were obtained from the patients before any chemotherapy. All specimens were reviewed by experienced pathologists to confirm the diagnosis. Informed consent was obtained from all patients for the use of their tissue samples. There were 32 males and 21 females with a median age of 20 (range 4–57 years). The median follow-up duration was 72 months (8–200 months). According to the AJCC system, there were 40 tumors of stage IIA and 13 of stage IIB. The site of involvement was the femur in 26 cases, tibia 18, humerus 3, fibula 3, and radius 3. "Proximal" referred to the humerus or femur and "distal" to the radius, tibia, or fibula. All patients completed the standard therapeutic regimen including neoadjuvant chemotherapy and surgical resection with wide or radical margin followed by adjuvant chemotherapy. The effect of neoadjuvant chemotherapy was defined histologically as the percentage of dead cells. The patients received various chemotherapeutic regimens. Cisplatin, doxorubicin, high-dose methotrexate, and ifosfamide were used in 32 patients; cisplatin, doxorubicin, and high-dose methotrexate in 17; and other combinations in 4. Fifty (94 %) of 53 cases underwent limb-sparing surgery with a wide margin. No cases developed local recurrence.

Immunohistochemistry

Tumor samples obtained prior to chemotherapy were fixed with 10 % formalin for 24 h and embedded in paraffin. Paraffin specimens were cut at 8-μm thickness. After deparaffinization and rehydration, specimens were immersed three times in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked with 3 % hydrogen peroxide in methanol for 10 min at room temperature and rinsed in PBS. Then the slides were soaked for 10 min in 10 % normal goat serum as a blocking agent. Then slides were incubated at room temperature for 1 h with primary rabbit antibody for human EMMPRIN (34-5600; Invitrogen, Carlsbad, CA; 1:500 dilution). After rinsing with PBS, biotinylated anti-rabbit IgG conjugated with peroxidase was applied as the second antibody. The reaction products were observed using 3,3′-diaminobenzidine tetrahydrochloride. Slides were counterstained with hematoxylin, dehydrated, and mounted. Staining positivity was evaluated by two independent observers (H.U. and N.F.) without any knowledge of the clinicopathological information. Because there was a large difference in

numbers of each group according to the metrics in previous reports [16, 19–21], staining positivity was divided into four groups: 0 % for positive stainable cell number (negative), 1 to 39 % (low), 40 to 79 % (moderate), and 80 to 100 % (strong), on four different high-power fields without necrosis. Using these criteria, both observers finally agreed on the degree of positivity or negativity of each case. We statistically analyzed the correlation of EMMPRIN expression with various clinical variables, including necrotic rate after neoadjuvant chemotherapy and patient survival.

Given that EMMPRIN has crucial roles in the induction of MMPs, we analyzed the correlation of EMMPRIN positivity with that of MMP-2, MMP-9, and MT1-MMP, which has been reported previously [8]. Staining positivity of MMPs were divided into four groups: 0–10 % for positive stainable cell number (negative), 11 to 25 % (low), 26 to 49 % (moderate), and 50 to 100 % (strong), on four different high-power fields without necrosis according to this previous report [8].

Statistical analysis

Clinical data was collected from the patients' database of our institutions. Association with EMMPRIN expression and various clinicopathological characteristics were analyzed using the χ^2 test. Because there were no cases of local recurrence, two endpoints were examined for survival analysis, disease-free survival (DFS), and overall survival (OS). Survival times were counted from the date of presentation to the date of death or last follow-up time, and the disease-free period was counted from the date of operation to the date of detection of the first relapse. Survival rate was determined with Kaplan-Meier and statistically analyzed with the log-rank method between groups. For the multivariate analysis, confidence intervals (CIs) for relative risks of survival and metastasis were carried out with the Cox regression method. P values of <0.05 were considered statistically significant.

Results

Immunohistochemical study revealed positive expression of EMMPRIN in neoplastic cells in 46 cases (87 %), with the expression levels of EMMPRIN varying widely. Ten cases showed strong positive immunostaining, 19 moderate, 17 weak, and 7 negative. Thirteen of 18 (72 %) cases with distant metastasis after surgery and 7 of 9 (78 %) cases whose final status was dead of disease (DOD) showed moderate-strong immunoreactivity for EMMPRIN (Table 1). Representative immunohistochemical results are shown in Fig. 1. EMMPRIN expression was not associated with age (younger than 20 versus 20 years or older), gender, anatomic location (proximal versus distal), necrosis after neoadjuvant chemotherapy (>90 versus ≤90 %), or surgical stage (AJCC stage IIA versus stage IIB) (Table 2).

Table 1 Expression of EMMPRIN and clinical outcome

Positivity of staining	Cases Distant metastasis	Cases Dead of disease
Negative (0 %); 7 cases	0	0
Low (1–39 %); 17 cases	5	2
Moderate (40–79 %); 19 cases	7	3
Strong (80–100 %); 10 cases	6	4
Total; 53 cases	18	9

At the last follow-up, 26 (49 %) of the 53 patients remained continuously disease free. There was no local recurrence. Eighteen patients (34 %) had distant metastases and nine (17 %) of them died of the disease. Disease-free and overall survivals at 5 years were 67 and 83 %, respectively. The estimated disease-free survival at 5 years was 54 % in patients with higher (strong-moderate) EMMPRIN expression and 80 % in patients with lower (low-negative) expression (Fig. 2a) (P=0.024), and the estimated overall survival at 5 years was 74 % in patients with higher expression and 92 % in patients with lower (weak-negative) expression (Fig. 2b) (P=0.037).

Higher EMMPRIN expression was found to be a significant risk factor for disease-free survival on both univariate (P=0.024) and multivariate (hazard ratio (HR) 3.52, 95 % CI 1.18–10.50, P=0.024) analyses (Table 3).

On univariate analysis of overall survival, age over 20 years, distal location, and higher EMMPRIN expression were found to be significant poor prognostic factors (P=0.001, P=0.004, and P=0.037, respectively; Table 4). On multivariate analysis, age (HR 12.52, 95 % CI 2.25–69.8, P=0.004), distal location (HR 6.77, 95 % CI 1.03–44.46, P=0.046), and higher EMMPRIN expression (HR 9.40, 95 % CI 1.13–77.95, P=0.038) were found to be independent poor prognostic factors (Table 4).

Co-expression analyses showed more meaningful results for prediction of prognosis for patients with extremity osteosarcoma. As reported in a previous study that MT1-MMP is a poor prognostic factor in patients with osteosarcoma [8], strong/strong expression of EMMPRIN/MT1-MMP had a significant poor prognostic value (P=0.056 for DFS, P=0.006 for OS, compared to other patients) (Fig. 3).

Discussion

There have been several reports on the association of EMMPRIN with malignant tumors and the prognosis of patients with malignancies. A previous study reported that EMMPRIN expression can be used to differentiate malignant

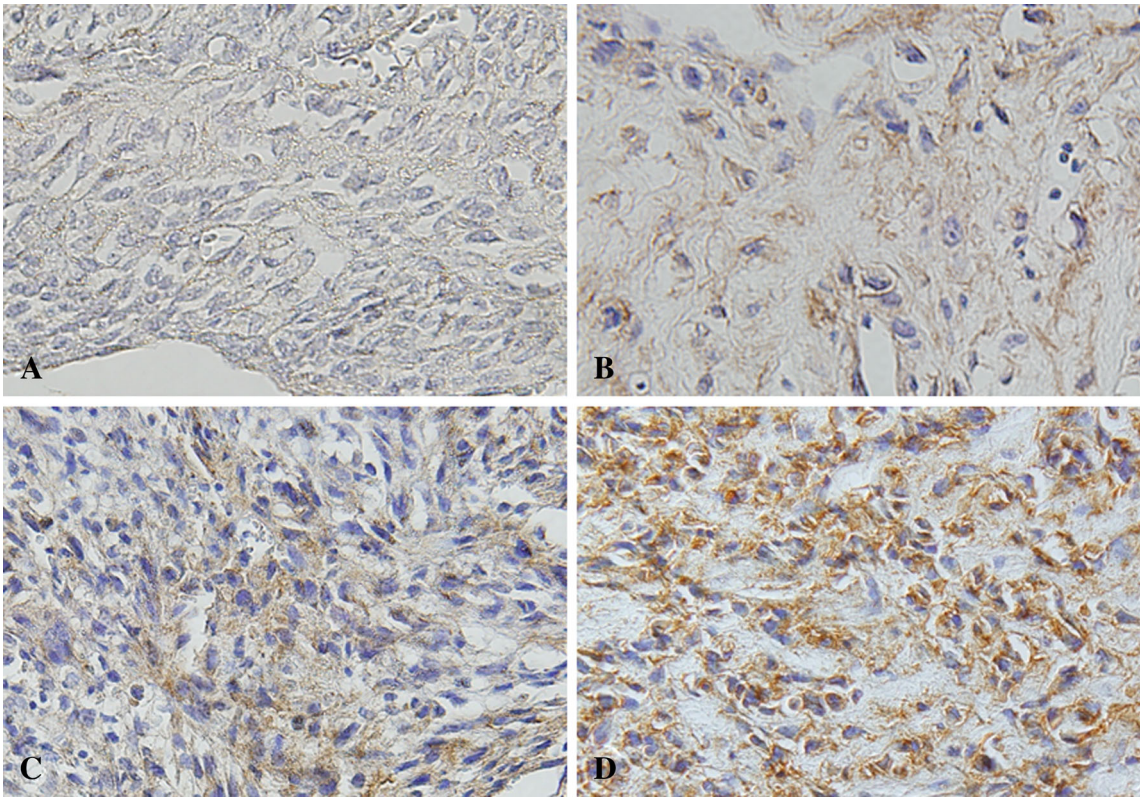

Fig. 1 Representative immunohistochemical staining for EMMPRIN. Osteosarcoma tissues showed varied stainability for EMMPRIN (**a** negative, **b** low, **c** moderate, **d** strong) (original magnification ×400)

brain tumors from normal tissues [22]. Other studies reported that EMMPRIN expression correlates with the prognosis of patients with colon cancer and adenoid carcinoma [19, 23].

Table 2 Correlation of EMMPRIN expression with clinicopathological variables

	Case	EMMPRIN expression				*P* value
		Negative	Low	Moderate	Strong	
Age						0.565
<20	36	5	10	15	6	
≥20	17	2	7	4	4	
Gender						0.107
Male	32	5	8	15	4	
Female	21	2	9	4	6	
Location						0.779
Proximal	32	3	11	12	6	
Distal	21	4	6	7	4	
Necrosis rate						0.447
<90 %	34	6	11	10	7	
≥90 %	19	1	6	9	3	
AJJC stage						0.079
IIA	40	5	16	14	5	
IIB	11	2	1	5	5	

AJCC American Joint Committee on Cancer

Two previous studies described the correlation of EMMPRIN with the prognosis of patients with osteosarcoma. Zhou et al. reported that co-expression of EMMPRIN and VEGF significantly correlated with poor overall survival in patients with osteosarcoma [21]. They analyzed 65 patients with osteosarcoma, of whom 48 (74 %) died of it. The outcome noted in their study, however, differs markedly from the present clinical outcome for osteosarcoma in developed countries, suggesting that the results of the study are not applicable worldwide. Lu et al. reported significant relationships between EMMPRIN expression and OS/DFS in univariate analysis [20]. In their study, although patients with distant metastasis were included, 42 (76 %) of 55 patients succumbed to osteosarcoma with a mean duration of 32 months. Extracted prognostic factors in the study did not seem to reflect the latest cohort of osteosarcoma patients. Compared to the two previous studies [20, 21] reporting the association of EMMPRIN with the prognosis of patients with osteosarcoma, our study was limited to patients with extremity osteosarcoma without distant metastasis, and the 5-year overall survival was 83 %, indicating that the cohort of osteosarcoma patients was identical in the current study, and the treatment outcome may have better reflected the latest one in developed countries. Moreover, the median duration of our study was much longer (72 months) than that of the previous studies (32 and 32 months). EMMPRIN is thought to facilitate invasion and

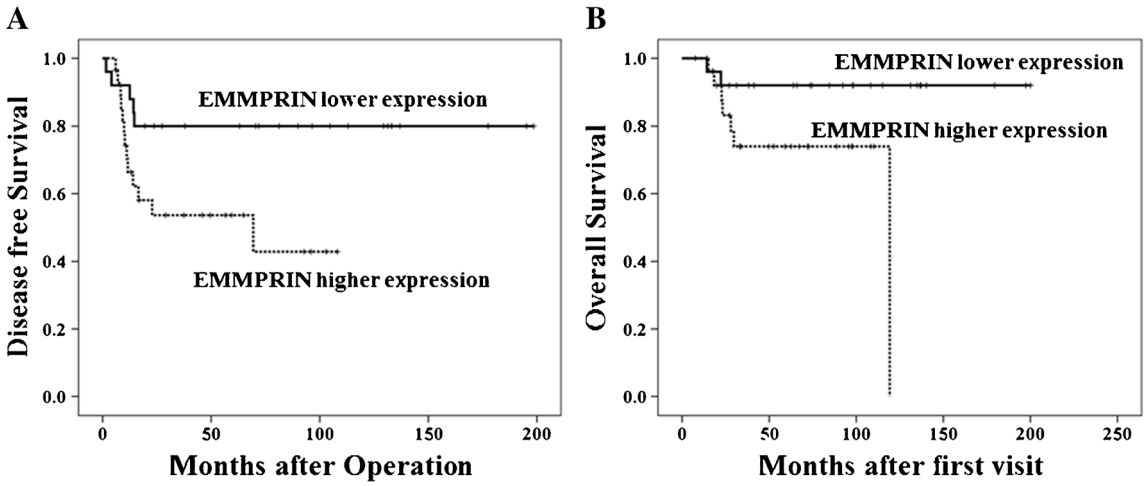

Fig. 2 Kaplan-Meier survival curves for EMMPRIN expression in osteosarcoma. **a** DFS of conventional osteosarcoma patients with higher EMMPRIN expression and lower EMMPRIN expression (N=53, log rank P=0.024). **b** OS of conventional osteosarcoma patients (N=53, log rank P=0.037)

metastasis indirectly via the induction of matrix-degrading enzymes, MMPs. MMPs that can be up-regulated by EMMPRIN in vitro include MMP-1, MMP-2, MMP-3, and MT1-MMP [13, 24, 25], while the MMPs up-regulated by EMMPRIN differ among cell types [24, 26–28]. Although two recent studies demonstrated that EMMPRIN expression correlates with the prognosis of patients with osteosarcoma [20, 21], the correlation between EMMPRIN and MMP expression including MMP-2, MMP-9, and MT1-MMP was not investigated in those studies. The current study reported for the first time the correlation of EMMPRIN and MMP expression in osteosarcoma and the prognostic significance of co-expression. Our study demonstrated that patients with strong/strong expression of EMMPRIN/MT1-MMP had a significantly worse prognosis. A cohort with dismal prognosis could be identified not only from the EMMPRIN expression but more significantly from the MMP expression.

The results of the current study suggest the potential of EMMPRIN as a therapeutic target. Several studies suggest that EMMPRIN may become a therapeutic target in malignant

Table 3 Univariate and multivariate analyses of disease-free survival

	Univariate analysis		Multivariate analysis		
	5-year DFS (%)	*P* value	HR	95 % CI	*P* value
Age					
≤20	74	0.140	1	0.98–7.45	0.055
>20	51		2.70		
Gender					
Male	64	0.484	1	0.25–2.36	0.653
Female	71		0.77		
Location					
Proximal	74	0.214	1	0.57–4.69	0.366
Distal	56		1.63		
Necrosis rate					
≤90 %	64	0.686	1	0.41–3.40	0.756
>90 %	72		1.18		
EMMPRIN expression					
Lower	80	0.024	1	1.18–10.50	0.024
Higher	54		3.52		
AJCC stage					
IIA	69	0.297	1	0.49–4.56	0.486
IIB	59		1.49		

DFS disease-free survival, *HR* hazard ratio, *CI* confidence interval

Table 4 Univariate and multivariate analyses of overall survival

	Univariate analysis		Multivariate analysis		
	5-year OS (%)	*P* value	HR	95 % CI	*P* value
Age					
≤20	97	0.001	1	2.25–69.8	0.004
>20	55		12.52		
Gender					
Male	79	0.229	1	0.09–3.32	0.512
Female	90		0.55		
Location					
Proximal	96	0.004	1	1.03–44.46	0.046
Distal	64		6.77		
Necrosis rate					
≤90 %	94	0.399	1	0.35–14.65	0.389
>90 %	78		2.27		
EMMPRIN expression					
Lower	92	0.037	1	1.13–77.95	0.038
Higher	74		9.40		
AJCC stage					
IIA	84	0.777	1	0.06–2.83	0.363
IIB	79		0.41		

OS overall survival, *HR* hazard ratio, *CI* confidence interval

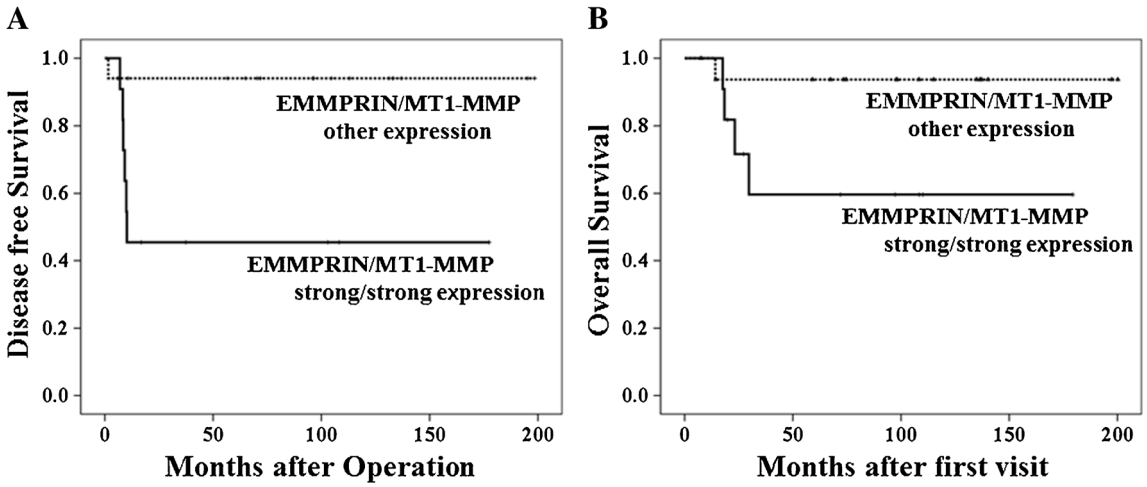

Fig. 3 Kaplan-Meier survival curves for co-expression of EMMPRIN and MT1-MMP in patients with osteosarcoma. **a** DFS of osteosarcoma patients, as a function of EMMPRIN strong/MT1-MMP strong (N=11) and EMMPRIN/MT1-MMP other expressions (N=17, P=0.056). **b** OS curves for EMMPRIN strong/MT1-MMP strong patients and EMMPRIN/MT1-MMP other expressions (P=0.006)

tumors. Newman et al. showed that forced expression of EMMPRIN in head and neck carcinoma cells increased MMP-9 expression in vitro and positively correlated with tumor growth, while negatively correlating with survival of the animals in vivo. Inhibition of EMMPRIN by small interfering RNA (siRNA) improved the prognosis of tumor-bearing mice significantly [15]. Other studies have also demonstrated anti-tumorigenic effects of siRNA for EMMPRIN in malignant melanoma and adenoid cystic carcinoma [29, 30].

Survival of patients with osteosarcoma has improved with the introduction of chemotherapy, although a considerable number still develop metastatic disease possibly due to the presence of chemoresistant cells. Several previous reports demonstrated the relationships between EMMPRIN expression and chemoresistance. Collaboration of EMMPRIN with the hyaluronan-CD44 complex plays a significant role in drug resistance [31]. Qin et al. reported that EMMPRIN played cooperative roles with lymphatic vessel endothelia hyaluronan receptor-1 (LYVE-1) and a drug transporter in chemoresistance of lymphoma cells [18]. Zhu et al. reported that down-regulation of EMMPRIN expression increased the tumor sensitivity of cisplatin in laryngeal carcinoma cells [32], suggesting that EMMPRIN could be a possible therapeutic target as a chemosensitizer.

In our study, older age and distal location were significant poor prognostic factors. Mankin et al. and Lee et al. reported that older patients showed a worse outcome [5, 33]. In contrast, studies from the Memorial Sloan-Kettering Cancer Center and Rizzoli Institute have shown unfavorable prognoses for patients aged 14 and younger [1, 34]. Bielack et al. and Szendroi et al. reported that tumor location in a proximal extremity is associated with a poor prognosis [2, 35], and Glasser et al. reported that humerus location gave better results [3]. As reported above, controversial results were described previously regarding age and location.

This study has a number of limitations. First, the number of analyzed patients was relatively low, reducing the statistical power. However, as compared to the two previous reports describing EMMPRIN expression of osteosarcoma [20, 21], this study had an identical cohort (limited to extremity involvement and without metastasis at the initial referral), which makes the results more meaningful. Second, the results of immunohistochemical analyses may vary according to the sensitivity of the antibodies and/or protocol used. However, the results of immunohistochemistry should be stable if techniques of immunohistochemistry improve, and the results of the immunohistochemical analyses were in fact reproducible in the current study.

In conclusion, this study demonstrates that higher EMMPRIN expression correlated with poor prognosis in patients with osteosarcoma, with co-expression of EMMPRIN and MT1-MMP having an even more significant impact on prognosis than EMMPRIN expression alone. These results suggest that EMMPRIN may be a novel therapeutic target in patients with osteosarcoma solely or in combination with MMPs to inhibit tumorigenicity and/or to stimulate chemosensitivity.

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Conflicts of interest None

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