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Expression of CXCR4 and MMP-2 is associated with poor prognosis in patients with osteosarcoma

Chen Gong[1], Kai Sun[2], Hui-hua Xiong[1], Tal Sneh[3], Jing Zhang[1], Xiao Zhou[1], Peng Yan[1] and Jian-hua Wang[1]

[1]Department of Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, [2]Department of Orthopedics, Renmin Hospital of Wuhan University, Wuhan, P.R. China and [3]Center for Molecular Design and Biomimetics, The Biodesign Institute, Arizona State University, Tempe, AZ, USA

Summary. Backgroud. Osteosarcoma is a primary malignant tumor with a high tendency to form metastasis and poor prognosis. Consequently, finding effective early indicators of metastases is crucial for identifying and treating high-risk patients. CXCR4 and MMP-2 have been found to strongly correlate with invasion and metastasis of malignant tumors, including osteosarcoma. Materials and Methods. Our study evaluated CXCR4 in conjunction with MMP-2 as an important clinicopathological prognostic predictor for metastasis and overall survival of osteosarcoma. 73 patients' clinical data and pathological samples were retrieved for the study. A median time of 36 months follow-up was performed to evaluate for tumor metastasis and patient survival. CXCR4 and MMP-2 proteins in tumor tissues were detected by immunohistochemistry on paraffin-embedded tissue sections. Results. The positive expression rate of CXCR4 and MMP-2 was 68.5% and 54.8% respectively, and of the 45 patients who developed distal metastasis, 33 and 28 patients had positive expression of CXCR4 and MMP-2 respectively. The median metastasis-free survival was 72.00 months in the CXCR4-negative group and 14.00 months in the CXCR4 positive group. Furthermore, median overall survival was 73.77 and 24.00 months in these same two groups. Further, the median metastasis-free survival was 66.51 months in the MMP-2 negative group and 9.00 months in the MMP-2 positive group. The median overall survival was 75.07 and 19.00 months in these same two groups. MMP2 and metastasis remained the significant and independent prognostic factors for metastasis-free survival and overall survival by using the COX regression model adjusted for the multivariate predictors of survival. Conclusion. Our results suggest that metastasis and MMP-2 are both independent prognostic indicators for metastasis-free and overall survival of osteosarcoma patients.

Key words: Osteosarcoma, CXCR4, MMP-2, Metastasis, Survival

Offprint requests to: Jian-hua Wang, Department of Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, P.R. China. e-mail: superman.4812@163.com

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Introduction

Osteosarcoma is the most common primary malignant bone tumor among young adults and adolescents, with conventional osteosarcoma being the most common pathological type (Sun et al., 2017). With the development and application of comprehensive therapies including neo-adjuvant chemotherapy, radical operation and adjuvant chemotherapy, the 5-year survival rate has approached 70% (Misaghi et al., 2018). However, 20% of osteosarcoma patients already present with lung metastasis at initial diagnosis, and 40-50% of them develop it at a later stage due to osteosarcoma's high ability to invade and metastasize (Qu and Liu, 2015). Up to 80% of patients develop micro-metastasis and metastasis at the early stage (Huang et al., 2016). Fewer than 28 percent of patients with systemic metastases live more than 5 years, despite advanced

multidisciplinary therapy (Oda et al., 2006). One of the biggest reasons that micro-metastasis cannot be detected and treated in a timely manner is due to the lack of a detectable and effective biological markers for osteosarcoma metastasis. Therefore, it is imperative to determine testable and reliable biological markers for early detection of tumor metastasis that could significantly improve the prognosis of many osteosarcoma patients in the clinic.

Tumor metastasis is a complex process of cell migration, implantation and growth, which involves degradation of the basement membrane and extracellular matrix (ECM), detachment from the primary tumor, cell migration through basement membranes, entrance into the circulation, dissemination, and arrest at select distant organs (Ren et al., 2016). It is well known that matrix metalloproteinases (MMP) are involved in degradation of the basement membrane and ECM, and further promote tumor growth by increasing the activity of growth factors residing in the ECM (Bjornland et al., 2005). In addition, MMPs play an important role in tumor angiogenesis and epithelial to mesenchymal transition (EMT) (Huang et al., 2016). MMP-2 is an important member of the MMP family which also plays an important role in tumor invasion and metastasis in osteosarcoma, ovarian cancer, colorectal cancer and non-small cell lung cancer (Li et al., 2013; Liu, 2016; Zhang et al., 2018). More importantly, Wen et al. suggested that levels of MMP-2 expression are relative to tumor size, lymph node metastasis, distant metastasis and survival (Wen et al., 2014).

The CXC chemokine receptor 4 (CXCR4) has been proven to play a crucial role in the metastases of a variety of tumors, including breast cancer, prostate cancer, colon cancer, lung cancer, and pancreatic cancer (Bai et al., 2011). Stromal derived factor-1 (SDF-1) is the only known ligand of CXCR4 and is constitutively expressed in several organs including the lungs, liver, skeletal muscle, and the brain (Ren et al., 2016). Moreover, tumor cells that highly express CXCR4 are attracted by stromal cells with high levels of SDF-1, which suggests that SDF-1/CXCR4 pathway might be relative to organ-specific metastasis, although its molecular mechanism is not clear. It's worth noting that CXCR4 has been shown to be involved in lymph node metastasis and distant metastasis of several types of cancer (Oda et al., 2006).

In our study, we examined the expression of MMP-2 and CXCR4 in non-metastatic and metastatic primary osteosarcoma samples to elucidate the relationship among them and associated clinical features.

Material and methods

Patient samples and clinical information

We analyzed medical records and pathological samples retrospectively at Renmin Hospital of Wuhan University from 2010 to 2013. Only pathological samples taken before chemoradiotherapy were collected, that is, all patients that received chemoradiotherapy were excluded from the study. The study population consisted of 73 patients diagnosed with osteosarcoma, including 42 males and 31 females. Histopathology confirmed the diagnosis as osteosarcoma *via* the World Health Organization's classification of bone tumor by two different pathologists for all samples. The current study required patients to conduct standardized follow-up every 3 months for the first 2 years, every 6 months for the next 2 years and every 6-12 months for years 5-10 (Casali et al., 2018). The contents of the follow-up included chest low-dose computer tomography, abdominal color doppler ultrasound and primary site magnetic resonance imaging.

Immunohistochemical analysis

Tumor biopsy samples were fixed, decalcified, and embedded. The paraffin-embedded blocks were cut into 4μm-thick sections, then deparaffined with xylene, rehydrated with a graded series of ethanol and endogenous peroxidase activity quenched with 3% H_2O_2. The samples were incubated with primary antibody at 1:100 dilution (rabbit anti-MMP-2 and anti-CXCR4 polyclonal antibody, Santa Cruz) at 4°C in a humidified chamber overnight, then incubated with secondary antibody (Biotin-labeled goat-anti-rabbit IgG antibody, Boster, China) for 0.5 h at room temperature. Phosphate-buffered saline (PBS) was used as negative control. The subsequent operations were performed per instructions of the Ready-to-use SABC-POD (rabbit IgG) kit (Boster, China, SA1029). DAB reaction was performed using DAB color developing kit (Boster, China, AR1022).

Each section was evaluated independently by three oncologists with no prior knowledge of the patients' clinical information. We selected 100 cells total from five fields (at ×400 magnification) of each section and evaluated for MMP-2 and CXCR4 expression. The semi-quantitative results were based on the intensity of cytoplasmic staining and percentage of positive cells. The staining intensity was scored to four groups (Ren et al., 2016): 0=negative, 1=weakly staining, 2=moderately staining and 3=strongly staining. The percentage of positive cells in total cells was also scored to four groups (Han et al., 2012): less than 25%=0, 26-50%=1, 51-75%=2, and more than 75%=3. The score of intensity plus the score of the percentage was considered as the total score. As previously reported, scores of 0 to 3 were regarded as negative and scores of 4 to 6 as positive (Zhang and Zhang, 2015; Ren et al., 2016).

Statistical analysis

Metastasis-free survival (MFS) is defined as the elapsed time from the initial diagnosis until the occurrence of metastasis. Overall survival (OS) is defined as the elapsed time from initial diagnosis until death or

last follow-up if the patient was still alive. All statistical analyses were carried out using SPSS 21.0. Log-rank test was used for single factor analysis and Cox regression model was used for multi-factor analysis.The test level a=0.05, that is, P<0.05 has statistical significance.

Results

Patient clinical characteristics

The patients (n=73) of osteosarcoma were included in the retrospective study. The patients' clinicopathological characteristics are summarized in Table 1. The patients comprised 42 male and 31 female patients, ranging in age from 8 to 61 years (average age 23 years, median age 19 years). The pathological diagnosis of all patients included 48 of osteoblastic, 13 of chondroblastic and 12 of other types. All the patients received neoadjuvant chemotherapy before the surgical procedure, and all patients with extremity osteosarcoma underwent limb salvage surgery. Doxorubicin combined with cisplatin was used in neoadjuvant chemotherapy. The postoperative adjuvant chemotherapy was performed according to the tumor necrosis rate and the

Table 1. Clinicopathologic characteristics.

Feature	Amount	CXCR				MMP-2			
		Positive	Negative	X^2	P	Positive	Negative	X^2	P
Gender									
Male	42	32	10	2.715	0.257	26	16	2.019	0.364
Female	31	18	13			14	17		
Age									
≥19 y	41	28	13	0.002	0.999	24	17	0.529	0.768
<19 y	32	22	10			16	16		
Location									
Thigh	31	21	10	0.056	1.000	13	18	4.987	0.289
Crus	19	13	6			14	5		
Brachium	10	7	3			6	4		
Cubitus	6	4	2			3	3		
Other	7	5	2			4	3		
Histological classification									
Osteoblastic	48	33	15	0.528	0.768	29	19	1.839	0.399
Chondroblastic	13	8	5			6	7		
Others	12	9	3			5	7		
Metastasis									
Yes	44	33	11	2.173	0.337	28	16	3.496	0.174
No	19	17	12			12	17		

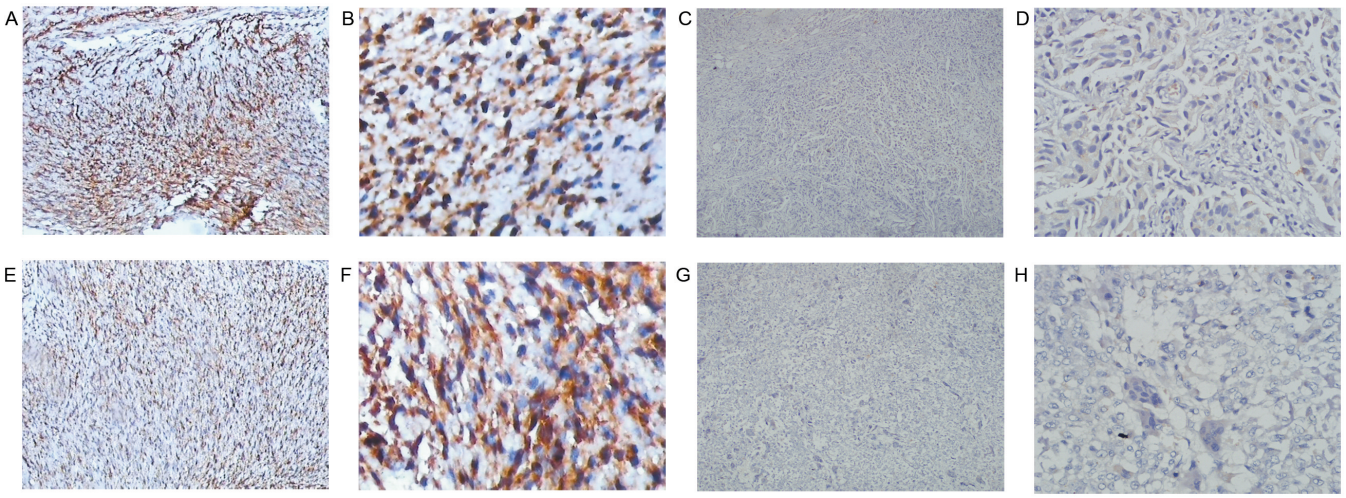

Fig. 1. Immunohistochemical CXCR4 expression in primary osteosarcoma tissue. CXCR4 positive staining **(A, B)** and negative staining **(C, D)** in patient with metastasis. CXCR4 positive staining **(E, F)** and negative staining **(G, H)** in patient without metastasis. A, C, E, G, x 100; B, D, F, H, x 400.

patient's tolerance to chemotherapy drugs.

Follow-up data was obtained from hospital records and correspondence with the patient's attending physician, and ranged from 3 to 97 months (average 42 months, median 36 months). During follow-up 44 (60.27%) had distal metastasis, 47 patients (64.38%) died of tumor-related causes during the study. The median OS and MFS of all patients were 47 months (95% confidence interval, 27.852-66.148 months) and 24 months (95% confidence interval, 13.070-34.930 months) respectively.

Clinicopathologic correlation of CXCR4 and MMP-2 expression in osteosarcoma

Positive reaction of CXCR4 and MMP-2 were mainly present on the osteosarcoma cancer cell in Fig. 1A,B,E,F and Fig. 2A,B,E,F, and the negative staining is shown in Fig. 1C,D,G,H and Fig. 2C,D,G,H. As shown in Tables 1 and 2, the positive expression cases of CXCR4 and MMP-2 were 50 (68.5%) and 40 (54.8 %) respectively, and of the 44 patients (60.3%) who developed distal metastasis, 33 (45.21%) and 28 (38.36%) patients had positive expression of CXCR4 and MMP-2 respectively. Also, 37 (50.7 %) patients had positive expression of both CXCR4 and MMP-2, 16 (21.9 %) patients were negative for either CXCR4 or

Table 2. Condition of CXCR4 and MMP-2.

Immunohistochemical phenotype	MMP-2 positive	MMP-2 negative
CXCR4 positive	37	13
CXCR4 negative	3	20
χ^2 23.63		
P 0.00007		

Table 3. Univariate survival analysis about MFS.

Feature	Amount	Median survival time	95%CI	χ^2	P
Sex					
Male	42	23.00	6.25 ~ 39.75	0.061	0.805
Female	31	24.00	9.53 ~ 38.47		
Age					
<19	32	34.00	13.54 ~ 54.46	1.897	0.168
≥19	41	18.00	3.18 ~ 32.82		
Tumor location					
Thigh	31	47.00	4.54 ~ 89.46	4.985	0.289
Crus	19	14.00	5.47 ~ 22.53		
Brachium	10	11.00	0.00 ~ 35.79		
Cubitus	6	17.00	8.60 ~ 25.40		
Other	7	52.00	22.02 ~ 81.98		
Metastasis					
No	29	80.90	70.36 ~ 91.44	52.268	0.000**
Yes	44	9.00	6.26 ~ 11.74		
AJCC staging					
I	8	82.75	63.96 ~ 101.54	71.887	0.000**
II	22	51.00	13.40 ~ 88.60		
III	37	12.00	4.05 ~ 19.95		
IVa	6	0.00	0.00 ~ 0.000		
MMP2					
Negative	33	65.51	52.80 ~ 78.22	24.429	0.000**
Positive	40	9.00	6.58 ~ 11.42		
CXCR4					
Negative	23	72.00	44.50 ~ 77.12	6.815	0.009**
Positive	50	14.00	16.06 ~ 31.94		
MMP2 CXCR4					
Double negative	20	64.74	48.19 ~ 81.29	23.606	0.000**
Double positive	37	9.00	5.50 ~ 12.50		
Single positive	16	48.36	34.96 ~ 61.75		

**P<0.01.

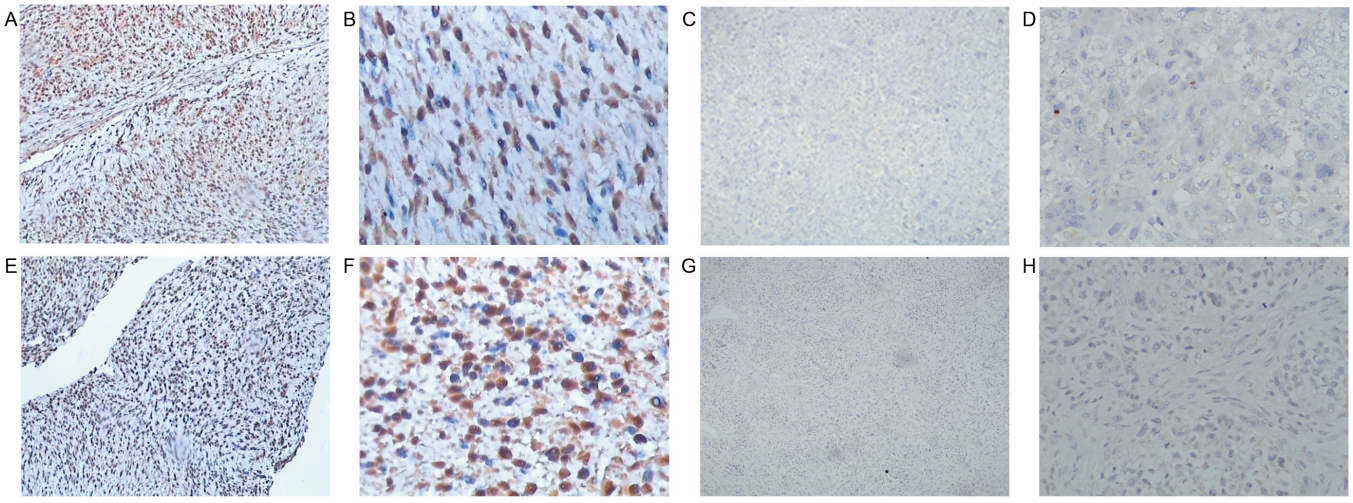

Fig. 2. Immunohistochemical MMP-2 expression in primary osteosarcoma tissue. MMP-2 positive staining **(A, B)** and negative staining **(C, D)** in patient with metastasis. MMP-2 positive staining **(E, F)** and negative staining **(G, H)** in patient without metastasis. A, C, E, G, x 100; B, D, F, H, x 400.

MMP-2, and 20 (27.4 %) patients were negative for both CXCR4 and MMP-2.

The results indicated that CXCR4 and MMP-2 positive expression rates were significantly correlated with distal metastasis. Also, the results showed that the expressions of CXCR4 and MMP-2 had no correlation with gender, age or tumor location.

High expression of CXCR4 and MMP-2 were correlated with poorer prognosis

In order to detect the relationship between the expression of CXCR4 or MMP-2 and patient survival, we tested the relationship between CXCR4 and MMP-2 expression and MFS and OS (Fig. 3). The median MFS was 72.00 months (95% CI 44.50 ~ 77.12 months) in the CXCR4-negative group and 14.00 months (95% CI 16.06 ~ 31.94 months) in the CXCR4 positive group (Fig. 3A). Furthermore, median OS was 73.77 (95% CI 62.37 ~ 85.17 months) and 24.00 months (95% CI 16.06 ~ 31.94 months) in these same two groups (Fig. 3D). Further, the median MFS was 66.51 months (95% CI 52.80 ~ 78.22 months) in the MMP-2 negative group and 9.00 months (95% CI 6.58 ~ 11.42 months) in the MMP-2 positive group (Fig. 3B). The median OS was 75.07 (95% CI 66.06 ~ 84.08 months) and 19.00 months (95% CI 15.22 ~ 22.78 months) in these same two groups (Fig. 3E). The 2-year MFS and OS rate was 73.7% and 95% in the CXCR4 and MMP-2 negative expressed group, and 32.8% and 28.1% in the CXCR4 and MMP-2 positive group (Fig. 3C,F). Our results suggested metastasis, AJCC staging, MMP2, CXCR4, coexpression of MMP2 and CXCR4 were significant

Table 4. Univariate survival analysis about OS.

Feature	Amount	Median survival time	95%CI	χ^2	P
Sex					
Male	42	36.000	10.84 ~ 61.16	0.000	1.000
Female	31	52.000	24.18 ~ 79.82		
Age					
< 19	32	52.000	12.90 ~ 91.10	1.717	0.190
≥19	41	35.000	14.20 ~ 55.80		
Tumor location					
Thigh	31	67.000	37.44 ~ 96.56	4.654	0.326
Crus	19	24.000	16.89 ~ 31.11		
Brachium	10	29.000	0.00 ~ 82.72		
Cubitus	6	24.000	0.00 ~ 54.01		
Other	7	56.143	29.56 ~ 82.72		
Metastasis					
No	29	81.305	70.94 ~ 91.67	39.403	0.000**
Yes	44	21.000	17.01 ~ 24.99		
AJCC staging					
I	8	88.000	78.83 ~ 97.17	27.686	0.000**
II	22	70.000	46.23 ~ 93.77		
III	37	24.000	15.66 ~ 32.34		
IVa	6	14.000	6.16 ~ 21.84		
MMP2					
Negative	33	75.073	66.06 ~ 84.08	34.006	0.000**
Positive	40	19.000	15.22 ~ 22.78		
CXCR4					
Negative	23	73.766	62.37 ~ 85.17	13.356	0.000**
Positive	50	24.000	16.06 ~ 31.94		
MMP2 CXCR4					
Double negative	20	78.294	68.09 ~ 88.5	35.217	0.000**
Double positive	37	20.000	15.17 ~ 24.83		
Single positive	16	54.375	44.20 ~ 64.55		

**P<0.01.

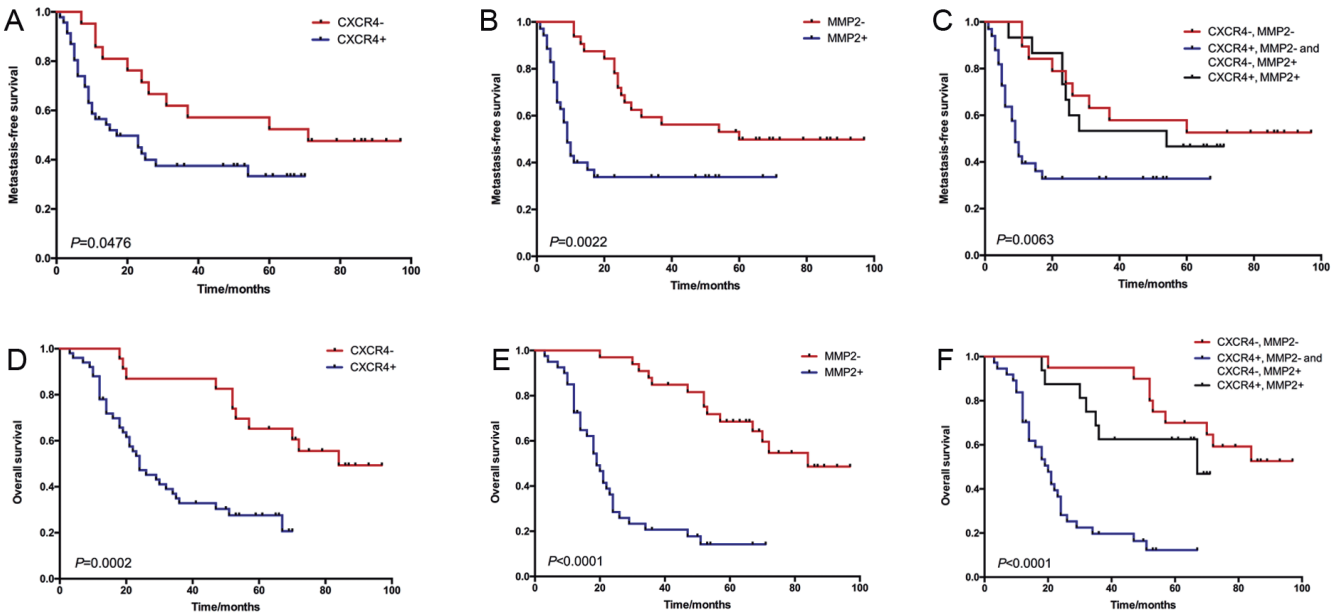

Fig. 3. Survival curve of patients with osteosarcoma according to CXCR4 and MMP-2 expression. Comparisons of MFS and OS by CXCR4 (**A, D**), MMP-2 (**B, E**), combined CXCR4 and MMP4 (**C, F**).

Table 5. Cox regression analysis about MFS.

Feature	B	SE	Wald	P	HR	95.0% CI for HR	
						Lower	Upper
Metastasis	4.029	0.620	42.216	0.000**	56.181	16.665	189.390
MMP2	2.825	0.517	29.864	0.000**	16.869	6.123	46.472

**P<0.01.

Table 6. Cox regression analysis about OS.

Feature	B	SE	Wald	P	HR	95.0% CI for HR	
						Lower	Upper
Metastasis	3.244	0.554	34.343	0.000**	25.634	8.663	75.858
MMP2	3.031	0.543	31.100	0.000**	20.710	7.138	60.083

**P<0.01.

prognostic negative indicators for MFS and OS using the Kaplan-Meier log-rank test (Tables 3, 4). However MMP2 and metastasis remained significant and independent prognostic factors for MFS and OS using the COX regression model adjusted for the multivariate predictors of survival (Tables 5, 6).

Discussion

Osteosarcoma is the most common primary malignant bone tumor in young adults and is characterized by the production of osteoid tissue or immature bone (Han et al., 2012). The prognosis of patients with osteosarcoma has significantly improved, reaching 70%, but a great barrier to improving this clinical outcome further is osteosarcoma metastasis. There are no effective systematic or surgical therapies to prolong life and improve life quality in patients with osteosarcoma metastasis, and as a result the 5-year survival of these patients remains at a dismal 10% to 20%, compared to 60% to 70% for those without metastases (Ren et al., 2016; Li et al., 2017; Wang et al., 2017). In order to identify those patients at highest risk of osteosarcoma metastasis and provide necessarily more aggressive treatments for this group, it is necessary to find reliable prognostic biomedical markers to achieve the above objectives. As shown in table 3 and 4, the histological classification, sex, age and tumor location were not significantly correlated with MFS, but metastasis, staging, MMP2 expression and CXCR4 expression were significantly correlated with MFS, which was consistent with OS research. The results strongly suggested that the expressions of MMP2 and/or CXCR4 might be potential prognostic predictors.

CXCR4 is an important chemokine receptor, and is widely studied in HIV and hemopathy (Li et al., 2017). More and more studies find that CXCR4 plays a crucial role in invasion and metastasis malignant tumors derived from different tissues, such as the prostate, thyroid, pancreas, lung, colon, cartilage and breasts (Bai et al., 2011). The complementary C-X-C motif chemokine 12,also known as SDF1, is expressed in many organs and tissues such as the lung, bone and lymph node (Bai et al., 2011), the lung being the most common metastatic site of osteosarcoma. The CXCR4/SDF1 axis was found to play an important role in the metastatic processes of osteosarcoma (Perissinotto et al., 2005). Li et al. (2017) found that the expression of CXCR4 was significantly associated with higher rate of metastasis and higher clinical stage (III-IV according to the AJCC stage system), and was not significantly associated with gender, age and tumor primary site, suggesting that CXCR4 was an indicator for poor overall survival in bone and soft tissue sarcomas, including osteosarcoma. Oda et al. (2006) proved that CXCR4 expression has an important role in the metastatic process in osteosarcoma. Sand et al. (2015) reported that CXCR4 signaling was a potential targetable pathway and inhibition of CXCR4 in Ewing sarcoma in vitro and in xenografts had been shown to reduce tumor migration and angiogenesis. Ren et al. (2016) suggested that the expression of CXCR4 was found to be associated with lung metastasis, and that CXCR4 could be a potential biomarker for predicting osteosarcoma lung metastasis, shown to be a prognosticc factor of overall and metastasis free survival in osteosarcoma patients. Bai et al. (2011) found that all human chondrosarcoma cells were positive for antibodies directed toward CXCR4 epitopes in both the cytoplasm and nucleus, and CXCR4 expression levels increased in high-grade chondrosarcoma cells compared with low-grade specimens. In addition CXCR4 expression levels increased in the metastatic samples compared with the primary lesion in the same patient, which also suggested that expression level of CXCR4 correlates with prognosis and potential metastatic development (Bai et al., 2011). However our results suggested that CXCR4 was correlated with MFS and OS in univariate analysis, but was not an independent risk factor in multifactorial analysis, which is different to what Ren et al reported (Ren et al., 2016). We think the reasons for this difference may be as follows: first, it is related to the small number of initial metastasis cases in the included cases; second, CXCR4 is significantly correlated with metastasis, which is not an independent predictor of metastasis. The relationship between CXCR4 and osteosarcoma prognosis needs further study to clarify.

MMP-2 is a main member of the MMP family and primarily hydrolyzes type IV collagen. Many studies have shown that MMP-2 is highly expressed in tissues of various human cancer, implying a critical role in tumor growth, tissue invasion, angiogenesis and distant metastasis (Wen et al., 2014; Sudhakar et al., 2014; Ren et al., 2018). High levels of MMP2 expression are correlated with larger tumors, lymph node metastasis, distant metastasis and tumor invasion, which suggest

that a high MMP2 expression could be a potential biomarker for poor prognosis (Wen et al., 2014; Salem et al., 2016; Chan et al., 2017). Chan et al. (2017) found that MMP-2 is correlated with poor survival in patients with bladder cancer, and patients whose bladder tumors express the biomarkers may benefit from early radical treatment and/or neoadjuvant or adjuvant therapies. Salem et al. (2016) found that MMP-2 was a significant independent predictor of disease-free survival and disease-specific survival, and MMP-2 expression patterns provide useful prognostic information in colorectal carcinoma, while predicting the patients at high risk for recurrent disease. Zhang and Zhang (2015) found that the expression of MMP-2 was associated with pulmonary metastasis, and was related to the prognosis of osteosarcoma, which suggested MMP-2 could act as an independent predictor of metastases in osteosarcoma patients. Wen and colleagues performed a meta-analysis using the original data from five studies that indeed confirmed that osteosarcoma patients with high MMP2 expression have poorer prognosis compared with those with low MMP2 expression (Wen et al., 2014). The single-factor significant variables about MFS, including staging, AJCC staging, MMP2, CXCR4 and MMP2 CXCR4, were entered into the Cox regression model, the score was 73.950 (P=0.000), and the χ^2 value of log-likelihood ratio test was 49.251 (P=0.000), which indicated that at least one independent variable in the model has an HR value that does not equal 1, and the whole model had statistical significance (Table 5).The single-factor significant variables about OS, including staging, AJCC staging, MMP2, CXCR4 and MMP2 CXCR4, were entered into the Cox regression model, the score was 79.051 (P=0.000), and the χ^2 value of log-likelihood ratio test was 45.835 (P=0.000), which indicated that at least one independent variable in the model has an HR value that does not equal 1, and the whole model had statistical significance (Table 6). Our study found that MMP2 was an independent factor for MFS (Table 5) and OS (Table 6), which suggested that CXCR4 might be a important independent prognostic predictor of osteosarcoma.

In conclusion, CXCR4 and MMP2 are correlated with the survival of patients with osteosarcoma. According to our current results, MMP2 is an independent prognostic predictor factor, and whether CXCR4 is an independent predictor of metastasis remains to be further studied.

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