Int J Clin Exp Pathol 2015;8(11):14965-14970
www.ijcep.com /ISSN:1936-2625/IJCEP0009886

Original Article

Association of MMP-2 expression and prognosis in osteosarcoma patients

Miao Zhang*, Xuran Zhang*

*Department of Fuxin Central Hospital, Fuxin, Liaoning Province, China. *Equal contributors.*

Received May 4, 2015; Accepted June 23, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Objective: Our aim was to investigate the MMP-2 expression and its prognostic value in osteosarcoma patients. Methods: we performed RT-qPCR to detect the expression of MMP-2 in 45 paired osteosarcoma tissues and adjacent noncancerous tissues. Immunohistochemical staining assay was used to verify the expression of MMP-2 protein in osteosarcoma patients. Independent-sample T test was used to analyze the difference of MMP-2 expression level between osteosarcoma and control groups. The relationship between clinicopathologic factors and MMP-2 expression was analyzed by chi-square test. Kaplan-Meier was performed to analyze the association of MMP-2 expression and overall survival rate. The prognostic value of clinicopathologic factors and MMP-2 was estimated via Cox regression analysis. Results: RT-qPCR revealed that the expression of MMP-2 was up-regulated in osteosarcoma group compared with the control group. Besides MMP-2 expression was influenced by pulmonary metastasis ($P<0.05$) while gender, age, tumor site and Enneking stage showed no obvious impact ($P>0.05$). Kaplan-Meier curve revealed that patients with positive MMP-2 expression had a shorter survival time than those with negative MMP-2 expression, and the survival rates were 18.5% (5/27) and 44.4% (8/18), respectively. Cox regression analysis indicated that pulmonary metastasis and expression of MMP-2 gene were important factors in the prognosis of osteosarcoma. Conclusion: The expression of MMP-2 was associated with pulmonary metastasis, and was related to the prognosis of osteosarcoma. MMP-2 could act as an independent prognostic marker in osteosarcoma patients.

Keywords: MMP-2, osteosarcoma, prognosis

Introduction

Osteosarcoma (OS) is one of the most common malignant bone tumors in children and adolescent with the characteristic of the direct formation of immature bone or osteoid tissue [1]. The peak age of OS patients is from 10 to 20 [2, 3]. Clinical diagnostic data shows that over 80% of patients have appeared pulmonary metastasis, which is the main characteristic of OS [4]. Once metastasis occurs, the 5-year survival is only at 20% [5]. With the improvement of neoadjuvant chemotherapy and positive operation intervention, the 5-year survival rate of patients with OS has been raised to 80% [6]. Based on this, amputation for OS patients has been replaced by limb salvage operation, however, there are still at least 40% OS patients died from the recurrence and metastasis of tumors [7]. Moreover, the molecular mechanisms involved in osteosarcoma progress remain poorly understood. Therefore, it is of critical importance to explore the mechanisms to make the complex etiology of OS clear and develop a new strategy for diagnosis, treatment and prognosis of this disease.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases and a kind of hydrolase which can degrade all ingredients in extracellular and basilar membrane [8]. Moreover, based on its function, MMPs play vital roles on the cell growth and tumor metastasis especially MMP-2 and MMP-9 [9, 10]. Among them, *MMP-2* is confirmed to be associated with the invasion and metastasis of many tumors [11]. It is reported that *MMP-2* mainly participates in the destruction of extracellular matrix and is involved in a series of physiological processes including repairing and healing the inflammation and trauma, and promoting the generation of embryo. In normal tissues, the regulation of *MMP-2* gene is inhibited by natural inhibitors including tissue inhibitors

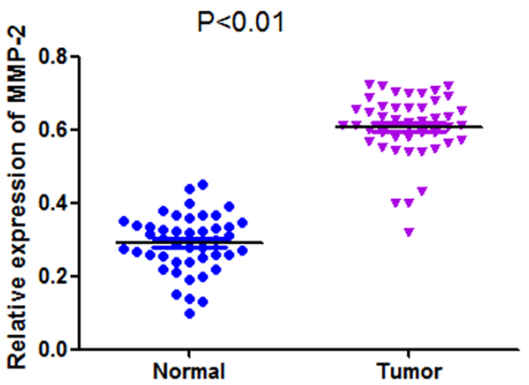

Figure 1. *MMP-2* was up-regulated in osteosarcoma. The expression of *MMP-2* was determined by RT-qPCR in 45 pairs of osteosarcoma and adjacent non-cancerous tissue (Normal).

of MMP (TIMP), RECK and a2 macroglobulin. However, in OS tissues, MMP-2 gene contributes to the invasion of endothelial cells via destroying collagens of extracellular [12]. Lana et al., found that the expression of MMP-2 in OS tissues was higher than in other tissues [13]. However, there were still not enough studies investigating the association of MMP-2 expression with OS metastasis and prognosis.

In our study, we aimed to detect the expression of MMP-2 in 45 OS patients, and estimate the prognostic value of *MMP-2* expression and clinicopathologic factors in the patients with OS.

Materials and methods

Patients and tissue samples

45 patients diagnosed as OS including 27 males and 18 females were collected from the Department of Orthopaedics in Fuxin Central Hospital. Paired resected surgical specimens from primary tumor and adjacent nontumor sites were obtained from osteosarcoma patients who were aged from 6 to 26 (mean age was 14.3). Inclusion criteria contained: (1) with malignant OS in the limb; (2) not received any chemical treatment and physical therapy prior to surgical resection; (3) available for immunohistochemical detection. Among the 45 cases, 24 cases were with lesions in distal femur, 17 in proximal tibia, 2 in proximal humerus, 1 in proximal fibula and 1 in pelvis. According to Enneking surgical stage [14], patients with OS were respectively divided into Stage I (5 cases), Stage II (21 cases), and Stage III (19 cases). According to the situation of pulmonary metastasis, OS samples were categorized into patients with and without pulmonary metastasis (20 cases; 25 cases). Besides, written informed consents were obtained from all the individuals involved in the trial. According to the ethical and legal standards, all specimens were handled and made anonymous. The follow-up information of all the participants was updated every 2 months for 3 years.

RNA extraction and RT-qPCR

Total RNA was extracted using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription reaction was carried out to synthesis cDNA, with 10 ng total RNA, 50 nmol/L RT primer, 1X buffer, 3.33 U/μl MultiScribe reverse transcriptase and 0.25 U/μl RNase inhibitor (Sigma-Aldrich). The reaction system was initially incubated at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and maintained at 4°C. qPCR was performed on 7500 Real-Time PCR detection system (Life Technologies, Foster City, CA, USA). The cycling conditions contained 95°C, 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 60 sec. U6 was used as an internal control. Each sample was measured in triplicate.

Immunohistochemical analysis

All the specimens were routinely decalcified, fixed with 40 g/L of formaldehyde solution, embedded with paraffin, and cut into 3 μm-thick sections. PV 6000 immunohistochemistry (PV 6000 SP kit and DAB kit, Beijing Zhong Shan Golden Bridge Biotec Co., LTD) was used to testify the expression of *MMP-2* protein in OS tissues. Then added into antibody with a concentration of 1:100. PBS buffer solution was taken as the control (Rabbit Anti-Human RECKmAb reagent, American Santa Cruz Biotec, Co., LTD; *MMP-2* Rabbit Anti-Human mAb reagent, Wuhan Boster Biotec, Co., LTD). All the operations were strictly implemented in accordance with instructions. Positive signals in *MMP-2* mainly located in cytoplasms presenting as claybank granulars. Cells were observed though high power lens, and 5-10 visions were randomly selected (at least two hundred cells were observed in a vision). The results were determined according to the percentage of positive cells in total and staining degree of cells. Firstly, we graded 0, 1, 2, 3 points respectively represented cells without coloration, with light yel-

Table 1. Association between *MMP-2* expression and clinicopathologic factors

Clinicopathologic feature	Case	*MMP-2* (+)	*MMP-2* (-)	χ^2	*P*
Gender					
Male	27	16	11	0.015	0.901
Female	18	11	7		
Age					
≤18	29	16	13	0.792	0.373
>18	16	11	5		
Tumor site					
Distal femur	24	13	11	0.872	0.647
Proximal tibia	17	11	6		
Others	4	3	1		
Enneking staging					
Stage I	5	3	2	0.819	0.664
Stage II	21	14	7		
Stage III	19	10	9		
Situation of pulmonary metastasis					
With pulmonary metastasis	20	16	4	6.000	0.014
Without pulmonary metastasis	25	11	14		

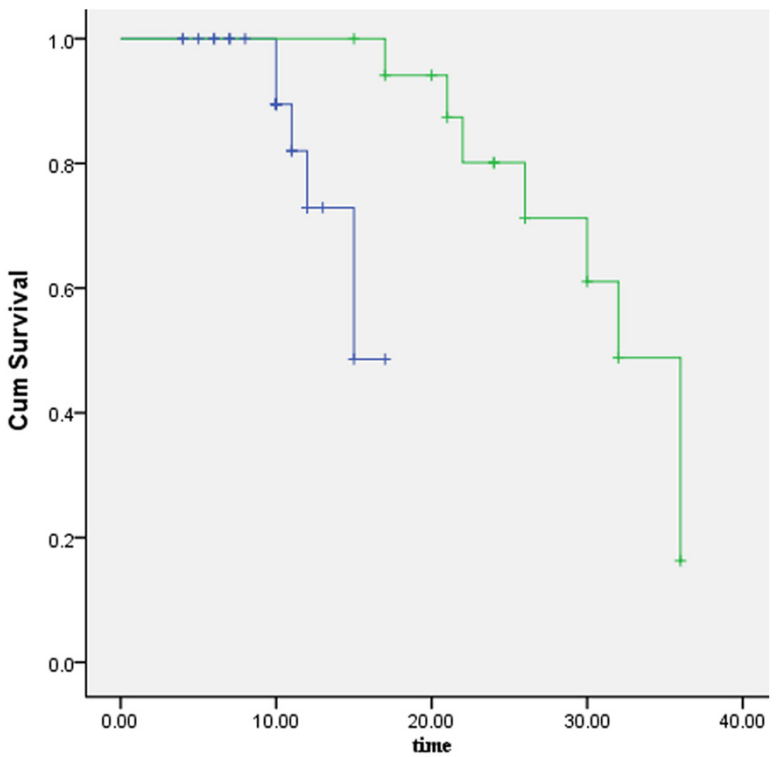

Figure 2. Analysis of survival curves in OS patients by Kaplan-Meier. Osteosarcoma patients were divided into two groups by positive and negative expression of *MMP-2*. The patients with positive expression of *MMP-2* had significant shorter survival time (*P*<0.01) than those with negative *MMP-2* expression.

low, claybank, and sepia according to the staining degree in the section. Secondly, we graded according to the percentage of positive cells in total cells. The percentage less than 30%, 30%-70%, and more than 70% were expressed as 0, 1, and 2 points, respectively. The score in first item plus with the score in second item was considered as the total score of the product, and 0 to 2 points stood for negative (-), and at least 3 points for positive (+).

Statistical analysis

Statistical analysis was preceded by using SPSS statistical software and GraphPad Prism 5.0 software. Moreover, it was considered to be significant difference when *P*<0.05. Statistical methods adopted in our study included T test, chi-square test, Kaplan-Meier survival curve, and Cox regression analysis.

Results

MMP-2 was up-regulated in the human OS tissues

To confirm *MMP-2* expression was different between tumor tissues and adjacent noncancerous tissues, we examined 45 pairs of human osteosarcoma samples. As shown in **Figure 1**, the results of RT-qPCR showed that *MMP-2* expression was up-regulated in osteosarcoma tissues compared with the adjacent noncancerous tissues (*P*<0.01).

Association between MMP-2 gene and clinicopathologic factors

No significant differences were found among *MMP-2* expression and gender, age, tumor site,

Table 2. Analysis of Cox proportional hazards model

Variable	HR	95% CI	*P*
Pulmonary metastasis	26.064	1.222-555.911	0.037
MMP-2 expression	18.166	1.239-266.278	0.034

Enneking stage ($P>0.05$). Nevertheless, with the absence or presence of pulmonary metastasis, the *MMP-2* expression showed an obvious difference ($\chi^2=6.00$, $P=0.02$), as displayed in **Table 1**.

Association between MMP-2 expression and the 3-year survival rate of OS patients

According to Kaplan-Meier analysis, we discovered that 3-year survival rate of OS patients with positive *MMP-2* expression were 18.5% (5/27) while those with negative expression were 44.4% (8/18), respectively. The result demonstrated that OS patients with positive *MMP-2* obviously lived shorter compared to those with negative *MMP-2* ($\chi^2=8.24$, $P=0.004$), as displayed in **Figure 2**.

Analysis of Cox regression

Cox regression was used to analyze the effects of multiple clinical factors on OS prognosis. High expression of *MMP-2* (HR=18.166, $P=0.034$) and pulmonary metastasis (HR= 26.064, $P=0.037$) showed significant difference in OS patients. And they might be as independent risk factors for prognosis of patients with OS, as shown in **Table 2**.

Discussion

During the past years, the prognosis for patients with OS has significantly improved with the development of aggressive chemotherapy treatment [15-18]. However, there are still plenty of patients died of OS with pulmonary metastasis, approximately 10-20% of OS patients present with metastatic disease at initial diagnosis [19, 20], and 30-40% of patients with initially non-metastatic OS eventually develop lung metastasis [21, 22]. The prognosis of patients with OS may not be easily predicted, and only a few prognostic factors in OS have been identified such as age, grade, tumor site and size [23]. Besides, some molecular in OS cells has also been shown to be prognostic factors, including P-glycoprotein [24], telomerase [25]. Previous several studies have demonstrated that the expression of MMPs plays crucial role in OS development and to be significant prognostic factor for the disease free survival of patients with OS, for example, *MMP-9* expression has been reported to be associated with OS [26, 27]. Jin et al. found *MMP-2* could be suppressed by *miR-218* then inhibited osteosarcoma cell migration and invasion [28]. The down regulation of *MMP-2/-9,* protein kinase B (PKB) and PKC signaling pathways combining with Gallic acid could inhibit migration and invasion in human osteosarcoma cells [29]. In the study of Zhang et al., the proliferation of osteosarcoma cell was inhibited by piperine via increased expression of TIMP-1/-2 and down-regulation of *MMP-2/-9* [30]. However, the prognosis value of *MMP-2* as well as its related factors in osteosarcoma was still unclear.

In our study, we analyzed the expression of *MMP-2* and its correlation with clinical outcome. The results of RT-qPCR demonstrated that *MMP-2* expression was higher in osteosarcoma group than the control group. Besides, the expression of *MMP-2* was associated with pulmonary metastasis ($P<0.05$), which was accordance with the previous studies that plenty of OS patients died with pulmonary metastasis [19-22]. But no relation was observed with age, gender, tumor site, and Enneking surgical stage ($P>0.05$).

To further evaluate the association of *MMP-2* with overall survival of OS patients, Kaplan-Meier and Cox regression analysis were taken. According to Kaplan-Meier analysis, we discovered that survival rate of OS patients with positive *MMP-2* expression was 18.5% (5/27) while those with negative *MMP-2* expression was 44.4% (8/18). The overall survival rate manifested that patients in negative *MMP-2* expression group lived longer than those in positive *MMP-2* expression group. And log rank test showed a significant difference between two groups ($P<0.01$). The expression of *MMP-2* gene had a significant impact on postoperative survival time of OS patients. Cox regression analysis indicated that *MMP-2* expression and pulmonary metastasis were significant difference in the prognosis of osteosarcoma. So we inferred that pulmonary metastasis and expression of *MMP-2* gene were influence factors for

the prognosis of patients with OS. And they might be independent prognostic markers in osteosarcoma. In addition, the expression of *MMP-2* might be closely related to the occurrence and development of OS.

MMP-2 primarily hydrolyzes collagen and many researchers have reported it is overexpressed in various human cancer tissues [31, 32] *MMP-2* expression is implicated in tumor initiation, invasion, angiogenesis, and metastasis. Naohisa F et al. [33] have demonstrated that co-expression of EMMPRIN and MT1-MMP could well predict the prognosis of patients with extremity OS. Thus we inferred that the role of *MMP-2* in prognosis of OS might relate with EMMPRIN, but the mechanism of the relationship still need to be further illustrated.

In conclusion, the prognosis of OS was affected by multiple factors including the expression of *MMP-2* gene and pulmonary metastasis. Besides, the abnormal expression of *MMP-2* gene might play an important role in the occurrence and development of OS according to the relationship between clinicopathologic factors and *MMP-2*. Nevertheless, the specific mechanism of *MMP-2* influencing OS remains unclear and needs to be further investigated.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Miao Zhang, Department of Fuxin Central Hospital, 74 Zhonghua Road, Haizhou District, Fuxin, Liaoning Province, China. E-mail: zhmiao523@yeah.net

References

[1] Picci P. Osteosarcoma (osteogenic sarcoma). Orphanet J Rare Dis 2007; 2: 6.

[2] Chou AJ, Geller DS and Gorlick R. Therapy for osteosarcoma: where do we go from here? Paediatr Drugs 2008; 10: 315-327.

[3] Ek ET, Ojaimi J, Kitagawa Y and Choong PF. Outcome of patients with osteosarcoma over 40 years of age: is angiogenesis a marker of survival? Int Semin Surg Oncol 2006; 3: 7.

[4] Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, Zoubek A, Jurgens H and Winkler K. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 2002; 20: 776-90.

[5] Chen L, Wang Q, Wang GD, Wang HS, Huang Y, Liu XM and Cai XH. miR-16 inhibits cell proliferation by targeting IGF1R and the Raf1-MEK1/2-ERK1/2 pathway in osteosarcoma. FEBS Lett 2013; 587: 1366-1372.

[6] Ham SJ, Schraffordt Koops H, van der Graaf WT, van Horn JR, Postma L and Hoekstra HJ. Historical, current and future aspects of osteosarcoma treatment. Eur J Surg Oncol 1998; 24: 584-600.

[7] Meyers PA and Gorlick R. Osteosarcoma. Pediatr Clin North Am 1997; 44: 973-989.

[8] Oh J, Takahashi R, Kondo S, Mizoguchi A, Adachi E, Sasahara RM, Nishimura S, Imamura Y, Kitayama H, Alexander DB, Ide C, Horan TP, Arakawa T, Yoshida H, Nishikawa S, Itoh Y, Seiki M, Itohara S, Takahashi C and Noda M. The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. Cell 2001; 107: 789-800.

[9] Han I, Lee MR, Nam KW, Oh JH, Moon KC and Kim HS. Expression of macrophage migration inhibitory factor relates to survival in high-grade osteosarcoma. Clin Orthop Relat Res 2008; 466: 2107-2113.

[10] Hicklin DJ and Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 2005; 23: 1011-1027.

[11] Loukopoulos P, Mungall BA, Straw RC, Thornton JR and Robinson WF. Matrix metalloproteinase-2 and -9 involvement in canine tumors. Vet Pathol 2003; 40: 382-394.

[12] Patterson ML, Atkinson SJ, Knauper V and Murphy G. Specific collagenolysis by gelatinase A, MMP-2, is determined by the hemopexin domain and not the fibronectin-like domain. FEBS Lett 2001; 503: 158-162.

[13] Lana SE, Ogilvie GK, Hansen RA, Powers BE, Dernell WS and Withrow SJ. Identification of matrix metalloproteinases in canine neoplastic tissue. Am J Vet Res 2000; 61: 111-114.

[14] Enneking WF, Spanier SS and Goodman MA. A system for the surgical staging of musculoskeletal sarcoma. 1980. Clin Orthop Relat Res 2003; 4-18.

[15] Saeter G, Alvegard TA, Elomaa I, Stenwig AE, Holmstrom T and Solheim OP. Treatment of osteosarcoma of the extremities with the T-10 protocol, with emphasis on the effects of preoperative chemotherapy with single-agent high-dose methotrexate: a Scandinavian Sarcoma Group study. J Clin Oncol 1991; 9: 1766-1775.

[16] Bacci G, Mercuri M, Longhi A, Ferrari S, Bertoni F, Versari M and Picci P. Grade of chemotherapy-induced necrosis as a predictor of local and

systemic control in 881 patients with non-metastatic osteosarcoma of the extremities treated with neoadjuvant chemotherapy in a single institution. Eur J Cancer 2005; 41: 2079-2085.

[17] Smeland S, Muller C, Alvegard TA, Wiklund T, Wiebe T, Bjork O, Stenwig AE, Willen H, Holmstrom T, Folleras G, Brosjo O, Kivioja A, Jonsson K, Monge O and Saeter G. Scandinavian Sarcoma Group Osteosarcoma Study SSG VIII: prognostic factors for outcome and the role of replacement salvage chemotherapy for poor histological responders. Eur J Cancer 2003; 39: 488-494.

[18] Fuchs N, Bielack SS, Epler D, Bieling P, Delling G, Korholz D, Graf N, Heise U, Jurgens H, Kotz R, Salzer-Kuntschik M, Weinel P, Werner M and Winkler K. Long-term results of the co-operative German-Austrian-Swiss osteosarcoma study group's protocol COSS-86 of intensive multidrug chemotherapy and surgery for osteosarcoma of the limbs. Ann Oncol 1998; 9: 893-899.

[19] Meyers PA, Heller G, Healey JH, Huvos A, Applewhite A, Sun M and LaQuaglia M. Osteogenic sarcoma with clinically detectable metastasis at initial presentation. J Clin Oncol 1993; 11: 449-453.

[20] Kager L, Zoubek A, Potschger U, Kastner U, Flege S, Kempf-Bielack B, Branscheid D, Kotz R, Salzer-Kuntschik M, Winkelmann W, Jundt G, Kabisch H, Reichardt P, Jurgens H, Gadner H and Bielack SS. Primary metastatic osteosarcoma: presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. J Clin Oncol 2003; 21: 2011-2018.

[21] Ward WG, Mikaelian K, Dorey F, Mirra JM, Sassoon A, Holmes EC, Eilber FR and Eckardt JJ. Pulmonary metastases of stage IIB extremity osteosarcoma and subsequent pulmonary metastases. J Clin Oncol 1994; 12: 1849-1858.

[22] Aljubran AH, Griffin A, Pintilie M and Blackstein M. Osteosarcoma in adolescents and adults: survival analysis with and without lung metastases. Ann Oncol 2009; 20: 1136-1141.

[23] Davis AM, Bell RS and Goodwin PJ. Prognostic factors in osteosarcoma: a critical review. J Clin Oncol 1994; 12: 423-431.

[24] Pakos EE and Ioannidis JP. The association of P-glycoprotein with response to chemotherapy and clinical outcome in patients with osteosarcoma. A meta-analysis. Cancer 2003; 98: 581-589.

[25] Ulaner GA, Huang HY, Otero J, Zhao Z, Ben-Porat L, Satagopan JM, Gorlick R, Meyers P, Healey JH, Huvos AG, Hoffman AR and Ladanyi M. Absence of a telomere maintenance mechanism as a favorable prognostic factor in patients with osteosarcoma. Cancer Res 2003; 63: 1759-1763.

[26] Foukas AF, Deshmukh NS, Grimer RJ, Mangham DC, Mangos EG and Taylor S. Stage-IIB osteosarcomas around the knee. A study of MMP-9 in surviving tumour cells. J Bone Joint Surg Br 2002; 84: 706-711.

[27] Himelstein BP, Asada N, Carlton MR and Collins MH. Matrix metalloproteinase-9 (MMP-9) expression in childhood osseous osteosarcoma. Med Pediatr Oncol 1998; 31: 471-474.

[28] Jin J, Cai L, Liu ZM and Zhou XS. miRNA-218 inhibits osteosarcoma cell migration and invasion by down-regulating of TIAM1, MMP2 and MMP9. Asian Pac J Cancer Prev 2013; 14: 3681-3684.

[29] Liao CL, Lai KC, Huang AC, Yang JS, Lin JJ, Wu SH, Gibson Wood W, Lin JG and Chung JG. Gallic acid inhibits migration and invasion in human osteosarcoma U-2 OS cells through suppressing the matrix metalloproteinase-2/-9, protein kinase B (PKB) and PKC signaling pathways. Food Chem Toxicol 2012; 50: 1734-1740.

[30] Zhang J, Zhu X, Li H, Li B, Sun L, Xie T, Zhu T, Zhou H and Ye Z. Piperine inhibits proliferation of human osteosarcoma cells via G2/M phase arrest and metastasis by suppressing MMP-2/-9 expression. Int Immunopharmacol 2015; 24: 50-58.

[31] Egeblad M and Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002; 2: 161-174.

[32] Nelson AR, Fingleton B, Rothenberg ML and Matrisian LM. Matrix metalloproteinases: biologic activity and clinical implications. J Clin Oncol 2000; 18: 1135-1149.

[33] Futamura N, Nishida Y, Urakawa H, Kozawa E, Ikuta K, Hamada S and Ishiguro N. EMMPRIN co-expressed with matrix metalloproteinases predicts poor prognosis in patients with osteosarcoma. Tumour Biol 2014; 35: 5159-5165.