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 Based on this, amputation for OS patients has been replaced by limb salvage operation, however, there are still at least 40% OS patients died from the recurrence and metastasis of tumors [7].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
MMP-2 expression and prognosis of osteosarcoma

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 α2 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 RECK mAb 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 claysbank 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-
MMP-2 expression and prognosis of osteosarcoma

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

<table>
<thead>
<tr>
<th>Clinicopathologic feature</th>
<th>Case MMP-2 (+)</th>
<th>MMP-2 (-)</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27</td>
<td>16</td>
<td>11</td>
<td>0.015</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>11</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤18</td>
<td>29</td>
<td>16</td>
<td>13</td>
<td>0.792</td>
</tr>
<tr>
<td>&gt;18</td>
<td>16</td>
<td>11</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal femur</td>
<td>24</td>
<td>13</td>
<td>11</td>
<td>0.872</td>
</tr>
<tr>
<td>Proximal tibia</td>
<td>17</td>
<td>11</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Enneking staging</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>0.819</td>
</tr>
<tr>
<td>Stage II</td>
<td>21</td>
<td>14</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>19</td>
<td>10</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Situation of pulmonary metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With pulmonary metastasis</td>
<td>20</td>
<td>16</td>
<td>4</td>
<td>6.000</td>
</tr>
<tr>
<td>Without pulmonary metastasis</td>
<td>25</td>
<td>11</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

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.

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

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 (+).

low, claybank, and sepia according to the staining degree in the section. Secondly, we graded

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

Table 2. Analysis of Cox proportional hazards model

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary metastasis</td>
<td>26.064</td>
<td>1.222-555.911</td>
<td>0.037</td>
</tr>
<tr>
<td>MMP-2 expression</td>
<td>18.166</td>
<td>1.239-266.278</td>
<td>0.034</td>
</tr>
</tbody>
</table>

Enneking stage (P>0.05). Nevertheless, with the absence or presence of pulmonary metastasis, the MMP-2 expression showed an obvious difference (χ²=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 (χ²=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...
MMP-2 expression and prognosis of osteosarcoma

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

[16] Bacci G, Mercuri M, Longhi A, Ferrari S, Bertoni F, Versari M and Picci P. Grade of chemotherapeutic-induced necrosis as a predictor of local and
MMP-2 expression and prognosis of osteosarcoma


