Increased expression of membrane-type matrix metalloproteinase-1 is correlated with poor prognosis in patients with osteosarcoma

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Received August 29, 2005; Accepted October 17, 2005

Abstract. Numerous studies have demonstrated a correlation of matrix metalloproteinase (MMP) overexpression with the prognosis of various kinds of cancer. The current study investigated whether the expression of MMPs is correlated with the prognosis of osteosarcoma. Expression levels of MMP-2, -9, MT1-MMP and TIMP-2 were examined immunohistochemically in samples from 47 patients with osteosarcoma. Correlation of the positivity of staining with prognosis was analyzed with the Kaplan-Meier method, and statistically analyzed with log-rank test. Co-localization of MMP-2, MT1-MMP and TIMP-2 was determined by double staining with fluorescence-conjugated antibodies. Activities of gelatinases in representative tissues were examined with gelatin zymography. MMP-2 was expressed strongly in 60% of cases (28/47), and MMP-9, MT1- MMP and TIMP-2 was strongly positive in 61% (29/47), 45% (21/47), and 91% (43/47), respectively. Increased MT1-MMP expression was associated significantly with poor prognosis in overall survival (P=0.0480). In cases of overexpression for both MMP-2 and MT1-MMP, there was a tendency for poor prognosis (P=0.0969). In 36 cases who underwent neoadjuvant chemotherapy, wide resection of the tumors and post-operative adjuvant chemotherapy, increased expression of MT1-MMP resulted in a significant negative prognostic factor for disease-free survival (P=0.0143). Also, co-overexpression of MT1-MMP and MMP-2 showed a tendency to correlate to the reduced disease-free survival (P=0.0502). Increased gelatinase activity was observed in tissues of co-overexpression of MT1-MMP and MMP-2. The results of this study demonstrate the correlation of MT1-MMP expression and the oncological outcome of osteosarcoma patients, suggesting the prognostic significance of these proteins in osteosarcoma patients.

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Abbreviations: MMP, matrix metalloproteinase; MT1-MMP, membrane-type matrix metalloproteinase-1; TIMP, tissue inhibitor of matrix metalloproteinase

Key words: osteosarcoma, matrix metalloproteinase, prognosis, immunohistochemistry, tissue inhibitor of metalloproteinase

Introduction

Osteosarcoma is the most frequent primary malignant bone tumor in children and adolescents (1-3). In the last two decades, the combination of chemotherapy with surgical treatment dramatically improved the clinical outcome of the patients with osteosarcoma. However, there are still a certain number of patients who do not benefit from these improvements. Attempts have been made to identify prognostic factors to predict outcome, and to determine those patients who should undergo more intensive treatment, however, further work is required.

Malignant tumor cell invasion is believed to involve a complex series of integrated events consisting of tumor cell adhesion, extracellular matrix (ECM) proteolysis, and cell migration within the microenvironment (4). Among the proteases contributing to ECM degradation, matrix metalloproteases (MMPs) are thought to play significant roles in tumor invasion and metastasis (5,6). Synthesized as either secreted or membrane-bound latent enzymes, MMPs require proteolytic activation involving cleavage of propeptide domain to exhibit enzymatic activity (7). Cell-mediated pro-MMP-2 activation involves cleavage by MT1-MMP on cell membranes (8,9). The activity of these MMPs is regulated by the endogenous specific tissue inhibitor of metalloproteinases-2 (TIMP-2), which in excess can bind both MT1-MMP and MMP-2 in an inhibitory manner. But, at lower concentrations, TIMP-2 is also essential for the efficient activation of pro-MMP-2. Due to the dual nature of TIMP-2, a small shift in the balance between TIMP and MMP levels may greatly modulate the invasive phenotype in tumor cells (10).

Overexpression of MMP-2 and MT1-MMP has been demonstrated in a variety of malignant tumors. Their expressions are associated with tumor invasion and increased metastatic potential (11-15). In soft tissue sarcomas, MMP-2 reactivity and lack of TIMP-2 expression is correlated with poor prognosis (16). However, although several studies concerning MMP-9 expression in osteosarcoma patients have been conducted (17,18), MMP-2, MT1-MMP and TIMP-2 expression have been described in only a few studies. The objective of the present study was to evaluate the immunohistochemical expression of MMP-2, MT1-MMP and TIMP-2 as well as MMP-9, and to determine whether their expression is correlated with the prognosis of osteosarcoma patients. The co-localization of these MMPs was examined by double staining with fluorescence-conjugated antibodies. Also, activities of gelatinases in the osteosarcoma biopsy samples were determined with gelatin zymography.

Patients and methods

Patients and specimens. Tumor tissue samples were obtained from 47 patients with osteosarcoma identified from surgical pathology and the tumor registry records at Nagoya University Hospital and Aichi Cancer Center Aichi Hospital. Biopsy specimens or surgical specimens without chemotherapy were recruited for this study. All patients gave signed consent. Patients were ineligible if they had multicentric primary disease, parosteal osteosarcoma, periosteal osteosarcoma, or recurrent disease. All slides of the cases were reviewed to confirm the diagnosis. There were 28 males and 19 females with a median age of 25.6 (range 8-75) years. According to American Joint Committee on Cancer Staging System (AJCC), there were 20 tumors of grade II-A, 23 of grade II-B, and 4 of grade IV-A. Follow-up data were obtained in April 2004 allowing a median follow-up period of 43.3 months (range 8-113). The demographic data are summarized in Table I. Most patients had been treated according to the regime of our institutions based on cisplatin, ifosfamide, adriamycin and methotrexate (Table II). Although patients of stage IV and extraskeletal osteosarcoma patients were included in this study, analysis of survivorship was also accomplished after exclusion of these patients.

Table I. Patient characteristics at initial diagnosis and treatment.

Characteristics	Value (range) or no. of patients (%)
Sex	
Male	28 (60)
Female	19 (40)
Age, years	
Median (range)	25.6 (8-75)
Anatomic site	
Femur	25 (53)
Tibia	5 (10)
Humerus	7 (15)
Other limbs[a]	4 (9)
Body trunk[b]	4 (9)
Soft tissue[c]	2 (4)
Surgical stage (AJCC[d])	
II-A	20 (42)
II-B	23 (49)
III	0 (0)
IV-A	4 (9)
IV-B	0 (0)

[a]Other primary sites include the radius (n=2) and fibula (n=2). [b]Body trunk includes the clavicle (n=1) and pelvis (n=3). [c]The two cases of the extraskeletal osteosarcoma occurred in the thigh. [d]AJCC, American Joint Committee on Cancer Staging System.

Immunohistochemistry. Conventional immunohistochemical studies were performed using a streptavidin-biotin complex technique on the formalin-fixed, paraffin-embedded sections (8 μm thickness) to detect MMP and TIMP expression. Deparaffinized and rehydrated sections were immersed 3 times in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min at room temperature and rinsed in PBS. The slides were then soaked 30 min in 10% of normal rabbit serum as a blocking agent. Then slides were incubated at room temperature for 1.5 h with primary monoclonal mouse antibodies for an oligopeptide of VTPRDKPMGPLLVATF (residue 468-483) on human MMP-2 (500 μg/ml mouse IgG/κ, F-68; 42-5D11, Daiichi Fine Chemical Co., Toyama, Japan, 1:500 dilution), an oligopeptide of RSASEVDRMFPGVPLDTHD (residue 626-644) on human MMP-9 (500 μg/ml mouse IgG/κ, F-69; 56-2A4, Daiichi Fine Chemical Co., 1:500 dilution), an oligopeptide of REVPYAYIREGHEK (residue 160-173) on human MT1-MMP (500 μg/ml mouse IgG/κ, F-86; 114-6G6, Daiichi Fine Chemical Co., 1:100 dilution) and an oligopeptide of YRGAAPPKQEFL DIED (residue 178-193) on human TIMP-2 (500 μg/ml mouse IgG/κ, F-70; 67-4H11, Daiichi Fine Chemical Co., 1:500 dilution) were applied. After rinsing with PBS, biotinylated anti-mouse rabbit IgG conjugated with peroxidase was applied as a second antibody, and the antibody binding was detected using the Vectastain Elite peroxidase kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin, dehydrated and mounted. Substituting the primary antibody with nonimmune mouse serum performed negative controls. Two observers without knowledge of clinicopathological information evaluated the results of the immunohistochemical staining semiquantitatively on the basis of a four-point scale: 0-10% for positive stainable cell number (negative; -), 11-25% (weak; ±), 26-49% (moderate; +), 50-100% (strong; ++). For statistical analysis, the cutoff used was 25%; negative/weak staining was classified as low expression and moderate/strong staining as high expression of the respective antigens. Using these criteria both observers agreed on the degree of positivity or negativity of each case.

Table II. Management and clinical outcome of patients.

Treatment and effectiveness	No. of patients (%)
Chemotherapy before and after surgery	40 (86)
Chemotherapy before surgery	0 (0)
Chemotherapy after surgery	2 (4)
Chemotherapy only	3 (6)
Surgery only	2 (4)
Chemotherapy	
C/D/M/I	20 (43)
C/D/M	18 (38)
Others	7 (15)
No chemotherapy	2 (4)
Chemotherapy response	
Complete response	0 (0)
Partial response	21 (47)
No change	16 (36)
Progressive disease	8 (17)
Surgery	
Amputation	2 (4)
Limb salvage	42 (90)
No surgery	3 (6)
End result	
DOD	11 (23)
AWD	4 (9)
NED	2 (4)
CDF	30 (64)
Mean follow-up, months (range)	43.3 (8-113)

C, cisplatin; D, doxorubicin; M, methotrexate; I, ifosfamide; DOD, dead of disease; AWD, alive with disease; NED, no evidence of disease; CDF, continuous disease-free.

Double immunofluorescent staining. To evaluate the co-localization between MMP-2, -9, MT1-MMP, and TIMP-2, double immunofluorescent staining was performed. Briefly, sections were deparaffinized, rehydrated, and then incubated with the mixture of 10% rabbit serum and 10% normal horse-serum for 30 min at room temperature. Next, they were incubated with a mixture of a mouse anti-human monoclonal antibody (MT1-MMP or TIMP-2: Daiichi Fine Chemical Co.) and a goat anti-human polyclonal antibody to the carboxy terminus of human MMP-2 (c-19, sc-6838; 200 μg/ml goat IgG/κ, or to the carboxy terminus of human MMP-9 M-17, sc-6841; Santa Cruz Biotec. Inc., Santa Cruz, CA) for 1.5 h at room temperature. After washing with PBS, the sections were incubated with a mixture of the two secondary antibodies (FITC-conjugated rabbit anti-goat IgG and Texas red conjugated horse anti-mouse IgG, Vector Laboratories) for 1 h at room temperature. After washing, they were mounted and examined with a confocal microscope (MRC-1024: Bio-Rad, Hercules, CA). In the preparations stained as described above, a green fluorescence indicated either MMP-2 or MMP-9; a red fluorescence showed MT1-MMP or TIMP-2. An autofluorescence, not indicative of positive stain, was observed in some macrophages and red blood cells. This autofluorescence was recognized by its presence in unstained sections and in immunohistochemically-negative control preparations.

Preparation of osteosarcoma tissue extracts. Osteosarcoma tissues were minced to 1-3 mm^3 fragments, followed by the addition (1:1, w/v) of tissue lysis/extraction reagent (CelLytic™ MT: Sigma, St. Louis, MO) along with the following protease inhibitor cocktail (Complete, EDTA-free: Roche, Penzberg, Germany). The mixture was homogenized on ice using a tissue dounce homogenizer and centrifuged at 1,000 g for 30 min at 4˚C, and supernatants were pooled, aliquoted, then frozen at -80˚C. Concentration of the extracts was determined by Bio-Rad protein assay.

Gelatin zymography. To determine the gelatinase activity in osteosarcoma tissue extracts, gelatin zymography was performed. Tissue extracts (7.5 μl) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions in 10% polyacrylamide gel containing gelatin (1 mg/ml). After removal of SDS from the gel treated twice with 2.5% Triton-X for 15 min, the gel was incubated in gelatinase activating buffer (5 mM $CaCl_2$, 1 μM $ZnCl_2$, 200 mM NaCl, 50 mM Tris-HCl, pH 7.6) with gentle agitation for 24 h at 37˚C. After incubation, the gel was stained with 0.25% Coomassie brilliant blue R-250 in 30% methanol and 7% acetic acid. The gels were destained in 30% methanol and 7% acetic acid, and zones of gelatinolytic activity were noted by negative staining. The observed gelatinolytic activity was confirmed since these bands failed to demonstrate gelatinolytic activity in replicate gels incubated in the presence of 10 mM EDTA.

Statistical methods. Clinical data were collected from the patients' clinical records, and survival times were counted (months) from the date of diagnosis to the date of death or last follow-up time before study closure. Kaplan-Meier product-limit method was used to estimate the disease-free survival and the overall survival for the group and to illustrate the effect of each protein expression. The log-rank test was used to evaluate the differences between survival curves. P-value of <0.05 was considered to indicate significance.

Results

Clinical features and treatment of study group. The clinical characteristics and treatment of 47 patients with osteosarcoma are listed in Tables I and II, respectively. Ninety-one percent of patients presented without distant metastasis. The majority of the cases had extremity bones as primary sites (41/47), with 4 cases of trunk and 2 cases of soft tissue in the remaining cases. Forty cases completed the standard therapeutic regimen

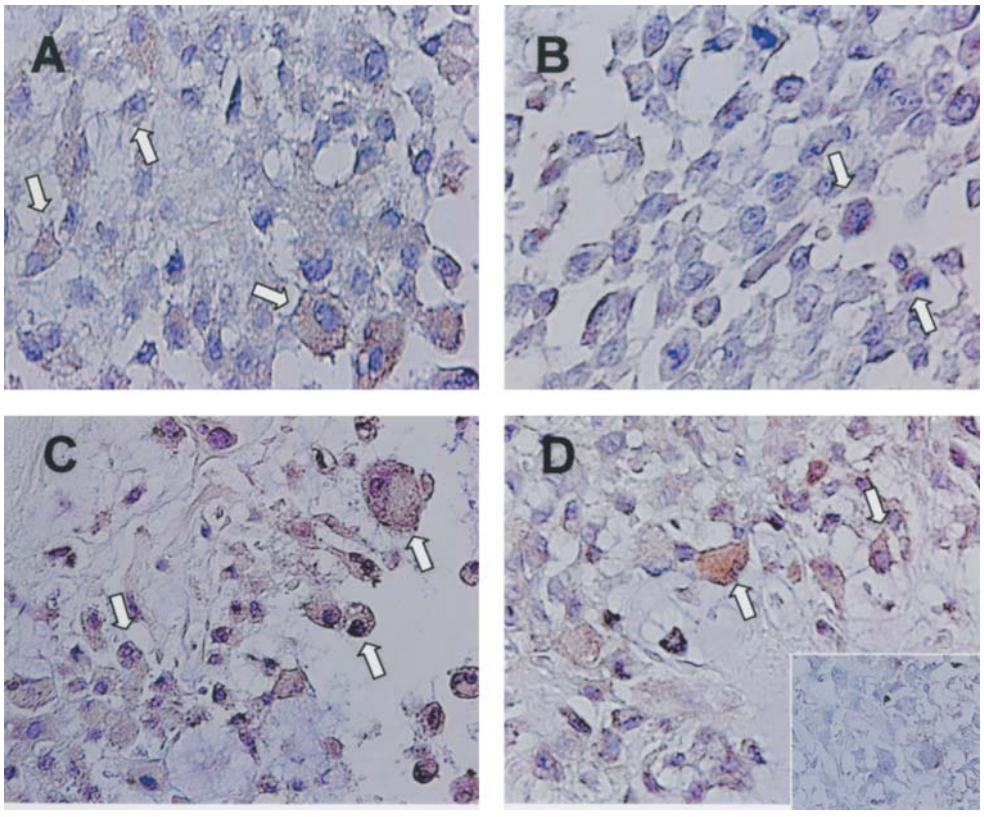

Figure 1. A representative case of immunohistochemistry. A 24-year-old male diagnosed with chondroblastic osteosarcoma of the left distal femur. He developed lung metastasis and died of disease 22 months after diagnosis. Immunostaining was performed with antibodies to MMP-2 (A), MMP-9 (B), MT1-MMP (C), and TIMP-2 (D). MT1-MMP showed strong immunoreactivity, whereas MMP-2 showed moderate immunoreactivities. And, MMP-9 and TIMP-2 showed weak expression. Negative control is shown in (D, inset). The number of positive cells was counted for semi-quantification (original magnification x400). Open arrows indicate positive staining of the tumor cells.

including neoadjuvant chemotherapy and surgical resection with curative margin, followed by continued adjuvant chemotherapy. However, three of these cases could not achieve initial disease-free period because of development of multiple lung metastasis. The other 37 cases, including one case of soft tissue osteosarcoma, achieved an initial complete remission (Table II). The patients received a variety of chemotherapeutic regimens, most frequently, however, cisplatin, doxorubicin, high-dose methotrexate, and ifosfamide were used. Only two patients of cases did not receive chemotherapy because of their religious reason or renal function failure. Forty-seven percent of the patients showed partial response and 36% of the cases evidenced no remarkable change in the radiological examination. Seventeen percent of the cases showed disease progression despite chemotherapy. Ninety percent of the cases underwent limb-sparing surgery with curative margin against the primary site. Four percent of the patients underwent amputation of the limb affected, and 6% of the patients could not undergo the surgery. At the end of follow-up, 64% of the cases remain continuously disease-free and 23% of them died of the disease.

Immunohistochemical analysis of MMP-2, -9, MT1-MMP and TIMP-2. In specimens from 47 patients with osteosarcoma, the expression levels of MMPs and TIMPs were variable and involved intracytoplasm and/or the cellular membrane of tumor cells (representative case is shown in Fig. 1). MMP-2, MT1-MMP and TIMP-2 were localized within the cytoplasm and membrane of numerous tumor cells. However, MMP-9 was expressed by some of the tumor cells and the multi-nuclear giant cells. All MMPs were expressed on endothelial cells or on the stromal fibloblasts surrounding tumor cells, but the number of positive cells was small. Given that the specimens were heterogeneous in each case, three fields in magnification x400 were randomly selected in each case, and the positivity of the staining was evaluated. The average positivity of three fields was considered as representative of each case.

Of the 47 tumors studied, 28 cases (60%) showed moderate to strong positive immunostaining for MMP-2, 29 (61%) for MMP-9, 21 (45%) for MT1-MMP, and 43 (91%) for TIMP-2, respectively. Eighteen cases (38%) showed a positive staining for both MMP-2 and MT1-MMP. All the cases stained with non-immune mouse serum (negative control) did not show any immuno-reactivity. Table III summarizes MMP and TIMP expression.

Fifteen patients developed metastasis, and 11 patients died of the disease. Thirty patients were continuously disease-free. Among the 15 cases who developed metastasis, 73, 47, 67, and 87% of them showed increased expression of MMP-2, -9, MT1-MMP and TIMP-2, respectively. Among those cases who died of the disease, 73, 64, 73 and 82% revealed increased expression of MMP-2, -9, MT1-MMP and TIMP-2, respectively. In those cases free from the disease, 50, 33, 70

Table III. Expression of MMPs and TIMP-2 in relation to clinical outcome.

Positive cell ratio	No. of cases	No. of metastases	No. of deceased	No. of disease-free
MMP-2				
Weak				
- 0-10%	6	2	2	4
± 11-25%	13	2	1	11
Strong				
+ 26-49%	12	3	3	7
++ 50-100%	16	8	5	8
MMP-9				
Weak				
- 0-10%	2	2	2	0
± 11-25%	16	6	2	10
Strong				
+ 26-49%	22	5	4	16
++ 50-100%	7	2	3	4
MT1-MMP				
Weak				
- 0-10%	4	0	0	4
± 11-25%	22	5	3	17
Strong				
+ 26-49%	19	8	6	9
++ 50-100%	2	2	2	0
TIMP-2				
Weak				
- 0-10%	0	0	0	0
± 11-25%	4	2	2	2
Strong				
+ 26-49%	17	6	3	10
++ 50-100%	26	7	6	18

and 7% showed weak expression of MMP-2, -9, MT1-MMP and TIMP-2, respectively.

Relationship between MMP expression and survival rate. For all 47 cases of osteosarcoma, overall survival (OAS) rate was estimated using the Kaplan-Meier method, and the results were statistically examined by log-rank test. OAS at 5 years in all cases was 75%. The OAS rate was 58% for patients with strong expression of MT1-MMP and 89% for patients with weak expression of MT1-MMP at 5 years. A significant association was observed between the strong expression of MT1-MMP and the shortened survival of patients, compared with the weak expression group (log-rank, P=0.0480: Fig. 2C). Whereas, there was no significant difference for OAS in MMP-2 and -9 expression between strong and weak group staining (log-rank P=0.2544 and P=0.8260, respectively: Fig. 2A and B). Analysis for TIMP-2 expression was not performed due to the small number of cases showing weak expression (n=4) of TIMP-2 compared with the number of strong expression (n=43).

Among the strong group of MMP-2 expression (28 cases), 18 cases also showed strong MT1-MMP expression. The estimated OAS of the group expressing strongly both MMP-2 and MT1-MMP was 57%, and that for the rest of the group was 87%. There was a tendency for low OAS in group of strong expression of both MMP-2 and MT1-MMP (log-rank, P=0.0969: Fig. 2D).

Osteosarcoma patients who develop lung metastasis during therapy have a very grave prognosis, adjuvant and neo-adjuvant chemotherapies positively affect the prognosis of osteosarcoma patients, and extraskeletal osteosarcoma may differ from skeletal osteosarcoma in prognosis. Excluding these cases, 36 skeletal osteosarcoma of 47 cases, who completed neo-adjuvant, adjuvant chemotherapy and wide resection, were re-evaluated. The estimated OAS at 5 years was 70% and 92% for the patients with strong and weak expression for MT1-MMP, respectively. There was no statistical relationship between MT1-MMP and OAS (log-rank P=0.1245: Fig. 3C). Also, MMP-2 and -9 showed no association with OAS (log-rank P=0.8967 and P=0.7327, respectively: Fig. 3A and B). Strong expression for both MMP-2 and MT1-MMP did not show association with OAS (log-rank P=0.4251: Fig. 3D).

We also analyzed the disease-free survival (DFS) rate of these 36 cases using the Kaplan-Meier method. The estimated DFS at 5 years was 60% for those patients with strong expression and 81% with weak expression of MT1-MMP. The difference between the two curves was statistically significant (log-rank, P=0.0143: Fig. 4A). However, the expression level of MMP-2 and -9 did not associate with DFS (log-rank P=0.4918, P=0.2748, respectively, data not shown). Among those cases with strong expression of MMP-2, 13 tumors also showed strong expression of MT1-MMP. In these 13 cases, the estimated DFS at 5 years was 62%, whereas in the rest of the cases, it was 78%. These 13 cases showed a tendency for poor prognosis (log-rank, P=0.0502: Fig. 4B).

Co-localization of MMPs and TIMP-2. Increased expression of MT1-MMP results in activation of MMP-2 on the cell surface that is required for cell invasion, and TIMP-2 plays important roles in controlling MMP-2 activation. The complex of TIMP-2 and MT1-MMP is also necessary for activating pro-MMP-2. These suggest the significance of evaluating the co-localization of MMP-2, MT1-MMP and TIMP-2 protein. Co-localization of MMP-9 with other molecules was also investigated. Results of double immunofluorescence labeling demonstrated that MMP-2 co-localized with MT1-MMP (Fig. 5A-C) and/or TIMP-2 (Fig. 5E-G). However, MMP-9 did not co-localize with MT1-MMP (Fig. 5D), but occasionally co-localized with TIMP-2 (data not shown).

Activities of gelatinases. Gelatin zymography identified gelatinase activity in fresh tissue samples of osteosarcoma.

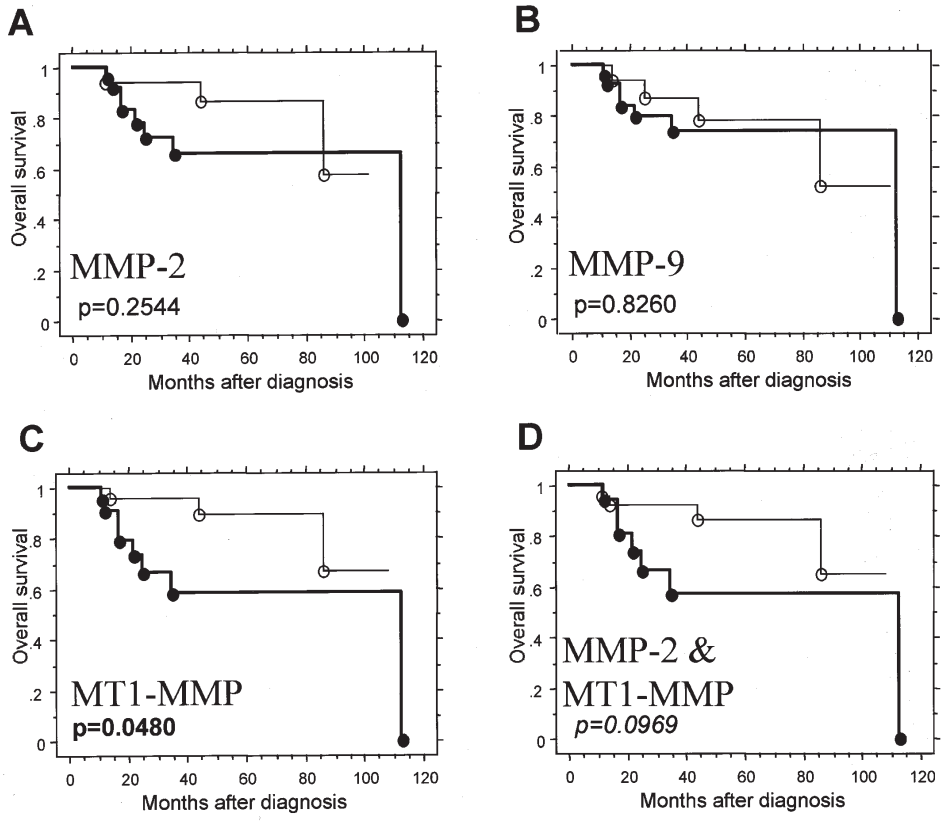

Figure 2. Cumulative overall survival rate of all patients (n=47) using Kaplan-Meier method. Open circles indicate weak group, and closed circles indicate the strong group. (A), Subdivided according to MMP-2 expression. (B), Subdivided according to MMP-9 expression. (C), Subdivided according to MT1-MMP expression. (D), Subdivided according to expression for both MMP-2 and MT1-MMP or others.

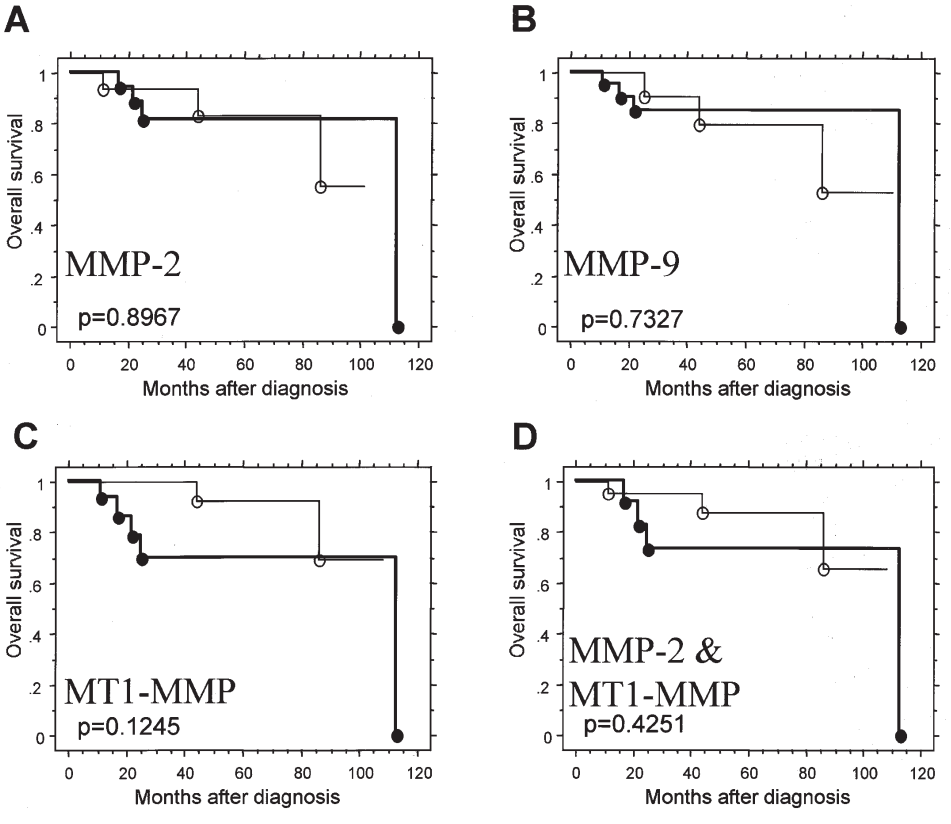

Figure 3. Cumulative overall survival rate of patients who achieved initial complete remission after neoadjuvant chemotherapy and curative surgery followed by adjuvant chemotherapy (n=36) using Kaplan-Meier method. Open circles indicate weak group, and closed circles indicate the strong group. (A), Subdivided according to MMP-2 expression. (B), Subdivided according to MMP-9 expression. (C), Subdivided according to MT1-MMP expression. (D), Subdivided according to expression for both MMP-2 and MT1-MMP or others.

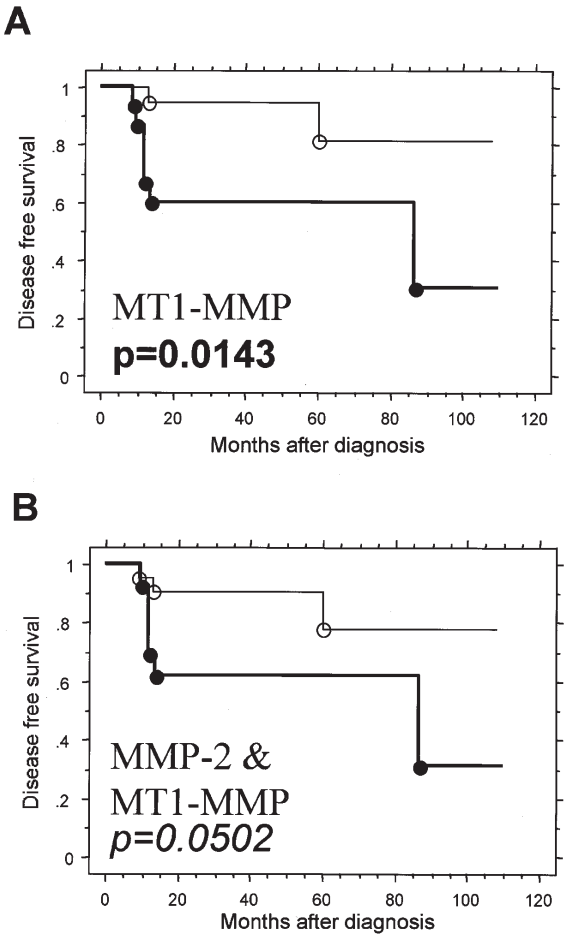

Figure 4. Cumulative disease-free survival rate of patients who achieved initial complete remission after neoadjuvant chemotherapy and curative surgery followed by adjuvant chemotherapy (n=36) using Kaplan-Meier method. Open circle indicates weak group, and closed circle indicates the strong group. (A), Subdivided according to MT1-MMP expression. (B), Subdivided according to expression for both MMP-2 and MT1-MMP or others.

Representative gelatin zymograms are shown in Fig. 6. Patients with lung metastasis showed an activated MMP-2 band at 67 kDa. The patient with lung metastasis who died of the disease showed a stronger band of activated MMP-2 (Fig. 6A). This patient also exhibited both MMP-2 and MT1-MMP overexpression in the immunohistochemical analysis. Another patient with lung metastasis but who remained alive with disease showed a rather weak but prominent band of activated MMP-2 (Fig. 6B). In immunohistochemical analysis, this patient showed strong MMP-2 expression, but MT1-MMP expression was rather weak. The patient with no metastasis and no recurrence did not show activated band of MMP-2 (Fig. 6C). The results of gelatinase activity were consistent with the immunohistochemical analysis.

In all cases MMP-9 was detected at 92kDa and 83 kDa, but these expressions were not correlated with prognosis but rather depended on each subtypes of pathologic appearance. Osteoblastic type expressed most strongly MMP-9 activity and fibloblastic or chondroblastic type showed rather weak activity (data not shown).

Discussion

The prognosis for patients with osteosarcoma has been improved over the last two decades because of the advent of aggressive chemotherapy regimens, but 30-50% of patients still die of pulmonary metastasis (19,20). The prognosis of patients with osteosarcoma may not be easily predicted. Several prognostic risk factors have been proposed, including tumor size, tumor grade, site, age, and response to chemotherapy (21). The presence or absence of some molecules in osteosarcoma cells has also been shown to be prognostic factors, as for example P-glycoprotein (22) or telomerase (23). Given that MMP is a family of proteolytic enzymes involved in the degradation of extracellular matrix components, and play crucial roles in tumor invasion, angiogenesis and metastasis, several studies have investigated the expression of MMPs in osteosarcoma as prognostic factors. MMP-9 expression has been shown to express in osteosarcoma (18), to be a significant prognostic factor for the development of metastatic disease, and to be associated with the disease-free survival of patients with osteosarcoma (17). These studies did not investigate other MMPs such as another gelatinase, MMP-2. In soft tissue sarcomas, increased MMP-2 expression and lack of TIMP-2 expression have been reported to be significant factors for poor prognosis (16). The present study analyzed for the first time the expression of not only MMP-9 but also MMP-2, MT1-MMP and TIMP-2 in osteosarcoma tissues immunohistochemically and their correlation to clinical outcome.

Among the many techniques that analyze MMP expression in tumor samples, immunohistochemical evaluation, although it cannot distinguish between latent and active forms of MMPs, has some advantages compared to other methods such as determination of mRNA expression. It can be performed on paraffin-embedded specimens, which is routinely practical in clinical assessment. Expression of mRNA is not guaranteed for translation.

Expression of MT1-MMP and activated MMP-2 are known to have a strong correlation with tumor invasion and metastasis in a variety of tumors (11-15). MMP-2 is secreted as a latent pro-enzyme, and its activation is considered to be essential for pericellular degradation of the extracellular matrix during cell migration. In 1994, Sato *et al* cloned the cDNA of MT1-MMP and showed that MT1-MMP activates pro-MMP-2 but not pro-MMP-9 (24). *In vitro*, the human osteosarcoma cell line MG-63 has been shown to express not only MMP-2 but also MT1-MMP (25), and decreased MMP-2 secretion contributes to the reduction of the invasive potential of osteosarcoma cells (26). In the current study, increased expression of MT1-MMP is associated with reduced prognosis. There was a tendency for low OAS and DFS in the group with strong expression of both MMP-2 and MT1-MMP. Furthermore, results of double immunofluorescence labeling demonstrated that MMP-2 co-expressed with MT1-MMP and/or TIMP-2. These results agree with the above-mentioned previous studies.

Endogenous specific inhibitor, TIMP-2, which in excess can bind cell surface MT1-MMP and both latent and active MMP-2 in an inhibitory manner, regulates the activity of these MMPs. However, TIMP-2 is also essential at lower

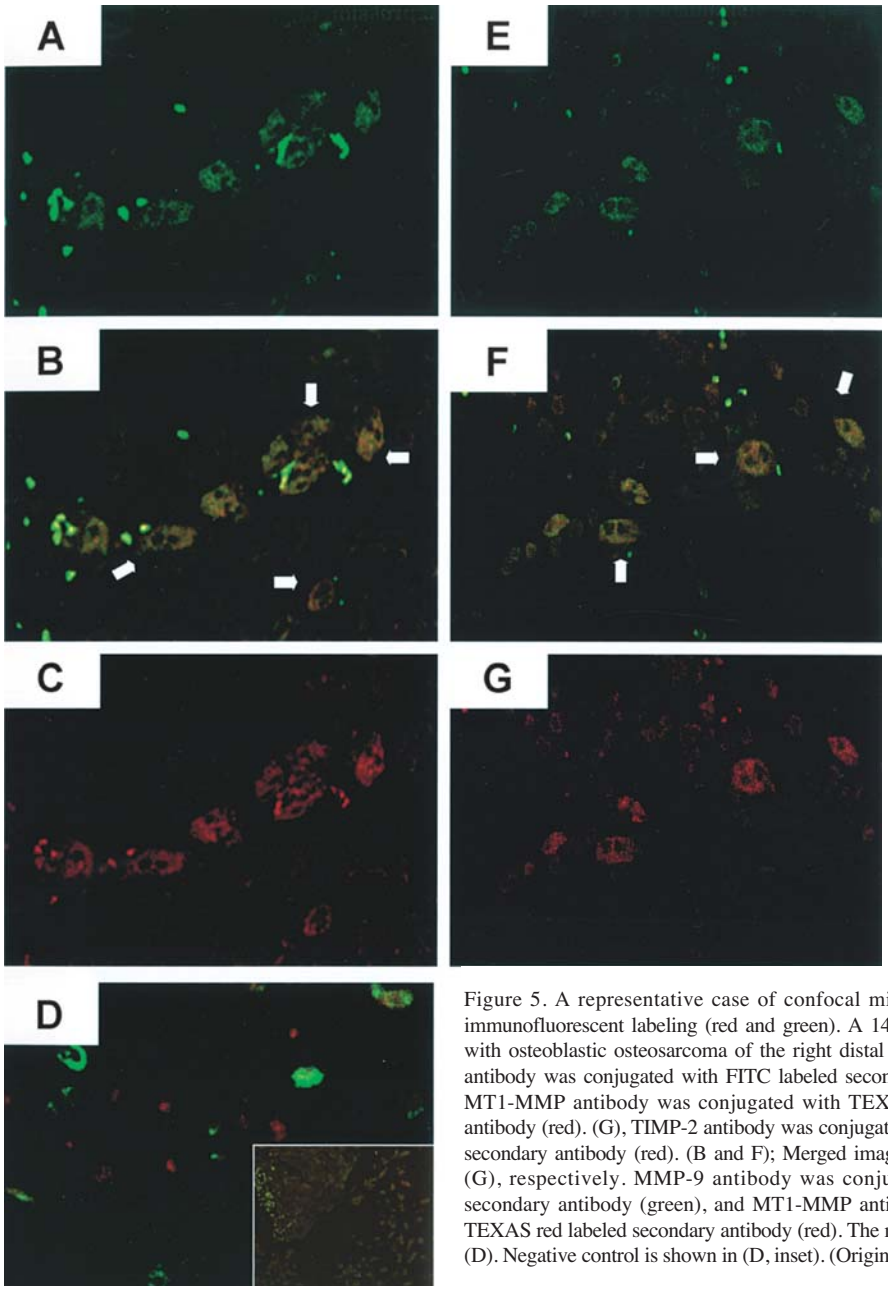

Figure 5. A representative case of confocal microscopic images of dual immunofluorescent labeling (red and green). A 14-year-old female diagnosed with osteoblastic osteosarcoma of the right distal femur. (A and E), MMP-2 antibody was conjugated with FITC labeled secondary antibody (green). (C), MT1-MMP antibody was conjugated with TEXAS red labeled secondary antibody (red). (G), TIMP-2 antibody was conjugated with TEXAS red labeled secondary antibody (red). (B and F); Merged images of (A) and (C), (E) and (G), respectively. MMP-9 antibody was conjugated with FITC labeled secondary antibody (green), and MT1-MMP antibody was conjugated with TEXAS red labeled secondary antibody (red). The merged images are shown in (D). Negative control is shown in (D, inset). (Original magnification x200).

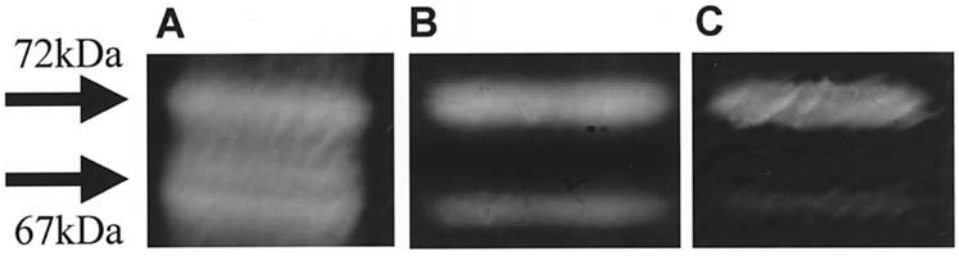

Figure 6. Characterization of gelatinolytic activity. Same concentration of 7.5 μl aliquots of tissue extract of fresh osteosarcoma samples were applied. Protein concentration of the tissue extract was determined by Bio-Rad Protein assay. (A), A 51-year-old female diagnosed with osteoblastic osteosarcoma of the right distal femur. Lung metastasis existed at the first visit and the patient died of disease at 12 months after diagnosis. (B), A 61-year-old female diagnosed with fibloblastic osteosarcoma of the right distal femur. She appeared with lung metastasis at the first visit and was alive with disease at the latest visit. (C), A 41-year-old male diagnosed with fibloblastic osteosarcoma of the right distal femur. He remains free from local recurrence and distant metastasis after neo-adjuvant chemotherapy and curative surgery for 60 months after diagnosis. The patients with metastasis and/or poor prognosis (A and B, respectively) showed remarkable expression of activated MMP-2 at 67 kDa.

concentrations for the efficient activation of MMP-2, acting as a bridge between MT1-MMP and MMP-2 on the cell surface to allow a second TIMP-free MT1-MMP molecule to cleave the propeptide domain of pro-MMP-2 (27). We

demonstrated the expression of MMP-2, MT1-MMP and TIMP-2, and their co-localization in human osteosarcoma tissue for the first time. However, expression of MMP-2 or TIMP-2 showed no significant prognostic value in this study, compared to MT1-MMP. One explanation is that antibodies used in this study conjugate with both latent and active MMP-2 (28). A given activity of MMP-2 was determined by zymogram and showed a stimulated level in poor prognosis patients. Although there are few fresh samples available for zymogram, determination of the activity by zymogram might be a better predictor for prognosis than immunohistochemistry. However, MT1-MMP overexpression showed a significant correlation with poor prognosis and MT1-MMP co-localized with MMP-2 and TIMP-2, suggesting that activated MMP-2 might also play important roles in the progression of osteosarcoma.

We could not, however, detect a relationship between the overexpression of MMP-9 and prognosis. The reason why there is this discrepancy from a previous study would be that the samples we used were before chemotherapy, whereas Foukas *et al* (17) used samples after neoadjuvant chemotherapy. Another reason might be the difference in sensitivity of monoclonal antibodies between this study and the previous one.

It seems difficult to improve the current response rates of chemotherapy with further dose escalation to overcome drug resistance, as resistant tumor cells are able to withstand the cytotoxic agents. In addition, the associated cytotoxic effects on normal tissues and organs remain a serious drawback. Therefore, we need a new approach for osteosarcoma patients with low cytotoxic agents. Recently, bisphosphonates have been shown to down-regulate MMPs and reduce the invasive potential of osteosarcoma cells (25,26). Bisphosphonates or other new drugs, including highly selective matrix metalloprotease inhibitors, may be new candidates for altogether new approaches to the treatment for osteosarcoma.

In conclusion, although the patient number was small and the study was less definitive, we demonstrated that increased expression of MT1-MMP correlates with a poor prognosis in osteosarcoma patients, and MT1-MMP co-localizes with MMP-2 and/or TIMP-2. These results support our speculation that MT1-MMP could be one of the key enzymes among MMPs in the process of osteosarcoma progression. Further investigation of the MT1-MMP and MMP-2 in osteosarcoma tissues will provide better insight into determining the optimal treatment plan, and also may help find as a novel therapeutic target involving fewer cytotoxic agent.

Acknowledgements

This work was supported by Grant-in-Aid 16591483 for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. All authors disclose that there is no financial and personal relationship with other people or organizations that could inappropriately influence the present study. We thank Eri Ishihara for secretarial assistance.

References

1. The JOA Committee of Tumors: Osteosarcoma. In: General Rules for Clinical and Pathological Studies on Malignant Bone Tumors. Kanehara, Tokyo, pp6-9, 2002.
2. Dorfman HD and Czerniak B: Osteosarcoma. In: Bone Tumors. Dorfman HD and Czerniak B (eds). Mosby, St. Louis, MO, pp128-252, 1998.
3. Unni KK: Osteosarcoma. In: Dahlin's Bone Tumors. 5th edition. Unni KK (ed.) Lippincott-Raven, Philadelphia, PA, pp143-183, 1996.
4. Stetler-Stevenson WG, Aznavoorian S and Liotta LA: Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 9: 541-573, 1993.
5. Stetler-Stevenson WG: Dynamics of matrix turnover during pathologic remodeling of the extracellular matrix. Am J Pathol 148: 1345-1350, 1996.
6. De Clerck YA: Interactions between tumour cells and stromal cells and proteolytic modification of the extracellular matrix by metalloproteinases in cancer. Eur J Cancer 36: 1258-1268, 2000.
7. Woessner JF and Nagase H: MMP sequences. In: Matrix Metalloproteinases and TIMPs. Woessner JF and Nagase H (eds.) Oxford University Press, New York, NY, pp11-41, 2000.
8. Deryugina EI, Bourdon MA, Luo GX, Reisfeld RA and Strongin A: Matrix metalloproteinase-2 activation modulates glioma cell migration. J Cell Sci 110: 2473-2482, 1997.
9. Seiki M: Membrane-type matrix metalloproteinases. APMIS 107: 137-143, 1999.
10. Lu KV, Jong KA, Rajasekaran AK, Cloughesy TF and Mischel PS: Upregulation of tissue inhibitor of metalloproteinases (TIMP)-2 promotes matrix metalloproteinase (MMP)-2 activation and cell invasion in a human glioblastoma cell line. Lab Invest 84: 8-20, 2004.
11. Tokuraku M, Sato H, Murakami S, Okada Y, Watanabe Y and Seiki M: Activation of the precursor of gelatinase A/72 kDa type IV collagenase/MMP-2 in lung carcinomas correlates with the expression of membrane-type matrix metalloproteinase (MT-MMP) and with lymph node metastasis. Int J Cancer 64: 355-359, 1995.
12. Yamamura T, Nakanishi K, Hiroi S, Kumaki F, Sato H, Aida S and Kawai T: Expression of membrane-type-1-matrix metalloproteinase and metalloproteinase-2 in nonsmall cell lung carcinomas. Lung Cancer 35: 249-255, 2002.
13. Maatta M, Soini Y, Liakka A and Autio-Harmainen H: Differential expression of matrix metalloproteinase (MMP)-2, MMP-9, and membrane type 1-MMP in hepatocellular and pancreatic adenocarcinoma: implications for tumor progression and clinical prognosis. Clin Cancer Res 6: 2726-2734, 2000.
14. Kurahara S, Shinohara M, Ikebe T, Nakamura S, Beppu M, Hiraki A, Takeuchi H and Shirasuna K: Expression of MMPS, MT-MMP, and TIMPs in squamous cell carcinoma of the oral cavity: correlations with tumor invasion and metastasis. Head Neck 21: 627-638, 1999.
15. Sakakibara M, Koizumi S, Saikawa Y, Wada H, Ichihara T, Sato H, Horita S, Mugishima H, Kaneko Y and Koike K: Membrane-type matrix metalloproteinase-1 expression and activation of gelatinase A as prognostic markers in advanced pediatric neuroblastoma. Cancer 85: 231-239, 1999.
16. Benassi MS, Gamberi G, Magagnoli G, Molendini L, Ragazzini P, Merli M, Chiesa F, Balladelli A, Manfrini M, Bertoni F, Mercuri M and Picci P: Metalloproteinase expression and prognosis in soft tissue sarcomas. Ann Oncol 12: 75-80, 2001.
17. 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 84: 706-711, 2002.
18. Himelstein BP, Asada N, Carlton MR and Collins MH: Matrix metalloproteinase-9 (MMP-9) expression in childhood osseous osteosarcoma. Med Pediatr Oncol 31: 471-474, 1998.
19. Glasser DB, Lane JM, Huvos AG, Marcove RC and Rosen G: Survival, prognosis, and therapeutic response in osteogenic sarcoma. The Memorial Hospital experience. Cancer 69: 698-708, 1992.
20. Rosen G, Huvos AG, Mosende C, Beattie EJ Jr, Exelby PR, Capparos B and Marcove RC: Chemotherapy and thoractomy for metastatic osteogenic sarcoma. A model for adjuvant chemotherapy and the rationale for the timing of thoracic surgery. Cancer 41: 841-849, 1978.
21. Davis AM, Bell RS and Goodwin PJ: Prognostic factors in osteosarcoma: a critical review. J Clin Oncol 12: 423-431, 1994.
22. 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 98: 581-589, 2003.

23. 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 63: 1759-1763, 2003.
24. Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E and Seiki M: A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature 370: 61-65, 1994.
25. Heikkila P, Teronen O, Hirn MY, Sorsa T, Tervahartiala T, Salo T, Konttinen YT, Halttunen T, Moilanen M, Hanemaaijer R and Laitinen M: Inhibition of matrix metalloproteinase-14 in osteosarcoma cells by clodronate. J Surg Res 111: 45-52, 2003.
26. Cheng YY, Huang L, Lee KM, Li K and Kumta SM: Alendronate regulates cell invasion and MMP-2 secretion in human osteosarcoma cell lines. Pediatr Blood Cancer 42: 410-415, 2004.
27. Itoh Y, Takamura A, Ito N, Maru Y, Sato H, Suenaga N, Aoki T and Seiki M: Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion. EMBO J 20: 4782-4793, 2001.
28. Fujimoto N, Mouri N, Iwata K, Ohuchi E, Okada Y and Hayakawa T: A one-step sandwich enzyme immunoassay for human matrix metalloproteinase 2 (72-kDa gelatinase/type IV collagenase) using monoclonal antibodies. Clin Chim Acta 30: 221: 91-103, 1993.