

## Laboratory Investigation

# Possible Role of Matrix Metalloproteinase in Osteolytic Intracranial Meningiomas

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**Objective** : Abnormalities of the bone are frequently encountered in patients with meningioma, and hyperostosis and endostosis are common bone alterations in these tumors. Extensive bony destruction is very unusual in patients with meningioma. We report six cases of intracranial meningioma associated with an osteolytic lesion of the skull and discuss the underlying mechanisms that may be responsible for bone destruction in patients with meningioma.

**Methods** : Six patients were classified into three groups, severe, moderate and mild, according to the degree of osteolytic bony destruction. The tumor was classified as intracranial or extracranial, depending on its location. We investigated the potential role of matrix metalloproteinase (MMP) in meningioma-associated osteolysis. The levels of MMP expression were determined by gelatin zymography, reverse transcription-quantitative PCR analysis (RT-PCR) and immunohistochemical analysis.

**Results** : Complete surgical removal of the lesion was performed in each patient. Histological examination revealed benign meningioma in four cases, and two cases of atypical meningioma. Patients did not have a poor prognosis except one case of recurred atypical meningioma. Gelatin zymography and RT-PCR detected high levels of MMP-2 in almost all extracranial masses in comparison with the intracranial masses and MMP-9 in two. There was no difference in the severity of bone destruction. Immunohistochemical analysis revealed MMP-2 expression in the vicinity of the bone destruction, and a few MMP-9-positive stainings were observed.

**Conclusion** : Osteolysis of the skull in patients with meningiomas might not be indicative of malignant pathological features and poor prognosis. Invasion to the extracranial portion and osteolysis might be associated with MMP-2 expression in meningioma.

**KEY WORDS** : Matrix metalloproteinase · Meningioma · Osteolytic.

## INTRODUCTION

Hyperostosis of the skull is one of the characteristic features of meningioma. It is seen in 4.5% of all meningiomas and observed frequently in meningioma en plaque, with an incidence of 13 to 49%<sup>5,11</sup>. However, osteolysis in meningioma is rare. Some authors believe that osteolytic meningiomas have malignant features and that osteolysis indicates a poor prognosis in patients with meningioma<sup>2,7,16,25</sup>. Russel and Rubinstein<sup>20</sup> emphasized that meningiomas that present with osteolysis are more aggressive than others. Taveras and Wood<sup>22</sup> have proposed that purely osteolytic or

destructive changes are more often associated with meningiomas that are primarily sarcomatous.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that are required for the degradation of the extracellular matrix during normal embryogenesis and in tissue remodeling. They also play an important role in tumor growth, invasion and metastasis<sup>3,23</sup>.

Bone destruction is very unusual in patients with meningioma. We report six cases of intracranial meningioma associated with osteolytic lesions of the skull and discuss the underlying mechanisms that may be responsible for bone destruction in patients with meningiomas associated with MMP.

## MATERIALS AND METHODS

### Patient tissue

Six patients with meningioma associated with osteoblastic

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destruction were surgically treated. The six patients with intracranial meningioma associated with an osteolytic lesion of the skull were divided into mild, moderate and severe destruction groups according to the severity of osteolytic destruction. During the operation, tumor tissues from extradural (within the area of osteolytic destruction) and intradural masses were obtained separately, snap-frozen in liquid nitrogen and stored in a deep freezer at  $-70^{\circ}\text{C}$  until MMP analysis.

### RNA extraction

Total RNA was extracted from frozen human tumor tissue using Trizol (Invitrogen), according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). After isolating the RNA, the RNA pellets were frozen and stored at  $-80^{\circ}\text{C}$  until use.

### RT-PCR

Total RNA was extracted as described earlier. Reverse transcription was performed with Improm-II reverse transcriptase (Promega) and oligo dT primer in accordance with the manufacturer's instructions for making cDNA. MMP-9 and MMP-2 were amplified in order to compare their expression in the samples. The forward primer (GACCTCAAGTGGCACCACCA) and reverse primer (GTGGTACTGCACCAGGGCAA) for MMP-9 as well as the forward primer (GCCATCCCCGATAACCTGGATG) and reverse primer (GCAGCCCAAAGCAGGGCTGCG) for MMP-2 were designed base on their reference sequences in the NCBI database. To account for differences in starting material, PCR was also carried out for each cDNA sample using housekeeping, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### Protein extraction and gelatin zymography for MMP-2 and MMP-9

The frozen tissues were pulverized in liquid nitrogen and homogenized for 10-20 s in protein extraction buffer [50 mM Tris-HCl (pH 7.5), 10 mM  $\text{CaCl}_2$ , 200 mM NaCl] and then centrifuged at 10,000 rpm at  $4^{\circ}\text{C}$  for 10 min. The supernatant was pooled for assays of MMP-2 and MMP-9 activities in zymography. The protein concentration of the supernatant was determined by the Bradford protein assay (Bio-Rad). Then, 40  $\mu\text{g}$  of total protein from the homogenate supernatant was mixed with sample buffer (50 mM Tris-Cl, 2% SDS, 0.1% bromophenol blue, 10% glycerol) prior to electrophoresis. The sample was electrophoresed on 8% denaturing SDS-polyacrylamide gels containing 2 mg/mL of gelatin (Type A, Sigma). Each gel was washed 3 times in 2.5% Triton X-100 for 30 minutes each and then

incubated for 20 hours at  $37^{\circ}\text{C}$  in 50 mM Tris-HCl (pH 7.5), 10 mM  $\text{CaCl}_2$  and 200 mM NaCl. The gel was stained with Coomassie brilliant blue R-250 (0.2% with Coomassie brilliant blue R-250, 20% methanol, 10% acetic acid in  $\text{H}_2\text{O}$ ) and then destained (20% methanol, 10% acetic acid in  $\text{H}_2\text{O}$ ).

### Immunohistochemical analysis of MMP-2 and MMP-9

Immunohistochemistry for MMP-9 (mouse monoclonal antibody at 1 : 500 dilution from Abcam, Inc, UK) and MMP-2 (rapid polyclonal antibody at 1 : 700 dilution from Abcam, Inc, UK) was performed in formalin-fixed, paraffin embedded tissues. Before processing the next steps, the samples used to detect MMP-9 were cooked in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) at  $94^{\circ}\text{C}$  for 30 min and cooled at room temperature (this process was not applied for MMP-2). The paraffin-embedded sections of benign meningioma tissue were deparaffinized in xylene. The endogenous peroxidase activity was quenched with 3%  $\text{H}_2\text{O}_2$  in methanol, and the nonspecific binding sites were blocked by treatment with 3% albumin bovine (Sigma) in PBS for 20 min at room temperature. A primary antibody was then added at a previously determined optimum dilution. Samples were incubated at room temperature for 2 hours for MMP-2 and overnight at  $4^{\circ}\text{C}$  for MMP-9. After washing with immuno buffer (ScyTek Laboratories, USA), biotin-labeled secondary antibody (Dako North America, Inc, USA) was added, and the samples were incubated at room temperature for 1 hour. A streptavidin-horseradish peroxidase (Dako North America, Inc, USA) detection system was then applied to the capillary channels, followed by 20 min of incubation at room temperature. The tissue sections were ready for chromogen reaction with 3-amino-9-ethylcarbazole. Counterstaining was performed using Harris hematoxylin. Control experiments were performed in all specimens at the time of immunostaining using only the secondary antibody.

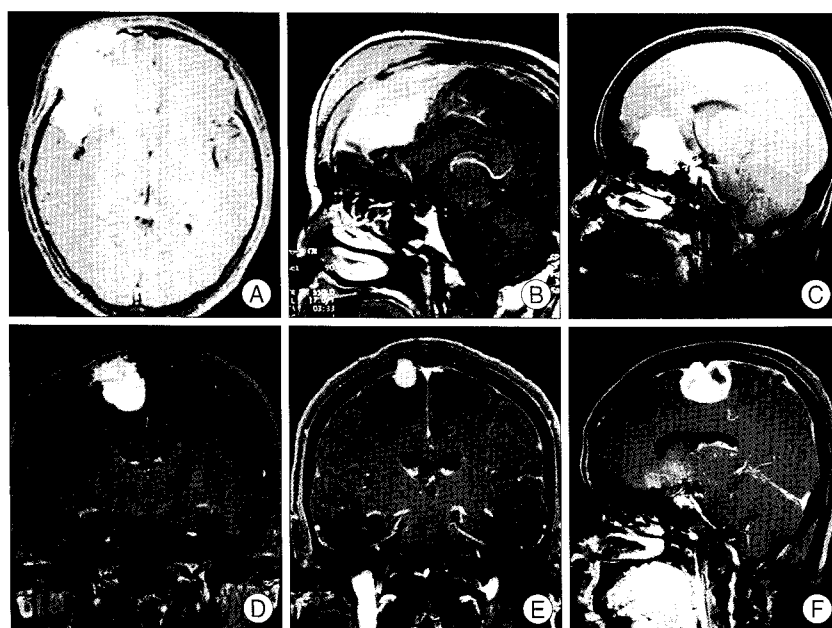
## RESULTS

### Summary of patients

Based on radiologic and intraoperative findings, the patients were divided into 3 groups according to the state of extracranial extension. In the severe destruction group, MRI showed convexity and parasagittal meningioma with extensive extracranial expansion over the skull (Fig. 1A, B). In the moderate destruction group with olfactory groove and convexity meningioma, the mass extended into the outer table of the skull (Fig. 1C, D). In the mild group with

convexity and parasagittal meningioma, the mass extended into the inner table and diploic space (Fig. 1E, F). Intraoperatively, extensive osteolysis of the skull was observed in the patients in the severe groups (Fig. 2A). The osteolysis was localized into the skull in the moderate destruction group and into the diploic space and inner table within the skull in the mild group (Fig. 2B, C).

The clinical data of the patients is summarized in Table 1. Complete tumor resection (Simpson grade I and II) was achieved in all patients, and histologic examination revealed benign meningioma in four cases, and two cases of atypical meningioma. The Ki-67 labeling index was less than 5%. None of the patients received postoperative adjuvant treatment. In one case of recurred tumor, reoperation was performed and followed the radiotherapy. One patient was followed up for more than 8 years, and there was no recurrence during the follow-up period. Most of the osteolytic meningiomas except one did not have a poor prognosis.



**Fig. 1.** Radiologic findings. A : Severe destruction group : convexity meningioma. B : Severe destruction group : parasagittal meningioma. C : Moderate destruction group : olfactory groove meningioma. D : Moderate destruction group : convexity meningioma. E : Mild destruction group : convexity meningioma. F : Mild destruction group : parasagittal meningioma.

## Results of gelatin zymography and RT-PCR

The individual tumors were divided into three groups according to location : intradural, extradural and within the osteolytic lesion. MMP-2 and -9 activities were nearly ubiquitously present in all meningioma tissue, irrespective of the severity of destruction and location. The results of gelatin zymography and RT-PCR were similar. In the severe and mild destruction groups, the level of MMP-2 expression in extradural masses increased in comparison to that in the intradural masses (Fig. 3). However, MMP-9 expression was higher in the intradural masses than in the extradural masses. MMP-2 exhibited a similar expression pattern in the moderate destruction groups, while the pattern of MMP-9 expression in the moderate destruction groups differed in comparison with the severe and mild groups. The levels of MMP-2 and MMP-9 expression were higher in the extradural masses than in the intradural masses. In one case, in which the location of the mass was more superficial, the levels of MMP-2 and MMP-9 expression were increased (Fig. 4). Gelatin zymography and RT-PCR revealed a higher level of MMP-2 expression in almost all extradural masses in comparison with the intradural masses and MMP-9 in two.

## Immunohistochemical staining for MMP-2 and MMP-9

MMP-2 and -9 were localized in the cytoplasm of the tumor cells. The extracranial mass, including the osteolytic bone, was selected, and immunostaining was performed with MMP-2 and -9. The extradural mass was immunopositive, and the immunopositivity for MMP-2 was observed adjacent to the osteolytic bone (Fig. 5A, B). Immunohistochemical analysis demonstrated MMP-2 expression, especially in the vicinity of the bone

**Table 1.** Summary of the clinical characteristics of the patients

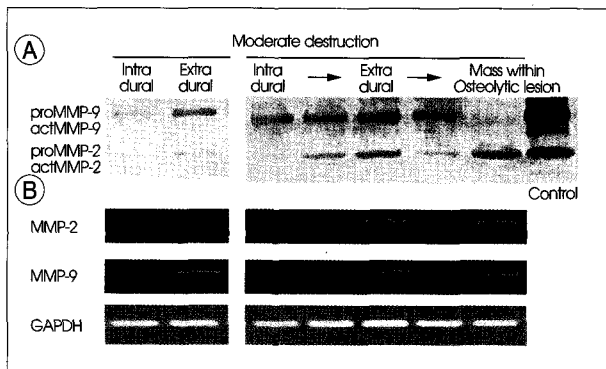
No.	Age	Sex	Location	Severity of osteolysis	Simpson Grade	Pathology	Ki-67 LI	Postop adjuvant Tx	Recurrence	Follow up duration before recurrence
1	14	F	Convexity	Severe	Gr I	Atypical	5	-	-	8Y7M
2	66	F	Parasagittal	Severe	Gr II	Syncytial	1-2	-	-	8M
3	45	F	Olfactory groove	Moderate	Gr II	Atypical	< 1	-	+	14M
4	41	F	Convexity	Moderate	Gr I	Transitional	2-3	-	-	21M
5	68	F	Convexity	Mild	Gr I	Fibroblastic	< 1	-	-	19M
6	48	F	Parasagittal	Mild	Gr II	Fibroblastic	< 1	-	-	21M



**Fig. 2.** Operative findings. A : Severe destruction with extensive osteolysis of the skull. B : Moderate destruction into the skull. C : Mild destruction into the diploic space and the inner table within the skull.



**Fig. 3.** Results of gelatin zymography and reverse transcription-quantitative PCR analysis (RT-PCR) in the severe and mild destruction groups. A : Gelatin zymography, B : RT-PCR; The expression of matrix metalloproteinase (MMP) -2 activity is higher in the extradural masses than in the intradural masses. The expression of MMP-9 is higher in the intradural masses than in the extradural masses (One case with severe destruction was not divided into intra and extradural portion).



**Fig. 4.** Results of gelatin zymography and reverse transcription-quantitative PCR analysis (RT-PCR) in the moderate destruction group. A : Gelatin zymography, B : RT-PCR; Extradural masses show higher levels of matrix metalloproteinase (MMP)-2 and MMP-9 expression when compared with intradural masses.

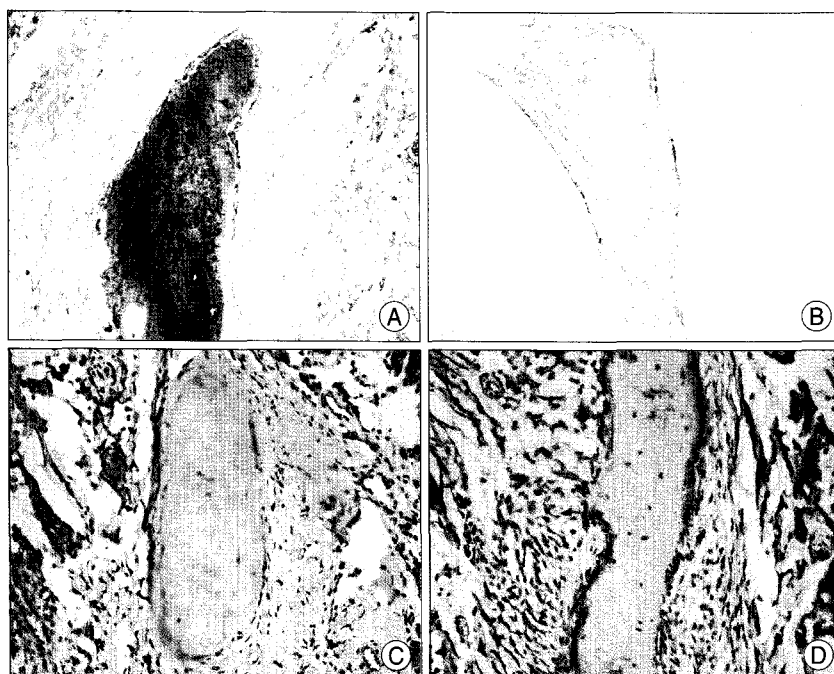
destruction, and a few MMP-9-positive staining was noted (Fig. 5C, D).

## DISCUSSION

Osteoblastic changes are fairly common in patients with

meningioma, but tumor cell invasion is rare<sup>1)</sup>. In contrast, osteolytic changes are mainly caused by tumor invasion, and they are potentially more malignant than osteoblastic changes, as demonstrated in other malignant tumors, such as metastatic brain tumors, multiple myelomas, and sarcomas<sup>16)</sup>. In our study, almost all of the osteolytic meningiomas were benign, except for one atypical meningioma, and the Ki-67 labeling index was less than 5 percent. The patient with atypical meningioma was followed up for more than 6 years, and no recurrence was noted. Therefore, meningiomas with osteolytic changes might not be related to malignant pathology, and they may not be an aggressive behavior of potentially malignant tumor cells. In our study, patients did not have a poor prognosis except one case of recurred atypical meningioma.

Matrix metalloproteinases are proteolytic enzymes that have the ability to breakdown basal membrane and connective tissue. Such enzymes are important for tissue breakdown during the process of invasive growth<sup>8,18)</sup>. MMP-2 and MMP-9 are two of the most common and most widely studied MMPs<sup>12,13)</sup>. The role of MMPs in meningioma has mainly been studied by comparing their expression with that observed in other brain tumors or in normal brain tissue. The expression of MMP-2, -9, -11, -12 and -14 has been demonstrated in meningiomas<sup>6,9,17)</sup>. MMP-2 and -9 expression in benign meningiomas has been observed *in vivo* and *in vitro*<sup>4,19)</sup>. However, increased MMP-9 expression has been observed in atypical and anaplastic meningiomas, while there was no difference in MMP-2 expression in meningiomas of different histological grade<sup>14)</sup>. In contrast, no difference in MMP-9 expression has been observed between atypical and benign meningiomas, while increased MMP-2 expression has been described in atypical and anaplastic meningiomas compared with benign meningiomas<sup>15)</sup>. High levels of MMP-2 and -9 expression in atypical and anaplastic meningiomas and a possible relationship between MMP-2 and -9 and invasion of meningiomas have been demonstrated in previous studies<sup>4,14)</sup>. One study de-



**Fig. 5.** Immunohistochemical staining with matrix metalloproteinase (MMP)-2 and MMP-9. A and B : The extradural mass was immunopositive, and the immunopositivity for MMP-2 is observed adjacent to the osteolytic bone (Original magnification  $\times 200$ ). C and D : A few MMP-9-positive staining is noted adjacent to the osteolytic bone (Original magnification  $\times 200$ ).

monstrated an association between MMP-2 and -9 expression and meningioma recurrence<sup>15</sup>). In our study, gelatin zymography and RT-PCR revealed higher levels of MMP-2 expression in almost all extracranial masses when compared with intracranial masses and MMP-9 in only two. Based on these results, we suggest that invasion to the extracranial portion might be related to MMP-2 expression in meningiomas.

Osteolytic lesions evolve through interactions between tumor cells and the bone microenvironment in a process known as the 'vicious cycle'<sup>21</sup>). Tumor cells secrete parathyroid hormone-related protein (PTHrP), which stimulates osteoblasts to produce a membrane bound RANK ligand (RANKL) and osteoprotegerin (OPG), a soluble decoy receptor for RANKL and a member of the TNF receptor family. The ratio of RANKL to OPG regulates osteoclast activation through its receptor for RANKL. Activated osteoclasts degrade bone matrix-releasing embedded growth factors, including the IGFs and TGF- $\beta$ , which in turn stimulate tumor cells to produce more PTHrP. Additional cycles have been reported, including tumor cell production of IL-11, resulting in osteoblast secretion of PGE2 and osteoclast activation<sup>10</sup>). Other factors released by bone metastatic tumor cells that may influence the microenvironment include MMPs, growth factors, inflammatory stimuli and angiogenic factors, and these are potential palliative and therapeutic strategies. MMP inhibitors reduced breast

osteolytic metastases<sup>24</sup>).

However, meningioma cells might differ from metastatic tumor cells because of its benign pathology. We used immunohistochemical staining to determine the location of the MMPs in the tumor tissue. The activities of the MMPs differed according to the location of the tumor, and MMP-2 showed the positive expression in the adjacent dura and osteolytic bone when compared with the brain parenchyma. In addition, MMP-2 expression was noted in the vicinity of bone destruction. Based on these results, we suggest that invasion to the extracranial portion and osteolysis might be related to MMP-2 expression in meningiomas, but further evaluation is needed in order to elucidate the mechanisms behind this activity.

## CONCLUSION

Osteolysis of the skull in meningioma might not be indicative of malignant pathological features and poor prognosis. Invasion to the extracranial portion and osteolysis might be related to MMP-2 expression in meningiomas.

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