

ORIGINAL

Matrix metalloproteinase 2 and 9 expression correlated with cavernous sinus invasion of pituitary adenomas

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Abstract : *Object:* Matrix metalloproteinase (MMP) 2 and 9 are important for tissue breakdown in the process of tumor invasion. The aim of this study is to evaluate the relationship between the expression of MMP-2, MMP-9, MIB-1 LI and cavernous sinus invasion in pituitary adenomas. *Methods :* Tissue samples from 54 patients with pituitary adenomas were studied. Expression of MMP-2, MMP-9, and MIB-1 labeling index (LI) were evaluated by immunohistochemical method. In sixteen cases, the expression of MMP-2 and MMP-9 mRNA was also examined by RT-PCR assay.

Results : Thirty-four patients were women and 20 were men, with a mean age of 49.9 years old (range 18-76 years). There were 12 cases with cavernous sinus invasion, and 42 were noninvasive cases. MMP-2 and MMP-9 score of invasive case (3.9 ± 0.5 , 4.1 ± 0.4) were significantly higher than those (2.3 ± 0.2 ; $p < 0.01$; 2.6 ± 0.2 ; $p < 0.01$) without invasion. The MIB-1 LI of this study presented no significantly difference between the invasive and noninvasive pituitary adenomas. The percentage of MMP-2 mRNA/ β -actin mRNA and MMP-9 mRNA/ β -actin mRNA were also observed significantly higher in invasive pituitary adenomas (68.2 ± 15.3 %; 59.7 ± 12.5 %) than noninvasive pituitary adenomas (21.8 ± 8.2 % , $p < 0.05$; 33.3 ± 5.4 % , $p < 0.05$).

Conclusions : Our study suggests that the expression of MMP-2 and MMP-9 may have a value to assess the invasive pituitary adenomas, and proliferation and invasion of pituitary adenomas may present a different mechanism. *J. Med. Invest.* 52 : 151-158, August, 2005

Keywords : MMP-2 ; MMP-9 ; Ki-67 ; pituitary adenomas ; tumor invasion

INTRODUCTION

Pituitary adenomas are generally benign tumor, and the majority of pituitary adenomas do not metastasize to distant sites, but about 30% of them invade to the local tissues, such as cavernous sinus, sphenoid sinus, orbit and clivus (1-4). The radical surgical treatment of the invasive pituitary adenoma is more difficult than the noninvasive tumors. Therefore, the tumor re-

urrence may be seen in a short period after operation.

Tumor invasion into surrounding tissues is a complex phenomenon and characteristic of more aggressive and often malignant tumor behavior. The invasive factors involve the heparinase, serine proteinases, cathepsins, and matrix metalloproteinases (5). MMPs are proteolytic enzymes that have the capability of breaking down basement membrane and connective tissue, and are important for tissue breakdown in the process of tumor invasion (6, 7). Among these invasive factors, MMP-2 and MMP-9 have been widely studied in lung cancers, breast cancers and other malignant neoplasms (8). Several studies examined the expression of MMPs in malignant gliomas (9-12). However, the expression of MMP-2 and MMP-9 in the invasive

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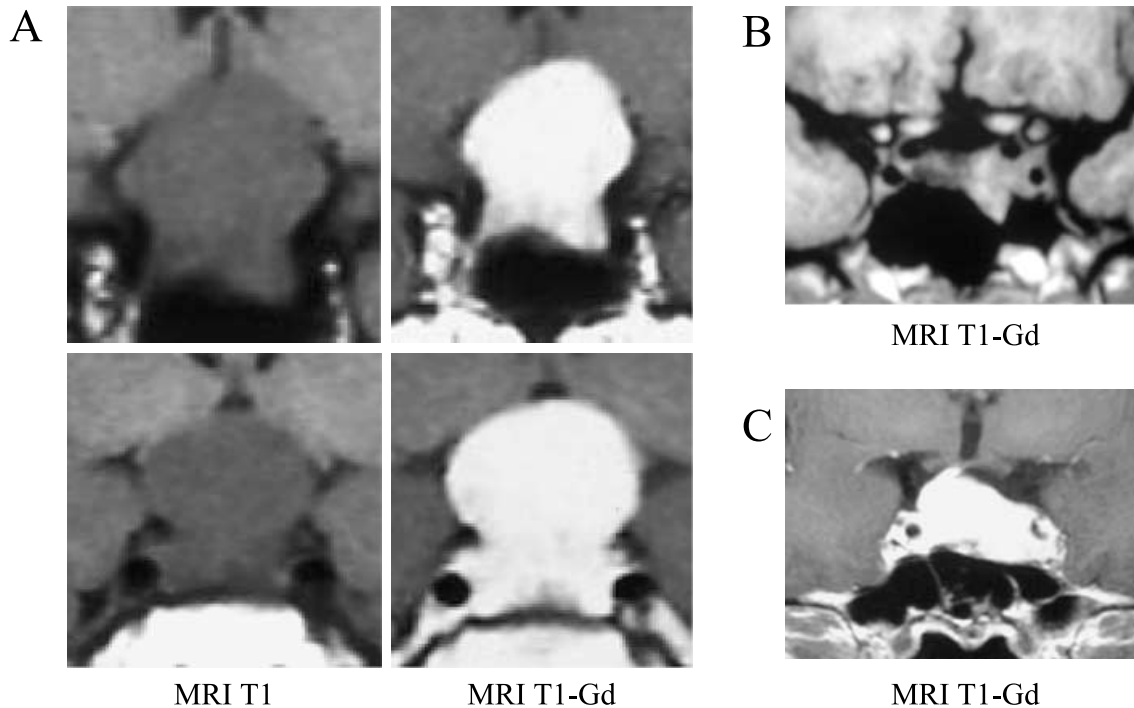


Figure 1. MRI imaging of invasive pituitary adenomas. Non-invasive pituitary adenomas are disguised from invasive adenomas by a medial tangent, the intercarotid line-through the cross-sectional centers-and a lateral tangent on the intra and supracavernous internal carotid arteries. Case A; The pituitary adenoma is huge but is classified in non-invasive type. Case B and C; The adenomas are not large but are considered as invasive type.

pituitary adenomas showed different results in the previous studies. This may be related to different methods, or different value of assessing the data.

The aim of this study was to determine whether expression of MMP-2 and MMP-9 might play a role in cavernous sinus invasion of pituitary adenomas. The possible mechanisms was investigated in cavernous sinus invasion of pituitary adenomas.

MATERIALS AND METHODS

Samples of pituitary adenomas were obtained from 54 patients who had undergone surgery from October 1984 to September 2001 at the Kagawa Medical University Hospital. Thirty-four patients were women and 20 were men, with a mean age of 49.9 years (range 18-76 years). There were 12 cases with cavernous invasion (Fig. 1), and 42 cases without invasion. There were 12 growth hormone (GH)-producing adenomas, 11 prolactin (PRL)-producing adenomas, 4 adrenocorticotropic hormone (ACTH)-producing adenomas, one thyroid stimulating hormone (TSH)-producing adenomas, one follicle-stimulating hormone (FSH)-producing adenomas, and 25 nonfunctioning (NF) pituitary adenomas. Invasion to cavernous sinus space of pituitary adenomas was defined on the basis of the

classification by Knops. Invasive and non-invasive adenoma are distinguished from each other by a medial target, the intercarotid line, through the cross-sectional centers (Fig.1) (13).

IMMUNOHISTOCHEMISTRY

MMP-2 and MMP-9 expression and proliferative potential were examined immunohistochemically on formalin-fixed, paraffin-embedded sections using monoclonal antibodies (MAbs) against MMP-2 (Ab-2; Fuji-chemistry, Japan), MMP-9 (Ab-2; Fuji-chemistry, Japan) and MIB-1 MAb (Immunotech, Marseille, France).

Six-micron sections were deparaffinized in xylene, rehydrated through graded alcohols, and immersed for 15 min in phosphate-buffered saline (PBS). For antigen retrieval, the sections were microwaved in 0.01 M citrate buffer (pH 6.0) for 20 min. After microwave pretreatment, the endogenous peroxidase activity was blocked with 3% hydrogen peroxidase in methanol for 10 min, and nonspecific staining was then blocked by 20-min incubation with normal horse serum. The sections were then incubated overnight at 4 °C with primary antibodies (MMP-2, ×100; MMP-9, ×100; MIB-1, ×50) in a humidity chamber. The sections were treated for 30 min with biotinylated horse secondary

antibody against mouse immunoglobulins (ABC Elite, Vector Lab., Burlingame, CA, USA) and for 30 min with avidin-biotin complex (ABC Elite, Vector Lab.), followed by 0.06 % diaminobenzidine (Sigma, MO, USA) with 0.01% hydrogen peroxidase for 5 min. The slides were lightly counterstained with hematoxylin. Controls were performed by omitting the primary antibody.

A semi-quantitative assessment of the immunohistochemistry of MMP-2 and MMP-9 was employed by grading the staining as (0) negative, (1) < 30%, (2) 30-60%, (3) 60-100% of tumor cells showing positive staining. The intensity of the immunostaining was also graded as (0) negative, (1) weak, (2) moderate, and (3) strong. The total immunohistochemical score was determined as the sum of the frequency and intensity scores for tumor cells (14). Twenty representative fields were counted, and the immunohistochemistry scores were determined by concordance among the scores of two independent reviewers. The score was divided into 0-6 for each sample.

The MIB-1 LI was calculated as the percentage of immunostained nuclei, excluding nuclei of vascular components and hematogenous cells, by counting approximately 200 tumor nuclei.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

RNA was extracted from the frozen tissue sample by using ISOGEN reagent (Nippon Gene, Toyama, Japan). Briefly, 100 mg of pituitary adenomas tissue was homogenized in 1 ml of ISOGEN. Subsequently, 0.2 ml of chloroform was added and the mix was centrifuged. This separated the solution into an aqueous phase containing RNA, an interphase containing DNA, and an organic phase containing protein. The aqueous layer was aspirated and added to 0.5 ml of isopropanol for RNA precipitation. Following this, the solution was centrifuged and the pellet was washed with 70% ethanol and centrifuged. After that, RNA was collected into 50 μ l of diethylpyrocarbonate (DEPC)-treated water. RT-PCR was performed using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech UK Limited). Two microlitre of total RNA (2 μ g) was added to 5.5 μ l of the RT mixture. After mixing, the samples were incubated at 37 °C for 45 min, 95 °C for 5 min, and 4 °C for at least 5 min. 17.5 microlitre of PCR-mixture containing 12.5 μ l primers and Taq DNA polymerase (Amersham Pharmacia Biotech UK Limited) was added to the RT products. Initial denaturation for 2 min at 94 °C was followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C, and a final extension

for 6 min at 72 °C. The PCR products were separated on 2% agarose gels, and ethidium bromide-stained bands were recorded by Mupid-2R (Cosmo-Bio, Tokyo, Japan). The primer sequences were as follows :

MMP-2 : forward primer : 5' primer : 5'-GCTGAT ACTGACACTGGTACTG-3', reverse primer : 5'-CAA TCTTTTCTGGGAGCTC-3'.

MMP-9 : forward primer : 5' primer : 5'-AAGGAT GGTCTACTGGCAC-3', reverse primer : 5'-AGAGA TTCTCACTGGGGC-3' (15).

β -actin : forward primer : 5' primer : 5'-ATCACCA TTGGCAATGAGGG-3', reverse primer : 5'-TTGAA GGTAGTTTCGTGGAT-3'.

The results of percentage of MMP-2 mRNA/ β -actin mRNA and MMP-9 mRNA/ β -actin mRNA were assessed by NIH software using computer.

Statistical analysis

Student's t-test was used to evaluate the relationships among various parameters. The effect of various parameters, i.e., tumor size, MMP-2 score, MMP-9 score, MIB-1 LI and percentage of MMP-2 mRNA/ β -actin mRNA and MMP-9 mRNA/ β -actin mRNA was assessed, using the STAT VIEW for windows software performed statistical analysis.

RESULTS

Immunohistochemistry findings

The cytoplasm and/or nuclear of tumor cells were stained as brown color in pituitary adenomas (Fig. 2). In this study, Forty-six cases (82 %) of 54 pituitary adenomas were observed positive immunoreactivity, whereas, staining intensity was variable. MMP-2 and MMP-9 positive staining could be seen in all 12 cases of invasive pituitary adenomas. The MMP-2 score of invasive pituitary adenoma (3.9 ± 0.5) (mean \pm SE) were significantly higher than those without invasion (2.3 ± 0.2) ($p < 0.01$). The MMP-9 score of invasive pituitary adenoma (4.1 ± 0.4) were significantly higher than those without invasion (2.6 ± 0.2 ; $p < 0.01$) (Table 1). We found that invasive pituitary adenomas were significantly more likely to express MMP-2 and MMP-9 compared with noninvasive pituitary adenomas. The MIB-1 LI tended to be higher in invasive pituitary adenomas (1.2 ± 0.4 %) than that (0.6 ± 0.2 %) in noninvasive tumors, however, there was no significant difference ($p=0.1528$). There was no correlation between the MMP-2 and MIB-1 LI ($r=-0.05$, $p=0.72$), MMP-9 and MIB-1 LI ($r=0.004$, $p=0.98$). According to tumor size, we divided the 54 pituitary adenomas into 4 groups

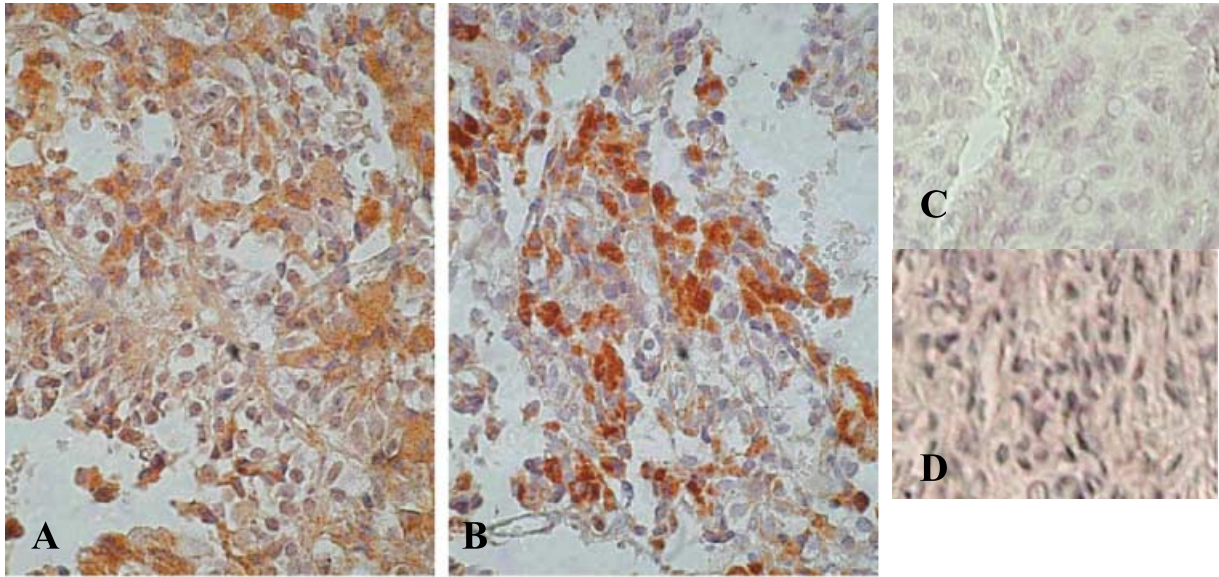


Figure 2. Immunohistochemical staining of MMP-2 (A) and MMP-9 (B) in invasive pituitary adenomas. Photograph showing cytoplasmic and/or nuclear immunoreactivity of the tumor cells. Negative control for MMP-2 (C) and MMP-9 (D) (magnification × 400)

(10 mm , > 10 mm to 20 mm , > 20 mm to 40 mm, and > 40 mm), and compared the expression of MMP-2, MMP-9, and MIB-1 LI in the different groups. There were no significant differences among the 4 groups (Table 2). There are also no correlation in MMP-2, and MMP-9 expression among ACTH, PRL, or GH producing adenomas and NF pituitary adenomas (Table 3).

Table 1. The score of MMP-2 and MMP-9 expression in invasive and noninvasive pituitary adenomas

Group	No. of patients	Age	Score of MMP-2	Score of MMP-9	MIB-1 LI
Invasion	12	52.7 ± 3.6	3.9 ± 0.5**	4.1 ± 0.4**	1.2 ± 0.4
Noninvasion	42	48.9 ± 2.6	2.3 ± 0.2	2.6 ± 0.2	0.6 ± 0.2

Patients age, score of MMP-2, MMP-9 expression and MIB-1 LI are expressed as means ± SE.

** : $P < 0.01$ vs noninvasion group LI : labeling index.

Table 2. Correlation with the expression levels of MMP-2 and MMP-9 and the tumor size

Group	Tumor size(mm)	No. of patients	Age	Score of MMP-2	Score of MMP-9	P value
1	10	10	31.6 ± 4.5	2.1 ± 0.5	2.1 ± 0.5	NS
2	>10 to 20	20	52.2 ± 3.2	3.0 ± 0.4	3.0 ± 0.3	NS
3	>20 to 40	21	56.3 ± 2.9	2.7 ± 0.4	3.2 ± 0.4	NS
4	>40	3	48.7 ± 6.7	2.3 ± 0.3	3.0 ± 0.6	NS

Patients age, and score of MMP-2, MMP-9 expression are expressed as means ± SE.

NS : not significant

Table 3. Correlation with the expression of MMP-2 and MMP-9 and the hormone secreting pituitary adenomas

Group	No. of patients	Score of MMP-2	Score of MMP-9	P value
ACTH	4	2.0 ± 0.7	2.5 ± 0.9	NS
GH	12	2.8 ± 0.5	3.1 ± 0.4	NS
PRL	11	2.7 ± 0.5	2.9 ± 0.6	NS
NF	25	2.7 ± 0.3	3.0 ± 0.3	NS

The score of MMP-2, MMP-9 expression are expressed as means ± SE.

NS : not significant. NF : nonfunction

mRNA Expression of MMPs

We examined MMP-2 and MMP-9 mRNA by RT-PCR from 16 cases including 4 invasive and 12 non-invasive pituitary adenomas. The expression of MMP-2 and MMP-9 mRNA was also observed in the invasive pituitary adenomas and noninvasive tumor (Fig. 3). The percentage of MMP-2 mRNA/β-actin mRNA was observed significantly higher in invasive pituitary adenomas (68.2 ± 15.3 %) than those without invasion (21.8 ± 8.2 % ; $p < 0.05$). The percentage of MMP-9 mRNA/β-actin mRNA was observed significantly higher in invasive pituitary adenomas (59.7 ± 12.5 %) than those of noninvasive adenomas (33.3 ± 5.4 % ; $p < 0.05$) (Fig. 4). There are significant correlation between mRNA and immunohistochemistry score in MMP-2 and MMP-9 ($r=0.582, p < 0.05$; $r=0.669, p < 0.01$) (Fig. 5).

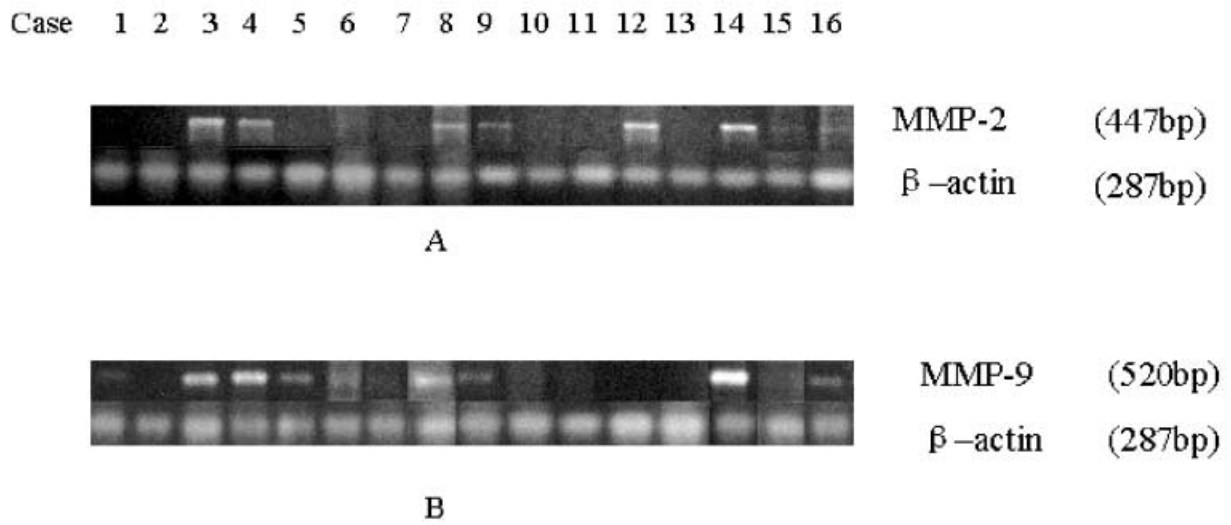


Figure 3. Expression of MMP-2 (A), MMP-9 (B) mRNA by RT-PCR with β-actin serving as a control for equal loading in pituitary adenomas. (A) Positive bands are observed in the invasive cases (case 3, 8, 12, and 14). (B) Positive bands are found in the invasive cases (case 3, 8, 12, and 14). The levels of MMP-2 were quantitated by using NIH image (version 1.61).

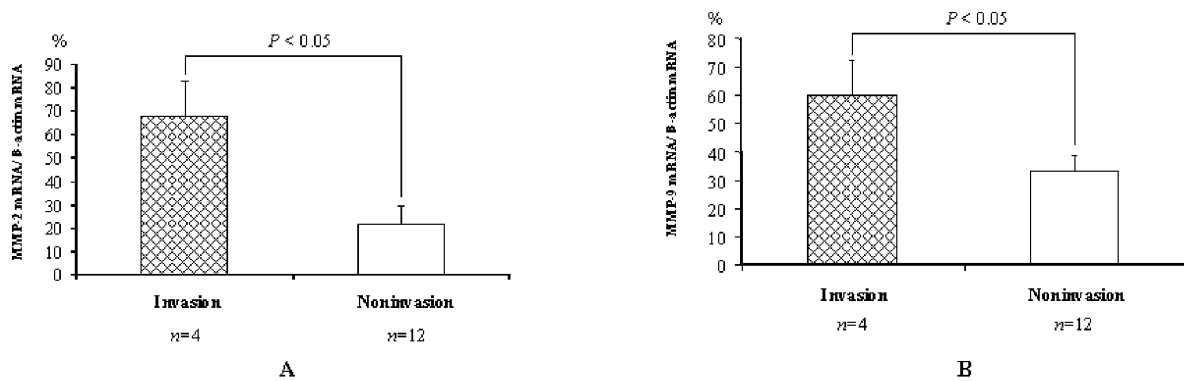


Figure 4. The graph showed the significant differences of MMPs mRNA expression between in the invasive cases and in the noninvasive cases ($*p < 0.05$). (A) MMP-2. (B) MMP-9.

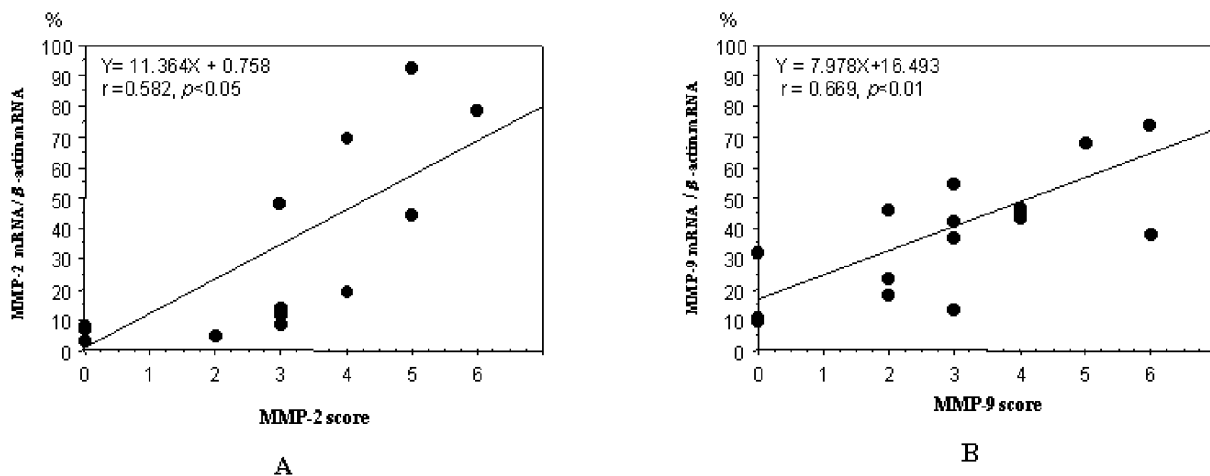


Figure 5. The graph showed that there are significant correlation between mRNA and immunohistochemistry score in MMP-2 and MMP-9. (A) MMP-2 and MMP-2 mRNA ($r=0.582, p < 0.05$); (B) MMP-9 and MMP-9 mRNA ($r=0.669, p < 0.01$)

DISCUSSION

MMPs are a family of zinc-dependent endopeptidases that regarded as promoting of invasion and metastasis (16). The family includes five subclasses : collagenases, gelatinases, stromelysins, metalloelastase and membrane-type metalloproteinases. MMPs have been implicated in various physiological and tissue morphogenesis as well as tumor cell invasion and metastasis (17). The MMP-2 and MMP-9 are the common studied gelatinases and can degrade the basement-membrane type collagen. Tumor cell invasion utilizes numerous proteases *in vitro*, of which the predominant ones may be gelatinase-A and -B (MMP-2 and MMP-9 respectively) (18). Nakada, *et al.* (19) demonstrated that the production levels of MMP-2 are significant higher in glioblastomas multiforme than in other grades of astrocytic tumors. Several studies indicated that there presented significant correlation between the MMP-2 and MMP-9 expression and invasion in the malignant tumors (20-25).

The majority of pituitary adenomas are benign and do not metastasize. However, it always doubted the neurosurgeons that parts of pituitary adenomas are locally invasive and more than 20% cases recur (26-28). The invasive behavior of pituitary adenomas is poorly understood. In present study, we evaluated the expression of MMP-2 and MMP-9 by immunohistochemistry and MMP-2 and MMP-9 mRNA level by RT-PCR assay in pituitary adenomas. We found that invasive pituitary adenomas have a significantly higher expression of MMP-2 and MMP-9 protein, and mRNA than noninvasive tumors. Turnere, *et al.* (9) found that invasive macroprolactinomas were significantly more like to express MMP-9 than noninvasive tumor. Kawamoto *et al.* described that the incidence of tumor cells secreting MMP-9 was significantly higher in invasive pituitary adenomas than in noninvasive ones (11). Pereta, *et al.* found the high expression of MMPs and low levels of TIMP-1 in pituitary adenomas, and the active form of the MMP-2 was found in some pituitary tumors (29). They also showed that high level of MMP activity could be to contribute the regulation of proliferation and hormone secretion in pituitary adenomas (28). We failed to find the significant difference of MMPs expression among the hormone (GH, PRL, and ACTH) secreting adenoma and NF pituitary adenomas. Our data suggest that proteases, MMP-2/-9, may play a role in tumor invasiveness of pituitary adenomas and did not correlate with hormone secreting activity.

A number of studies have been widely considered

that Ki-67 LI is the best means to evaluate cell proliferation in clinical practice. It is also considered a useful tool for assessing the recurrence of the tumor (30, 31). We have previously shown that no correlation between MMP-2/-9 expression and the Ki67 LI expression in gliomas (32). Mastronardi's study showed that the Ki-67 LI was a useful marker in determining the invasive behaviour of pituitary adenomas, and his results also seem to exclude significant correlations between MIB 1 LI and tumor size of pituitary adenomas (33, 34). For the first time, we have demonstrated that there was no difference between MIB1 LI of invasive pituitary adenomas and that of noninvasive adenomas, and no correlations were found between the expression of MMP-2/-9 and MIB-1 LI, MMP-2/-9 expression and tumor size. The present data suggested that the expression of MMP-2 and MMP-9 were the index markers of tumor invasion, whereas the expression of MMP-2 and MMP-9 had no correlation with the proliferation of the pituitary adenomas. Some researcher (6-9) found that the expression of MMP-9 was related with the invasiveness in pituitary adenomas, however few authors examined the MMP-2 expression in pituitary adenomas. Our study demonstrated the correlation between the expression of MMP-2, as well as MMP-9, and cavernous invasion in pituitary adenomas. We also found that some of the tumors, in spite of their small size, invade the cavernous sinus or the surrounding area, whereas some huge pituitary adenomas did not invade the surrounding structures. Our data may explain this phenomenon. Turner's study the adenomas removed when they recurred were more likely to express MMPs (9). On the other hand, Knappe *et al.* found no differences in expression of MMP9 in recurrent adenomas (35). Sampling of tumor tissue is done to the opening in the intrasellar parts of the tumor during transsphenoidal surgery and the areas of lateral invasion are cleaned by the suction rather than excision. Therefore, we could not investigate differences of expression of MMP-2/-9 in distinct areas of possible dural invasiveness, nor compare them with central intrasellar areas of the tumors.

Although there may be many biochemical mechanisms about the invasion of tumors, the present data can partially explain the invasive behavior of pituitary adenomas. Our study indicated that the expression of MMP-2 and MMP-9 had a value to assess the invasion in pituitary adenomas, while the MIB-1 LI was not the index of tumor invasion.

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