Reversion-Inducing Cysteine-Rich Protein with Kazal Motifs and Matrix Metalloproteinase-9 are Prognostic Markers in Skull Base Chordomas

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Abstract

Prognosis of chordomas is difficult to predict based solely on histological findings. The purpose of this study was to assess expressions of reversion-inducing cysteine-rich protein with kazal motifs (RECK) and matrix metalloproteinase (MMP)-2, MMP-9 in skull base chordomas and to find out their correlations to outcome. Immunohistochemical study was performed in 19 samples (initial, n=11; recurrent, n=8) from 11 patients. The correlations among expression of RECK, MMP-2, MMP-9 and their prognostic values were analyzed. Significant correlation between RECK and MMP-9 was found, but there was no correlation found between MMP-2 and MMP-9. Higher MMP-9 expression significantly influenced outcome. Furthermore, MMP-9/RECK ratio showed significant correlation to outcome, showing their inverse relationship in the disease progress of skull base chordoma. RECK and MMP-9 can be valuable markers to predict prognosis in skull base chordomas.

Key Words: skull base chordomas, RECK, MMP-2, MMP-9

Introduction

Chordomas are rare nonepithelial malignant tumors with locally invasive behavior. Management remains a challenge because of their location and involvement with delicate vascular and nervous structures and also because of their high recurrence rates.[3,4] Chordomas are also resistant to conventional radio- and chemotherapies. Although they are generally regarded as benign, slow growing lesions, approximately 20% recur as early as 1 year after surgery, with a tumor doubling time of just a few months.[4,20]

These tumors are some of the most common tumors of axial skeleton, although in the skull they are still rare, comprise approximately 0.1 to 0.2% of all primary intracranial neoplasms.[3,20] No typical symptoms are associated with skull base chordomas because they depend on tumor location and size.[3] The molecular basis for the clinical behavior of these neoplasm is unknown. Histologic features of chordomas have not been found to be reliably helpful in predicting tumor behavior.[7] Some studies have attempted to correlate the clinical behavior of this tumor by immunohistochemical markers, volumetric analysis and proliferation markers, structural protein determination, and cytogenetic studies.[2,23,28] As yet, however, results remained controversy.

Matrix metalloproteinases (MMPs) play an important role in tumor invasion and recurrence. Some studies of other types of brain tumor have pointed out significant role of MMPs.[9,10,11,13,15,16,18,19] There are only two studies found concerning MMPs in chordomas.[15,16] A major function of MMPs in metastasis is to facilitate the breakdown of the extracellular matrix, including the basement membrane, and they also play a substantial role in the maintenance of a microenvirontment that facilitates the growth and angiogenesis of a tumor. MMPs degrade various types of collagen, especially type IV collagen which is a chief constituent of the basement membrane of

brain vessels.[9,10,11] Recent studies have indicated that expression of MMPs correlates with invasiveness of certain brain tumors.[9,10,11,13,15,16,18,19]

In the present study, we examined MMP-2 and MMP-9 expression immunohistochemically in initial and recurrent surgeries of skull base chordomas. We also examined the role of RECK (reversion-inducing cysteine-rich protein with Kazal motifs), a membrane-anchored regulator of matrix metalloproteinase which is widely expressed in healthy tissues, whereas it is expressed at lower levels in many tumor-derived cell lines.[1,5,14,17,21,22,25,26,27] However, it has never been studied before in chordomas. Analysis of relationship of RECK and MMPs, and their prognostic values in skull base chordomas were also performed.

Materials and Methods

Patients and tissue samples

This study included 11 patients with skull base chordomas who were treated at Shinshu University Hospital between 1978 and 2005 **(Table 1).** The patients consisted of 5 men and 6 women, aged 9 to 71 (mean 48.8) years at the initial operation. Median **preoperative karnofsky performance status (KPS) was 80 (range: 60-90).** The mean follow-up period was 12.8 (4.6-28.6) years. Tumor resection was performed totally 61 (mean 5.5) times in 11 patients. In 8 of 11 patients, tumor excision was performed more than twice because of staged operations or tumor regrowth. Six patients underwent fractionated radiotherapy. Gamma knife surgery was performed for 14 lesions in 7 patients. Four patients died due to tumor growth and 7 were alive. They were retrospectively examined for their initial tumor sizes, tumor volume doubling times (Td) and clinical outcomes.

Nineteen samples from 11 patients (11 from initial surgery, 8 from recurrent surgery) were examined immunohistochemically for the expressions of RECK, MMP-2,

and MMP-9. Samples from recurrent surgery were obtained at the start point of the study, and they maybe from the second, third or fourth surgery. Samples from initial surgery were retrospectively collected from patients` paraffin blocks from the initial surgery. **Patients were divided into two groups, the disease-free survival group (patients with no recurrence more than 2 years after the final treatment), and the disease-ongoing group (patients with recurrence within the past 2 years, including patients who died of tumor-related causes). Two years were used as a cut-off point for recurrence because we took the effect of previous surgery, chemotherapy, and radiotherapy into account before the diagnosis of recurrence could be established. Outcome of these groups would be weighed against the expression of RECK, MMP-2, and MMP-9.**

Immunohistochemistry

Paraffin-embedded sections were examined for immunohistochemistry. The deparaffinized 4-um-thick sections were immunostained after antigen retrieval with microwave heating in 0.01 M citrate buffer (pH 6.0). The sections were incubated with anti-MMP-2 mouse monoclonal antibody (Daiichi Fine Chemicals, Japan), anti-MMP-9 mouse monoclonal antibody (Daiichi Fine Chemicals, Japan), and goat polyclonal antibody against RECK (C-16, Santa Cruz Biotechnology, Inc, CA). For secondary antibody, Envision+ (DAKO, Golstrup, Denmark) was used, which is a dextran polymers conjugating a large number of goat antibodies against mouse immunoglobulins and horseradish peroxidase. Counterstaining was performed with hematoxylin.

Staining and coding were done by pathologist (JN) and expressions of markers were done blindly by a separate examiner (NR).The expressions of RECK, MMP-2, and MMP-9 were evaluated by immunohistochemical staining frequency

(0-100%). Ten representative fields were counted. **Tumor cells were identified based on distinct morphological appearance compared to normal cells. Macrophage and infiltrating cells were excluded from counting.** Hematoxylin and Eosin staining was used as control. Examiner was blinded to surgical diagnosis and outcome.

Statistical Analysis

Because of limited number of cases, nonparametric study was used to analyze the results. Analysis of correlation between RECK, MMP-2 and MMP-9 expressions was done with Spearman`s Coefficient Correlation test, and their prognostic values were analyzed using Wilcoxon Signed Rank test**. Adjustment to age, initial tumor size and sex were also conducted.** The mean value, standard deviation and p-value were calculated. **Survival analysis was not conducted due to lack of number.** All of the analyses were performed using the program SPSS 16 for Macintosh.

RESULTS

Immunostaining and Location

Of 11 initial samples, we observed 3 samples of RECK, 5 samples of MMP-2 and 1 sample of MMP-9 which were negative for staining. Whereas of 8 recurrent samples, we observed 3 samples of RECK, 3 samples of MMP-2 and 2 samples of MMP-9 which were not stained. Intensity of staining varied in every sample, from weak to very strong staining. Most of MMP-2 samples showed positive staining around the cytoplasm, where as MMP-9 and RECK samples showed variation of stained location, such as the membrane, extracellular matrix or the cytoplasm (Figure 1).

Some samples also revealed positive staining on normal epithelial tissues. We also observed tumor cell infiltration to host bone in some samples. In general, we observed a high degree of heterogeneity of RECK, MMP-2 and MMP-9 expressions in

skull base chordoma tissues.

RECK, MMP-2, MMP-9 Expression and Their Correlations with Clinicopathological Parameters

The proteinase scores in initial and recurrent lesions were summarized in Table 1. No significant correlations were observed between the expression of RECK, MMP-2 and MMP-9 with age, sex, and size. The mean initial tumor size was 49.0 mm^3 (range, 6.1-161.9 mm^3). The mean TD was 16.3 months (range, 1.7-82.0 months). There was no significant correlation between MMP-2, MMP-9, RECK expressions and tumor doubling time.

When initial and recurrent markers expressions were examined using nonparametric study for 2 dependent variables, there was significant decrease of initial and recurrent RECK (p= 0.043), and tendency of increase of MMP-9, however was not significant (p= 0.091) (Figure 1).

Relationships among Proteinases

Relationships among proteinases were investigated using the initial samples. Significant correlation was observed between RECK and MMP-9 expression (p= 0.036). No correlation was observed between MMP-2 and MMP-9 (p= 0.78), or between RECK and MMP-2 (p= 0.193) (Table 2).

Prognostic Values

RECK, MMP-2 and MMP-9 expressions were also analyzed to determine their effects on outcome between patients with no recurrence more than 2 years after the final treatment (disease-free survival group) and patients with recurrence within the past 2 years (disease-ongoing group; including patients who had died of tumor-related causes; **Table 3**). The disease-free survival group consisted of patients 5, 6, 7, 10 and 11, and

the disease-ongoing group consisted of patients 1, 2, 3, 4, 8 and 9. **In the latter group, series of surgeries, chemotherapy and/or radiotherapy were given to patients regarding the tumor growth. Detail was explained in case illustration (Figure 2).** Initial samples were used because we would like to find out whether samples from initial surgery can predict the progression of the disease.

MMP-9 was shown to have influence on outcome even after being **adjusted to age, sex, and tumor size (p= 0.040), however MMP-2 did not show any influence on outcome.** RECK showed possibility of negative correlation to outcome, however it was not significant (p= 0.079).

We assumed that ratios between RECK and those of MMPs have prognostic values, because of correlated mechanism of action towards disease malignancy. To see the impact of MMP/RECK ratio on patient`s outcome, Wilcoxon Signed Rank test was performed. Our results revealed that MMP-9/RECK significantly influenced outcome (p= 0.018) (Table 3).

Case Illustration

47-year-old man was admitted to ear nose and throat (ENT) department with paranasal tumor in 1981, and the tumor was partially removed. Histological result showed chondromatous chordoma, and the patient was then referred to our service. One year later he underwent a transsphenoidal surgery for mass reduction, then followed by another two procedures before he underwent radiation therapy of 26 Gray. Eight years later, he underwent another 6 procedures (transfacial and transcranial) to reduce the size of the tumor, and during this clinical course facial paresis and deafness on the right side remained. He also underwent the second radiotherapy of 40 Gray, before two years later, in 1998, he had to undergo another surgery because of tumor regrowth. Subtemporal transpetrosal approach

was chosen for partial removal of the tumor. He underwent another procedure before he passed away because of disease progression one year later.

Discussion

RECK, an inhibitor to MMP recently has been the topic of researches in various fields. Reduced expression of RECK in prostat, lung, gastric, breast, bone and pancreatic cancers showed worse prognosis.[5,14,21,25,26,27] In lung cancer, RECK was significantly reduced in adenocarcinoma but not in non-small cell lung cancer, because RECK expression was insufficient to inhibit much higher MMP expression in non-small cell lung cancer.[27] In gastric cancer, reduced RECK expression was significantly correlated with more infiltrative macroscopic tumor growth, more metastasis into regional lymph nodes and more advanced staging.[25] However, to our knowledge, this is the first report to investigate RECK role in skull base chordomas and its relationship to MMPs. Results from our initial samples showed significant correlation between RECK and MMP-9 ($p= 0.040$), and there was no significant correlation between RECK and MMP-2. The reason for this to happen is because RECK is a membrane anchored MMP inhibitor. Membrane anchoring allows RECK to be concentrated on the plasma membrane and effectively regulate local proteolytic events at the cell surface, and it has been shown in this and other reports that MMP-9 was frequently observed in the membrane of tumor cells.

There is accumulating evidence that matrix metalloproteinases play a key role in the pathogenesis of neurological diseases. In astrocytic tumor, meningiomas, pituitary adenomas, multiple sclerosis, and meningitis they were proven to be significant increased of matrix metalloproteinases expression.[9,10,11,12,13,18] In skull base chordomas, there is only one report mentioning about MMP expression.[16] Naka et al. examined that expression of MMP-1, MMP-2, MMP-9, and found that higher MMP-1

expression was correlated with worse prognosis in skull base chordomas.[16] In a previous study of Naka et al., MMP-1 and MMP-2 were frequently expressed in a small series of non-skull base chordomas.[15] In glioma, MMP-9 expression was thought to be regulated by inflammatory cytokines such as tumor necrosis factor α and interleukins.[10] The presence of MMP-9 protein-positive neutrophils and macrophages in the vicinity of blood brain barrier might also indicate the involvement of MMP-9 in tumor infiltration, whereas MMP-2 was expressed by many cell types and was most likely regulated at the level of proenzyme activation.[10] In meningioma, different results appeared. Okada et al. mentioned that MMP-2 and MMP-9 were significantly correlated with histological malignancy,[18] whereas Kriches et al. concluded that the gene transcription and gelanolytic activity of MMP-2 and MMP-9 were insufficient to play a major role in tumor growth.[8] In pituitary adenomas, MMP-9 levels were found to be significantly higher in invasive adenomas than in noninvasive ones.[6] On the contrary, Yokoyama et al. found no difference in expression of MMP-9 in non-secreting adenomas with or without enhancement of the carotid artery.[29] In non-neurological cancers, various results regarding MMP and RECK were observed. For example in pancreatic cancer, MMP-2 showed strong correlation with RECK, but there was no correlation seen between MMP-9 and RECK.[14]

In our study, higher expression of MMP-9 showed worse outcome, and lower expression of RECK showed tendency towards disease progression. However, MMP-2 expression did not seem to have any significant effect on outcome. We assumed that ratio MMP to RECK in a tumor would affect outcome. Takemoto et al. found out that MMP-2/RECK ratio in adenocarcinoma of the lung was a significant prognostic factor.[27] When we performed analysis of MMP/RECK ratio to outcome, higher MMP-9/RECK ratio caused worse outcome. It may mean that RECK does not only act independently in affecting outcome, but it works antagonistically with MMP-9. Our

Conclusion

In this study significant correlation between RECK and MMP-9 was found, and higher MMP-9 expression significantly influenced outcome. MMP-9/RECK ratio also showed significant correlation to outcome, showing their inverse relationship in the disease progress of skull base chordomas. Our results have revealed the important role of RECK and MMP-9 as prognostic markers for skull base chordomas.

results suggest that the RECK and MMP-9 are associated with tumor recurrence and poor prognosis.

Expression of RECK was significantly lowered from initial to recurrent samples, and expression of MMP-9 also showed tendency to increase. Perhaps with more number of patients, significant result can be achieved. **Unfortunately we could not perform any analysis on the value of proteinase expression in each repeated surgery, despite the great possibility that it might show changes of expression.** Leppert et al. in their study of MMP expression in multiple sclerosis found out there was a sustained increase of MMP-9 in clinically stable multiple sclerosis, which supports the concept that multiple sclerosis is associated with ongoing proteolysis that may result in progressive tissue damage.[12] Whether this theory is applicable or not in skull base chordoma and whether expression of MMP will increase with increased number of surgeries will need further study.

Another interesting point is about tumor infiltration of host bone. Naka et al. showed that the ones with tumor infiltration to host bone had higher expression of MMP-1 and MMP-2.[16] Seven out of 11 samples in our study revealed tumor infiltration to host bone, and it had a tendency towards worse outcome in samples with infiltration, but we did not perform analysis of host bone infiltration in this study.

Our study lacked number of patients. Interpretation of the results presented here should be done with cautions. Bigger size study is needed to confirm our findings. **In this study we only evaluated protein expression, whereas enzymatic activity analysis might be able to show the definite expression and activity of the markers.** However, our results have demonstrated important findings for skull base chordomas. Novel tumor markers can be used in the future to predict prognosis of this highly invasive tumor.

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Table 1. Clinical characteristics and percentage of expressions of MMP-2, MMP-9, and RECK in 11 patients.

Table 2. P values for correlations among staining scores of proteinase expression

Table 3. P values of disease-free and disease-ongoing groups

Figure 1. Expressions of MMP-2, MMP-9 and RECK at initial and recurrent surgery

RECK expression showed significant decrease from initial to recurrent surgery ($p=0.043$). MMP-9 showed tendency of increasing value, however was not significant ($p=0.091$).

Figure 2. Case Illustration

(A-C) Initial histological images of Case 4. (A) negative expression of MMP-2, (B) slight expression of MMP-9, (C) strong expression of RECK, (D) initial CT scan showing isodense mass mainly occupying the clivus. (E-G) Recurrent histological images, (E) weak expression of MMP-2, (F) strong expression of MMP-9, (G) decreased expression of RECK compared with initial stage, and (H) T2-weighted MR image of recurrent stage showing the mass occupying not only the clivus but the adjacent areas as well.