Objective: Glioma is the most common type of primary central nervous system tumor. This study was aimed at investigating the expression of matrix metalloproteinase-9 in astrocytic glioma samples and its association with clinicopathological characteristics as well as survival of patients.

Methods: Astrocytic glioma samples from 272 patients who had not received chemotherapy or radiotherapy were collected, in which matrix metalloproteinase-9 expression was assessed by immunochemistry assays. The association of staining evaluation results with clinicopathological characteristics was analyzed by appropriate statistical analysis. Kaplan–Meier analysis and Cox proportional hazards regression models were used to investigate the association between matrix metalloproteinase-9 expression and survival of patients.

Results: Results showed that matrix metalloproteinase-9 expression is increased in astrocytic glioma and associated with tumor progression as its expression increased from Grade II to Grade IV glioma ($P < 0.001$). Kaplan–Meier analysis showed that patients with glioma with higher matrix metalloproteinase-9 expression tend to have shorter overall survival time ($P < 0.001$). In multivariate analysis, matrix metalloproteinase-9 expression was proved to be an independent prognostic factor for patients with astrocytic glioma ($P < 0.001$).

Conclusions: This study confirmed the overexpression of matrix metalloproteinase-9 and its association with tumor progression in astrocytic glioma. It also provided the first evidence that matrix metalloproteinase-9 expression in glioma was an independent prognostic factor of patients, which might be a potential diagnostic and therapeutic target of astrocytic glioma.

Key words: MMP-9 – glioma – sonic hedgehog – immunohistochemistry – prognosis

INTRODUCTION

Glioma is the most common primary malignancy in the human central nervous system with a tendency to invade the surrounding brain tissue, which is classified into astrocytic and oligodendroglial gliomas (1,2). According to the classification by World Health Organization (WHO), gliomas are histologically classified into four grades: low-grade astrocytomas (WHO Grade I–II), anaplastic astrocytomas (WHO Grade III) and glioblastoma (GBM, WHO Grade IV) (3,4). Now, postoperative radiation therapy and
chemotherapy has been considered indispensable for most patients with a high-grade glioma. However, despite recent advances in surgery, radiotherapy and chemotherapy, survival of glioma patients remains poor because of tumor heterogeneity and strong therapeutic resistance (5,6). The 5-year survival rates of low-grade glioma are 30–70% depending on histology (7). GBM, as the most aggressive type, is rapidly growing and highly infiltrative making complete surgical removal impossible. Thus, patients with GBM usually have the worst prognosis with a median survival of 9–12 months even after surgical resection, radiation therapy and chemotherapy (8). Although WHO grade is a well-accepted prognostic factor in patients with glioma, the prognosis of patients within different grades may be different. Thus, the identification of prognostic markers might help to assess more precisely the prognosis and to address more clearly the use of adjuvant therapy.

Matrix metalloproteinases (MMPs) are a group of zinc-dependent proteases that have been viewed as key modulators of tumor invasion and metastasis because of their extracellular matrix (ECM) degrading capacity, which could enhance tumor cell invasion (9–12). However, MMPs exhibit considerable promiscuity with respect to their substrates, leading to considerable redundancy in biologic functions. It has been proved that expression of MMPs is associated with tumor aggressiveness in various human malignancies (13–15). MMP-9, also known as 92 kDa type IV collagenase, 92 kDa gelatinase or gelatinase B, has been found to be activated or enhanced by oncogenic proteins in many cancer types, such as breast cancer, prostate cancer, bladder cancer and pancreatic cancer (16–21). Previous study has also detected MMP-9 expression in glioma, which may facilitate the invasion and progression of multiple biological events required for glioma progression, such as invasion, migration and dissemination of glioma cells. In addition, MMP-9 also regulates tumor angiogenesis and may be required for the angiogenic switch that occurs during tumor neovascularization (22). Thus, efforts to better understand the prognostic role of MMP-9 in glioma may provide clinical insights into the efficacious therapeutic strategy. However, to our knowledge, the prognostic value of MMP-9 in astrocytic glioma is still unclear.

In this study, we investigated the protein expression level of MMP-9 in astrocytic glioma and analyzed its association with prognosis of patients.

**PATIENTS AND METHODS**

**PATIENTS AND SPECIMENS**

This research has been approved by the ethics committee of Tangdu Hospital and General Hospital of PLA Chengdu Military Region. Patients with WHO Grade I glioma had been excluded as they have extremely good prognosis (23). None of these patients had received radiotherapy or chemotherapy prior to surgery. Eligible patients with WHO Grade III glioma received stereotactic fractionated radiotherapy with a median total dose of 36 Gy postoperatively; eligible WHO Grade IV glioma patients received stereotactic fractionated radiotherapy with a median total dose of 55 Gy and a median of three courses of carmustine given at 4-week intervals postoperatively. In addition, normal brain tissue samples were taken from 71 patients who underwent surgery for reasons other than malignancy, such as cerebral trauma; these normal control samples were collected by partial resections of normal brain tissue required as decompression treatment for severe head injury to reduce increased intracranial pressure. The histomorphology of all the tissue specimens was confirmed by the Department of Pathology, Tangdu Hospital. The specimens were fixed in 10% formaldehyde and imbedded in paraffin for histological sections. Patients’ clinical information, such as age, sex, Karnofsky performance status (KPS) score and WHO grade, was collected and stored in a database. Follow-up information of all the eligible patients was updated every 3 months by telephone visit and questionnaire letters. Overall survival was calculated from the date of the initial surgical operation to death. Patients who died of diseases not directly related to glioma had been excluded from this study. Death of participants was ascertained by reporting from the family and verified by review of public records.

**IMMUNOHISTOCHEMICAL ASSAYS**

Four-micrometer-thick sections cut from the paraffin-embedded glioma samples were deparaffinized and rehydrated. For antigen retrieval, sections were placed in citrate buffer and heated at 90°C in a microwave oven for 15 min. The sections then were treated with 3% hydrogen peroxide for 15 min at room temperature. After washing in phosphate-buffered saline (PBS), endogenous biotin was blocked with 30% egg white for 20 min at room temperature. After washing, non-specific binding was blocked with normal goat or rabbit serum. These sections were then incubated overnight with rabbit polyclonal MMP-9 antibody (1:200, Abcam) in PBS at 4°C. After rinsing with PBS, secondary goat anti-rabbit IgG (1:400, Sigma) was added, and the sections were incubated for 1 h at room temperature. After rinsing with PBS, ready-to-use streptavidin–horseradish peroxidase complexes were added and incubated for 20 min at 37°C. The color was developed by 0.05% 3',3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) plus 0.01% hydrogen peroxide for 5 min at room temperature. The nucleus was counterstained with hematoxylin for 2 min at room temperature. Negative controls were performed by replacing the primary antibody with pre-immune rabbit
serum. Appropriate positive and negative controls were performed in each run of immunohistochemistry assay.

**Evaluation of Staining**

The tissue specimens were viewed separately by two pathologists without prior knowledge of the clinical or clinicopathological status of the specimens. The staining was evaluated by scanning the entire tissue specimen under low magnification (×40) and then confirmed under high magnification (×200 and ×400). An immunoreactivity score (IRS) system based on the proportion and intensity of positively stained cancer cells was applied (24). The extensional standard: (A) a number of positive stained cells of ≤5% scored 0, 6–25% scored 1, 26–50% scored 2, 51–75% scored 3 and >75% scored 4 (B) intensity of stain: colorless scored 0, pallide-flavens scored 1, yellow scored 2 and brown scored 3. Multiplying A and B, the staining grade was stratified as absent (−, 0 score), weak (+, 1–4 score), moderate (+++, 5–8 score) and strong (+++, 9–12 score). The concordance of MMP-9 scoring between the two pathologists was 0.94 (j = 0.66; P < 0.001), indicating substantial agreement. Specimens will be rescored if difference in scores from two pathologists was more than 3 (24).

**Statistical Analysis**

All statistic analyses were performed using the SPSS® version 13.0 software (SPSS Inc., Chicago, IL, USA). Associations between MMP-9 expression and categorical variables were analyzed by the Mann–Whitney test or the Kruskal–Wallis test, as appropriate. Overall survival was calculated from the day of initial surgery until death or the end of follow-up. The cumulative survival after tumor removal was calculated according to Kaplan–Meier, with differences in survival distributions evaluated by the log-rank test. Cox proportional hazards regression with a stepwise selection procedure was performed in order to calculate unadjusted and adjusted hazard ratios (HRs). Differences with a P value of 0.05 or less were considered to be statistically significant.

**RESULTS**

**MMP-9 Staining and Its Association with Clinicopathological Characteristics**

MMP-9 staining was found localized mainly in the cytoplasm of neoplastic astrocytes (Fig. 1), which is consistent with previous study (25). On the basis of IRS system on MMP-9 staining, 40 samples were defined as strong positive staining (+++), 61 samples were defined as moderate positive staining (++), 72 samples were defined as weak positive staining (+) and 99 samples were defined as negative staining (−) of MMP-9 among all the eligible 272 glioma specimens. By contrast, 67 samples were defined as negative staining (−) and 4 samples were defined as weak positive staining (+) of MMP-9, while no moderate positive staining (+++) or strong positive staining (+++) was detected among 71 normal brain tissues. Thus, the difference in MMP-9 staining between glioma and normal brain was statistically significant (P < 0.001), indicating that MMP-9 expression in glioma is increased compared with normal brain.

We further analyzed the association between MMP-9 staining and clinicopathological characteristics of patients. Results showed that the positive ratio of MMP-9 tends to increase from Grade II to Grade IV glioma and strong positive MMP-9 staining was more frequently detected in high-grade glioma, suggesting that MMP-9 staining was significantly associated with WHO grade of glioma (P < 0.001; Table 1). Furthermore, it is also found that the positive ratio of MMP-9 was statistically higher in patients with KPS <80 in comparison with patients with KPS ≥80 (P = 0.021; Table 1). However, the positive ratio of MMP-9 was not found to be associated with patient sex (P = 0.833) or age (P = 0.774; Table 1).

**Relationship Between MMP-9 Staining and Prognosis of Patients**

During the follow-up period, 162 of the 272 patients with glioma (59.6%) had died. Kaplan–Meier postoperative analysis was used to analyze the survival rate of patients with glioma of hierarchical MMP-9 staining. Results showed that patients with glioma of higher MMP-9 expression tend to...
have poorer overall survival (log-rank test: \(P < 0.001\); Fig. 2). The median survival time of patients with negative (−) expression of MMP-9 could not be estimated for all patients who survived for a time more than the overall median level. While the postoperative median survival time of patients with weak positive (+) expression of MMP-9 was 33 months [95% confidence interval (CI): 22.8–43.2], and those of patients with moderate positive (+++) and strong positive (++++) of MMP-9 were 17 months (95% CI: 12.5–21.1) and 11 months (95% CI: 8.5–13.3), respectively. When unadjusted HR was considered with MMP-9-negative (−) staining as a reference, patients with glioma of strong positive MMP-9 staining had a 7.41-fold higher risk of death compared with those with glioma of negative MMP-9 staining (95% CI: 3.66–15.21; \(P < 0.001\)), and the unadjusted HR of moderately positive (++) and weak positive (+) groups was 4.56 (95% CI: 3.42–11.01; \(P < 0.001\)) and 2.56 (95% CI: 1.43–5.02; \(P = 0.006\)), respectively. As far as clinicopathological characteristics were considered, WHO grade was proved to be associated with overall survival since patients with a high-grade glioma tend to have worse overall survival and higher risk of death compared with those with a low-grade glioma (\(P < 0.001\)). Moreover, KPS score was also proved to be associated with survival of patients since patients with glioma of KPS score <80 tend to have worse overall survival and higher risk of death (\(P = 0.032\)). However, sex or age had no prognostic value on overall survival of patients with glioma (Table 2).

### DISCUSSION

Despite that great progress has been made in surgical, radioactive and chemical modalities, the prognosis of glioma remains poor. And the molecular mechanism for the aggressiveness of glioma and the suboptimal response to therapies is still not clear. Recent studies suggested that a diversity of biological changes in glioma cells may account for the poor overall survival of patients with glioma (26). And several clinicopathological features have been considered important prognostic factors for gliomas, such as WHO grade and KPS score. However, these factors may not estimate prognosis in glioma patients accurately because patients’ heterogeneity for the outcome of patients in each grade is highly variable and genetic differences among them may contribute to their different survival. Thus, regular treatments do not benefit all patients equally and adverse effects of the treatments may also dramatically deteriorate the quality of life of some patients. Thus, it is hoped that a greater understanding of molecular mechanism involved in glioma survival will lead to new insights into
accurate prediction of patient prognosis, which is critical to the selection of appropriate therapeutic approaches.

In this study, we investigated MMP-9 protein expression in 272 cases of astrocytic glioma specimens and its association with clinical data as well as prognosis. The results showed that MMP-9 protein expression is increased in glioma compared with that in normal brain. When considering clinicopathological characteristics, MMP-9 expression was also proved to be associated with WHO grade for high expression of MMP-9 was more likely to be detected in high-grade glioma, which might lie behind the aggressiveness of high-grade glioma. Our results also showed a close relationship between MMP-9 expression and KPS score of patients for MMP-9 expression were higher in patients with KPS 80 compared with those with KPS 80, suggesting that the function of MMP-9 on tumor progression might affect clinical behavior of patients with glioma. These results indicated that MMP-9 may play an oncogenic role in glioma and related to tumor progression.

Moreover, Kaplan–Meier analysis of overall survival showed that patients with glioma of higher MMP-9 expression tend to have worse overall survival compared with patients whose tumors have lower MMP-9 expression, suggesting that MMP-9 was significantly associated with overall survival of patients with glioma. When the Cox proportional hazards model was adjusted for sex, age, KPS, WHO grade and treatment, results showed that MMP-9 was a marker of poor overall survival independent of the known clinical prognostic indicators. Therefore, it could constitute a molecular prognostic marker identifying who are more likely to have higher risk of death, and therefore, good candidates to receive more aggressive treatment. The function of MMP-9 facilitating the initiation and progression of multiple biological events required for glioma progression, such as migration, invasion, proteolytic digestion and dissemination of glioma cells, due to its capacity of digesting and degrading components of ECM may account for the prognostic value of MMP-9 in glioma.

In conclusion, this study proved that MMP-9 expression was increased in astrocytic glioma and associated with tumor progression, indicating that MMP-9 may be a mediator for the functions of oncogene in the progression of astrocytic glioma. It is also demonstrated that MMP-9 was an independent prognostic factor of patients with astrocytic glioma. These findings suggested that MMP-9 may be a potential diagnostic and even a potential therapeutic target in patients with astrocytic glioma.

### Funding

This work was supported by the National Natural Science Foundation of China (31070940).
Conflict of interest statement

None declared.

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