Abstract. Glioma is the most common human brain cancer and has poor prognosis. Messenger RNA profiling identified that sineoculis homeobox homolog 1 (Six1) is dysregulated in glioma tumor progenitor cells from glial progenitor cells isolated from normal white matter. However, the expression and role of Six1 in glioma remains unclear. The purpose of the present study was to investigate the expression level of Six1 in glioma tissues and the association between Six1 expression and clinicopathological characteristics and prognosis of gliomas. The Six1 protein was detected by immunohistochemistry in 163 glioma tissues of distinct malignancy grades, and Kaplan-Meier survival analysis was performed to assess the prognosis of the patients. The Six1 protein was stained in 49.1% (80 out of 163) of the glioma tissues, including 34.2% of low-grade (World Health Organization (WHO) I/II) gliomas and 80.8% of high-grade (WHO III/IV) gliomas. Normal brain tissues rarely expressed the Six1 protein. In addition, Six1 expression was significantly associated with WHO grade (P<0.001). According to the log-rank test and Cox regression model, Six1 may be suggested as an independent prognostic factor, in addition to the WHO grade. Overall, Six1 protein expression varies between different grades of glioma and is associated with the WHO grade. Upregulation of Six1 is more frequent in high-grade glioma and is an independent prognostic factor of poor clinical outcome.

Introduction

Glioma is the most common brain tumor and markedly affects patient survival due to the high metastasis and recurrence rate (1,2). According to the World Health Organization (WHO), there are 4 malignancy grades, consisting of grade I, which may develop in a benign pattern; grades II-III, which may invade the adjacent brain tissues and gradually develop into highly aggressive grade IV glioma, which is also termed glioblastoma (3,4). Despite developments in therapies that involve surgical resection, radiation therapy and chemotherapy, the prognosis of glioma has not been significantly improved over the past few decades (5). The overall survival rate of high-grade gliomas is only 40% at 1 year, and the 5-year survival rate is <10% (6). Thus, understanding the molecular etiology of glioma may aid the development of more effective treatments.

Sineoculis homeobox homolog 1 (Six1) is a mammalian homolog of the Drosophila sine oculis gene, and the gene is highly conserved between Drosophila and humans (7,8). The correct expression of this gene is crucial for the development of multiple organs, including the brain, eye, ear, craniofacial structures and kidney sensory structures (9-11). In addition to the involvement of Six1 in the early development of organs, the gene is often misexpressed in diverse tumors, including breast cancer (12), ovarian cancer (13,14), cervical cancer (15,16), Wilms' tumors (17), rhabdomyosarcomas (18) and hepatocellular carcinoma (19). Additionally, the misexpression of Six1 in cancer may induce developmental programs out of context, contributing to tumor onset and progression (20,21).

However, the association between Six1 expression and glioma remains unknown. The present study aimed to investigate the expression of Six1 in gliomas with distinct clinicopathological features and to analyze the effect on the prognosis of glioma patients.

Materials and methods

Patients and tissues. The present study enrolled 163 patients with glioma, who had been clinically and histopathologically diagnosed and were retrieved for tissue microarray (TMA) construction and immunohistochemical (IHC) analysis from the Department of Neurosurgery of Inner Mongolia People's Hospital (Hohhot, Inner Mongolia, China). In accordance with the WHO classification, all cases were classified as shown in Table II, with 44 patients diagnosed with grade I disease,
67 patients diagnosed with grade II disease, 21 patients diagnosed with grade III disease and 31 patients diagnosed with grade IV disease. The mean age of patients at diagnosis was 45.26±10.43 years (range, 9-70 years), with 99 male and 64 female patients. Follow-up data were available for 153 patients (range, 4-77 months; mean, 40.9±19.95 months). The study protocol was performed with approval from the Ethics Committee of the Inner Mongolia People's Hospital, and informed consent was obtained from all patients.

**TMA construction and immunohistochemistry (IHC).** Representative sections of glioma or normal brain tissues in the pre-existing paraffin-embedded tissue blocks were determined according to the overlaid hematoxylin and eosin staining slides. The TMA was constructed by excising a 1.0 mm diameter cylinder from the representative section of each block using a needle, and placing the cylinders into an array on a recipient paraffin block. Subsequently, multiple 5-µm thick sections were cut from the TMA block and mounted on microscope slides for IHC analysis. The TMA consisted of a total of 163 cases of glioma and 16 cases of normal control paraffin-embedded tissue. The clinical characteristics of the patients are summarized in Table I. The TMA slide was dried overnight at 37˚C, deparaffinized in xylene, rehydrated in graded alcohol solutions, and then immersed in 3% hydrogen peroxide for 10 min to inactivate peroxidase activity. Antigen retrieval was performed by microwave heating with 0.01 mol/l citrate buffer at 100˚C for 15 min, and the slides were then cooled for 30 min at room temperature to expose antigenic epitopes. The slides were pre-incubated with 5% normal goat serum (Jetyaw Biotech, Co., Ltd., Guangzhou, China) at room temperature for 30 min to reduce the non-specific reaction. The primary rabbit anti-Six1 polyclonal antibody (cat. no. HPA001893; Atlas Antibodies, Stockholm, Sweden) was diluted (1:1,500) with 1X phosphate-buffered saline (PBS) and applied overnight in a humidity chamber at 4˚C. The slide was sequentially incubated with a polymer peroxidase-labeled secondary antibody (1:1,500; ZDR-5306; ZSGB-Bio, Beijing, China) for 30 min at room temperature, and then visualized by catalysis of 3,3'-Diaminobenzidine Horseradish Peroxidase Color Development kit (Beyotime Institute of Biotechnology, Haimen, China). Finally, the sections were counterstained by hematoxylin. A known IHC-positive slide was used as a positive control, and PBS replaced the anti-Six1 primary antibody in the condition that was used as a control.

**Evaluation of IHC.** Immunoreactivity for the Six1 protein was scored using the staining intensity and positive percentage. Tissue sections were classed as expressing Six1 if cells showed immunoreactivity in the nucleus or cytoplasm when observed by an evaluator that was blinded to the clinical history and outcome. In total, 10 low-power fields were randomly selected per tissue, and the cells were counted under a high-power field. The positive percentage scores were then acquired. Positive percentage scores were assessed according to the following scale: 0, 0% cells; 1, 0-25% cells; 2, 25-50% cells; and 3, >50% cells. Staining intensity was then also scored semiquantitatively as follows: 0, None; 1, mild; 2, moderate; and 3, intense. A total score ranging between 0 and 9 was then obtained by multiplying the positive percentage score and intensity score for each research section. From the total scores, 0, 1-3, 4-6 and 7-9 were recorded as -, +, ++, and ++++, respectively. These scores were defined as no or low expression when the score was <4; positive or high expression when the score was ≥4. The scores were accepted if two investigators agreed with the values. Otherwise, the values were re-estimated until a consensus was reached. The investigators were in complete agreement in 80% of the cases, which indicated that the scoring method was highly reproducible.

**Statistical analysis.** Statistical analysis was performed using the SPSS statistical software program, version 18.0 (SPSS, Inc., Chicago, IL, USA). The association between Six1 protein expression and the clinicopathological data of patients with glioma was estimated using the χ² test. The association between survival and each variable was determined using the Kaplan-Meier method. Differences between survival rates were analyzed using the log-rank test and Cox regression analysis. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Six1 expression in 163 glioma tissues.** To identify the Six1 protein expression level, IHC staining was performed. Six1 expression was identified in 49.1% (80 out of 163) of all gliomas. According to the WHO grade, Six1 expression was identified in 34.2% of low-grade (WHO I/II) gliomas and 80.8% of high-grade (WHO III/IV) gliomas, respectively (Table I). Overall, the Six1 level in the high-grade tumors was significantly higher compared with the level in low-grade tumors (P<0.001; Table I), and the Six1 expression level in all normal brain tissues was also markedly lower compared with the level in glioma tissues (P<0.05; Table I). At the same time, the IHC staining revealed that the Six1 protein was mainly expressed in the cytoplasm (Fig. 1).

**Six1 expression and pathological indicators.** As shown in Table II, Six1 expression was significantly associated with the WHO grade (P<0.001), indicating that the status of Six1 expression was upregulated in high-grade glioma patients. No association was identified between Six1 expression and age. Also, no association was observed between Six1 expression and gender, indicating that Six1 expression was not dependent on the gender of the patients. Of the 4 grades, grade I exhibited the lowest expression level. However, no significant difference was observed between the expression of Six1 in grade III and IV gliomas (P=0.084).

**Six1 expression was associated with the prognosis of patients.** To evaluate the association between Six1 protein expression and the prognosis of patients, all glioma patients were allocated to two groups, the low and high Six1 expression groups. A log-rank test and Kaplan-Meier analysis were performed to assess the effect of Six1 expression on the patient survival. Out of the 163 patients, the survival data of 153 patients were available, among which 10 patients were still alive at follow-up and were censored. The high expression of Six1 in all 141 gliomas exhibited a significant difference from 21 patients with low expression (P<0.001; Fig. 2). As shown in Table III, the
The median of overall survival time in all patients was 41.0 months \([n=163; 95\% \text{ confidence interval (CI), 38.954-47.046}]\); the median survival time of patients with low Six1 expression was only 27.0 months (95\% CI, 13.892-40.108), whereas the median survival time of those with high Six1 expression was 46.0 months (95\% CI, 40.708-51.292). The log-rank test revealed that patients with low Six1 expression had a significantly shorter overall survival time compared with patients with high Six1 expression \((\chi^2=15.668; P<0.001; \text{Table III})\).

In addition, multivariate analysis was also performed to investigate whether Six1 was an independent prognostic factor for patient survival. As shown in Table IV, multivariate analysis identified Six1 expression \((P=0.045)\) and WHO grade \((P<0.001)\) as independent prognostic factors, instead of age and gender.

**Discussion**

Homeobox genes encode transcription factors that are essential for the development of numerous organs and control...
processes, such as proliferation, apoptosis, migration, and invasion (9,21-23). The Six1 homeoprotein is a member of the Six family of homeodomain transcription factors and has been found to be upregulated in multiple cancers, including breast cancer (12,20,24), rhabdomyosarcomas (18,25,26), hepatocellular carcinomas (19), ovarian cancer (13) and Wilms’ tumors (17). In addition, Six1 plays a role in cellular migration and invasion during embryogenesis (22,27-30) and in breast cancer (31,32). Notably, a recent study demonstrated that messenger RNA profiling of Six1 is dysregulated in A2B5+ glioma tumor progenitor cells from A2B5+ glial progenitor cells isolated from normal white matter (33).

Overexpression of vascular endothelial growth factor C (VEGF-C) has been detected in numerous cancers (34-37), and the role of VEGF-C in promoting lymphatic metastasis has been demonstrated in several VEGF-C overexpression animal models of mammary carcinoma (38-40). Previous study revealed that Six1 could coordinate with transforming growth factor β (TGF-β) to promote the expression of VEGF-C, thereby facilitating lymphatic metastasis in glioma (33). The combination of Six1 and TGF-β may provide a new therapeutic target for glioma treatment.

Table IV. Multivariate analysis of various prognostic indicators in patients with glioma, performed using the Cox regression model.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients, n</th>
<th>RR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (≤45 years/&gt;45 years)</td>
<td>81/82</td>
<td>0.728</td>
<td>0.522-1.016</td>
<td>0.062</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>99/64</td>
<td>1.015</td>
<td>0.733-1.406</td>
<td>0.928</td>
</tr>
<tr>
<td>WHO grade (I+II/III+IV)</td>
<td>111/52</td>
<td>2.695</td>
<td>1.838-3.952</td>
<td>0.000</td>
</tr>
<tr>
<td>Six1 expression (low/high)</td>
<td>141/22</td>
<td>1.670</td>
<td>1.011-2.760</td>
<td>0.045</td>
</tr>
</tbody>
</table>

RR, risk ratio; CI, confidence interval; WHO, World Health Organization; Six1, sineoculis homeobox homolog 1.
growth factor-β (TGF-β) to increase the expression of VEGF-C through two pathways. Firstly, SIX1 enhances TGF-β signaling by upregulating TGF-β receptor 1 (TβR1) expression, which promotes the activation of SMAD family member 2/3 (SMAD2/3) and its binding to the VEGF-C promoter, thus increasing the expression of VEGF-C. Secondly, SIX1 may cooperate with SMAD2/3 to bind to the VEGF-C promoter and modulate VEGF-C expression. In tumor cells without SIX1 expression, the expression of VEGF-C was not notably affected by TGF-β stimulation, although SMAD2/3 was phosphorylated and was able to bind to the VEGF-C promoter. Therefore, SIX1 is necessary for TGF-β to induce increased expression of the VEGF-C gene (41). In addition, overexpression of SIX1 significantly enhances the activity of the cyclin D1 promoter in pancreatic cancer and promotes cell cycle progression and proliferation (42). Furthermore, SIX1 overexpression is positively correlated with the disease-free survival and 5-year overall survival rates of patients with breast cancer (43). However, the expression model and prognostic value of SIX1 in the gliomas were rarely reported. Therefore, it was hypothesized in the present study that SIX1 may be expressed and play a role in gliomas of different malignancy grades.

In the present study, the expression of the SIX1 protein was detected in glioma tissues of various malignancy grades, and it was found that the level of SIX1 expression in all glioma tissues was significantly higher than the expression level in normal brain tissues. Furthermore, SIX1 expression was found to be associated with the WHO grade, but not with age and gender. The present results indicated that SIX1 expression in glioma is responsible for glioma progression. In order to investigate the effect of SIX1 expression on the prognosis of glioma patients, 163 patients were followed up subsequent to surgery. SIX1 was identified as an independent factor to significantly predict the overall survival time of glioma patients. Firstly, the log-rank test revealed that patients with high SIX1 expression possess a significantly shorter median overall survival time of 27 months, compared with the median of 46 months in the low expression group. Secondly, Cox regression analysis identified that SIX1 may act as an independent prognostic factor, in addition to the WHO grade. This indicated that SIX1 may be recommended as a useful marker associated with a worse prognosis in glioma patients.

In conclusion, SIX1 is differently expressed in different grades of glioma and is associated with the WHO grade of disease, indicating a worse prognosis in patients with glioma. In addition, the SIX1 protein may be suggested as a useful prognostic biomarker for glioma, including glioblastoma.

Acknowledgements

The authors thank Dr Hongdian Zhang (Department of Neurosurgery, Affiliated Bayi Brain Hospital, General Hospital of Beijing Military Region, Beijing, China) for his assistance with writing the manuscript.

References


