Clinical significance of B7-H6 protein expression in astrocytoma

Jian-gui Guo1,#
Cheng-cheng Guo2,#
Zhen-qiang He2
Zhi-gang Liu3
Yang Wang2
Yong-gao Mou2

1Department of Radiation Oncology, 2Department of Neurosurgery, Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangzhou, People’s Republic of China; 3Department of Radiation Oncology, Key Laboratory of Translational Radiation Oncology, Hunan Cancer Hospital, The Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha, People’s Republic of China

#These authors have contributed equally to this work

Abstract: Currently, immunotherapy by blocking the immune checkpoint inhibitors, such as anti-PD-1, has been carried out in many clinical studies on recurrent glioma, and the preliminary results are satisfactory, which provides a rationale for the exploration of immune checkpoint inhibitors in glioma. B7-H6 is a newly discovered member of the B7 family, which triggers antitumor of natural killer cell cytotoxicity and cytokine secretion by binding the NKp30 receptor. B7-H6 mRNA and protein expressions, which are not detected in normal tissues, are expressed mainly on the cell surface of various primary tumors and cell lines. However, up until now, there is no data about the clinical significance of B7-H6 expression in astrocytoma patients. The present study provides an investigation on the relationship between prognostic and clinical value of B7-H6 protein in astrocytoma tissues. All the astrocytic glioma tissues were stained for B7-H6. Immunohistochemistry stain of 122 astrocytoma samples showed that immunoreactivity of B7-H6 was seen predominantly in the cytoplasm. The B7-H6 expression did not show significant relevance with patient age, sex distribution, Karnofsky performance status score, extent of resection, and tumor location in astrocytoma patients, but B7-H6 positive expression is significantly associated with World Health Organization grade (P=0.046). However, the survival rate after operation presented no significant difference of B7-H6 expression in astrocytoma patients. Kaplan–Meier analysis and the log-rank test revealed that B7-H6 expression cannot predict the overall survival. In all, it seems that the B7-H6 expression might be a marker to differentiate the World Health Organization grade level of astrocytoma, but the prognosis value of B7-H6 in astrocytoma should be studied in detail.

Keywords: B7-H6, astrocytoma, glioma, immunotherapy

Introduction

Astrocytic glioma is the most common type of primary malignant brain tumor.1 The 5-year survival rate in patients with glioma is among the lowest for all cancers.2 Conventional therapies, such as surgery, chemotherapy, and radiotherapy, play an important role in the treatment of malignant gliomas; however, the prognosis of malignant gliomas is still poor.3,4 Since astrocytoma patients face a dismal prognosis and have limited therapeutic options, developing a new treatment modality is necessary. Immunotherapy with immune checkpoint inhibitors, such as ipilimumab and nivolumab, has provided relevant clinical improvements in other advanced tumors for which conventional therapies have had limited success, making immunotherapy an appealing strategy in glioma, which provides a rationale for the exploration of immune checkpoint inhibitors in glioma.

The B7 family members, which played critical roles in the control and fine tuning of antigen-specific immune responses, have great implications for the treatment of cancer.5 At present, several B7 family members have been found in glioma. B7-H6 is
a newly discovered member of the B7 family, which triggers
the antitumor of natural killer cell cytotoxicity and cytokine
secretion by binding the NKp30 receptor. Recent studies
showed that B7-H6 mRNA and protein expressions have
not been detected in most normal adult tissues, while B7-H6
cell surface expression is observed in tumor cell lines from
various origins, such as lymphoma, leukemia, melanoma, and
carcinoma as well as on primary tumor blood cells, which
indicates that its expression may take an important part in
tumor prognosis. However, up until now, no data about
the clinical significance of B7-H6 expression in patients of
astrocytoma have been reported.

In this article, we investigated the B7-H6 expression in
tumor specimens collected from a large cohort of astrocytoma
patients. We then confirmed the correlation of intratumoral
B7-H6 expression with various clinicopathological param-
eters and patient survival to investigate whether B7-H6 acts as a novel identified prognostic marker in astrocytoma
patients.

Materials and methods
Paraffin-embedded tumor samples were obtained from 122
astrocytoma patients who underwent surgery at the Sun
Yat-sen University Cancer Center, Guangzhou, People’s
Republic of China, between 2000 and 2008. Patients with
autoimmune diseases were excluded. None of the patients
had received anticancer treatments prior to surgery. The
follow-up dates of the patients in this study are available and
complete. Overall survival (OS), which was defined as the
time from operation to patient death or the last follow-up, was
used as a measure of prognosis. This study was approved by
the Ethics Committee of the Sun Yat-sen University Cancer
Center, and written informed consent was obtained from
each patient.

Immunohistochemical staining
Immunohistochemical staining was performed using a two-
step method (Envision™). Paraffin-embedded tissues were
cut into 5 μm serial sections, transferred onto adhesive
slides, and dried at 65°C for 30 minutes. The sections were
deparaffinized with xylene and rehydrated through graded
alcohols. Endogenous peroxidase activity was blocked with
0.3% hydrogen peroxide solution for 30 minutes, and anti-
gen retrieval was performed at 100°C for 30 minutes in a
citrate buffer (10 mmol/L, pH 6.0). After being washed three
times with phosphate-buffered saline (PBS) for 5 minutes
each, the sections were incubated with 10% normal goat
serum to block nonspecific binding. The slides were then
incubated overnight at 4°C with rabbit anti-human B7-H6
(Abcam, Cambridge, MA, USA; dilution 1/100). The slides
were incubated with horseradish peroxidase (ChemMate™,
DAKO Envision™ Detection Kit, Dako, Glostrup, Denmark)
at room temperature for 30 minutes. After the slides were
washed in PBS, the visualization signal was developed with
3,3′-diaminobenzidine solution, and all slides were counter-
stained with hematoxylin, dehydrated in graded alcohol,
and mounted with a neutral resin. Negative controls were
prepared by replacing the primary antibody with PBS. Human
gallbladder tissue was used as a positive control.

The B7-H6 immunostaining score was calculated as
both the percentage of positively stained tumor cells and the
staining intensity. The percent positivity was scored as “0”
(<5%, negative), “1” (5%–25%, sporadic), “2” (25%–50%,
focal), or “3” (>50%, diffuse). The staining intensity was
scored as “0” (no staining), “1” (weakly stained), “2” (moder-
ately stained), or “3” (strongly stained). Both the percentage
of positive cells and the staining intensity were evaluated
under double-blind conditions. Two independent pathologists
examined and scored each sample without any knowledge
of the patient outcome (double-blinded). The B7-H6 score
was calculated as the percentage positive score × the staining
intensity score and ranged from 0 to 9. Based on the B7-H6
expression levels, the astrocytoma patients were divided into
two groups: the low B7-H6 expression group (score 0–4) and
the high B7-H6 expression group (score 6–9).

Statistical analysis
The statistical significance of the correlations between B7-H6
expression and the clinicopathologic features was analyzed
using the chi square (χ²) test. Univariate and multivariate
survival analyses were performed using the Kaplan–Meier
analysis and log-rank test. All statistical analyses were
performed with the SPSS software (Version 19.0; StataCorp
LP, College Station, TX, USA). For all tests, a P-value
of ≤0.05 was considered statistically significant.

Results
Study population
The patient characteristics are presented in Table 1. Of the 122
patients examined, 91 (74.6%) had died before the end of the
observation period. The median age of the study population
was 42 years (range: 2–75 years). The majority of patients
(90/122, 73.8%) underwent total tumor resection. There were
41 cases (33.6%) of grade II, 32 cases (26.2%) of grade III,
and 49 cases (40.2%) of grade IV astrocytoma, according to
the World Health Organization (WHO) classification criteria.
The median follow-up for the entire cohort was 35.3 months (range: 2.0–135.0 months). The 2-year survival rate for the entire study population was 43.4%.

### Immunohistochemical characteristics

B7-H6 expression was immunohistochemically assessed in 122 glioma specimens, of which 102 (83.6%) showed low B7-H6 expression and 20 (16.4%) exhibited high B7-H6 expression. In the positive specimens, immunoreactivity of B7-H6 was seen predominantly in the cytoplasm (Figure 1).

### Relationships between B7-H6 and clinicopathological characteristics

Table 2 summarizes the associations between B7-H6 protein expression and the clinicopathological characteristics of human astrocytoma cases. The B7-H6 protein expression presented no significant association with patient age, sex distribution, KPS performance status score, extent of resection, and tumor location (Table 2, $P>0.05$). However, the B7-H6 expression was significantly associated with the tumor WHO grade (Table 2, $\chi^2=6.142, P=0.046$).

### Correlation of intratumoral B7-H6 expression with patient survival

Kaplan–Meier analysis and the log-rank test were used to evaluate the effect of the B7-H6 expression on OS in astrocytoma patients. The prognostic value of B7-H6 for OS was evaluated by comparing the patients with high and low B7-H6 expression. According to the Kaplan–Meier survival analysis, the different OS between the high and low B7-H6 expression did not have statistical significance (Figure 2, Log-rank [$P>0.05$]). The separate analysis of the OS of B7-H6 expression with the different WHO grades also showed no statistical significance (Figure 3, Log-rank [$P>0.05$]). Univariate and multivariate analyses were conducted using Cox proportional hazards model to examine the impact of B7-H6 expression and other clinicopathological parameters in astrocytoma patients. Univariate analysis showed that the WHO grades were significant prognostic factors (Table 3, $P<0.05$). Multivariate Cox regression analyses showed that the WHO grade was an independent prognostic factor (Table 3, $P<0.05$), whereas univariate and multivariate analyses revealed that B7-H6 was not an independent prognostic factor (Table 3, $P>0.05$). Thus, the survival analysis did not confirm the prognostic significance of B7-H6 expression in astrocytoma.

### Discussion

Primary nervous system tumors account for 1.4% of all cancers and cause 2.4% of all cancer deaths in the US. Although a lot of progress has been made in reducing mortality rates owing to both earlier detection and improved therapies, including chemotherapy and radiotherapy, glioma still is a major threat to human health. It would therefore be valuable to develop more approaches that could improve survival rates in glioma patients. Nowadays, immunotherapy is an important and effective combination detection for malignancy. Immunotherapy such as blocking the immune checkpoint molecule that can recover the capability of T-cells to discover and attack cancer cells and promote their anticancer response is a promising strategy in cancer therapy. Antibody-based blockade of CTLA-4 ligation on T lymphocytes is associated with enhanced antitumor immunity in animal models of cancer and in patients with advanced melanoma. Early-stage clinical trials reported that blocking the immune checkpoint molecule programmed death 1 (PD-1) and its ligand (PD-L1) could induce impressive durable responses in patients with advanced cancer. The introduction of immune checkpoint inhibitors has dramatically changed the prognosis for some advanced tumors. The US Food and Drug Administration first approved ipilimumab (a checkpoint inhibitor targeted CTLA-4) to treat patients with late-stage melanoma in March 2011, and pembrolizumab and nivolumab (two checkpoint inhibitors that target PD1) for unresectable or metastatic...
melanoma in late 2014. More new agents targeting PD1 or PD-L1 have been investigated alone and in combination treatment for various cancers, including glioma. Because of the immunotherapy progress in other cancers and the current understanding of the interaction between the brain and the immune system, it provides a rationale for the exploration of immune checkpoint inhibitors in astrocytoma.

The B7 family members are transmembrane proteins which can produce a co-stimulatory signal or a co-inhibitory signal to regulate immune responses. They are widely expressed on tumor cells and the tumor cell microenvironment. The co-inhibitory B7-H ligands promote the suppression of host antitumor response, whereas the co-stimulatory molecules might affect the growth of the malignant tumor cells. The two primary members of B7-1 and B7-2 provide a balance of positive and negative signals required for appropriate priming of naïve T-cells by interacting with CD28 and CTLA-4. The other members, such as B7-H1, B7-DC, B7-H2, B7-H3, and B7-H4, described as B7 homologs, have a less
restricted distribution and their expression in cancer has been predictive of patient prognosis. B7-H6 is among the most recently identified members of the B7 superfamily, which is a PD-L1/B7-H3 homologue specifically binding the CTLA-4-homologous NK-effector molecule NKp30. Unlike other B7 family members, B7-H6 mRNA and protein expression are not detected in normal tissues while expressed mainly on the cell surface of various primary tumors and cell lines. B7-H6 and the expression of B7-H6 on tumor cells induces NKp30-dependent cell activation and cytotoxicity. Therefore, it seems that its expression may play an important role in tumor prognosis. If B7-H6 expression is specific and stable in tumor cells, immunotherapy based on the effective blockade of the tumor-associated B7-H6 could be a promising clinical strategy. The present study provides the first investigation about the relationship between prognostic and clinical value of B7-H6 protein in astrocytoma tissues. All the astrocytoma tissues were stained for B7-H6. Immunohistochemistry stain of 122 astrocytoma samples showed that immunoreactivity of B7-H6 was seen predominantly in the cytoplasm, which is consistent with other studies. The B7-H6 expression did not show significant relevance with patient age, sex distribution, Karnofsky performance status score, extent of resection, and tumor location in astrocytoma patients. However, B7-H6 positive expression is significantly associated with WHO grade, which demonstrated that B7-H6 expression might be a marker to differentiate the WHO grade level of astrocytoma. That suggested B7-H6 expression level was significantly associated with the degree of differentiation, whereas it was not correlated with other clinical parameters. This result is similar to the study in gastric carcinoma and lung cancer. This implied that high B7-H6 expression in astrocytoma patients may indicate more malignancy and calls for more aggressive treatment and close surveillance. However, the survival rate after an operation presented no significant difference of B7-H6 expression in astrocytoma patients. The separate analysis of the OS of B7-H6 expression with different WHO grades also showed no statistical significance. Univariate and multivariate analyses revealed that B7-H6 was not an independent prognostic factor. Thus, Kaplan–Meier analysis and the log-rank test revealed that B7-H6 expression may not be useful for predicting the OS of astrocytoma patients, which also have similar outcome in gastric carcinoma and lung cancer. Altogether, as detected by immunohistochemistry, the survival analysis did not confirm the prognostic significance of B7-H6 expression in astrocytoma. As the number of patients we

**Table 2** Correlation of B7-H6 expression with clinicopathological characteristics of astrocytoma patients

| Variable            | B7-H6 expression | P-value | χ² value |
|---------------------|------------------|---------|----------|
|                     | Low  | High |       |        |
| All cases           | 102  | 20   |        |        |
| Sex                 |       |      |        |        |
| Male                | 59   | 13   |        |        |
| Female              | 43   | 7    | 0.552  | 0.354  |
| Age (years)         |       |      |        |        |
| ≥ 40                | 48   | 10   |        |        |
| < 40                | 54   | 10   | 0.058  | 0.810  |
| KPS                 |       |      |        |        |
| ≥ 70                | 95   | 20   |        |        |
| < 70                | 7    | 0    | 0.496  | 0.464  |
| Extent of resection |       |      |        |        |
| Total               | 75   | 15   |        |        |
| Subtotal            | 27   | 5    | 0.891  | 0.019  |
| Location            |       |      |        |        |
| Supratentorial      | 96   | 17   |        |        |
| Infratentorial      | 6    | 3    | 0.338  | 0.919  |
| WHO grade           |       |      |        |        |
| II                  | 37   | 4    |        |        |
| III                 | 29   | 3    |        |        |
| IV                  | 36   | 13   | 0.046  | 6.142  |
| Survive time after surgery |       |      |        |        |
| ≥ 2 year            | 44   | 9    |        |        |
| < 2 year            | 58   | 11   | 0.878  | 0.024  |

**Note:** P-value < 0.05 was considered significant.

**Abbreviations:** KPS, Karnofsky performance status; WHO, World Health Organization.

**Figure 2** Kaplan–Meier survival curve in astrocytoma patients after surgery according to B7-H6 expression (n=122) (P=0.537).
Table 3  Univariate and multivariate Cox regression analyses of patient survival

| Covariate | Univariate | Multivariate |
|-----------|------------|--------------|
|           | HR | 95% CI | P-value | HR | 95% CI | P-value |
| Sex (male vs female) | 0.627 | 0.300–1.311 | 0.215 | 0.631 | 0.277–1.438 | 0.273 |
| Age (≥40 years vs <40 years) | 1.162 | 0.485–2.780 | 0.736 | 1.352 | 0.467–3.909 | 0.578 |
| KPS (≥70 vs <70) | 1.580 | 0.208–12.026 | 0.658 | 1.212 | 0.023–3.320 | 0.259 |
| Extent of resection (total vs subtotal) | 1.071 | 0.345–3.320 | 0.295 | 0.253 | 0.023–7.51 | 0.905 |
| Tumor location (supratentorial vs infratentorial) | 1.380 | 0.529–3.597 | 0.510 | 3.469 | 0.991–12.140 | 0.052 |
| WHO grade (II/III/IV) | 2.220 | 1.319–3.378 | 0.003 | 3.087 | 1.565–6.090 | 0.001 |
| B7-H6 (low vs high) | 1.308 | 0.552–3.097 | 0.542 | 0.911 | 0.352–2.360 | 0.849 |

Note: *P*-value <0.05.

Abbreviations: KPS, Karnofsky performance status; WHO, World Health Organization; CI, confidence interval; HR, hazard ratio.
undertaken to investigate the prognostic significance expression of B7-H6 in astrocytoma.

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Disclosure
The authors report no conflicts of interest in this work.

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