Association between small heat shock protein B11 and the prognostic value of MGMT promoter methylation in patients with high-grade glioma

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OBJECTIVE This study investigated the role and prognostic value of heat shock proteins (HSPs) in glioma.

METHODS Data from 3 large databases of glioma samples (Chinese Glioma Genome Atlas, Repository for Molecular Brain Neoplasia Data, and GSE16011), which contained whole-genome messenger RNA microarray expression data and patients’ clinical data, were analyzed. Immunohistochemical analysis was performed to validate protein expression in another set of 50 glioma specimens.

RESULTS Of 28 HSPs, 11 were overexpressed in high-grade glioma (HGG) compared with low-grade glioma. A univariate Cox analysis revealed that HSPB11 has significant prognostic value for each glioma grade, which was validated by a Kaplan-Meier survival analysis. HSPB11 expression was associated with poor prognosis and was independently correlated with overall survival (OS) in HGG. This study further explored the combined role of HSPB11 and other molecular markers in HGG, such as isocitrate dehydrogenase 1 (IDH1) mutation and O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation status. HSPB11 expression was able to refine the prognostic value of IDH1 mutation in patients with HGG. However, when combined with MGMT promoter methylation status, among patients with a methylated MGMT promoter, those with lower levels of HSPB11 expression had longer OS and progression-free survival than patients with higher levels of HSPB11 expression or with an unmethylated MGMT promoter. Moreover, within the MGMT promoter methylation group, patients with low levels of HSPB11 expression were more sensitive to combined radiochemotherapy than those with high levels of HSPB11 expression, which may explain why some patients with HGG with a methylated MGMT promoter show tolerance to radiochemotherapy.

CONCLUSIONS HSPB11 was identified as a novel prognostic marker in patients with HGG. Together with MGMT promoter methylation status, HSPB11 expression can predict outcome for patients with HGG and identify those who would most benefit from combined radiochemotherapy.

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KEY WORDS HSPB11; prognosis; MGMT promoter methylation; glioma; oncology

Glioma is the most common type of tumor in the CNS. The WHO classifies glioma on a scale of Grades I–IV, according to histopathologic features.28 Grades III and IV are progressive, resistant to treatment, and grouped together as high-grade glioma (HGG) for the purposes of clinical management. Glioblastoma and anaplastic glioma comprise the majority of HGG cases. Since the incorporation of alkylating agents such as temozolomide (TMZ) into routine chemotherapy, the prognosis for patients with HGG has significantly improved. The epigenetic silencing of O6-methylguanine-DNA methyltransferase (MGMT) (which encodes a DNA repair protein) by promoter methylation is associated with favorable prognosis in patients with HGG.35,40,41 Numerous studies have also shown that MGMT promoter methylation can be used to predict the extent to which patients

ABBREVIATIONS CGGA = Chinese Glioma Genome Atlas; GBM = glioblastoma; GO = gene ontology; GSEA = Gene Set Enrichment Analysis; HGG = high-grade glioma; HSP = heat shock protein; IDH1 = isocitrate dehydrogenase 1; KPS = Karnofsky Performance Scale; LGG = low-grade glioma; MGMT = O6-methylguanine-DNA methyltransferase; mRNA = messenger RNA; OS = overall survival; PCR = polymerase chain reaction; PFS = progression-free survival; REMBRANDT = Repository for Molecular Brain Neoplasia Data; SAM = significance analysis of microarrays; TMZ = temozolomide.


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Heat shock proteins (HSPs) are highly conserved proteins present in normal cells. HSP expression is upregulated in response to endogenous and exogenous stress. HSPs have various functions, including protection against cytotoxic stress and maintenance of cellular homeostasis. High levels of HSP expression are required for cells to survive lethal conditions such as radiation, exposure to chemical agents, and hypoxia. Upregulation of HSP expression has been detected in many types of cancer and is often associated with poor clinical outcome and resistance to chemotherapy. Previous studies have also reported high levels of HSPs (including members of the small HSP family, a group of chaperones with monomeric molecular weights ranging from 12 to 43 kD and heat shock-like properties) in malignant glioma. However, there is limited information on the role and predictive value of HSPs in glioma.

In this study, messenger RNA (mRNA) microarray expression data from 3 independent, large databases of samples comprising all grades of glioma were analyzed. The expression level and prognostic value of HSPs in glioma were evaluated. HSPB11 was the only HSP that had significant prognostic value for Grades II–IV. Its expression was associated with poor prognosis and served as an independent prognostic factor for patients with HGG. HSPB11 also enhanced the predictive value of MGMT promoter methylation status in patients with HGG.

Methods
Patients and Samples
A total of 295 samples from the Chinese Glioma Genome Atlas (CGGA) were included in this study as the discovery set, which contained whole-genome mRNA expression microarray data and corresponding clinical information (Supplemental Table S1). Tumor samples were obtained from patients with newly diagnosed glioma who were treated by the CGGA group. Each sample underwent histological analysis, results of which were independent and determined the percentage of positive cells. We chose the 5 most heavily stained high-resolution fields under ×200 magnification, determined the percentage of positive cells, and calculated the average of the percentages. Five categories were used to assess the staining intensity in cells: 0, no positive staining; 1, 1%–10% positive staining; 2, 11%–30% positive staining; 3, 31%–50% positive staining; and 4, >50% positive staining.

Evaluation of Isocitrate Dehydrogenase 1 Mutation Status by DNA Pyrosequencing
Genomic DNA was isolated from frozen tumor samples using the QIAamp DNA Mini Kit (QIAGEN). The genomic region spanning wild-type R132 of isocitrate dehydrogenase 1 (IDH1) was analyzed by pyrophosphate sequencing using the following primers: 5’-GCT TGT GAG TGG ATG GGT AAA AC-3’ and 5’-biotin-TGG CCA ACA TGA CTT ACT TGA TC-3’. The polymerase chain reaction (PCR) analysis was performed in duplicate in a 40-μl reaction volume, containing 1 μl of 10 μM each forward and reverse primer, 4 μl 10× buffer for 3.2 μl of 2.5 mM deoxynucleoside triphosphate, 2.5 U hotstart Taq (Takara), and 2 μl of 10 μM DNA. The PCR conditions were as follows: 95°C for 3 minutes, 50 cycles of 95°C for 15 seconds, 56°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes (ABI PCR system 9700). Single-stranded DNA was purified from the total PCR products and subjected to pyrosequencing on a PyroMark Q96 ID System (QIAGEN) using the primer 5’-TG TGG ATG GGT AAA ACC T-3’ and EpiTect Bisulfite Kit (QIAGEN).

Evaluation of MGMT Promoter Methylation by DNA Pyrosequencing
The MGMT promoter methylation status was detected by DNA pyrosequencing as previously described. Bisulfite modification of DNA was performed using the EpiTect Bisulfite Kit. Sequences of the primer set used to amplify the MGMT promoter region were as follows: 5’-GGT TTY GAT ATG TTY GGA TA-3’ and 5’-biotin-ACC AAA ACC TGA CTC ACC ACC AAA TC-3’. Pyrosequencing analysis was performed by Gene Tech. Obtained methylation values were averaged across 7 CpG loci within the MGMT promoter. Methylation was defined as samples with an average methylation value >10%.

Gene Ontology Analysis of Differential Genes in HGG With Methylated MGMT Promoter
Patients with HGG who had a methylated MGMT pro-
moter were stratified into 2 groups based on their median HSPB11 expression level. A significance analysis of microarrays (SAM) was performed to identify differential probes with threshold of false discovery rate = 0.1 and number of permutations = 100. A gene ontology (GO) analysis of different probes was performed using the Database for Annotation, Visualization and Integrated Discovery (Laboratory of Immunopathogenesis and Bioinformatics, SAIC-Frederick, Inc., http://david.abcc.ncifcrf.gov/home.jsp) functional annotation tool.22

**Gene Set Enrichment Analysis**

To obtain functional information on HSPB11, Gene Set Enrichment Analysis (GSEA) (Broad Institute, http://www.broadinstitute.org/gsea/index.jsp) was performed as previously described,26 which identified gene sets showing statistically significant, concordant differences between the 2 biological states based on the C5.bp GO gene set collection.

**Statistical Analysis**

SPSS (SPSS, Inc.) and GraphPad Prism 6 (GraphPad Software, Inc.) software were used for statistical analyses. Differences in HSP expression levels between groups were compared using Student’s t-test in microarray data and the chi-square test in immunohistochemical analysis. The normalization of gene expression was performed using the “normalize genes” and “normalize arrays” options in the purposes of normalization and increasing the comparability among these genes. A summary of mean differences and p values for each HSP based on the t-test in the CGGA database is shown in Supplemental Table S2. These findings suggest that specific HSPs play important roles in glioma progression and pathogenesis.

**High HSPB11 Expression Level Is Associated With Unfavorable Prognosis in Glioma**

To evaluate the prognostic value of HSPs in patients with glioma, a univariate Cox regression analysis was performed for each grade; the results are summarized in Table 1. Of the 28 HSPs, only HSPB11 was related to prognosis for each glioma grade (Grade II: HR = 2.5958, p = 0.0459; Grade III: HR = 2.4233, p = 0.0025; and Grade IV: HR = 1.5817, p = 0.0104).

The prognostic value of HSPB11 was further evaluated based on the median expression level using the Kaplan-Meier survival analysis and log-rank test. Patients with Grade II–IV HGG had different outcomes depending on HSPB11 expression level: patients with low HSPB11 expression level had longer OS and PFS than those in whom HSPB11 expression was upregulated (Fig. 1B–G). Similar analyses in the 2 validation sets (REMBRANDT and GSE16011) confirmed the results that HSPB11 overexpression confers a poor prognosis (Supplemental Fig. S1). Both the univariate Cox and Kaplan-Meier analyses showed that HSPB11 expression level was significantly associated with prognosis in patients with glioma.

**Expression Level of HSPB11 Is Associated With Progressive Glioma Malignancy**

According to the expression data in the CGGA database, HSPB11 expression was notably decreased in Grade II relative to Grades III (p < 0.0001) and IV (p < 0.0001) glioma (Fig. 2A), although expression levels were similar between Grades III and IV (p = 0.4669) (Fig. 2A), which are both considered HGG. A comparison of HSPB11 expression levels in LGG and HGG based on the CGGA and validated with the REMBRANDT and GSE16011 data sets revealed that HSPB11 was upregulated in HGG (Fig. 2B–D).

We further explored the expression level of HSPB11 in glioma specimens from 50 patients (20 with Grade II, 15 with Grade III, and 15 with glioblastoma (GBM) using immunohistochemical analysis (Fig. 2E–G). In concordance with the findings above, HSPB11 showed a higher expression status in HGG than in LGG (p < 0.0001, chi-square test), whereas no significant difference was observed between Grades III and IV (p = 0.4151, chi-square test) (Supplemental Table S3). The concordance in the results from the 3 independent data sets and the immunohistochemical analysis confirmed that a strong correlation exists between HSPB11 expression level and glioma malignancy.

**Level of HSPB11 Expression Is an Independent Prognostic Factor in HGG**

Given that HSPB11 is highly expressed in HGG, the prognostic value of HSPB11 expression level in patients with HGG was investigated. Dichotomization was used to
classify samples into 2 groups based on median $HSPB11$ expression level in patients with HGG. In the CGGA and the 2 validation data sets, the prognostic value of $HSPB11$ was enhanced in patients with HGG, and patients with high levels of $HSPB11$ expression had significantly reduced survival time compared with those with low expression levels (Figs. 3A and B and Supplemental Fig. S2). Next, a univariate Cox regression analysis was performed using clinical variables for 176 patients with HGG in the CGGA. Age, tumor grade, Karnofsky Performance Scale (KPS) score, radiotherapy, chemotherapy, and $HSPB11$ expression level were all associated with OS. Then, multivariate Cox regression analysis, evaluating factors that contribute to OS, found that $HSPB11$ expression level was independently correlated with OS in patients with HGG (HR = 2.155, $p = 0.008$) (Table 2) when age, tumor grade, KPS score, radiotherapy, and chemotherapy were considered.

The Expression Level of $HSPB11$ Confers Different Responses to Radiochemotherapy

Because there are great disparities among patients’ re-
sponses to treatment strategy, we attempted to explore the relationship between 
HSPB11 expression level and patients’ responses to chemotherapy, which is widely used in routine clinical management. Patients with HGG were classified into 2 groups according to the median expression level of HSPB11. Survival analysis revealed that patients with lower levels of HSPB11 expression who received radiotherapy survived significantly longer than those who received radiotherapy alone (p = 0.0013) (Supplemental Fig. S3A). However, in patients for whom HSPB11 was upregulated, the difference in prognosis between radiotherapy and radiochemotherapy groups was not statistically significant (p = 0.1278) (Supplemental Fig. S3B).

The Prognostic Association Between HSPB11 Expression Level and IDH1 Mutation Status in HGG

The IDH1 mutation status and MGMT promoter methylation status have been identified as stable prognostic and predictive indicators for patients with HGG in various studies. The IDH1 mutation status and MGMT promoter methylation status were analyzed in 147 randomly selected HGG samples by DNA pyrosequencing as described above. A total of 44 samples contained an IDH1 mutation, whereas 103 samples contained wild-type IDH1. The survival time of patients with an IDH1 mutation was significantly longer than that of patients without an IDH1 mutation (Supplemental Fig. S4), which was consistent with previous reports. Afterward, IDH1 mutation status combined with HSPB11 expression level was used for stratification, classifying patients into 4 subgroups: 22 (15.0%) samples with IDH1 mutation/low HSPB11 expression, 22 (15.0%) samples with IDH1 mutation/high HSPB11 expression, 51 (34.7%) samples with wild-type IDH1/low HSPB11 expression, and 52 (35.4%) samples with wild-type IDH1/high HSPB11 expression. Survival analysis indicated that OS and PFS were significantly different among these 4 subgroups (Fig. 3C and D). Although there were no statistical differences in clinical outcome between 2 subgroups with equal IDH1 mutation status, patients’ survival times were notably different according to variable HSPB11 expression between wild-type IDH1 subgroups. In addition, patients who had IDH1 mutation/low HSPB11 expression survived significantly longer than the 2 subgroups with wild-type IDH1 (Fig. 3C and D).

### Table 1. Correlation of HSPs with patients’ prognoses in each grade of CGGA database

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<th>WHO Grade III HR</th>
<th>p Value</th>
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Among 147 patients with HGG, DNA pyrosequencing data indicated that 76 patients had methylated MGMT promoter, whereas 71 patients had unmethylated MGMT promoter. Consistent with other studies, MGMT promoter methylation had significant prognostic value in patients with HGG (Supplemental Fig. S5). Subsequently, MGMT promoter methylation status and HSPB11 expression level were incorporated into the classification, resulting in 4 subgroups: 38 (25.9%) patients with methylated MGMT promoter/low HSPB11 expression, 38 (25.9%) patients with methylated MGMT promoter/high HSPB11 expression, 35 (23.8%) patients with unmethylated MGMT promoter/low HSPB11 expression, and 36 (24.5%) patients with unmethylated MGMT promoter/high HSPB11 expression. Regardless of treatment, OS and PFS varied across subgroups (p = 0.0006 for OS and p = 0.0012 for PFS) (Fig. 3E and F). Patients with a methylated MGMT promoter and low HSPB11 expression had the best prognosis, with a median OS of 883 days and PFS of 546 days, which were longer than in the other 3 subgroups. In patients with a methylated MGMT promoter, reduced HSPB11 expression was associated with a better prognosis, whereas patients with high HSPB11 expression had similar prognoses to those with an unmethylated MGMT promoter; in the latter, HSPB11 expression level did not affect prognosis.

The differences in prognosis among patients with a methylated MGMT promoter (who were presumed to be sensitive to chemotherapy) prompted an evaluation of the association of HSPB11 with cell cycle regulation and the maintenance of genomic integrity. For patients with a methylated MGMT promoter, responses to treatment varied according to HSPB11 expression levels; thus, the function of HSPB11 was evaluated. A SAM was performed for high and low HSPB11 expression subgroups of patients with HGG, with MGMT promoter methylation resulting in 294 probes (Supplemental Fig. S6). Differentially expressed genes were subjected to GO analysis, which revealed that HSPB11-expressing samples were associated with the cell cycle (Supplemental Table S4). GSEA was performed based on the level of HSPB11 expression (Supplemental Table S5). Genes associated with the maintenance of genomic integrity were enriched in the high HSPB11 expression group (Fig. 3I–M and Supplemental Table S4), suggesting that factors that affect cell cycle and maintain genomic integrity are responsible for patients’ variable responses to treatment.
HSPB11 and MGMT promoter methylation predict high-grade glioma

Discussion

Despite treatment advances in recent decades, the prognosis for patients with glioma remains poor, especially for HGG, with a median survival of 15 months and a 2-year survival rate of 27% after standard treatment consisting of maximal resection followed by adjuvant chemotherapy and radiotherapy. Due to its infiltrating nature, HGG cannot be completely excised and in most cases recurs locally. Given the high level of heterogeneity and inaccuracy of traditional clinicopathological factors such as age, grade, KPS score, and tumor resection for predicting prognosis, the identification of molecular markers in HGG that can help to distinguish among patients with different prognoses or clinical responses to specific therapies is essential.

HSPs are highly conserved proteins that are expressed at low levels under normal physiological conditions but are upregulated in response to cellular stress caused by heat, hypoxia, and toxicants. Cancer cells exist in a state of continuous stress induced by aberrant overproduction of oncoprotein, genomic instability, and local hypoxia and acidosis; HSPs are thus highly expressed in several cancer types, including malignant glioma. In the present study, 11 HSPs were upregulated in HGG compared with LGG, suggesting that cells are under continuous stress in glioma, which may be linked to tumor malignancy. In this study, a univariate Cox analysis of 28 HSPs across glioma grades identified only HSPB11 as having a significant prognostic value in each grade.

HSPB11 is a member of the small HSP protein family, and has intracellular localization, chaperoning, and oligomerization properties that are similar to HSP27. HSPB11 is present only in tumor cells and its expression level in...
creases with the aggravation of cell anaplasia.4,30 Similar to what is observed for HSP27, HSP72, and HSP90, a high HSPB11 expression level was associated with poor prognosis for patients with glioma.19 However, only HSPB11 had significant prognostic value for each glioma grade in 3 large sample databases, which was verified using Cox regression and Kaplan-Meier survival analyses based on respective median expression levels. Because of the large differences exhibited in HSPB11 expression levels among glioma grades, we focused on the survival analysis in specific groups of patients restricted by glioma grade. It was more reasonable to define an independent cutoff based on the median expression level of HSPB11 in the corresponding analysis for dichotomization rather than set a uniform cutoff for all analyses. The cytoprotective effects of small HSPs rely on their molecular chaperone functions and interactions with components of the programmed cell death machinery.2,11 Under experimental conditions, similar to other small HSPs, HSPB11 overexpression can induce resistance to cell death by inhibiting mitochondrial cytochrome c release and caspase activation to suppress apoptosis.5,24,31 However, in cancer cells that have abnormally high HSP expression levels, the cytoprotective function is commandeered to protect cancer cells from the toxic effects of oncprotein aggregation and chemo- and radio-therapy, which can explain why HSPs serve as negative prognostic markers.13,23

Based on the results of univariate Cox and Kaplan-Meier survival analyses, we indicated that the HSPB11 expression level was a determinant of patients’ prognoses, with a high HSPB11 expression level associated with reduced survival in Grades II–IV and higher in HGG. It has been well established that multiple factors severely affect the prognosis for patients with glioma. Considering the combined effects of multiple factors, univariate Cox analysis and Kaplan-Meier survival analysis were not able to fully clarify the prognostic value of HSPB11 in patients with HGG. Therefore, we conducted multivariate Cox regression analysis to verify the prognostic role of HSPB11, taking into consideration several critical clinical features, such as age, tumor grade, KPS score, radiotherapy, and chemotherapy. These results indicated that HSPB11 is an independent prognostic factor in patients with HGG. As one of the most popular molecular markers in glioma, IDH1 mutation typically occurred in LGG, anaplastic glioma, and secondary GBMs, but rarely in primary GBM.3,42 Previous reports also identified IDH1 mutation as a powerful prognostic marker in patients with HGG,43,45 and found that IDH1 mutation status was sufficient to delineate clinically distinct subclasses of glioma.37 However, patients exhibited various clinical outcomes, even with similar IDH1 status; thus, studies were needed to further refine the prognostic value of IDH1. In our study, patients with wild-type IDH1 had significantly different clinical outcomes according to their HSPB11 expression levels. Meanwhile, patients with IDH1 mutation/low HSPB11 expression always survived longer than patients with wild-type IDH1. Our results indicated that, when incorporated with IDH1 mutation status, HSPB11 could be introduced for accurate prediction of prognosis, although further study is needed to clarify the hidden mechanism.

In routine clinical management, patients treated with alkylating agents such as TMZ have better prognoses than those receiving radiotherapy alone.15 These agents cause cell death by cross-linking adjacent DNA strands.9 MGMT can rapidly reverse DNA alkylation; thus, the epigenetic silencing of MGMT by promoter methylation is a favorable prognostic marker for patients with HGG and confers sensitivity to chemotherapy, although this has been contested.12,18,29,38,39 In the clinical setting, patients with similar MGMT promoter methylation status have variable prognoses and responses to treatment, underscoring the need to refine its use as a predictive marker. In this study, patients with HGG were stratified into 4 subgroups according to MGMT promoter methylation status and HSPB11 expression levels. Patients with a methylated MGMT promoter and low levels of HSPB11 expression had longer OS and PFS than other patient subgroups. Only patients with a methylated MGMT promoter had a prognosis that differed according to level of HSPB11 expression.

It was hypothesized that the different prognoses of patients with MGMT promoter methylation reflected variable responses to specific treatments. Patients with low HSPB11 expression levels had different prognoses depending upon treatment with combined radiochemotherapy or radiotherapy only, with those receiving the former surviving longer than those receiving the latter. However, for patients who expressed high levels of HSPB11, the prognosis was similar for the 2 treatment strategies, suggesting that high HSPB11 expression is an indicator of chemo-resistance and can explain the unfavorable prognosis for some patients with a methylated MGMT promoter. Genes related to the cell cycle and the maintenance of genomic integrity were enriched in patients with HGG who had MGMT promoter methylation and high levels of HSPB11 expression. The close functional association between HSPB11 level and genomic maintenance indicates that even with promoter methylation, the response to DNA damage from alkylating agents and the relevant genomic integrity may also modulate the beneficial effect.7 We propose that HSPB11 can distinguish between responses to treatment in patients with MGMT promoter methylation due to the functional correlation with the maintenance of genomic integrity and cell cycle regulation; however, the detailed mechanism needs confirmation from in vivo and in vitro experiments. The results of this study indicate that HSPB11 expression level in conjunction with MGMT promoter methylation status can be used to identify patients who are suitable for combined radiochemotherapy. In addition, the HSPB11 expression profile can explain why a subset of patients with a methylated MGMT promoter are resistant to alkylating agents.

Conclusions

The expression of HSPB11 is upregulated in patients with HGG and is independently associated with poor prognosis. HSPB11 expression level can be used in conjunction with MGMT promoter methylation status to identify patients with HGG who can benefit from combined radiochemotherapy. Thus, HSPB11 is a novel biomarker and potential drug target with important therapeutic implications.
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References


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Supplemental Information
Online-Only Content
Supplemental material is available with the online version of the article.
Supplemental Tables S1–S5 and Supplemental Figures S1–S6.

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