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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will benefit from chemotherapeutic alkylating agents.¹⁸ However, even among patients with a methylated *MGMT* promoter, resistance to chemotherapy and unfavorable prognosis after standard treatment are observed. This suggests that additional factors predict response to treatment and determine clinical outcome.

Current cancer treatments aim to undermine cell viability and thereby enhance the cellular stress response. Heat shock proteins (HSPs) are highly conserved proteins present in normal cells. HSP expression is upregulated in response to endogenous and exogenous stress.²³ 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.^{10,16,23,25,26} Upregulation of HSP expression has been detected in many types of cancer¹³ and is often associated with poor clinical outcome and resistance to chemotherapy.^{6,47} 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.^{1,17,20} 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 independently confirmed by 2 neuropathologists based on the 2007 WHO classification guidelines.²⁸ All patients provided written informed consent. The study was approved by the ethics committees of participating hospitals. The Repository for Molecular Brain Neoplasia Data (REMBRANDT; National Cancer Institute, <http://cainegrator.nci.nih.gov/rembrandt>) and GSE16011 (Gene Expression Omnibus; National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16011>) databases were used as validation data sets.

Immunohistochemical Analysis

HSPB11 immunostains were done using formalin-fixed, paraffin-embedded tissues. Four-micrometer-thick

sections were cut from each paraffin block, dewaxed in xylene, rinsed in graded ethanol, and rehydrated in double-distilled water. The sections were then treated with 3% H₂O₂ for 5 minutes at room temperature to block endogenous peroxidase activity. For antigen retrieval, slides were pretreated by steaming in sodium citrate buffer (10 mM sodium citrate, pH 6.0) for 15 minutes at 100°C. After washing with phosphate-buffered saline for 3 minutes, the sections were manually immunostained with an anti-human *HSPB11* rabbit polyclonal antibody (Proteintech, 15732–1-AP) at 1:50 dilution with standard avidin-biotin-peroxidase. Staining for *HSPB11* was scored manually for 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-TTG 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 20 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'-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.^{43,44} 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'-GTT TYG GAT ATG TTG GGA TA-3' and 5'-biotin-ACC CAA ACA CTC 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-

motor 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,³⁶ 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 Cluster 3.0 (Human Genome Center, Institute of Medical Science, University of Tokyo) before transforming expression information into a heat map. Overall survival (OS) and progression-free survival (PFS) were used as clinical end points. The former was calculated from the date of diagnosis until death or the end of follow-up; the latter was defined as the time between diagnosis and the first unequivocal clinical or radiological sign of disease progression. A Cox regression analysis was performed to evaluate the prognostic value of HSPs in glioma. A Kaplan-Meier survival analysis was used to estimate the survival distribution, and the log-rank test was used to evaluate differences between stratified groups, using the median value as the cutoff. Statistical significance was defined as a 2-tailed *p* value < 0.05.

Results

Expression of HSPs Is Dysregulated in Glioma

Of 295 glioma samples in the CGGA that were included in this study, 119 (40.34%) samples were low-grade glioma (LGG) and 176 (59.66%) were HGG. The expression of 28 HSPs was compared between LGG and HGG cases using Student's t-test. A total of 18 HSPs were differentially expressed between LGG and HGG: *HSPB1*, *HSPB11*, *HSPA6*, *HSPB6*, *HSPA4*, *HSPA5*, *HSP90B1*, *HSPB7*, *HSPA9*, and *HSP90AA1* were upregulated in HGG as compared with LGG (*p* < 0.05), whereas *HSPB8*, *HSPA12A*, *HSPA2*, *HSP90AB1*, *HSPB5*, *HSPA12B*, and *HSPB9* were downregulated in LGG (*p* < 0.05). Prior to transforming HSP expression levels into a heat map for visualization (Fig. 1A), the "normalize genes" and "normalize arrays" options in Cluster 3.0 were performed for

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

FIG. 1. A heat map is shown, depicting HSP expression levels in LGG versus HGG. Genes were clustered into 3 groups (upregulated in HGG, downregulated in HGG, and no significant difference). Differences in expression levels were evaluated with the Student t-test (**A**). Survival analysis was performed to assess the prognostic value of *HSPB11* for each glioma grade in the CGGA database. The discriminative power of *HSPB11* was tested using the Kaplan-Meier analysis and log-rank test. *HSPB11* downregulation was associated with prolonged OS (**B–D**) and PFS (**E–G**) for each grade. Figure is available in color online only.

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-

TABLE 1. Correlation of HSPs with patients' prognoses in each grade of CGGA database

HSP	WHO Grade II		WHO Grade III		WHO Grade IV	
	HR	p Value	HR	p Value	HR	p Value
HSP90AA1	0.9783	0.9607	0.9020	0.7146	1.1704	0.4612
HSP90AB1	0.4575	0.1022	0.3993	0.0106	0.9123	0.6250
HSP90B1	1.3407	0.5623	0.8398	0.6413	1.0635	0.6957
HSPA12A	0.6434	0.1781	0.8364	0.3631	0.8454	0.2159
HSPA12B	2.0246	0.0364	0.8616	0.4899	0.8132	0.1248
HSPA13	0.4625	0.0894	0.7768	0.3238	1.0879	0.6379
HSPA14	0.6544	0.4419	0.7661	0.4179	1.1596	0.3762
HSPA1A	1.0449	0.8717	0.9845	0.9333	1.0717	0.5643
HSPA2	1.1505	0.4324	0.9972	0.9791	0.9954	0.9450
HSPA4	3.1513	0.1579	2.5368	0.1687	0.7745	0.2737
HSPA5	0.5610	0.3477	2.0172	0.0754	1.0664	0.6362
HSPA6	1.0381	0.8067	1.2289	0.0366	1.0956	0.1357
HSPA8	0.2978	0.0583	0.2234	0.0075	0.8206	0.2052
HSPA9	0.9256	0.8319	0.4834	0.0517	0.8425	0.3083
HSPB1	1.0139	0.9506	1.2685	0.1364	1.1284	0.2157
HSPB2	0.8318	0.3902	0.9614	0.8006	0.9815	0.8321
HSPB3	0.8558	0.1884	0.9369	0.4777	1.0202	0.7304
HSPB4	1.3117	0.4454	1.3233	0.3473	0.9395	0.5592
HSPB5	1.1547	0.5142	0.7640	0.0603	0.8664	0.0539
HSPB6	1.0599	0.8742	1.5056	0.0150	1.2719	0.0265
HSPB7	0.9467	0.8424	1.5297	0.0013	0.9861	0.8796
HSPB8	0.9508	0.7982	0.8590	0.1675	0.9358	0.3309
HSPB9	1.2264	0.6237	0.5693	0.2255	0.8419	0.3226
HSPB10	0.9718	0.9346	0.7222	0.1807	1.0363	0.6950
HSPB11	2.5958	0.0459	2.4233	0.0025	1.5817	0.0104
HSPD1	1.7655	0.3018	0.3246	0.0337	1.1139	0.5477
HSPE1	5.2043	0.0062	0.8487	0.6194	0.9666	0.8619
HSPH1	0.8539	0.5270	0.7479	0.1721	1.2608	0.0932

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 radiochemotherapy 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.^{18,35,40,41,45} 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).

FIG. 2. Relationship between *HSPB11* expression level and glioma grade analyzed using the Student t-test. *HSPB11* expression was downregulated in Grade II compared with Grades III ($p < 0.0001$) and IV ($p < 0.0001$), whereas expression was similar between Grades III and IV ($p = 0.4669$) (A). Differences in *HSPB11* expression between LGG and HGG were evaluated in the CGGA database and 2 independent validation data sets (B–D). Photomicrographs of immunohistochemical staining of *HSPB11* in Grade II (E), III (F), and IV (G) are shown. Original magnification $\times 200$. Figure is available in color online only.

Expression Level of *HSPB11* Refines the Prognostic Value of *MGMT* Promoter Methylation in HGG

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 predictive value of *HSPB11* in these patients ($n = 76$), who were stratified into 4 subgroups according to their *HSPB11*

expression level and treatment. The OS and PFS differed across subgroups ($p = 0.0139$ for OS and $p = 0.0163$ for PFS) (Fig. 3G and H). The longest OS and PFS rates were among patients with low *HSPB11* expression who received combined radiochemotherapy (Fig. 3G and H): their median OS and PFS were 970 and 716 days, respectively, compared with 455 and 341 days, respectively, among patients with low *HSPB11* expression who received radiotherapy alone. Among patients with high *HSPB11* expression, there was no difference according to treatment received. The interesting findings regarding the relationship between *HSPB11* expression and *MGMT* promoter methylation status further highlighted the role of *HSPB11* in predicting response to glioma treatment.

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.

FIG. 3. Low levels of *HSPB11* expression are associated with longer OS and PFS in patients with HGG (**A** and **B**). Although there was no statistical significance in survival time between 2 subgroups with equal *IDH1* mutation status, patients with wild-type *IDH1* had significantly different clinical outcomes according to their levels of *HSPB11* expression (**C** and **D**). Patients with a methylated *MGMT* promoter and low levels of *HSPB11* expression had the best prognoses; the prognoses of patients with a methylated *MGMT* promoter differed according to their *HSPB11* expression levels. Patients with an unmethylated *MGMT* promoter showed no significant differences in prognosis (**E** and **F**). Survival analysis was performed for patients with HGG who had *MGMT* promoter methylation. Patients with low levels of *HSPB11* expression who received radiochemotherapy had the best prognoses, whereas no differences were observed among the other groups (**G** and **H**). An asterisk denotes statistical significance between 2 groups at the end point. GSEA was performed in patients with HGG who had *MGMT* promoter methylation (**I–M**). Genes associated with cell cycle and maintenance of genomic integrity were enriched in patients with high levels of *HSPB11* expression. The horizontal bar in graded color from red to blue represents the rank ordering of patients based on decreasing levels of *HSPB11* expression. Vertical black lines represent the projection of individual genes constituting relevant gene sets. Figure is available in color online only.

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.³⁴ Due to its infiltrating nature, HGG cannot be completely excised and in most cases recurs locally.¹⁴ 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.^{8,21,27,32} 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.^{23,33,46} 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*.³ *HSPB11* is present only in tumor cells and its expression level in-

TABLE 2. Cox regression analyses of *HSPB11* expression and other characteristics related to OS in HGG

Variable	Univariate Regression		Multivariate Regression	
	HR	p Value	HR	p Value
Sex	0.962	0.826		
Age	1.021	0.005	1.006	0.558
Grade	2.119	<0.001	1.812	0.067
KPS score	0.974	<0.001	0.974	0.003
Resection	0.779	0.203		
Radiotherapy	0.489	0.002	0.435	0.013
Chemotherapy	0.463	<0.001	0.602	0.079
HSPB11 expression	1.826	<0.001	2.155	0.008

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 oncoprotein aggregation and chemo- and radiotherapy, 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 chemoresistance 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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Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Wu, Cheng. Analysis and interpretation of data: Cheng. Drafting the article: Cheng. Statistical analysis: Cheng, Li, Zhang, Cai, Wang. Administrative/technical/material support: Jiang. Study supervision: Li, Zhang, Cai.

Supplemental Information

Online-Only Content

Supplemental material is available with the online version of the article.

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