High-mobility group box 2 is associated with prognosis of glioblastoma by promoting cell viability, invasion, and chemotherapeutic resistance

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Background. The expression profile of high-mobility group box 2 (HMGB2) in patients with glioblastoma multiforme (GBM) and its clinical signature with underlying mechanisms were not fully explored.

Methods. HMGB2 protein levels were measured in 51 GBM patients by immunohistochemical studies. To clarify the precise role of HMGB2 on cell invasion and viability of 3 GBM cell lines, we did in vitro and in vivo analyses with lentivirus vectors and small interfering RNA. Transwell invasion assays and wound-healing assays were used to analyze the invasion of GBM cells. Expression of p53 and matrix metalloproteinase 2/tissue inhibitors of metalloproteinase 2 (MMP2/TIMP2) protein was analyzed by Western blot.

Results. HMGB2 protein expression was significantly higher in GBM than in controlled brain tissues (P < .0001). HMGB2 overexpression was significantly correlated with shorter overall survival time, which was the only independent prognostic factor for overall survival in a multivariate analysis (P = .017). HMGB2 knockdown by small interfering RNA decreased cell viability and invasion in vitro and significantly decreased tumor volume in vivo, which might be involved in the change of p53 expression and the balance of MMP2/TIMP2. Moreover, silencing of HMGB2 could significantly increase the sensitivity of GBM cells to temozolomide chemotherapy.

Conclusions. Our present data suggest that HMGB2 expression is a significant prognostic factor and might play an important role in cell invasion and temozolomide-induced chemotherapeutic sensitivity of GBM. This study highlights the importance of HMGB2 as a novel prognostic marker and an attractive therapeutic target of GBM.

Keywords: glioblastoma, high-mobility group box 2, prognosis, invasion, temozolomide.

Human glioblastoma multiforme (GBM) accounts for approximately 60% to 70% of malignant gliomas, which are the most common and most deadly brain tumors. The life expectancy of patients with GBM who undergo the current standard of care (maximal safe surgical resection, radiotherapy with concomitant temozolomide [TMZ] followed by ≤6 cycles of adjuvant TMZ) is improved from 12.1 to 14.6 months on average after diagnosis. Overall survival (OS) in the European Organisation for Research and Treatment of Cancer–National Cancer Institute of Canada trial was 27.2% in 2 years and 9.8% in 5 years with the combined treatment. It was promising that OS with O6-DNA methylguanine-methyltransferase (MGMT) promoter methylation was 48.9% at 2 years and 13.8% at 5 years with the combined treatment. However, not all GBM patients benefited from the addition of TMZ to radiation therapy. On the other hand, despite advances in understanding of the molecular pathogenesis of GBM, the poor level of OS is not dramatically improved, especially for those patients without MGMT promoter methylation.

The high-mobility group box (HMGB) family consists of HMGB1, HMGB2, HMGB3, and HMGB4, whose
proteins are ubiquitous in eukaryotic cells and nonspecifically bind to DNA, inducing large-angle DNA bends, enhancing the flexibility of DNA, and likely facilitating numerous important biological interactions.6–9 Although HMGB2 is a structural analogue of HMGB1 with an amino acid sequence > 85% identical to that of HMGB1, the HMGB family has different biological functions in the cell.10 HMGB1 overexpression has been reported as a novel prognostic marker and potential therapeutic target in a variety of human cancers.11–19 Despite extensive characterization of the diverse roles of HMGB1 in cancer,20,21 much less is known of HMGB2 in carcinogenesis and prognostic value, except for the prognostic significance of HMGB2 expression in hepatocellular carcinoma,22 squamous cell carcinoma in skin,23 and epithelial ovarian cancer.24

There have been few published reports evaluating the biological role of HMGB in human glioblastoma cells.21,25,26 HMGB1 has been considered as an autocrine factor capable of stimulating the growth and migration of human glioma cells.21 Recently, Balani et al26 confirmed a significant elevation of HMGB2 mRNA expression in glioblastoma cells compared with normal brain tissues by reverse transcription (RT)–PCR and then constructed a baculoviral vector expressing the herpes simplex virus thymidine kinase gene driven by the HMGB2 promoter. The Balani study26 demonstrated that transduction with the viral vector induced cell death in glioblastoma cell lines in vitro and in vivo. However, the role of HMGB2 in GBM is as yet not entirely clear, especially regarding the prognostic significance of HMGB2 expression in GBM.

The present study investigated the effects of HMGB2 knockdown on cell viability and invasion in vitro and in vivo and on TMZ-induced chemotherapeutic sensitivity. Furthermore, we explored the underlying mechanisms of HMGB2 knockdown, which might be involved in the expression of protein (p)53 and matrix metalloproteinase 2 (MMP2)/tissue inhibitors of metalloproteinase 2 (TIMP2). Lastly, we analyzed the prognostic significance of HMGB2 expression in a large number of patients with GBM by immunohistochemistry (IHC). We found that HMGB2 can be a novel prognostic factor and potential treatment target for GBM patients.

Materials and Methods

Cell Culture and Reagents

The human GBM cell lines U87, U251, and Shanghai human glioblastoma (SHG)66 were used in this study. SHG66 came from a 47-year-old male with a right parietal glioblastoma (World Health Organization grade IV).27 U87 and U251 cells were purchased from the Cell Bank of the Shanghai Branch of the Chinese Academy of Sciences. All cell lines were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 100 U/mL penicillin/streptomycin (Gibco) and were maintained in a humidified atmosphere with 5% CO₂ at 37°C. TMZ was purchased from Sigma.

Western Blot Analysis

Cell lysates were extracted with cell lysis buffer (Beyotime), and the protein concentration in the lysates was quantified using an enhanced bicinchoninic acid protein assay kit (Beyotime). Protein samples with 30–50 μg were loaded for immunoblotting using antibodies against p53, MMP2, TIMP2 (Epitomics), and actin (Kangwei).

Cell Viability Assay and Cell Cycle Analysis

Cell viability was assayed using the Cell Counting Kit-8 (CCK-8, Dojindo) as described by the manufacturer’s protocol.

Cells were harvested and fixed in 70% ethanol at –20°C overnight, then stained with propidium iodide (36 μg/mL; Sigma) containing 400 μg/mL RNase (Roche) with shaking for 1 h and analyzed by flow cytometry (CyAn ADP, Beckman Coulter) for cell cycle profile and apoptosis.

GBM Tissue Collection and Patient Follow-up

A total of 51 patients with GBM, who underwent surgical removal of tumor between 2008 and 2010 in the Department of Neurosurgery, Huashan Hospital, were enrolled in this retrospective study. No patient had a history of radiotherapy or chemotherapy preoperatively. The study protocol was approved by the local, independent ethics committee at Huashan Hospital. Clinical data were available for all patients and are summarized in Table 1. There were 30 males and 21 females, ranging in age from 9 to 80 years (mean, 51.7 y). After tumor removal, all patients had at least 3 cycles of TMZ chemotherapy with (n = 42) or without radiotherapy (n = 9). The median follow-up time for OS for all patients was 18.6 months (range, 5–36 mo). Additionally, 9 controlled brain tissues were obtained from adjacent brain tissues of contusion and laceration in traumatic brain injury patients.

Real-time Reverse Transcription PCR

Total RNA was extracted from GBM and normal brain tissues or from the cell lines using Trizol reagent (Invitrogen), according to the manufacturer’s instructions. The first-strand cDNAs were synthesized using a high-capacity cDNA archive kit. Each cDNA (2 μL) was amplified in SYBR Green Realtime PCR Master Mix (final volume, 20 μL) and loaded on the Applied Biosystems 7900 Real-time PCR Detection System. Thermal cycling conditions were as follows: the first step, 95°C for 10 min and the ensuing 40 cycles, 95°C for 15 s, 60°C for 60 s, and 72°C for 30 s. PCR primers used were as follows: HMGB2 (forward): 5’-TGGCCAAAAGATCGAAAGGAATACAC-3’ and HMGB2 (reverse): 5’-GCTGCCTTGAATCCATCTTCTTAG-3’.
Table 1. Relations between HMGB2 protein levels and clinicopathologic features in GBM

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*All patients received ≥3 cycles of TMZ chemotherapy.

Immunohistochemical Analysis

Human GBM formalin-fixed, paraffin-embedded tissue sections were provided and were IHC stained with HMGB2-specific antibody made against COOH-terminal peptide of human HMGB2 (Epitomics), using the Dako Cytomation EnVision+ System horseradish peroxidase (diaminobenzidine) [HRP (DAB)] detection kit. Briefly, the tissue sections in 5 microns were dehydrated and subjected to peroxidase blocking. HMGB2 antibody was added at a dilution of 1:400 and incubated at room temperature for 30 min on the Dako AutoStainer using the Dako Cytomation EnVision+ System HRP (DAB) detection kit. The slides were counterstained with hematoxylin. The stained slides were observed under a microscope, and images were acquired.

All the IHC stained sections were evaluated in a semi-quantitative fashion by 2 senior neuropathologists blinded to the clinical parameters. In cases of occasional scoring discrepancy, consensus was always achieved after discussion of findings. Nuclear staining was considered positive. To evaluate the expressions of HMGB2, 10 high-power fields (400×) within the tumor showing nuclear staining were selected. The extent of staining was calculated according to the percentages of the positive staining tumor cells in relation to the whole tumor cells. The extent of staining was classified as low (+ = <25%), medium (++ = ≥25% and <50%), high (+++ = ≥50% and <75%), or highest (++++) (≥75%). In this study, in order to analyze the prognostic relations between groups, staining extent <50% and ≥50% were considered to define the low-score group and the high-score group, respectively, and these cutoff values had been used in past studies.28–30

IHC staining of MGMT was the same, with MGMT-specific antibody (Abgent) at a dilution of 1:200. Nuclear staining of tumor cells was considered positive. Nuclear staining of vascular endothelial cells was the positive control. MGMT protein expression was classified into negative (−) and positive (+) according to the extent of nuclear staining of <5% and ≥5%, respectively.

Gene Silencing and Overexpression

Three GBM cell lines—U87, U251, and SHG66—were transfected with small interfering (si)RNA oligonucleotides using Lipofectamine 2000. Briefly, siRNA and Lipofectamine 2000 were each incubated separately with Opti-MEM for 5 min and mixed together for 20 min at room temperature, and then the mixture was applied to cells plated in 4 mL of medium (final concentration of siRNA, 60 nM). The sequences of siRNAs were as follows: for HMGB2, siHMGB2: 5′-CUGA ACAUCGCCCCAAAGAU-3′;22 for control scrambled siRNA, siControl: 5′-TTCTCCGAACGTGTCACGT TT-3′. All siRNAs were purchased from GenePharma.

Nucleotide sequence coding for HMGB2 was cloned into pCDH-CMV-MCS-EF1-Puro vector (System Biosciences) at the BamH and EcoRI site. A panel of human cancer cell lines were infected with protocadherin (pCDH)-HMGB2 or pCDH-Control for 72 h, then split for the next assays.

Transwell Invasion Assay and Wound-Healing Assay

Matrigel solution (Becton Dickinson) was prepared in serum-free cell culture medium at a dilution of 1:8, for coating in 24-well transwell chambers (8-μm pores; Corning Costar) overnight at 37°C before cell seeding. The cells were cultured in the chamber with serum-free media containing 1% bovine serum albumin in triplicate at 3 × 10^3 cells per well. After 24 h of cultivation, the media from the chamber and the transwell were removed and the chamber was gently wiped with a cotton swab. Migrated cells were fixed in 4% polyoxy-methylene for 15 min and then washed by phosphate buffered saline (PBS) twice for a total of 10 min. Staining was done with 0.1% crystal violet, and the cell numbers on the lower surface of the membrane were quantified.

Three GBM cell migrations (U87, U251, and SHG66) were measured by determining the ability of the cells to move into acellular space. The cells were treated siRNA against HMGB2 or scrambled siRNA as mentioned. After 72 h of culture, the confluent monolayers were wounded using a sterile pipette tip and evaluated under phase contrast microscopy at 0 and 24 h. Photographs
were taken, and the relative distance traveled by the cells at the acellular front was measured.

**Tumor Formation Assay**

Five-week-old female athymic nude mice were purchased from the Shanghai Experimental Animal Center. U87 cells transfected with siControl or siHMGB2 for 24 h were trypsinized, resuspended in PBS, and then subcutaneously injected into the right back for siHMGB2 or left back for siControl with $10^6$ cells per injection. Tumor size was measured by a vernier caliper weekly and calculated as $(\text{length} \times \text{width}^2)/2$. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**In silico REMBRANDT Analysis**

The relationship between patients’ prognosis and HMGB2 mRNA expression level was analyzed in silico using the Repository of Molecular Brain Neoplasia Data (the REMBRANDT database) of the National Cancer Institute (http://rembrandt.nci.nih.gov).

**Statistical Analysis**

All statistical analyses were carried out using the SPSS 16.0 software package. Continuous variables were expressed as mean ± SE. Correlation between IHC staining score and clinicopathologic variables was evaluated using a $\chi^2$ test. Kaplan–Meier survival curves were calculated using death as the end point. The difference in OS...
curves was examined by log-rank test. The significance of various variables for OS was analyzed by the Cox proportional hazards model in univariate and multivariate analyses to identify which factors were independent indicators for prognosis. Tumor cell invasion assays and cell viability assays were tested using independent t-tests. A 2-tailed P-value test was used with P < .05 considered statistically significant.

Results

**HMGB2 Silencing Suppressed the Growth of GBM Cells In vitro and In vivo**

To address the efficacy of HMGB2 on GBM cells, we knocked down HMGB2 in 3 GBM cell lines (U87, U251, and SHG66) and subjected these lines to cell growth analysis. As shown in Fig. 1, each GBM cell line transfected with HMGB2 siRNA showed efficient silencing of HMGB2 expression, as judged by immunoblot analysis and real-time RT-PCR (P < .05; Fig. 1A and B). HMGB2 mRNA expression significantly decreased 62.3%, 47.6%, and 87.3% in SHG66, U87, and U251 cells, respectively, by siRNA silencing compared with siControl cells. In cell viability assay, all GBM cell lines showed significant reduction of cell viability by HMGB2 silencing compared with siControl cells (Fig. 1C). At 96 h after transfected siRNA, cell viability reduced 48.3%, 26.1%, and 20.8% in SHG66, U87, and U251 cells, respectively. Furthermore, we found that tumor growth and weight in the siHMGB2 group were significantly inhibited compared with those in the siControl group in vivo (Fig. 1D and E). Tumor volumes were 171.4 ± 29.8 mm³ and 1731.4 ± 239.5 mm³ in the siHMGB2 group and the siControl group, respectively, at end point (P < .01). Furthermore, tumor weights were 0.75 ± 0.14 g and 6.38 ± 0.71 g in the siHMGB2 group and the siControl group, respectively (P = .0034). These findings showed that HMGB2 was required for the growth of GBM cells both in vitro and in vivo.

**HMGB2 Silencing Diminished the Migration and Invasion of GBM Cells**

To address the mechanism underlying the growth suppression of GBM cells by HMGB2 knockdown, we first determined the cell cycle profile of the HMGB2-silenced cells by propidium iodide staining. No obvious cell cycle arrest or apoptosis occurred (data not shown). Having established that HMGB2 knockdown did not induce cell cycle arrest and apoptosis in GBM cells, we next investigated whether HMGB2 knockdown inhibited invasion, as a novel cellular response, to regulate cell proliferation. HMGB2 knockdown resulted in significantly decreased migration in SHG66 cells in triplicate independent assays by 31.5%, 74.3%, and 66.6% (Fig. 2A) as well as in U87 and U251 cells (Supplementary Fig. S1). Moreover, in wound-healing assays, we found that HMGB2 knockdown diminished wound-healing migration in 3 GBM cells (Fig. 2B). The ability of migration was decreased by 14.4%–43.8% in SHG66 cells, 50.5%–82.6% in U87 cells, and 29.6%–44.2% in U251 cells (Fig. 2C).

**Effect of HMGB2 on Invasion-Related Biomarkers**

To further investigate the downstream molecules of HMGB2, we performed Western blot to examine the expression of invasion-related biomarkers, including p53, MMP2, and TIMP2. As shown in Fig. 3A, p53 and MMP2 expressions were significantly downregulated; meanwhile, TIMP2 was significantly upregulated in HMGB2-silencing cells. On the other hand, we also found that overexpression of HMGB2 upregulated the expression of p53 and MMP2, although no change of TIMP2 levels was observed (Fig. 3B).

**HMGB2 Silencing Sensitized GBM Cells to TMZ**

We then determined the potential chemoresistant effect of HMGB2 in GBM cells. We first constructed the pCDH-HMGB2 vector in order to exogenously upregulate the expression of HMGB2 in 3 GBM cells (Fig. 4A). We then confirmed that TMZ treatment indeed caused the growth inhibition in 3 GBM cell lines (the inhibitory rate of SHG66 cells was 50.5%, that of U87 cells was 39.8%, and that of U251 cells was 10.7% at 48 h; Fig. 4B). HMGB2 silencing was sufficient to increase chemosensitivity by 21.25%, 22.34%, and 16.74% at 48 h in SHG66, U87, and U251, respectively (Fig. 4B). On the contrary, as shown in Fig. 4C, GBM cells were more resistant to TMZ chemotherapy when HMGB2 was overexpressed. The resistance of SHG66, U87, and U251 cells to TMZ chemotherapy increased 17.1%, 25.1%, and 6.3%, respectively, compared with the negative control group at 48 h. On the other hand, HMGB2 down- or overexpression did not induce change of MGMT protein expression (data not shown). These findings demonstrated that HMGB2 silencing sensitized GBM cells to TMZ chemotherapy.

**Overexpression of HMGB2 Protein and Its Prognostic Significance in GBM Patients**

To investigate the protein expression profile of HMGB2 in GBM, IHC was initially performed in 51 formalin-fixed, paraffin-embedded tissue sections and 9 controlled brain tissues. Based upon the extent of staining, we classified the samples into 4 groups with increasing staining extent from the lowest (+) to the highest (++++) (Fig. 5A and B). As shown in Fig. 5A, HMGB2 protein was mainly localized to the nuclei of tumor cells (right panel). Average IHC scores of HMGB2 expression are shown in Fig. 5B (Supplementary Table S1). Controlled brain tissue had lower expression of HMGB2 protein compared with GBM tissues (11.6 ± 9.8 vs 55.9 ± 28.3, respectively, P < .0001). Moreover, HMGB2 expression in the tumor central zone was obviously higher than that in the invading zone (seen in Supplementary Fig. S2).
We further quantified the expression level of HMGB2 in GBM tissues compared with their controlled brain tissues (10 GBM vs 8 control) by immunoblot analysis. As shown in Fig. 5C, the level of HMGB2 in tumor tissues was significantly higher than that in the controlled brain tissues (43.8 ± 12.5 vs 11.8 ± 2.4, respectively, P = .039; Fig. 5D, Supplementary Table S2).

Thus, we analyzed the relationship between HMGB2 protein overexpression and patients' prognosis. A Kaplan-Meier survival curve was used to analyze
the prognostic significance of HMGB2 expression. Expression of HMGB2 protein levels was divided into a low-score group (25.7 ± 16.3, 95% confidence interval [CI]: 18.08–33.31) and a high-score group (75.5 ± 12.7, 95% CI: 70.83–80.13), which was significantly differentially expressed between the 2 groups (P < .01; Fig. 5E). The median OS time in patients with low or high HMGB2 expression was 20.1 ± 7.6 or 16 ± 5.9 months, respectively (P = .033). The log-rank test showed that patients with high HMGB2 protein expression had a significantly shorter OS time (log-rank test statistic = 6.724, P = .01; Fig. 5F).

The relationship between clinicopathologic features and HMGB2 protein expression in 51 GBM patients is summarized in Table 1. No correlation was observed between HMGB2 protein expression and age, gender, preoperative KPS score, tumor resection degree, treatment strategy, and MGMT protein expression. To determine whether the expression of HMGB2 was an important prognostic factor for OS among those 6 clinicopathologic features, a univariate Cox regression analysis was used in this 51-patient cohort analyzed by IHC; only a high HMGB2 protein level was identified as an important risk factor for OS (P = .017; Table 2). A multivariate analysis was also performed with the Cox proportional hazards model, which included those 6 clinicopathologic features. The results showed that HMGB2 protein expression level was the only independent prognostic factor of OS (probability for stepwise: entry = .05, removal = .05; Table 2).

Furthermore, we used the REMBRANDT database to examine the relationship between clinical prognosis of GBM patients and HMGB2 mRNA expression value. According to the results obtained from REMBRANDT, patients with high HMGB2 mRNA-expressing GBM (n = 130) showed statistically poorer prognoses compared with patients with intermediate HMGB2 mRNA-expressing GBM (n = 50, P = .015; Fig. 5G).

Taken together, these findings demonstrated that overexpression of HMGB2 protein predicted a poor prognosis in GBM patients.

**Discussion**

To the best of our knowledge, this is the first study to analyze the prognostic significance of HMGB2 expression in GBM patients. Moreover, we confirmed HMGB2 as a signature molecule correlated with the survival and invasion, as well as the TMZ-induced chemotherapeutic sensitivity, of GBM cells. Silencing of HMGB2 expression significantly reduced cell viability and invasion while increasing the sensitivity of TMZ chemotherapy, which indicates that HMGB2 could be a potential therapeutic target of GBM.

HMGB2 has been shown to have high-level expression in GBM. Balani et al. reported that HMGB2 mRNA levels were found to be 11- to 79-fold higher in primary glioblastoma tissue samples than in the normal human brain. In the recent study by Lin et al., massively parallel signature sequencing was used to find that HMGB2 was overexpressed in GBM compared with normal brain tissue in protein level (ratio of GBM/normal = 6.45) despite the lack of confirmation by Western blot or
IHC. In our study, we further confirmed that HMGB2 expression was significantly 5 times higher in GBM than in controlled brain tissues in protein level. To explore the biological function of HMGB2 overexpression in GBM, we downregulated the expression of HMGB2 by siRNA, which could significantly reduce the proliferation and viability in U87, U251, and SHG66 cells in vitro. Furthermore, downregulation of HMGB2 expression could also significantly reduce the invasion of those cells. In nude mice, U87 cell lines transfected with HMGB2-siRNA could significantly inhibit tumor growth in vivo. These observations support our conclusions that HMGB2 overexpression is correlated with cell survival and poor prognosis of GBM.

Since MMPs are involved in tumor invasion and metastasis, we explored whether levels of MMP2 and their inhibitors (TIMP2) were changed when HMGB2 down- or overexpression was regulated in GBM cells. Silencing of HMGB2 expression resulted in a decrease in the expression of MMP2 in GBM cell lines, while an increase in TIMP2 in the same cells indicated that invasion of GBM cells was significantly reduced. Conversely, exogenous overexpression of HMGB2 induced the upregulation of MMP2 and thus increased the invasion of GBM cells. In this condition, TIMP2 was not downregulated, which indicated that it was not always the case that an increase in the active form of MMP2 found in glioma cells was accompanied by a
decrease in TIMP2 in the same cells. On the other hand, it is now clear that p53 regulates the key stages of metastatic progression, such as cell migration and invasion. HMGB2 participates in DNA damage response by modulating p53 phosphorylation and thus interacts with p53. HMGB2 suppressed the transcriptional activity of p53 in osteosarcoma, whereas HMGB2 enhanced the transcriptional activity of p53 in HeLa cells, which indicated that HMGB2 possibly acted in a tissue-specific manner. In this study, ectopic overexpression of HMGB2 led to accumulation of the p53 protein, whereas HMGB2 knockdown with siRNA resulted in a substantial decrease in the p53 protein level in GBM cell lines. These results explain the underlying mechanism that HMGB2 overexpression is correlated with cell invasion and poor prognosis of GBM.

Fig. 5. HMGB2 was overexpressed in GBM and negatively correlated with patient survival. (A and B) IHC staining of human GBM tissues using HMGB2-specific antibody, as described in Material and Methods. HMGB2 protein is expressed in nucleus (A, right panel). Classification of samples according to the extent of staining of HMGB2 expression (n = 51). (C) Immunoblot analysis to determine expression of HMGB2 in GBM and controlled brain tissues. T: GBM tumor tissues (n = 10); C: controlled brain tissues (n = 8). (D) Quantification of HMGB2 expression in GBM and controlled brain tissues. (E) The extent of staining in the low- and high-score group of HMGB2. Low: low-score group of HMGB2 with staining extent < 50%; High: high-score group of HMGB2 with staining extent ≥ 50%. (F and G) Correlation analysis of HMGB2 protein expression (F, n = 51) and mRNA levels (G, n = 180) and patient OS. **P < .01.
Table 2. Summary of OS Analyses by Univariate and Multivariate Cox Regression Analysis (HR = Hazard Ratio)

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aAll patients received ≥3 cycles of TMZ chemotherapy.
bProbability for stepwise: entry = .05, removal = .05.

Several studies have demonstrated that HMGB was associated with the sensitivity of cancer cells to chemotherapy agents, including cytarabine, cisplatin, and oxaliplatin. Overexpression of HMGB2 resulted in more resistance of SK-Hep-1 and SH-J1 cells to cisplatin chemotherapy. However, other studies demonstrated that the introduction of the HMGB2 gene into human lung cancer cells increased cisplatin sensitivity, which indicates that the role of HMGB2 might be dependent on cancer type. TMZ, an oral alkylating agent, was most commonly used in GBM’s standard of care. We investigated the effects of HMGB2 down- or overexpression, in particular whether it would change the sensitivity of GBM cells to TMZ. This study confirmed that GBM cells with HMGB2 overexpression were more resistant to TMZ chemotherapy. On the contrary, downregulation of HMGB2 would obviously increase sensitivity to TMZ treatment. However, the magnitude of chemotherapeutic responses with modulation of HMGB2 expression was not great—the change in U87 and SHG66 was about 20% and in U251 was lower. One of the major reasons might be that HMGB2 down- or overexpression did not induce a change of MGMT protein expression. On the other hand, modulation of HMGB2 expression mainly changed the ability of GBM cells to invade, which resulted in a change in chemotherapeutic responses and cell proliferation to a certain degree. These results further support our conclusions that HMGB2 overexpression is correlated with the resistance of chemotherapy and poor prognosis in GBM.

In GBM, MGMT promoter methylation is correlated with improved progression-free survival and OS in patients treated with alkylating agents. Thus, it is widely accepted that MGMT promoter methylation is the strongest predictor for outcome and benefit from TMZ chemotherapy. However, it is controversial whether MGMT protein expression is also a prognostic factor, as is MGMT promoter methylation. Several studies confirmed that MGMT protein expression level was not a prognostic factor, including an interesting study involving Chinese GBM patients, because there was a poor correlation between the IHC staining results and the methylation status of the MGMT promoter in GBM samples. In our study, univariate Cox regression analysis for OS found that HMGB2 protein expression was an independent prognostic factor of outcomes, despite MGMT protein expression having the tendency to be near statistical significance (P = .092). In a multivariate Cox analysis, high HMGB2 protein expression was found to be the only independent poor prognostic factor for OS (P = .017), which further confirmed that HMGB2 protein expression had a significant correlation with the prognosis of GBM patients. Of course, MGMT promoter methylation should be further analyzed as a possible prognostic factor in patients with GBM in our series.

Taken together, our findings demonstrated that HMGB2 protein expression was significantly increased in GBM and that HMGB2 might play an important role by promoting invasion and proliferation of GBM cells. Moreover, our present data suggest that HMGB2 expression is involved in the sensitivity of TMZ chemotherapy and is a significant independent prognostic factor of GBM. This study highlights the importance of HMGB2 as a novel prognostic marker and an attractive therapeutic target of GBM.

Supplementary Material
Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).

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