Tim-3 protein expression with MGMT methylation status in glioblastoma and association with survival

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Abstract

Background Understanding the molecular landscape of glioblastoma (GBM) is increasingly crucial for its therapy. Immune checkpoint molecules motivated the emergence of immune checkpoint-targeting therapeutic strategies. However, the prognostic significance of the immune checkpoint molecule T cell immunoglobulin mucin-3 (Tim-3) on tumor-infiltrating immune cells (TIICs) and O-6-Methylguanine-DNA methyltransferase (MGMT) methylation status remains to be fully elucidated. We aimed to develop an MGMT methylation status-associated immune prognostic signature for predicting prognosis in GBMs.

Patients and Methods: A total of 84 patients with newly diagnosed GBM were involved. MGMT methylation status was retrospectively analyzed and the expression level of Tim-3 protein was investigated using immunohistochemistry (IHC). The correlation between Tim-3 protein expression and MGMT methylation status, and the prognosis was explored.

Results The obtained data showed that Tim-3 protein was expressed at different levels in GBMs. Mesenchymal expression of Tim-3 protein in these tissues was 73.81% (62/84), including low 15.48% (13/84), moderate 7.14% (6/84) and strong expression 51.19% (43/84), respectively. Of the 48 patients whose tumors tested positive for MGMT methylation, the remaining 36 patients was negative.

Conclusions We profiled the immune status in GBM with MGMT promoter methylation and established a local immune signature for GBM, which could independently identify patients with a favorable prognosis, indicating the relationship between prognosis and immune. MGMT promoter methylation with lower Tim-3 protein expression was statistically significantly associated with better survival.

Background

Glioblastoma is the most common and devastating primary brain tumor in adult [1]. Despite recent advances in the treatment measurements, only a few strategies are available for GBMs and the prognosis remains dismal [2]. There are few effective means for GBMs with strong risk of relapse and short survival periods. Because the biology of GBM on cellular and molecular levels is reasonably unknown, especially in relation to various treatments, the development of novel therapeutic approaches urgently requires a deeper understanding of the tumor’s nature [3]. In addition to standard treatment involving surgery, radiotherapy and chemotherapy in GBM, immunotherapy is rapidly identified as a promising treatment modality [4]. A number of immune-related parameters have been reported for predicting the outcomes of patients with GBM [5, 6]. MGMT promoter methylation status is significantly related to the prognosis of GBMs [7]. However, there is still a lack of studies which have systematically explored the MGMT promoter methylation status on immune microenvironment and their relationship with prognosis.

Tim-3 was widely expressed on mature T lymphocytes and macrophages [8]. Of note, with the exception of the immune response, increasing evidence has displayed that Tim-3 has functional roles in tumor biology [9]. Literatures suggests that Tim-3 is a promising target in glioma. But until now, no evidence has revealed the value of Tim-3 as a prognostic biomarker in GBM patients. The present study aimed to

investigate the influence of MGMT promoter methylation on the immune microenvironment and develop an MGMT-associated immune prognostic signature for predicting prognosis in GBMs.

Materials And Methods

Patients and specimens

A cohort of patients with histologically newly diagnosed GBM (WHO grade IV) was consecutively studied from July 2016 to January 2018. We only selected patients for

whom affirmatory MGMT promoter methylation status, treatment course, and survival outcomes were known. Patients with a mixed history of cancer other than GBM and previous adjuvant radiotherapy or chemotherapy were excluded. Patients who died of diseases unrelated to gliomas were also eliminated from the study. The age ranged from 18 to 70 years old at the time of diagnosis. A series of 84 eligible patients who had tumor tissue available for testing were involved in this study. These patients received standard subsequent therapy by the Stupp protocol [10]. Follow up was regularly carried out. The overall survival (OS) was defined as the interval from GBM diagnosis until death or the last known followup for those who were censored.

Immunohistochemistry (IHC)

Tim-3 was immunohistochemically stained by using a previously described standard technique [11]. Briefly, slides were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was done in tris-ethylenediaminetetraacetic acid (EDTA) pH 9.0 buffer at 95 °C for 20 min. Slides were incubated in Tris-buffered saline (TBS) for 5 min. Endogenous peroxidase blocking was done in 3% H_2O_2 for 10 min. Subsequently, the slides were incubated in a rabbit polyclonal antibody against Tim-3 (1:500, Abcam, Inc., Cambridge, MA) overnight at 4 °C. The slides were rinsed 5 times with 0.01 M phosphate-buffered saline (PBS; pH 7.4) for 10 min. Sections were incubated with primary antibodies against Tim-3 (1:1,000; cat. no. ab185703; Abcam) horseradish peroxidase (HRP) tagged secondary antibody (1:1,000; cat. no. sc-3836; Santa Cruz Biotechnology, Inc.) for another 1 hour at 37 °C. Subsequently, the slides were washed in PBS and stained with 3, 3-diaminobenzidine (DAB). Finally, the slides were counterstained, dehydrated and mounted.

IHC assessment

The IHC number of Tim-3 protein expression was independently reviewed by two neuropathologists. The number of stained cells was designated as either unexpressed (0), weak (1–5/HPF), moderate (5–10/HPF), and strong (> 10/HPF). The model of the microscope is BX53, OLYMPUS.

MGMT promoter methylation

We collected only patients who had documented MGMT promoter methylation testing of their initially resected tumor tissues. MGMT promoter methylation was confirmed by methylation-specific real-time PCR, according to our institutional practice.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.0.0. The correlation between Tim-3 protein expression intensity with MGMT promoter methylation status and their prognosis was calculated with the chi-square test. Spearman's correlation analysis and statistical significance were used to evaluate correlations with gene expression. Independent prognostic factors for OS were identified using the Cox's proportional hazards model. OS curve was plotted with Kaplan-Meier method and the log-rank test was employed to assess the resulting survival curves. A probability value of less than 0.05 ($p < 0.05$) was regarded as significant.

Results

Clinicopathological characteristics

Archival tissue samples from 84 patients with GBMs were enrolled in the study. Of the 84 GBMs patients, 43 were male (51.19%) and 41 female (49.81%). The median age at diagnosis was 41 years (range 18–70 years) and the median Karnofsky Performance Status (KPS) was 90 (range 70–100). All specimens were obtained from supratentorial area, identified by preoperative MRI. The follow-up duration ranged from 4 to 47 months and the median OS was 17.3 months.

Expression of Tim-3 protein

As the expression status of specific inhibitory receptors, Tim-3, programmed cell death 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), and lymphocyte-activation gene 3 (LAG3), are associated with T cell exhaustion and immune escape, the Oncomine database was used to investigate the mRNA levels of these molecules in GBMs and normal tissues (Fig. 1c). Figure 1a showed that Tim-3 expression was remarkably high in GBMs than the corresponding normal tissues. To validate the relationship of immune checkpoint molecules between Tim-3 and LAG3 as well as PD-1, the RNA-seq data in the TCGA database were exploited. As depicted in Fig. 1b, with a p-value threshold of 0.05, Tim-3 expression was significantly related with LAG3 and PD-1 in GBM. Tim-3 and other exhausting immune molecules (LAG3, PD-1, CTLA4, CD244, PD-1) exhibited significantly differential expression profiles between normal brain tissues and GBM tissues in TCGA database (https://cancergenome.nih.gov/), indicating their potential correlation with glioma progression (Fig. 1). According to the immunohistochemical analysis, mesenchymal expression of immune checkpoint Tim-3 protein in TIICs is unexpressed in 22/84 (26.19%), low in 13/84 (15.48%), moderate in 6/84 (7.14%) and strong in 43/84 (51.19%), respectively (Fig. 2, Table 1). Notably, the strong expression of Tim-3 protein was more frequently observed in GBMs.

Table 1
Tim-3 expression in 84 GBM patients.

No. of cases (84)	Tim−3 expression			
	NE (22)	Weak (13)	Moderate (6)	Strong (43)
Methylation	14	10	2	22
Non-methylation	8	3	4	21

MGMT methylation status in 84 GBMs

Among the 84 patients, MGMT methylation status was analysed respectively. All patients had MGMT promoter methylation testing results. Testing was performed via methylation specific real-time PCR at the time of diagnosis. 48 patients whose tumors tested positive for MGMT methylation were determined to be MGMT methylated (57.14%) and 36 patients whose tumors tested negative for MGMT promoter methylation were MGMT unmethylated (42.76%) (Fig. 3, Table 1).

Correlation between MGMT methylation status with Tim-3 protein expression and survival

The relationship between the expressions of Tim-3 protein and the MGMT promoter methylation status are shown in Table 1 and Fig. 3. The low expression of Tim-3 protein in TIICs was associated with the MGMT promoter methylation status. A univariate analysis revealed a significant correlation of low expression of Tim-3 protein in TIICs with MGMT promoter methylation and a better prognosis. We summarized the correlation between Tim-3 protein expression with MGMT promoter methylation and the prognosis of GBM patients (Fig. 4). Increased expression of Tim-3 protein with MGMT promoter non-methylation was significantly associated with poor prognosis. No significant correlation of Tim-3 protein expression level with gender ($P = 0.846$) or tumor location ($p = 0.447$) was observed. However, the moderate and strong expression of Tim-3 protein with MGMT promoter nonmethylation or with MGMT promoter methylation was negatively associated with good prognosis. No expression of Tim-3 protein with MGMT promoter nonmethylation have a similar effect on prognosis, compared to MGMT promoter methylation.

Multivariate Cox regression analyses confirmed that Tim-3 protein expression with MGMT promoter methylation status was an independent risk factor for survival in GBM patients. Strong expression of Tim-3 protein with MGMT promoter nonmethylation correlated significantly with shorter OS in the four subgroups ($p < 0.05$, Fig. 4). In the two subgroups, the median survival was 16.9 and 16.4 months for patients whose tumor had the unexpressed and moderate level of Tim-3 protein, whereas the median survival was 7.6 months for those with high expression of Tim-3 protein with MGMT promoter nonmethylation. In the patients with low expression of Tim-3 protein with MGMT promoter methylation have average survival time was 21.8 months.

Discussion

Glioblastoma is the most common and lethal primary brain tumor, with strong risk of recurrence and short survival periods, and the cure of this formidable disease seems impossible [2, 11]. Recent developments emphasize targeting the molecular characteristics and various approaches of immunotherapy. Although many new molecular markers have been identified, the MGMT promoter methylation status is commonly used in GBM studies [12].

The discovery of MGMT promoter methylation in GBMs as compared to its counterpart MGMT promoter nonmethylation, plays a key role in the understanding of glioma biology [13]. Increasing research displayed that the immunological tumor microenvironment of the gliomas differs based on their molecular property [14]. However, the mechanism controlling the association of MGMT promoter methylation status with the immune microenvironment is yet to be studied. The current study systemically investigated the prognostic impact of immune checkpoint molecule Tim-3 and MGMT promoter methylation status in TIICs GBMs. We identified Tim-3 protein expression with MGMT promoter methylation status as a novel prognostic parameter for GBMs. MGMT promoter methylation status was related to Tim-3 protein expression in immune cell-infiltrating GBMs. Our data demonstrated that Tim-3 protein was differently expressed in the most of GBM tissues. Then, we observed that the checkpoint molecule TIM-3 with MGMT promoter methylation status showed significant prognostic potential. Interestingly, strong expression of Tim-3 protein with MGMT promoter nonmethylation showed a poor effect on survival. Accordingly, expression of Tim-3 protein with MGMT promoter methylation status can be a potential prognostic predictor in immune cell-infiltrating GBMs.

The present study is the first, to the best of our knowledge, to report that Tim-3 protein expression with MGMT promoter methylation status is critical in prognosis for patients with GBM. Tim-3 is an immune regulatory molecule, which motivates downstream cascade events upon stimulation by its ligand [9, 15]. Emerging research has demonstrated the importance of Tim-3 in human tumorigenesis. However, no further investigations have been made with respect to the role of Tim-3 protein expression with MGMT promoter methylation status in GBM patient prognosis. Aberrant expression of Tim-3 protein has been noticed to boost tumor progression and be associated with unfavorable prognosis in many kinds of caners [9, 16–19].

Tim-3 has previously been recorded to be expressed at strong levels in prostate cancer, hepatocellular carcinoma and melanoma [9, 16–19]. The protein level of Tim-3 in the GBM tissues were initially examined using immunohistochemical analyses. We observed the expression of Tim-3 protein in the GBM interstitial tissue. In line with previous reports, the present study found that the expression of Tim-3 protein was significantly stronger in GBM samples without MGMT methylation. Tim-3 can efficiently predict the aggressive behavior of head and neck squamous cell carcinomas [20]. In prostate cancer, Tim-3 overexpression results in the attenuated level of tumor suppressor FLRT3 and increased expression of genes such as MMPs to trigger invasion and metastasis [21]. Tim-3 promotes glioma cell proliferation and increased level of Tim-3 enhances angiogenesis by inducing transdifferentiation of glioma stem cells

into endothelial cells and stabilization of vascular base membranes, which is implicated as a mechanism that Tim-3 furtherances the progression of gliomas [22]. But in that study, the authors did not explore the association between Tim-3 protein level and the prognosis of glioma patients, probably due to the rare number of glioma specimens. Therefore, the prognostic significance of Tim-3 in gliomas remains unclear.

Molecular genetic testing, in particular MGMT promoter methylation, is nowadays considered as a predictive factor for standard chemotherapy in GBM [23]. In the study, 57.14% of the responders has a routine determination of MGMT promoter methylation. The study concluded methylated patients had better results with temozolomide [24, 25]. A single center study observed that MGMT promoter methylation is an independent prognostic factor associated with a good outcome, including prolonged progression-free survival (PFS) and OS in GBM [26]. In order to look into this relationship between MGMT promoter methylation status and the expression of Tim-3 protein in GBMs, we retrieved 84 specimens from the tumor tissue bank. In line with previous findings, Tim-3 protein was expressed in different level in GBM tissues. High Tim-3 protein expression was more frequently found in GBMs without MGMT promoter methylated patients, which was capable of predicting poor prognosis of GBM patients. Among these 84 patients, the median OS was 17.3 months. The survival time for those GBM patients with low Tim-3 protein expression with MGMT promoter methylation was longer than those with mediate and strong Tim-3 expression. Subsequent multivariate cox regression analyses confirmed that Tim-3 protein expression with MGMT promoter methylation was an independent prognostic factor for GBM patients. The subgroup analysis revealed that strong Tim-3 protein expression with MGMT promoter non-methylation was remarkably correlated with the short survival for patients. The average survival time, however, hardly differs in those patients with unexpressed and mediate Tim-3 expression.

We also observed that high-risk GBM patients had higher levels of Tim-3 protein with unmethylated MGMT. An MGMT-associated immune prognostic signature, which was related to prognosis, was constructed. In addition, the immune prognostic signature provides novel insights into the GBM immune microenvironment and potential immunotherapies, which enabled us to classify patients into subgroups with distinct outcomes and immunophenotypes, signifying that it may be used to delimit the current prognostic model and facilitate further stratification of patients with GBM and improve prognosis. Therefore, we integrated the complementary value of molecular pathology and immune checkpoint molecule Tim-3 to develop a novel model to provide superior survival prediction.

Conclusions

Our data demonstrated that Tim-3 protein expression with MGMT promoter methylation status in GBMs correlated with the survival, which make Tim-3 a promising target. Our study assessed the association between the expression of Tim-3 protein with MGMT promoter methylation and clinical prognosis. For the first time, we have associated the high expression of Tim-3 protein without MGMT promoter methylation in GBM tissues with worse prognosis. Most importantly, the univariate and multivariate analyses revealed the high expression of Tim-3 protein with MGMT promoter methylation status as an absolute prognostic

factor for patients with GBM. Moreover, the checkpoint obviously associated with treatment response and prompt meaningful hints for selecting chemotherapeutic drugs.

Limitations
The current study had some limitation. First, it was a retrospective research. Second, the number of patients was limited. Third, selection bias could not be avoided completely. In addition, there might be other parameters not considered, which can influence study results.

Abbreviations

Tim-3: T cell immunoglobulin mucin-3; GBM: glioblastoma multiforme; OS: overall survival; IHC: immunohistochemistry

Declarations

Acknowledgment

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Availability of data and materials

The data during and/or analyzed during the current study are available from the corresponding author.

Authors' contributions

All authors contributed equally to the paper. JZ, XLW and SQY drafted the manuscript. LJL and YZ performed data collection and statistics. ZJC, WMH and JML supervised the data collection and revised this article. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was approved by the local ethic committee, and written informed consent was obtained from every participant.

Competing Interests

The authors report no conflicts of interest in this work

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Figures

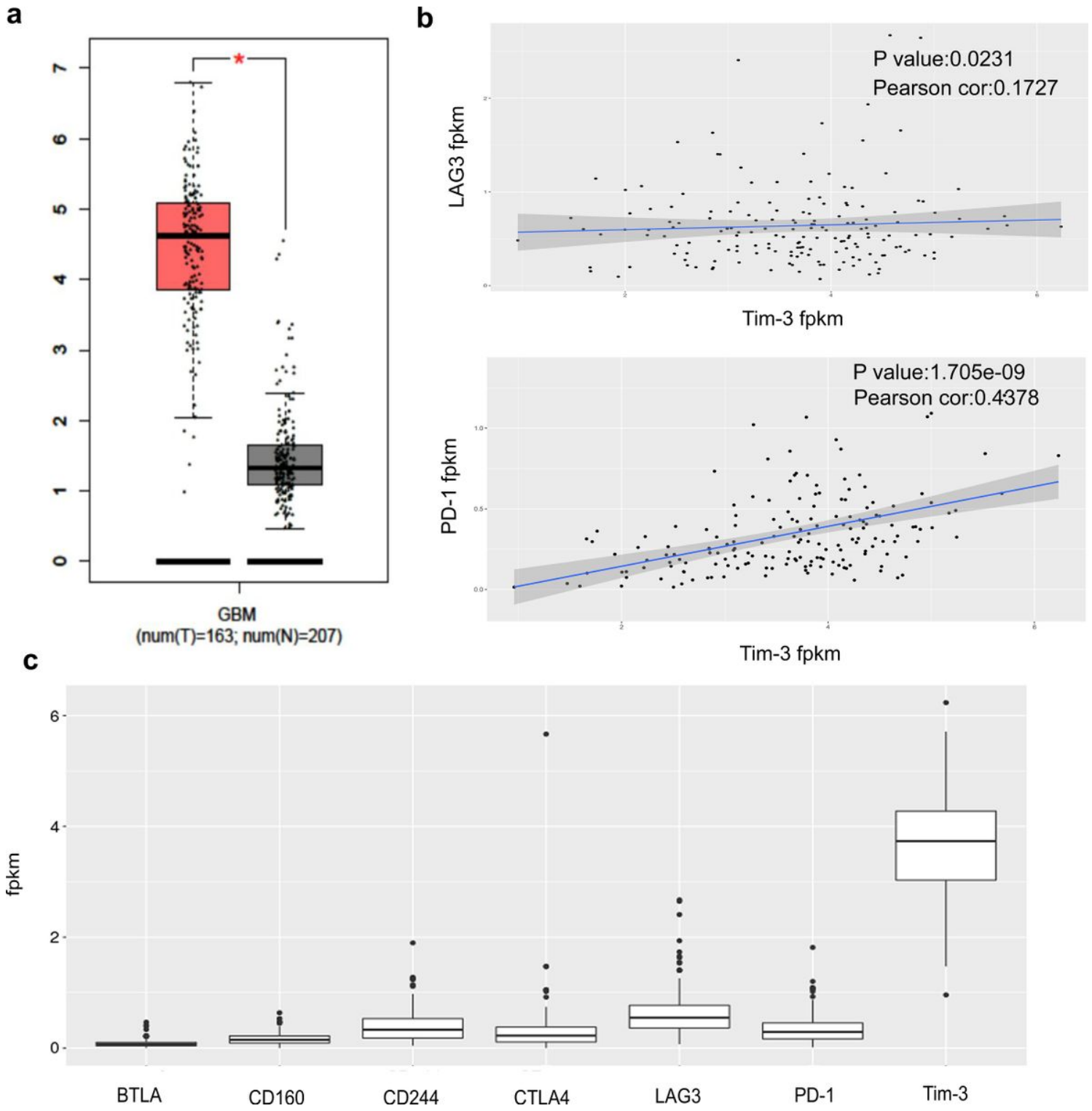

Figure 2

Differential expression of the four immune checkpoint molecules PD-1, CTLA4, TIM- 3 and LAG-3 in GBM tissues compared to corresponding normal adjacent tissues by TIMER analysis. Tim-3 protein expression in GBM compare with normal adjacent tissues (a). Tim-3 protein expression is significantly associated with LAGI and PD-1 in GBM in TCGA database (b). Tim-3 is one of exhausted genes in T cell in GBM in TCGA database (c).

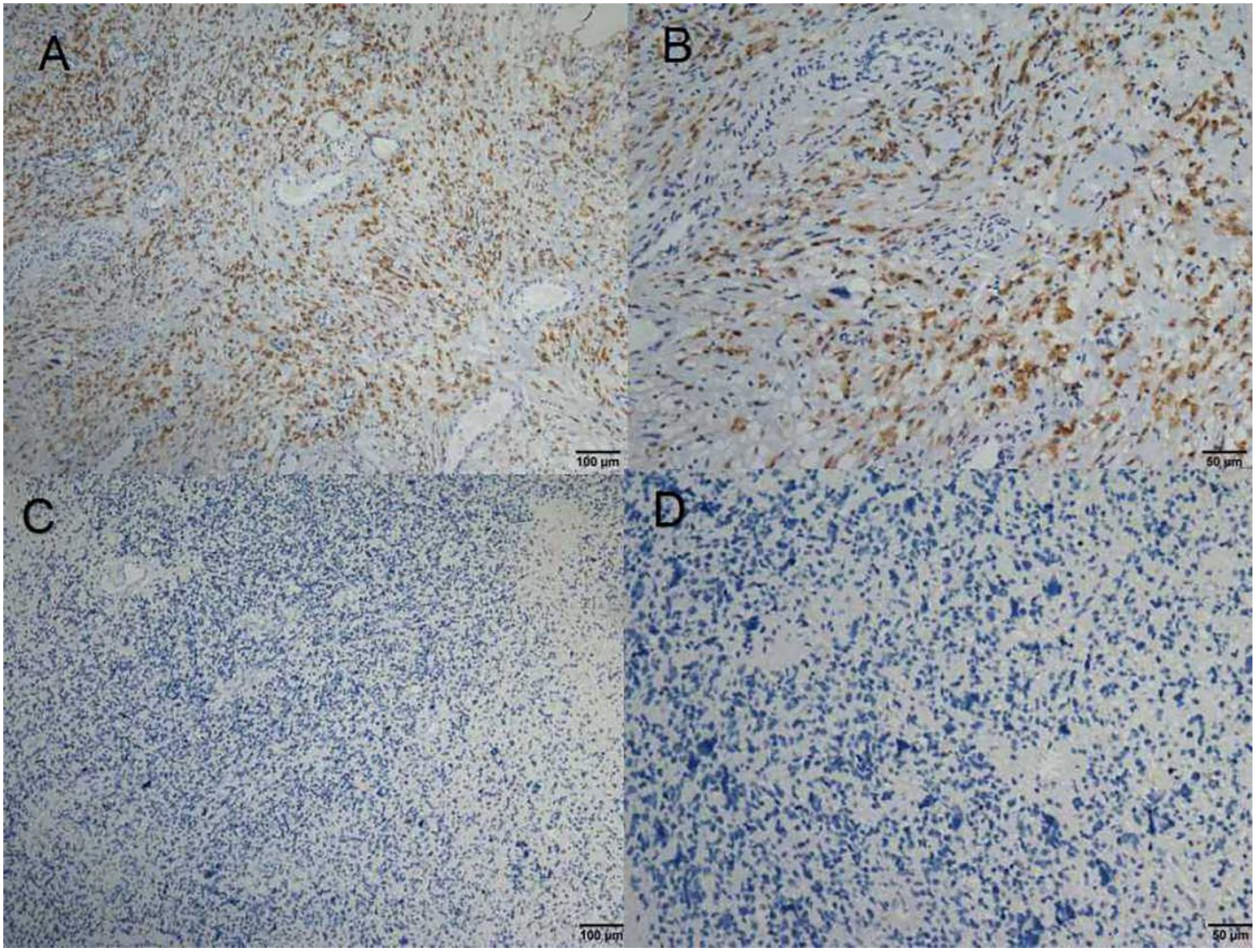

Figure 4

Immunohistochemical staining of Tim-3 protein expression in the formalin- fixed, paraffin-embedded GBM tissues.

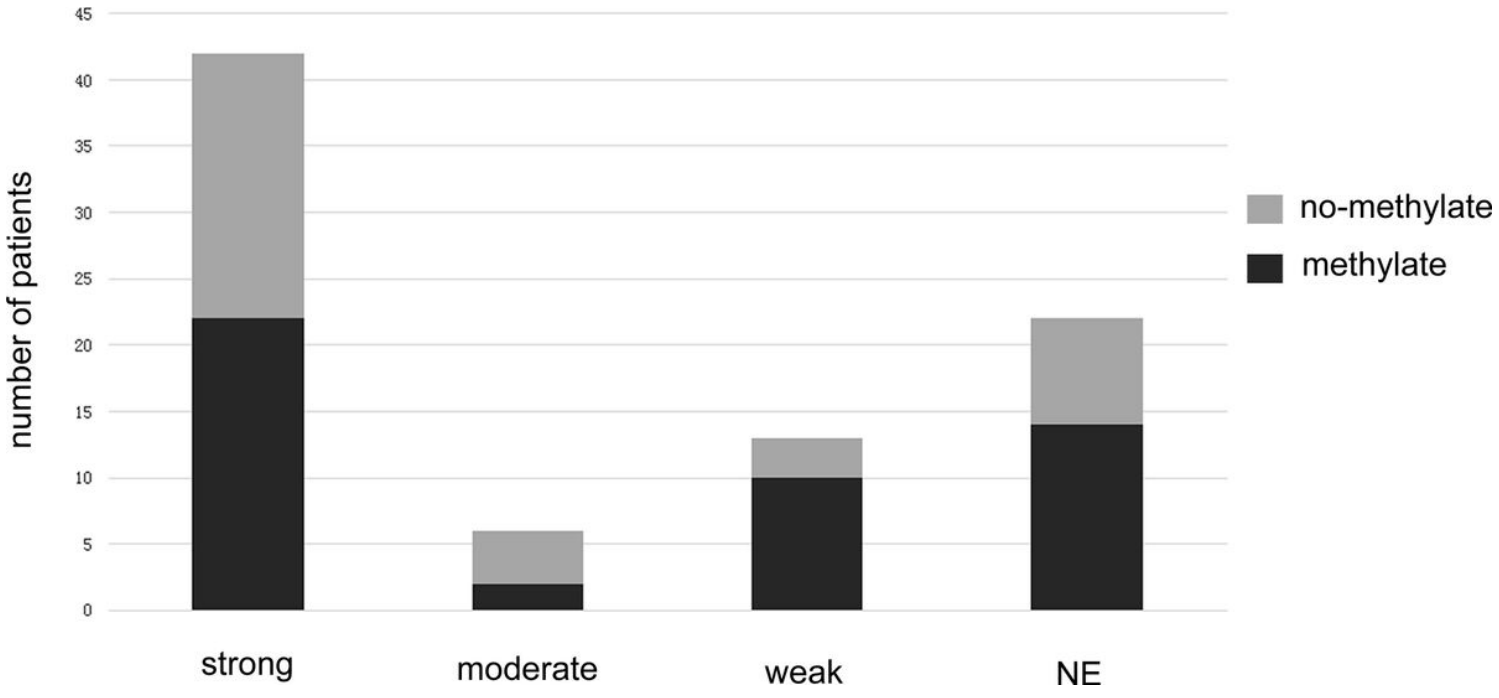

Figure 6

The bar graph shows methylated and unmethylated MGMT distribution in different Tim-3 protein expression group.

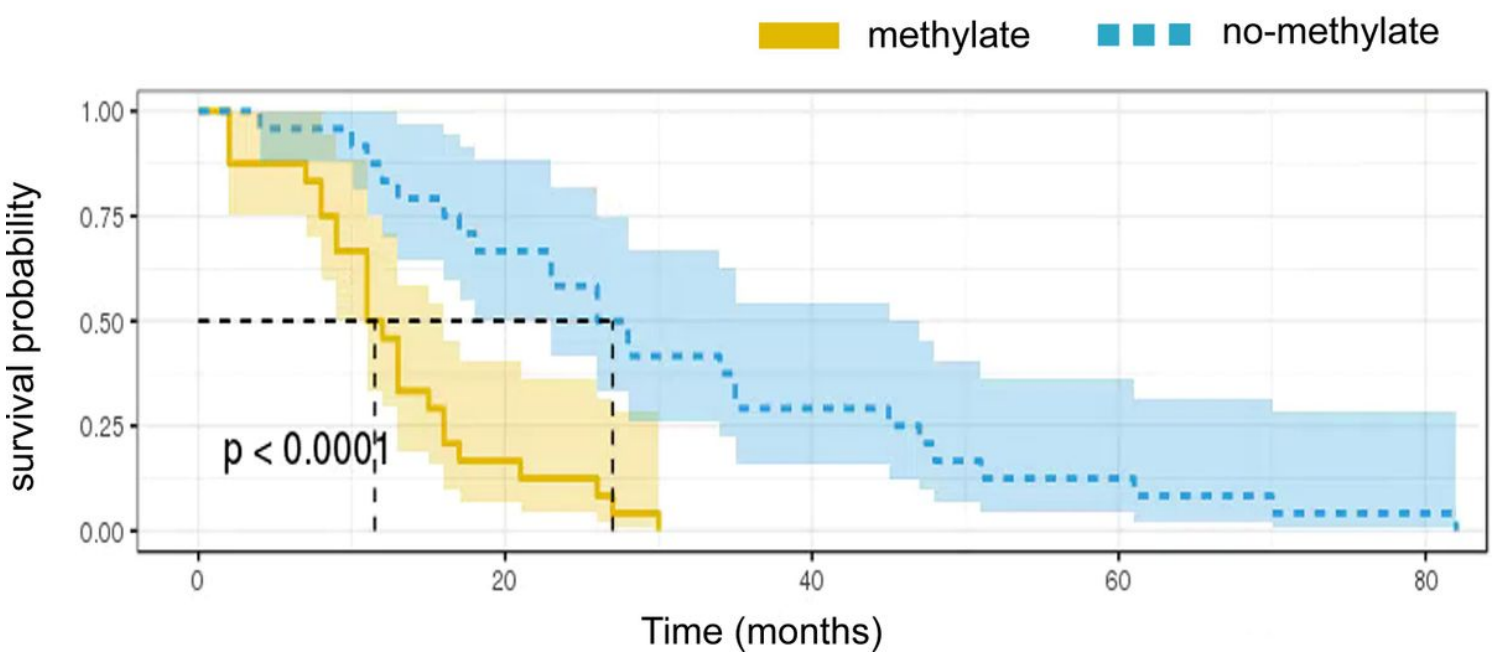

Figure 8

Kaplan-Meier survival curves for overall survival according to the MGMT promoter methylation status.

Supplementary Files

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