Increased *LGALS3* expression independently predicts shorter overall survival in patients with the proneural subtype of glioblastoma

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**Abstract**

In the current study, we tried to study the expression of *LGALS3* and *LGALS3BP*, their potential as prognostic markers and the possible genetic/epigenetic mechanisms underlying their dysregulation in different subtypes of glioblastoma (GBM). An in silico retrospective study was performed using large online databases. Results showed that *LGALS3* and *LGALS3BP* were upregulated at both RNA and protein levels in GBM tissue and were generally associated with shorter overall survival (OS) in GBM patients. However, in subgroup analysis, we only found the association in proneural subtype. The copy number alterations did not necessarily lead to *LGALS3/LGALS3BP* dysregulation. In the proneural subtype of GBM patients, hypermethylation of the two CpG sites (cg19099850 and cg17403875) was associated with significantly lower expression of *LGALS3*. In univariate and multivariate analysis, *LGALS3* expression independently predicted shorter OS in the proneural subtype of GBM (HR: 1.487, 95% CI: 1.229-1.798, *P* < 0.001), after adjustment of age, gender, *IDH1* mutations, temozolomide chemotherapy, radiotherapy and *LGALS3BP* expression. In comparison, *LGALS3BP* lost the prognostic value in multivariate analysis. Based on these findings, we infer that *LGALS3* expression serves as an independent biomarker of shorter OS in the proneural subtype of GBM, the expression of which might be regulated in an epigenetic manner.

**KEYWORDS**

glioblastoma, *LGALS3*, *LGALS3BP*, proneural subtype

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**1 | INTRODUCTION**

Glioblastoma multiforme (GBM) (WHO grade IV Astrocytoma), is the most malignant tumor that begins within the brain. It accounts for about 15% of all brain tumors, with overall median survival only around 15 months.¹ In the past decades, two prognostic biomarkers attracting significant interest were isocitrate dehydrogenase (*IDH*) mutations and O(6)-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation.²

*IDH* mutations lead to the loss of native enzymatic activity and subsequent abnormal production of 2-hydroxyglutarate (2-HG).³ 2-HG can inhibit the enzymic activity of DNA demethylases and result in increased DNA methylation. Due to this influence, a subset of GBM shows Glioma CpG Island Methylation Phenotype (G-CIMP).⁴ GBM patients...
with IDH1 mutations or those belonging to the G-CIMP pheno-
type have significantly improved OS.\(^5\) Besides, MGMT
promoter methylation is also a powerful and independent in-
dicator of therapeutic responses among GBM patients who
received chemotherapy with alkylating agents.\(^6\) However,
these markers have their own limitations in clinical use. IDH1
mutations are rare in primary GBM cases.\(^7\) In compari-
son, the prognosis value of MGMT promoter might depend
on the chemotherapeutic substances used \(^6\) and might lose the
value under some conditions.\(^8,9\) Therefore, it is quite mean-
ingful to further explore other potential prognostic markers in
GBM. However, GBM is a not a homogenous disease, but has
heterogeneous histological features.\(^10,11\) The Cancer Genome
Atlas (TCGA) researchers divided GBM into four subtypes
(Proneural, Neural, Classical and Mesenchymal) according to
their gene expression pattern.\(^12\) Besides the genomic differ-
ences, these subtypes varied significantly in survival length,
patient age and treatment response. Therefore, exploring spe-
cific prognostic marker of the GBM subtypes might support
better therapeutic management.

The galectins are a group of beta-galactoside–binding
proteins that involve in regulating cell-cell and cell-matrix
interactions.\(^13\) Till now, galectin-1, -2, -3, -4, -7, -8, -9, -10
and -12 have been identified in humans. Galectin-3 encoded
by LGALS3 gene plays an important role in cell adhesion,
growth, differentiation, apoptosis, as well as angiogenesis in
both normal and cancerous tissues.\(^14\) Its upregulation might
serve as a valuable prognostic marker in multiple cancers,
such as breast cancer,\(^15\) gastric cancer,\(^16,17\) colorectal cancer\(^18\)
and liver cancer.\(^19\) Ureguplated LGALS3 was also observed
in GBM tissues.\(^20,21\) Galectin-3–binding protein (Gal-3BP) is
a secreted glycoprotein encoded by LGALS3BP gene. In the
microenvironment of human neuroblastoma, Gal-3BP inter-
acts with Galectin-3 (Gal-3) in bone marrow mesenchymal
stem cells (BMMSC) and induces transcriptional upregulation
of IL-6, via the Gal-3BP/Gal-3/Ras/MEK/ERK signaling
pathway.\(^22,23\) These findings suggest that these two genes
may collaboratively participate in the pathological process of
cancer.

In the current study, we tried to study the expression of
LGALS3 and LGALS3BP, their potential as prognostic mark-
ers and the possible genetic/epigenetic mechanism underly-
ing their dysregulation in different subtypes of glioblastoma
(GBM), by using large online databases.

2 | PATIENTS AND METHODS

2.1 | Retrospective analysis using data from TCGA-GBM

The level-3 data in TCGA-GBM were obtained using the
UCSC Xena Browser (https://xenabrowser.net/heatmap/).\(^24\) The recurrent tumors and cases that had a history
of neoadjuvant treatment were excluded. Affymetrix Human
Genome U133 Array Strip (AffyU133a) data was used to
quantify gene expression. Based on these criteria, a total of
508 primary tumor cases and 10 adjacent normal tissue
cases were included in this study. The genomic, clinicopat-
ological and survival data of the included patients were
downloaded. Briefly, the data included IDH1 mutations,
gene expression subtypes, age at initial pathologic diagno-
sis, longest tumor dimension, gender, karnofsky performance
score (KPS), overall survival (OS) time, temozolomide
chemotherapy status, radiation therapy status, LGALS3/
LGALS3BP expression, LGALS3/LGALS3BP DNA copy
number alterations (CNAs, calculated by an algorithm called
Genomic Identification of Significant Targets in Cancer 2.0
[GISTIC2])\(^25\) and their DNA methylation (quantified by
Infinium HumanMethylation27 BeadChip). In the GISTIC2,
CNAs were defined as −2: homozygous deletion; −1: hete-
rozygous deletion, 0: copy-neutral, +1: low–level copy gain,
+2: high–level amplification.

MGMT-TP2 model,\(^26\) which includes two CpG sites
(cgi12434587 and cg12981137) was used determine the
MGMT promoter methylation status.

2.2 | Data mining in the R2 web-based
application

The association between LGALS3/LGALS3BP expression
and OS in GBM patients was also analyzed using genomic
and survival data in GSE16011 from GEO datasets,\(^27\) using
the R2 web–based application (https://hgserv1.ams.nl/cgi-
bin/r2/main.cgi). Only the GBM cases in GSE16011 were
included in Kaplan-Meier survival analysis. The best cutoff
was identified using scan model.

2.3 | Data mining in the HPA

LGALS3 and LGALS3BP protein expression in normal brain
tissues (typically cerebral cortex and hippocampus) and
GBM tissues was examined using data from the Human
Protein Atlas (HPA) (available fromwww.proteinatlas.
org).\(^28,29\) Immunochemical images and protein scoring data
of LGALS3 and LGALS3BP were retrieved.

In this database, protein expression score is based on the
combination of staining intensity (negative, weak, moderate
or strong) and fractions (<25%, 25%-75% or >75%) of the
immunohistochemical images. Protein expression score is
defined as: not detected (negative or weak <25%); low (weak
combined with either 25%-75% or 75%); medium (moder-
ate <25%—low; moderate combined with either 25%-75%
or 75%); and high (strong <25%—medium, strong combined
with either 25%-75% or 75%). Besides, protein expression
scores are manually adjusted as necessary when evaluated by
the expert annotators.\(^28,29\)
2.4 Statistical analysis

Statistical analyses were conducted using SPSS 25.0 software (SPSS, Chicago, IL) and GraphPad Prism 7.04 (GraphPad Inc, La Jolla, CA). One-way ANOVA with Bonferroni post hoc tests and Welch’s t test (unequal variances t test) were performed to assess the statistical differences. Kaplan-Meier OS curves were generated using GraphPad Prism 7.04. Receiver operating characteristic (ROC) analysis for death detection was performed to identify the best cutoff (Youden index) for gene expression in Kaplan-Meier curves. The difference between the curves was compared using the Log-rank test. The independent prognostic value of LGALS3/LGALS3BP expression in proneural subtype was assessed using the univariate and multivariate Cox regression models. P < 0.05 was considered as statistically significant.

3 RESULTS

3.1 Both LGALS3 and LGALS3BP RNA expression were upregulated in GBM tissues compared with adjacent normal tissues

In TCGA-GBM, 508 cases of primary GBM and 10 adjacent normal tissues were subjected to AffyU133a microarray analysis of gene expression. Using the array data, we compared the expression of LGALS3 and LGALS3BP in GBM and the adjacent normal tissues, as well as the different subtypes of GBM (Figure 1A). Results showed that both LGALS3 and LGALS3BP were significantly upregulated in GBM tissues compared with adjacent normal tissues (Figure 1A-C). Since the distinct molecular subtypes of GBM show varying prognosis and responses to aggressive chemotherapy and radiotherapy, we also examined the expression profiles of these two genes in the subtypes. Group comparison showed that LGALS3 expression varied significantly among the four subtypes, in which the mesenchymal and proneural subtypes had the highest and lowest expression, respectively (Figure 1D). In comparison, LGALS3 expression in the neural and proneural subtypes was significantly lower than that in the mesenchymal and classical subtypes (Figure 1E).

3.2 LGALS3 and LGALS3BP protein expression was not detectable in glial cells in normal brain tissues, but was detectable in GBM tissues

Using IHC staining images and protein expression scoring in the HPA, we examined LGALS3 and LGALS3BP protein expression in normal brain and GBM tissues. According to the data in the HPA, LGALS3 and LGALS3BP protein expression was not detectable in glial cells in normal brain tissues (Figure 2, left). In comparison, among 9 cases of GBM...
with LGALS3 examined, 8 cases showed positive LGALS3 staining (3 low and 5 medium) (Figure 2, right). In addition, 8 out of 10 GBM cases had positive LGALS3BP staining (1 low, 2 medium and 5 high). These findings confirmed that LGALS3 and LGALS3BP were expressed at the protein level in GBM tissues.

3.3 | **LGALS3 and LGALS3BP upregulation was associated with unfavorable OS in GBM patients**

By comparing LGALS3 and LGALS3BP expression between the deceased and living GBM cases, we found that the deceased group had significantly elevated LGALS3 and LGALS3BP expression ($P = 0.005$ and $P = 0.006$, respectively) (Figure 3A,B). Then, we tried to explore the association between the expression of these two genes and the survival outcomes by generating Kaplan-Meier curves, using the survival data in TCGA-GBM. Via setting the best cutoff identified in the ROC analysis for death detection, we found that the high LGALS3 expression group and the high LGALS3BP expression group had significantly shorter OS compared to the respective low expression group ($P = 0.007$ and $P = 0.013$, respectively) (Figure 3C,D). To verify these trends, we also used genomic and survival data in another study (Tumor Glioma French database, GSE16011 from GEO datasets 27), which included 156 cases of GBM out of 284 glioma cases. Results confirmed the association between...
the high gene expression and unfavorable OS in GBM patients (Figure 3E,F).

3.4 Kaplan-Meier OS analysis in the four molecular subtypes of GBM

Since we found that both LGALS3 and LGALS3BP expression varied significantly in the four subtypes of GBM, we then tried to explore whether the association between the high gene expression and unfavorable survival was consistent in the four subtypes. Subgroup analysis suggested that LGALS3 and LGALS3BP expression had no prognostic value in terms of OS in mesenchymal, classical and neural subtypes (Figure 4A,C,E,G). In comparison, the associations were confirmed in proneural subtype (Figure 4D,H).

3.5 LGALS3 expression was not correlated with its DNA CNAs, but was associated with IDH1 mutations in proneural GBM

Using genomic data in TCGA-GBM, we further investigated the potential genomic and epigenetic alterations associated with dysregulation of these two genes. The proneural subtype was prone to have LGALS3 heterozygous deletion (50/125, 40%) (Figure 5A). In comparison, LGALS3BP amplification (28/125, 22.4%) was more common than heterozygous deletion (6/125, 4.8%) (Figure 5B). However, these genetic alterations did not necessarily lead to LGALS3/LGALS3BP dysregulation (Figure 5C-D). Previous studies found that IDH1 mutation is an important prognostic marker in GBM patients and is associated with hypermethylation status of...
a series of genes. By checking the correlation between these two genes and IDH1 mutations, we found that the IDH1 mutation group had significantly lower LGALS3 expression ($P < 0.001$, Figure 5E). But this association was not observed in LGALS3BP expression ($P = 0.70$, Figure 5F).

### 3.6 LGALS3 expression is related to its DNA methylation status in proneural GBM

Since IDH1 mutations are generally associated with hypermethylation status, we then checked whether LGALS3 expression was related to its DNA methylation level. By examining the correlation between LGALS3 expression and its DNA methylation (methylation 27k data), we found that the group of patients with hypermethylation of the two CpG sites (cg19099850 and cg17403875) had significantly lower expression of LGALS3 (Figure 6A, green box). In comparison, this association was not observed in terms of LGALS3BP expression (Figure 6B). By separating the patients according to median LGALS3/LGALS3BP methylation, we found that the LGALS3 hypermethylation group had significantly better OS (Figure 6C). No significant association was observed between LGALS3BP methylation and OS (Figure 6D).

### 3.7 LGALS3 expression was an independent prognostic indicator of OS in proneural subtype, after the adjustment of IDH1 mutations

To explore the independent prognostic value of LGALS3 expression in terms of OS in proneural subtype, we performed
univariate and multivariate analysis based on COX regression model. The clinicopathological, genomic and survival data used were given in supplementary Table S1. Univariate analysis showed that older age, male patients, no IDH1 mutations, no temozolomide chemotherapy, no radiotherapy, increased LGALS3 expression and increased LGALS3BP expression were risk factors of shorter OS (Table 1). In multivariate analysis, increased LGALS3 expression was independently associated with shorter OS (HR: 1.487, 95% CI: 1.229‐1.798, \( P < 0.001 \)) (Table 1), after adjustment of other risk factors. However, LGALS3BP expression had no independent prognostic value (Table 1).

4 | DISCUSSION

A series of studies confirmed the oncogenic properties of LGALS3 expression in glioma. Galectin-3 expression is correlated with the malignant potential of tumors in the central nervous system.\(^{30}\) Its expression could be induced under hypoxic and nutrient deprived microenvironments, which protects cells from cell death.\(^{30}\) In addition, it also enhances GBM cell motility.\(^{31}\) However, previous studies found that LGALS3 expression varied significantly in different types of glioma, such as supratentorial pilocytic astrocytoma, anaplastic astrocytoma and glioblastoma.\(^{21,32}\) These findings suggest that Galectin-3 might have varying regulatory effects on different types of glioma. In terms of LGALS3BP, it is characterized as an onco-protein that regulates the malignant behaviors of multiple cancers.\(^{13,35}\) In this study, we confirmed the upregulation of LGALS3 and LGALS3BP at both RNA and protein level in GBM tissues. Besides, we found that LGALS3 expression was generally associated with shorter OS in GBM patients. Since we observed that these two genes had different expression in the 4 subtypes of GBM, we further performed subtype group analysis to check the robustness of the finding. In subgroup analysis, we only found the association between LGALS3/LGALS3BP expression and unfavorable OS in proneural subtype, but not in other subtypes.

Among the subtypes, the proneural subtype has the most frequent mutations in the IDH1 gene. In addition, the proneural subtype had the longest median OS, around 17 months.\(^{36}\) In this study, we found that among 60 cases of proneural subtype patients with IDH1 mutations examined, only 13 cases had mutations. Therefore, although IDH1 mutations might be a powerful prognostic marker, it is still necessary to explore other robust prognostic biomarkers for the large proportion of proneural patients without IDH1 mutations. In univariate analysis, we found that...
the promoter methylation status of MGMT might not be a prognostic marker in proneural subtype, when not considering the therapeutic strategy. This finding is consistent with previous studies that suggest the prognostic value of MGMT promoter methylation depends on the chemotherapeutic substances used.³⁶ By conducting univariate and multivariate analysis, we confirmed that LGALS3 expression was an independent prognostic indicator of shorter OS in the proneural subtype of GBM (HR: 1.487, 95% CI: 1.229-1.798, P < 0.001), after the adjustment of age, gender, IDH1 mutations, temozolomide chemotherapy, radiotherapy and LGALS3BP expression. In comparison, although LGALS3BP expression is a risk factor in univariate analysis, it lost the prognostic value in multivariate analysis. These findings suggest that LGALS3 expression might be a valuable prognostic biomarker in the proneural subtype of GBM.

LGALS3 methylation expression is associated with the loss of the galectin-3 expression in the mucinous colorectal carcinomas,³⁷ prostate cancer³⁸ and breast cancer,³⁹ suggesting that its expression is controlled in an epigenetic manner. Since IDH1 mutations lead to hypermethylation status and G-CIMP phenotypes⁴ and is associated with suppressed LGALS3 expression, we examined the correlation between the methylation status of LGALS3 and their expression. Results showed LGALS3 DNA hypermethylation was associated with decreased LGALS3 expression. This phenomenon suggests that DNA methylation is a potential epigenetic alteration leading to LGALS3 dysregulation in the proneural subtype of GBM.

This study also had some limitations. Firstly, some clinical information, such as surgical resection and the extent of resection were not recorded in TCGA-GBM. This might impair the credibility of the findings. Secondly, although the Tumor Glioma French database was used to validate the prognostic value of LGALS3, this database had no information of molecular subtypes. Therefore, more detailed validation analysis based on a large cohort of the proneural subtype is required in the future.

5 | CONCLUSION

LGALS3 expression serves as an independent biomarker of shorter OS in the proneural subtype of GBM, the expression of which might be regulated in an epigenetic manner.
CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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