

Original Article

RARRES1 is a novel immune-related biomarker in GBM

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Abstract: Immunotherapy is a promising route for the treatment of glioblastoma (GBM). Researchers have conducted a large number of studies on the pathogenesis of GBM; however, these studies are not comprehensive. High-throughput sequence analysis allows for insights into the pathogenesis of GBM. In this study, we used The Cancer Genome Atlas dataset to identify the function of RARRES1 enriched in GBM, especially in the WHO grade-IV cases. We discovered that RARRES1 is highly expressed in patients with mesenchymal subtype, unmethylated MGMT, IDH1 wild type, and non-G-CIMP, all of which are molecular characteristics of malignant GBM. Results of the immune microenvironment analysis showed that RARRES1 is strongly correlated with dendritic cells PD1, PDL2, TIM3, and CTLA4, which are the immune checkpoints in GBM. Furthermore, according to the overall survival and status analysis, a high expression of RARRES1 was found to be an unfavorable factor for prognosis. This indicates that RARRES1 may participate in the pathogenesis and immune-related processes in GBM, and may serve as a therapeutic target.

Keywords: Glioblastoma, TCGA, RARRES1, immune checkpoints, survival

Introduction

Gliomas are the most common primary tumors of the central nervous system, with an incidence of approximately 500,000 [1]. Among gliomas, pleomorphic glioblastoma (GBM) is the most malignant type, accounting for 50% of all adult primary brain tumors. The median survival time of untreated glioma patients is 14.6 months and the 3-year survival rate is improved by only 10% after surgical resection combined with radiotherapy and chemotherapy [2]. Therefore, it is critical to investigate the pathogenesis of GBM for effective treatment.

RARRES1, a retinoid acid receptor-responsive gene, has been reported to be dysregulated in many forms of cancers, such as nasopharyngeal carcinoma, colorectal adenocarcinoma, and melanoma [3-5]. Researchers have suggested that RARRES1 can regulate proliferation and migration of tumor cells; however, the role of RARRES1 in GBM remains unknown.

In this study, we analyzed the expression of RARRES1 between lower-grade gliomas (LGG)

and GBM (WHO grade IV). Moreover, we investigated the relationships among the subtypes, CIMP-status, mutation of isocitrate dehydrogenase 1 (IDH1), O6-methylguanine-DNA-methyltransferase (MGMT) status, and RARRES1. Furthermore, the functional enrichment analysis of RARRES1-related genes revealed that RARRES1 was mainly involved in immunomodulation. Moreover, the correlation between the immune status and RARRES1 suggested that RARRES1 is highly associated with immune checkpoints. Ultimately, the survival analysis showed that RARRES1 expression is associated with adverse clinical outcomes in GBM patients.

Material and methods

Data information

In the current study, the expression level of RARRES1 and clinical information of GBM were acquired from The Cancer Genome Atlas (TCGA) dataset (<https://portal.gdc.cancer.gov/>) [6]. Overall survival was calculated from the date of diagnosis to the date of death or last

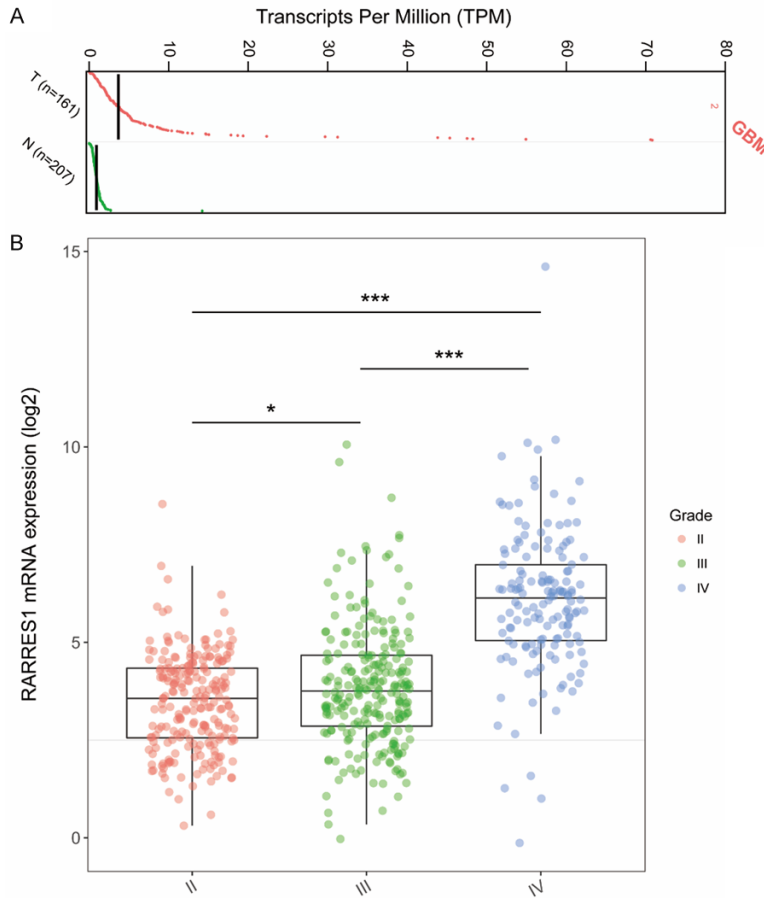


Figure 1. Expression level of RARRES1 in GBM. A. RARRES1 is remarkably overexpressed in GBM tissues compared with normal brain tissues (T: tumor; N: normal). B. RARRES1 is significantly upregulated in GBM (WHO grade IV) compared with WHO grades II and III in TCGA dataset. * and *** represent $P < 0.05$ and $P < 0.001$, respectively.

follow-up. Owing to the lack of expression data from normal brain tissues, we obtained information from the Gene Expression Profiling Interactive Analysis (GEPIA) website (<http://gepia.cancer-pku.cn/>) [7], which contained TCGA and the Genome-Tissue Expression data, to test the expression level of RARRES1 in normal brain.

Expression level of RARRES1

The expression level of RARRES1 was analyzed between different WHO-grade groups. Compared with traditional histopathological diagnosis, the correlation among molecular subtype analysis, IDH1 mutation status, glioma-CpG island methylator phenotype (G-CIMP) status, MGMT methylation status, and RARRES1 was calculated.

Function of RARRES1 in GBM

To explore the functional role of RARRES1 in GBM, RARRES1-related genes with an absolute value of Pearson's correlation coefficient higher than 0.3 were identified. Subsequently, the clusterProfiler R package was utilized to analyze the biological process, molecular function, and cellular component categories [8].

Immune-related analysis

Studies revealed that the immune microenvironment of GBM is crucial in GBM and is associated with the disease progression [9-11]. To better understand this, the correlation between RARRES1 and the immune score acquired by the ESTIMATE website was calculated (<https://bioinformatics.mdanderson.org/estimate/>). Furthermore, the Tumor Immune Estimation Resource (TIMER) web tool (<http://cistrome.org/TIMER/>) was utilized to examine the correlation between RARRES1 and major immunocytes [12]. In addition, the

relationship between RARRES1 and immune checkpoints, such as programmed cell death-1 (PD1), programmed death-ligand 1 (PD-L1), T-cell immunoglobulin, and mucin 3 (TIM3) were evaluated using Pearson's correlation analysis.

Survival analysis

Kaplan-Meier plot analysis was performed to analyze the overall survival and prognosis of patients with GBM.

Methylation status of RARRES1

The methylation status of RARRES1 was investigated using the MEXPRESS web tool (<https://mexpress.be/>) [13].

RARRES1, an immune marker in GBM

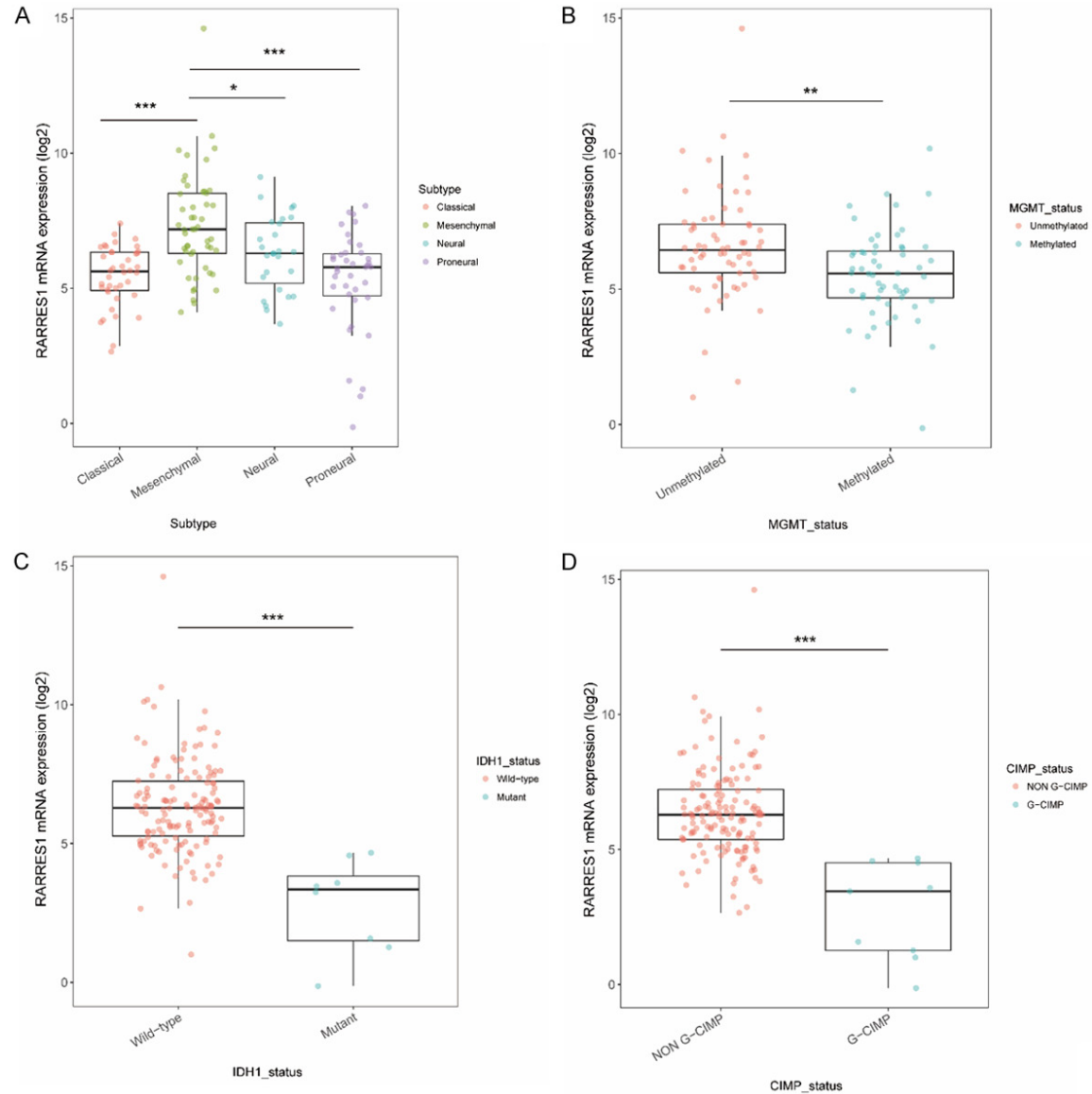


Figure 2. RARRES1 is highly correlated to molecular phenotype of GBM. A. RARRES1 is significantly enriched in mesenchymal subtypes. B. RARRES1 is markedly downregulated in MGMT methylated groups. C. RARRES1 is significantly increased in IDH1 wild type. D. RARRES1 is significantly decreased in G-CIMP group. *, **, and *** represents $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Statistical analysis

Statistical analyses were performed using SPSS20.0, with the calculation of a two-sided p value where a Fisher exact test was used for categorical data, a log-rank test for obtaining a Kaplan-Meier curve, and Pearson correlation coefficients for correlation analysis. A p -value of 0.05 was considered as the significant threshold in all analyses.

Results

RARRES1 is upregulated in GBM

First, we analyzed the expression level of RARRES1 in GBM (WHO grade IV) tissues and compared it with that of normal brain tissues using the GEPIA web tool; we observed a significant difference with $P < 0.05$ (Figure 1A). Subsequently, the expression level of RARRES1

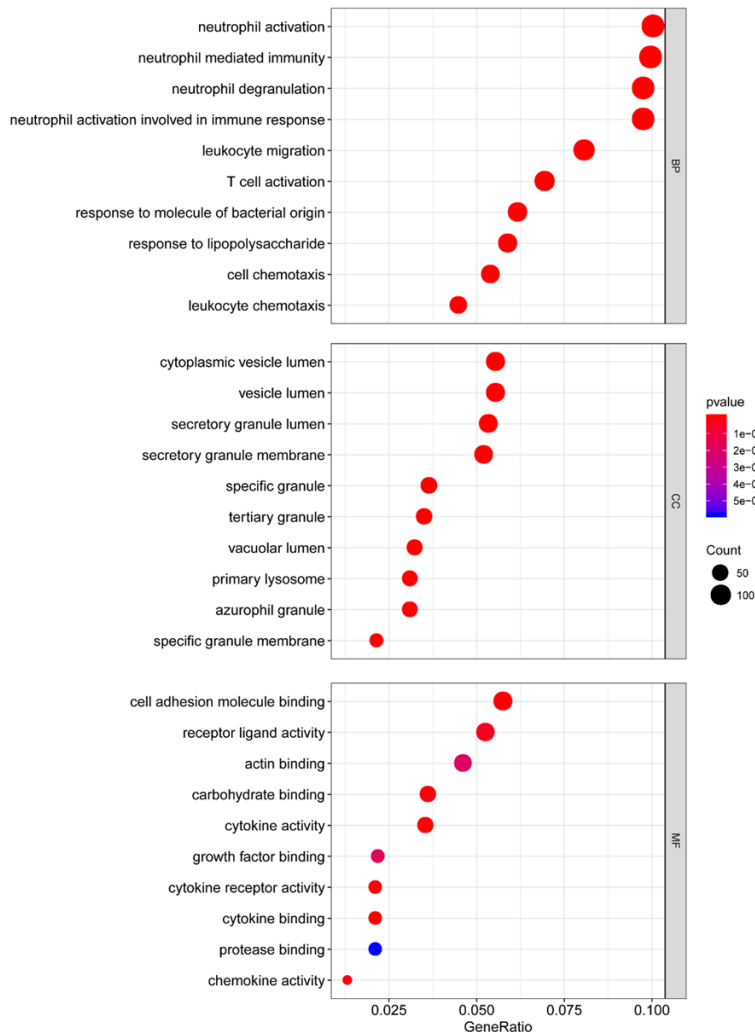


Figure 3. Functional enrichment analysis of RARRES1-related genes. Gene Ontology analyses, including biological process, molecular function, and cellular component were performed by clusterProfiler R package. The size of each spot represents the gene number represented by this term, while the color represents the *p*-value of each term.

among GBM tissues with different WHO grade levels was investigated. As expected, RARRES1 exhibited a greater expression in GBM (WHO grade IV) compared with WHO grades II and III (Figure 1B).

RARRES1 is correlated with molecular phenotype of GBM

Although the WHO grade classification of GBM is based on pathomorphological analysis, numerous studies have demonstrated that the prognosis of patients with the same WHO grade can vary owing to difference in molecular genetics background [14]. After in-depth research,

favorable biomarkers of GBM have been identified. For example, the presence of IDH wild-type and non-G-CIMP subtypes have been correlated with an unfavorable prognosis compared with other subtypes [15, 16]. In addition, the presence of methylated MGMT promoter in patients signifies that the tumor is more sensitive to chemotherapy and radiotherapy and thus has a favorable prognosis [17]. Furthermore, in 2010, GBM was classified into four subtypes (classical, mesenchymal, neural, and proneural) according to the gene map; it was identified that the mesenchymal subtype exhibited the worst clinical outcome [18]. Therefore, we investigated RARRES1 expression in GBM according to its molecular classification. In brief, RARRES1 is more enriched in mesenchymal subtypes than in classical, neural, and proneural subtypes (Figure 2A). Subsequently, regarding the MGMT methylation status, RARRES1 is significantly overexpressed in the methylated group compared with the unmethylated groups (Figure 2B). In addition, RARRES1 is more upregulated in patients with wild

type IDH1 than in those with mutant type IDH1 (Figure 2C). Furthermore, patients with G-CIMP have a lower expression of RARRES1 than those without G-CIMP (Figure 2D). These findings suggest that the role of RARRES1 is critical in GBM pathogenesis.

RARRES1 is involved in immune process of GBM

To investigate the function of RARRES1 in GBM, Pearson coefficient analysis was performed to identify the genes related to RARRES1. A total of 1557 genes were discovered as RARRES1-related genes according to our criteria men-

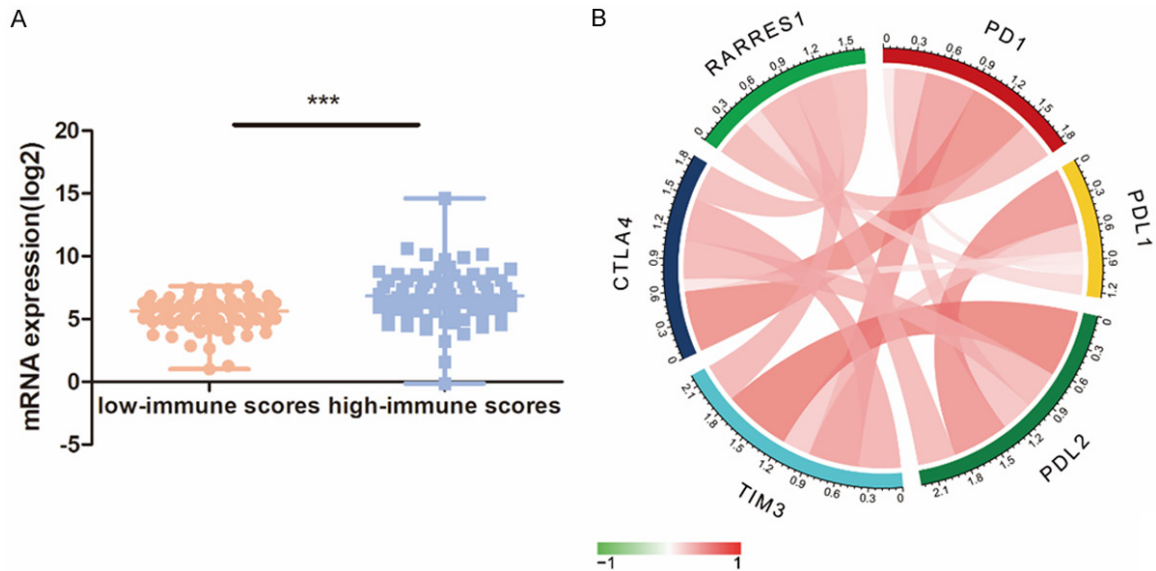


Figure 4. RARRES1 is highly correlated with the immune status of GBM. A. RARRES1 is highly expressed in samples with high immune scores. B. RARRES1 is positively correlated with immune checkpoints, including PD1, PDL1, PDL2, TIM3, and CTLA4. The color of each line between genes represents the Pearson coefficient. ***represents $P < 0.001$.

tioned above. Subsequently, a functional enrichment analysis of these RARRES1-related genes revealed that they were mainly involved in immune activation and regulation processes such as neutrophil activation, T-cell activation, and cytokine activity (**Figure 3**). Based on this, we further investigated the correlation among immune microenvironment, immune checkpoints, and RARRES1. Recent studies have shown that the immune microenvironment of GBM tumors is crucial in the prognosis of GBM; hence, we investigated the correlation between RARRES1 and the immune scores of each sample. We found that RARRES1 is highly expressed in groups with high-immune scores (**Figure 4A**). Subsequently, correlations between immune checkpoints including PD1, PD-L1, PD-L2, TIM3, CTLA4, and RARRES1 were evaluated. We found that RARRES1 had a positive correlation with the proteins mentioned above, especially with TIM3 ($r = 0.38$, $P < 0.001$) (**Figure 4B**).

RARRES1 is correlated with dendritic cell (DC) in GBM

Our previous results indicated that RARRES1 was closely related to the immune microenvironment in GBM. The microenvironment of glioma tissue is composed of tumor and immune as well as many factors secreted by them, such

as growth factors, chemokines, pro-inflammatory, and anti-inflammatory factors. They interact with each other to regulate the immune effects in the region and ultimately determine the outcome of the disease. Therefore, we further analyzed the correlation between infiltrated immune cells and RARRES1 by TIMER, a web tool to analyze infiltrated immune cells in the TCGA dataset. We discovered that RARRES1 has a negative correlation with the purity of GBM and is highly correlated to DCs (**Figure 5A**). To validate the correlation between RARRES1 and DCs, we investigated the correlation between RARRES1 and the markers of DCs (**Figure 5B**), which supports our previous hypothesis that RARRES1 is highly correlated with DCs.

RARRES1 is a marker for poor prognosis in GBM

As our results mentioned above indicated that RARRES1 exhibited an extensive role in GBM pathogenesis and microenvironment, we further evaluated the prognostic value of RARRES1 in GBM and its subtypes. Samples were divided into two groups according to the median value of RARRES1, and the Kaplan-Meier plot analysis showed that RARRES1 served as a marker for poor prognosis in both GBM (**Figure 6A**) and proneural subtype (**Figure 6B**).

RARRES1, an immune marker in GBM

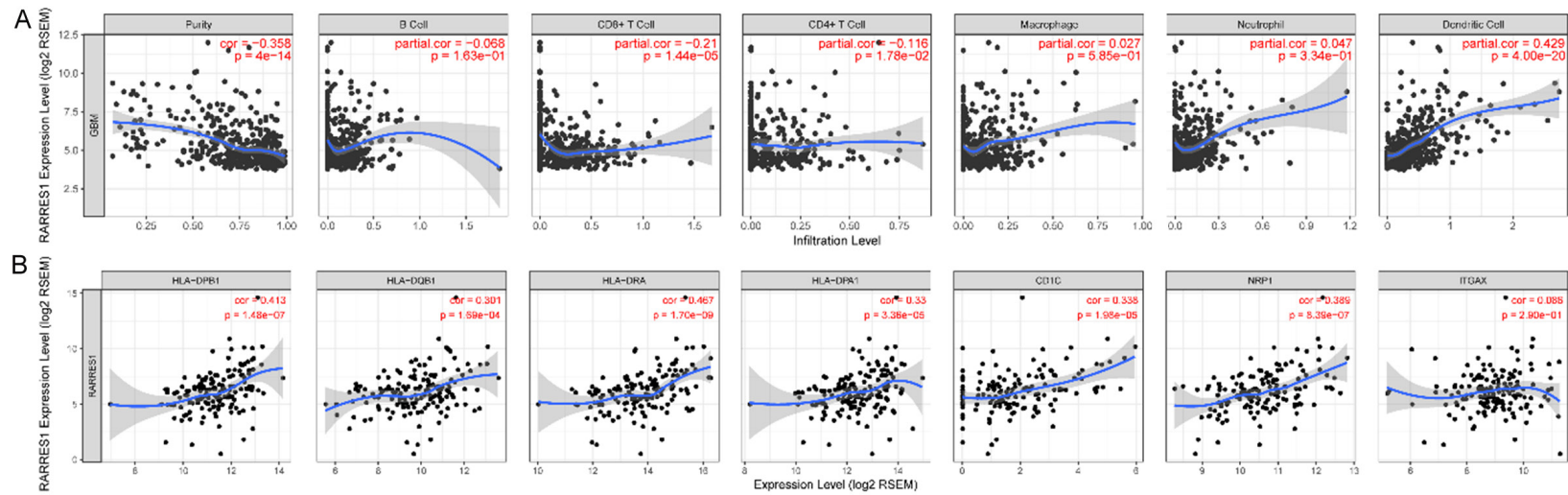


Figure 5. RARRES1 is highly correlated with dendritic cells. A. Correlation between RARRES1 and infiltrated immune cells in GBM. B. Correlation between RARRES1 and marker of dendritic cells.

RARRES1, an immune marker in GBM

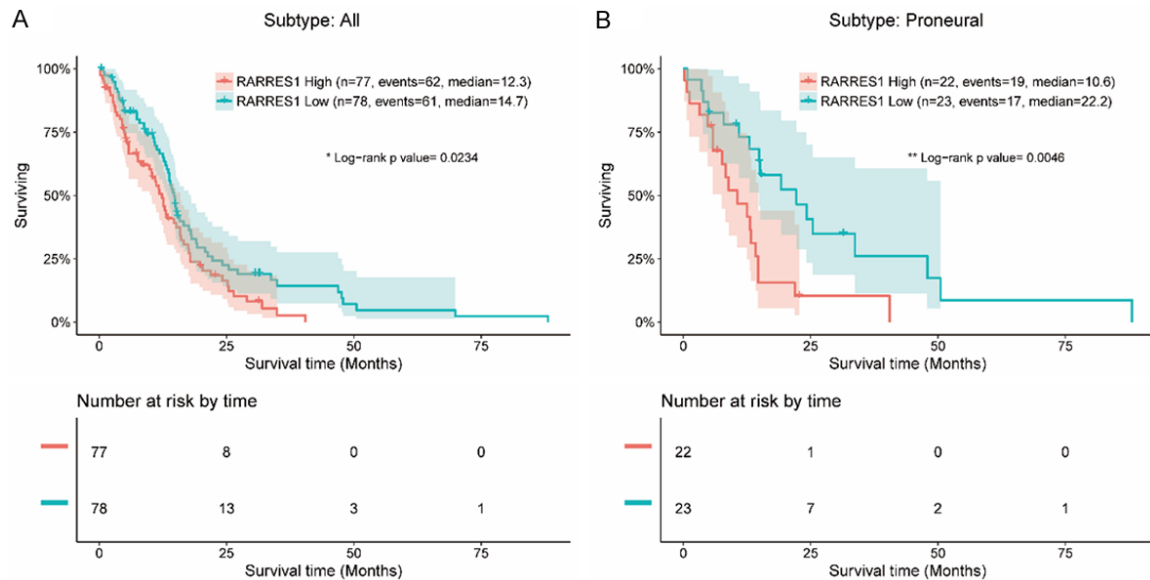


Figure 6. RARRES1 is an indicator of unfavorable clinical outcome in GBM. A. GBM patients with higher expressions of RARRES1 have poorer prognoses compared to those with lower expressions of RARRES1. B. Patients with higher RARRES1 have poor prognosis in proneural subtype.

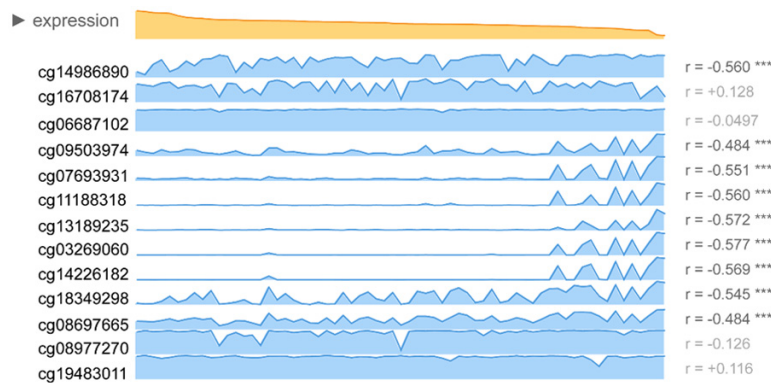


Figure 7. RARRES1 expression is negatively correlated with the methylation of its sites. Samples were ranked according to their RARRES1 expression. **represent p-value < 0.01.

RARRES1 expression correlated with its methylation

Previous research has demonstrated that the expression level of RARRES1 may be regulated by its methylation status. We explored the methylation status of the tumors and discovered that many methylated sites exhibited a negative correlation with RARRES1 expression, indicating that RARRES1 expression levels may be regulated by its methylation, as observed in other forms of cancers (Figure 7).

Discussion

In this study, we found that RARRES1 is upregulated in GBM compared with not only normal brain tissues but also tissues from LGG (WHO grades II, III), thus indicating its oncogenic role. Subsequently, we discovered that RARRES1 level is highly correlated with CIMP status, IDH1 mutation, and MTMT methylation, thus revealing that RARRES1 has a strong relationship with a malignant GBM molecular phenotype.

Considering that studies focusing on RARRES1 are limited, we applied functional enrichment analysis on RARRES1-related genes, and the results of this analysis revealed that most biological process categories were related to the activation and regulation of immune processes, indicating a strong relationship of RARRES1 with the immune microenvironment of GBM tumors.

The central nervous system had long been regarded an immune privileged area; however, recent findings have revealed that it is not an

“immune privileged organ”, as it not only exhibits complex immune response regulations, but can also produce corresponding immune responses [19-21]. Under physiological conditions, only a few circulating immune cells are present in the brain parenchyma owing to the blood-brain barrier [22]. However, when tumors occur, all types of immune cells can migrate to the tumor area through damaged or open blood-brain barriers and exert an anti-tumor effect [23]. Furthermore, the tumor cells can be induced by these infiltrated immune cells to produce immunosuppressive phenotypes and induce functional inhibition, thus promoting tumor growth. Glioma cells can escape from immune surveillance and exist in the body owing to three main reasons: 1) weak immunogenicity of glioma cells; 2) secretion of a large number of immunosuppressive factors by glioma cells; 3) expression of major histocompatibility complexes and co-stimulatory molecule B7 in glioma cells [24]. Therefore, the key to inducing glioma immunity is to effectively present the glioma antigen to effector cells.

Among the immune cells, DCs have the strongest ability to capture and present antigens. In the immune system, they can directly activate helper T-cells and cytotoxic T lymphocytes *in vivo* and cause B-cells to produce antibodies. The anti-tumor immune response of the body is closely related to the strong antigen-presenting function of DCs. Hence, DC-based immunotherapy has become a major form of tumor immunotherapy.

A high correlation between DCs and RARRES1 suggests that RARRES1 contributes to the regulation of DCs. We further verified the relationship between the markers of DCs and RARRES1 to confirm our hypothesis. In addition, survival analysis showed that high levels of RARRES1 may serve as an index of unfavorable clinical outcome in GBM, especially in the proneural subtype. In conclusion, RARRES1 may serve as an oncogene in the pathogenesis of GBM; hence, it can be considered a novel immune-related biomarker for GBM. In future studies, we will perform further experimental investigations to verify our results.

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Disclosure of conflict of interest

None.

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