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ORIGINAL RESEARCH

REST, not *REST4*, is a risk factor associated with radiotherapy plus chemotherapy efficacy in glioma

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Background/aim: Repressor element silencing transcription factor (*REST*) is a transcription repressor, expressed in several malignancies. This study aims to evaluate the prognostic values of *REST* and its splicing variant *REST4* in glioma, and investigate the potential correlation between *REST* and *REST4*.

Methods: *REST* and *REST4* expression values were evaluated by qRT-PCR in 89 patients with gliomas and 10 with normal brain tissues.

Results: Upregulation of *REST* was related to higher World Health Organization (WHO) grade, larger tumor size, higher ki67, and higher p53 positive rate. After radiotherapy+temozolomide (RT+TMZ) treatment, low *REST* expression patients could get better therapeutic efficacy (P=0.031). The positive rate of *REST4* expression was only 13.5% in glioma tissues, and *REST4* expression was not associated with clinical characteristics and *REST* expression in this study.

Conclusions: *REST* was a prognostic factor in glioma, while *REST4* was not. *REST* expression can be a predictor in evaluating the survival outcome of gliomas patients treated with RT+TMZ after surgery.

Keywords: *REST*, *REST4*, glioma, radiotherapy, chemotherapy, prognosis

Introduction

Glioma, one of the most common primary brain tumors, accounts for 74.6% of malignant brain and central nervous system (CNS) tumors.[1] According to the National Comprehensive Cancer Network (NCCN) guidelines of CNS cancers, maximum surgical resection followed with radiotherapy (RT), plus concomitant and adjuvant chemotherapy (temozolomide, TMZ) is the recommended therapeutic strategy for newly diagnosed glioma.[2] However, the median overall survival time of glioblastoma is still less than 15 months after treatment with this recommended therapy.[3] Recently, several genetic alterations, such as IDH1 mutation[4] and O-6-methylguanine-DNA methyltransferase (MGMT) methylation,[5] have been identified as the prognostic biomarkers of glioma, which impels us to find more genetic biomarkers to make it more precise for gliomas diagnosis and target therapy.

REST (repressor element silencing transcription factor), also known as neuron restrictive silencer factor (NRSF), is a transcription repressor. By binding to its target genes, *REST* can regulate gene expression in neural and non-neural cells depending on cell types. In neural cells and tumors, *REST* acts as an oncogene. However, in non-neural cells and tumors, such as lung,[6] breast,[7] and colon,[8,9] *REST* acts as a tumor suppressor.[10] In neural cells, the dysregulation of *REST* was reported to be associated with neurological dysfunction, such as Parkinson's disease,[11,12] epilepsy,[13] and ischemic stroke.[14] In glioblastoma cells, *REST* promoted cell proliferation and migration.[15]

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In medulloblastoma, *REST* expression was elevated, and high *REST* was correlated with poor prognosis.[16]

REST4, an alternative splicing variant of *REST*, was reported to be a nuclear target of *REST* because of its number five zinc finger motif.[17] Recently, *REST4* was demonstrated to be downregulated, and influence *REST* expression, in glioma.[18] However, *REST4* expression was only detected in eight glioma tissues, which needs further verification. Therefore, our work focuses on verifying the interaction between *REST* and *REST4*, and investigating whether *REST4* will be a prognostic biomarker in glioma.

REST expression has been confirmed to be associated with glioma grades.[18] In this study, we aimed to investigate the predictive and prognostic value of *REST* and its splicing variant, *REST4*, in glioma, and explore the influence of *REST* expression on RT and chemotherapy in glioma patients.

Materials and methods

Patients and tissue samples

A total of 89 primary glioma tissues and 10 normal brain tissues were enrolled in this study. All of these patients were recruited from Xiangya Hospital of Central South University from July 2015 to December 2016. Gliomas were diagnosed and graded by the Pathology Department of Xiangya Hospital according to the WHO (World Health Organization) grading system. In the present study, patients with chronic disease or other cancers were excluded. All participants gave written informed consent, and this study was approved by the Ethics Committee of the Institute of Clinical Pharmacology in Central South University. Tissue samples were stored in liquid nitrogen immediately after surgery until further experiments. The clinical characteristics, such as age, gender, MGMT promoter methylation, and IDH1 mutation were all collected from the case management system in Xiangya Hospital.

Treatment

According to the demand of patients, some of them chose the hospital near to their home to receive subsequent therapy, and only 40 patients received the RT plus TMZ concomitant and adjuvant chemotherapy in Xiangya Hospital. About 1 month after surgery, patients received a total dose of RT of 54–60 Gy, which was divided into 28–30 days, and 1.8–2.0 Gy daily. Concomitant and adjuvant chemotherapy (TMZ) was conducted with 120–150 mg per day during the RT. After these treatments for about 30 days, magnetic resonance imaging (MRI) was performed, and the short-term therapeutic efficacy was assessed according to the results of MRI. Based on the MRI, complete response (CR) was defined as the disappearance of all enhancing and non-enhancing tumor; partial response (PR) as a decrease of more than 50% of the diameter of the primary tumor; stable disease (SD) meant the remaining conditions or a small increase of under 25%; while progressive disease (PD) was defined as an increase of at least 25% in tumor size or the appearance of a new tumor.[19] Also, the adverse reactions were recorded during therapy.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from all tissues by trizol reagent (Code No 9108, Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. The quality and quantity of extracted RNA was measured by a NanoDrop Spectrophotometer (Shimadzu Biotech, Beijing China). The extracted RNA was determined to be pure only when the A260/A280 ratio is 1.8 to 2.1. For the reverse transcription reaction, 1 μg of total RNA was used by PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (Code No RR047A, Takara Bio Inc., Kusatsu, Japan). The qRT-PCR was conducted by Light Cycle@480 II (Roche, Basel, Switzerland) by using a SYBR® Premix DimerEraser™ (Perfect Real Time) (Code No: RR091A, Takara Bio Inc.) in a 20 μL mixture according to the manufacturer's protocol. The reaction was carried out at 95°C for 30 s for one cycle, and 95°C for 5 s, 55°C for 30 s, and 72°C for 30 s for 45 cycles. Primers used in these reactions are listed in Table 1. The relative expression of target gene was normalized to the endogenous gene β-actin, calculated by the $2^{-\Delta\Delta Ct}$ method. All of this qRT-PCR was conducted in duplicate.

Expression analysis in GEO and TCGA database

Four expression profiles (GSE4271,[20] GSE4412,[21] GSE7696,[22,23] and GSE4290[24]) were obtained from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/)

Table 1 Primer sequences used in this study

Gene	Sequence	Base
REST-F	ACTCAGCGTCGTAGAACCTCA	21
REST-R	CGAAAGGGTTTGGTCTTCGAG	21
REST4-F	ACTCATACAGGAGAACGCCCA	21
REST4-R	GGCTTCTCACCCATCTAGATCAC	23
β-actin-F	CCTGGCACCCAGCACAAT	18
β-actin-R	GGGCCGGACTCGTCATAC	18

database.[25] All of these series were based on Affymetrix Human Genome U133 Plus 2.0 Array platform (Affymetrix Inc., Santa Clara, CA, USA). In GSE4271, 77 primary high-grade astrocytomas, including WHO grade III and IV astrocytomas, were analyzed. A total of 24 grade III and 50 grade IV gliomas were used in GSE4412, 80 glioblastomas, and four non-tumor tissues in GSE7696, and 23 non-tumor tissues, 157 gliomas (including 45 grade II, 31 grade III, and 81 grade IV) data were obtained in GSE4290. The clinical information and gene expression data of 152 glioblastoma multiforme (GBM) and 460 LGG (low grade glioma) patients were downloaded from The Cancer Genome Atlas (TCGA) (Illumina HiSeq) (http://www.cancergenome.nih.gov/). In this dataset, most of the gliomas patients were treated with RT and/or TMZ chemotherapy.

Statistical analysis

All statistical analyses were performed using SPSS 18.0 (IBM Corp, Chicago, IL, USA) and Graph Pad Prism 5 (Graph Pad Software, Inc, San Diego, CA, USA). The measurement data between two groups were compared by Student's *t*-test, and performed with mean±standard deviation (SD). The goodness-of-fit chi-squared test was used to analyze the classified variable. Overall survival (OS) was deemed as the clinical end point of this study. The survival analysis was calculated by the Kaplan–Meier method and log-rank *t*-test to compare the difference between two groups, using the median value as the cutoff. Two-tailed $P<0.05$ was chosen for the criterion of statistical significance.

Results

REST is upregulated in glioma

Before experiments, we compared the expression difference of *REST* between glioma tissues and non-tumor brain tissues in the GSE4290 series of GEO datasets. As shown in Figure 1A, the expression level of *REST* was significantly elevated in glioma tissues compared with the normal brain tissues ($P<0.001$). The hierarchical cluster analysis also showed the upregulation of *REST* in glioma patients (Figure 2). Then, 89 glioma tissues and 10 normal brain tissues (six males and four females) were collected to verify *REST* expression by qRT-PCR. In this study, we found that *REST* was upregulated in gliomas compared to the normal brain tissues (Figure 1B, $P<0.001$), and this was in accordance with the analysis in GSE4290 and the reported manuscript.[18]

Association between *REST* and clinical characteristics in glioma patients

To investigate the correlation between *REST* expression and clinical characteristics in glioma patients, we divided these patients into two groups (low *REST* expression group, n=44; high *REST* expression group, n=45) based on the median *REST* expression level. As listed in Table 2, there were no significant changes of *REST* expression in age, gender, or preoperative KPS (Karnofsky Performance Score). After chi-square (χ^2) test, we found that *REST* expression was significantly associated with the WHO grade ($P=0.015$) and tumor size ($P=0.037$). *REST* expression was significantly

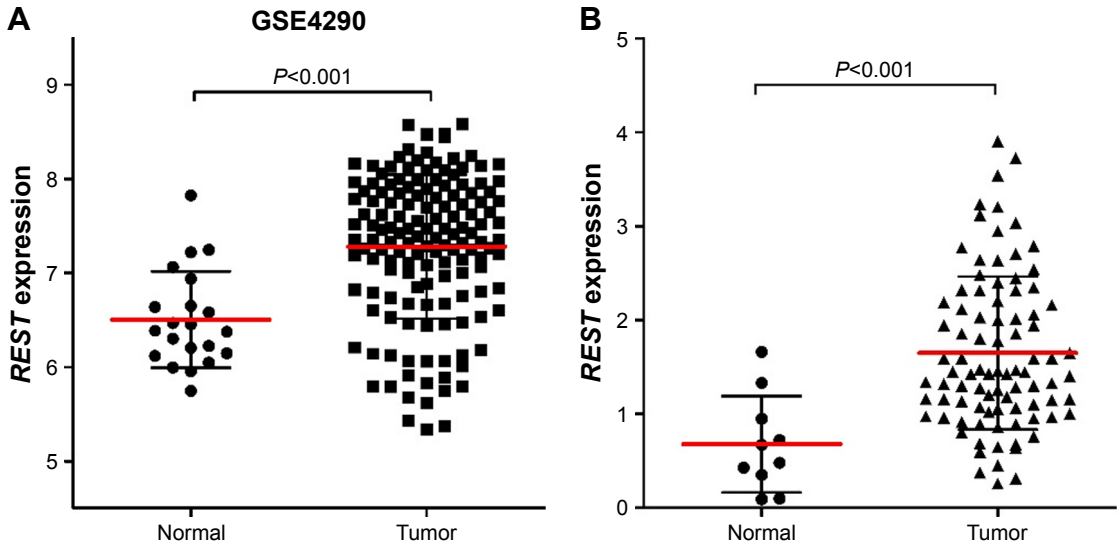

Figure 1 The scatter plot of *REST* mRNA expression level in non-tumor brain tissues and glioma in (**A**) the GEO dataset and (**B**) validated tissues.
Note: Data were analyzed by Student's *t*-test.
Abbreviations: mRNA, messenger RNA; GEO, Gene Expression Omnibus.

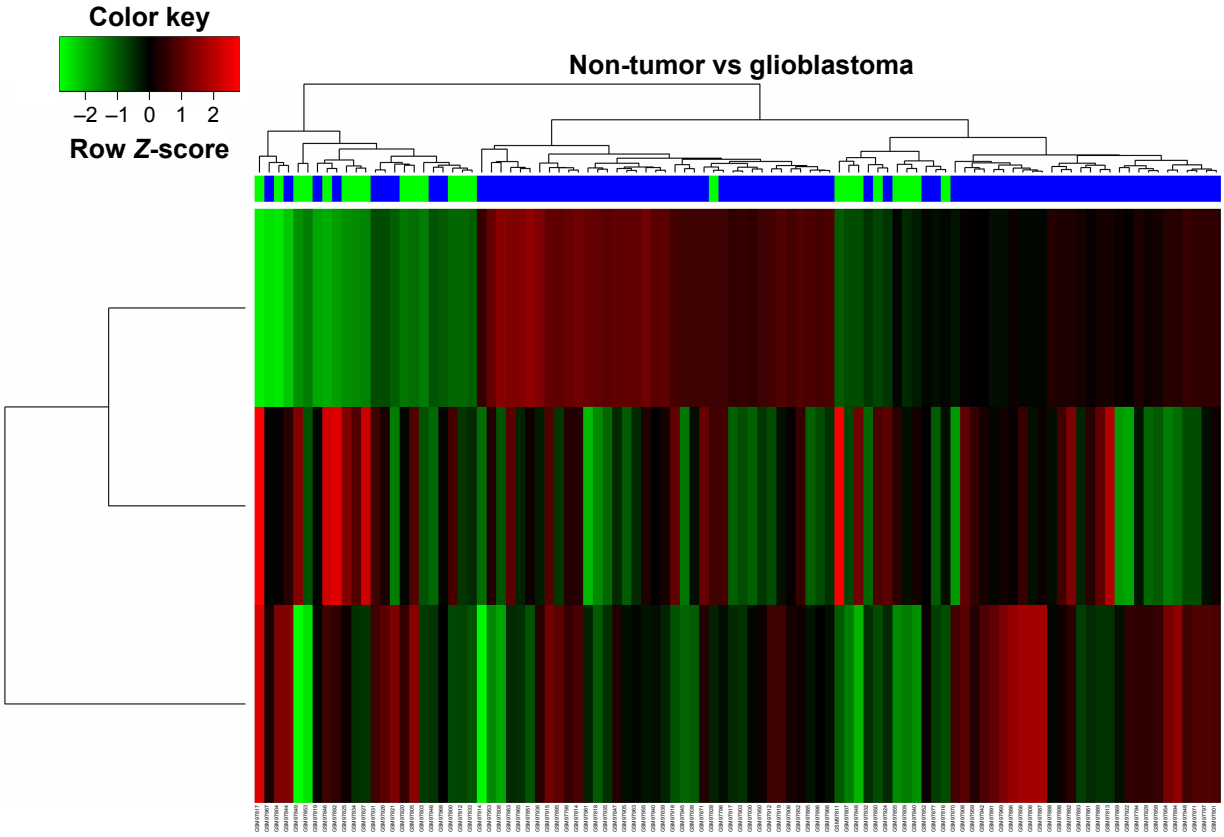

Figure 2 Clustering and heat map of three probe sets of *REST* in GSE4290.
Notes: Upregulated *REST* was clustered with red, while downregulated was clustered with green. The dendrogram reflects the relationship among samples; the blue bars represent glioblastoma samples and the green bars indicate non-tumor controls.

higher in the high grade gliomas compared with the low grade gliomas in the GSE4290 series (Figure 3A, $P<0.001$) and the verification samples (Figure 3B, $P=0.013$). In other words, the expression level of *REST* was positively correlated with the malignant degree of gliomas. Further, high *REST* gliomas tissues were detected with high positive rates of Ki67 ($P=0.026$) and p53 ($P=0.001$). However, the IDH1 mutation and MGMT promoter methylation were not associated with *REST* expression in these tissues ($P=0.604$ and $P=0.308$, respectively).

REST is a prognostic factor in glioma

To further determine the prognostic significance of *REST* in glioma, Kaplan–Meier analysis and log-rank *t*-test were carried out to analyze the relationship between *REST* expression and glioma patients' OS in GEO and TCGA datasets. As shown in Figure 4, patients with low *REST* expression level showed a better prognosis than high *REST* in all GEO datasets ($P=0.011$, HR=2.038, 95% CI=1.180–3.520 in GSE4271; $P=0.003$, HR=2.59, 95% CI=1.328–3.842 in GSE4412; $P=0.039$, HR=1.801, 95% CI=1.031–3.844 in GSE7696) and the TCGA dataset ($P<0.001$, HR=1.933, 95% CI=1.450–2.576 in TCGA).

Association between *REST* and RT+TMZ therapeutic efficacy in glioma patients

To further evaluate the association between *REST* expression and the therapeutic efficacy and toxicity after RT+TMZ treatment, 40 glioma patients treated with RT plus TMZ concomitant and adjuvant chemotherapy were enrolled. According to the MRI and the evaluated criteria mentioned in the method, 17 patients were categorized as CR+PR, and the response rate was 42.50% (17/40). Among these 17 patients, there were 12 in the low *REST* group and five in the high. The chi-square test showed that *REST* expression was associated with the therapeutic response after RT+TMZ treatment (Table 3, $P=0.031$). The survival analysis of TCGA datasets also showed that *REST* expression was significantly related to the OS of glioma patients treated with RT or chemotherapy ($P<0.001$, HR=1.995, 95% CI=1.423–2.797 in Figure 5A and $P<0.001$, HR=2.215, 95% CI=1.553–3.159 in Figure 5B, respectively). In other words, low *REST*

Table 2 Relationship between *REST* expression and glioma clinical characteristics in 89 patients

Characteristics	n	Low *REST* expression (n=44)	High *REST* expression (n=45)	*P*-value
Age (years)*	89	41.16±20.07	39.00±18.54	0.600
Gender				0.593
Male	46	24 (52.2%)	22 (47.8%)	
Female	43	20 (46.5%)	23 (53.5%)	
Preoperative KPS score				0.778
<80	4	2 (50.0%)	2 (50.0%)	
≥80	77	39 (50.6%)	38 (49.4%)	
WHO grade				0.015[a]
I–II	43	27 (62.8%)	16 (37.2%)	
III–IV	46	17 (40.0%)	29 (60.0%)	
Tumor size (cm)				0.034[a]
<3	13	10 (76.9%)	3 (23.1%)	
3–5	31	17 (54.8%)	14 (45.2%)	
≥5	45	17 (37.8%)	28 (62.2%)	
GFAP				0.544
Positive	86	42 (48.84%)	44 (51.16%)	
Negative	3	2 (66.7%)	1 (33.3%)	
Ki67				0.026[a]
<15%	46	28 (60.9%)	18 (39.1%)	
≥15%	43	16 (37.2%)	27 (62.8%)	
P53				0.001[a]
Positive	57	22 (38.6%)	35 (61.4%)	
Negative	32	22 (68.8%)	10 (31.2%)	
IDH1 mutation				0.604
Mutation	36	19 (52.8%)	17 (47.2%)	
Wild type	53	25 (47.2%)	28 (52.8%)	
MGMT promoter methylation				0.308
Methylated	9	3 (33.3%)	6 (66.7%)	
Not methylated	80	41 (51.3%)	29 (48.7%)	

Notes: Data were analyzed by chi-square (χ^2) test and *by Student's *t*-test, given as mean±SD. [a]Statistically significant values.

Abbreviations: KPS, Karnofsky Performance Score; WHO, World Health Organization; GFAP, Glial fibrillary acidic protein; MGMT, O-6-methylguanine-DNA methyltransferase; SD, standard deviation.

expression glioma patients could achieve better therapeutic efficacy of RT or/and chemotherapy compared with the high *REST* patients.

Apart from the therapeutic effects, we also observed the adverse reactions after RT+TMZ in these 40 glioma patients. As shown in Table 3, the expression level of *REST* was significantly associated with the occurrence of gastrointestinal reaction (P=0.002), but not associated with head discomfort and mucocutaneous lesion. In other words, it would be more probable for high *REST* glioma patients to have a gastrointestinal reaction after being treated with TMZ.

Expression of *REST4* in glioma

Apart from the expression of *REST*, we also detected the expression of *REST* splicing variant, *REST4* in glioma. First, there was no *REST4* expression value in TCGA and GEO datasets because *REST4* is a transcript of *REST*. Then, we detected *REST4* expression in 89 glioma tissues and nine non-tumor brain tissues by using qRT-PCR. In these specimens, the positive rate of *REST4* expression was 13.5% (12/77) based on the cut-off Ct value of 35.0. In the correlation analysis of *REST4*, we found there was no significant association between *REST4* expression and *REST* or the clinical characteristics (Table S1).

Discussion

In this study, we validated that *REST* is upregulated in both GEO datasets and the verified glioma tissues. High *REST* expression was significantly associated with high WHO grades, high Ki67, and high TP53 mutation. As we all know, Ki67 is a proliferation marker,[26] with scores also demonstrated to be associated with glioma patient's survival.[27] Also, it has been suggested as an important supplementary tool in the diagnostics and treatment of glioma.[28] Further, TP53 is a well-known cell cycle arrest marker in cells.[29,30] Based on these points, we can speculate that *REST* is a prognostic biomarker which associated with glioma cell's proliferation and cell cycle arrest. This speculation is in accordance with Zhang et al's[15] study that *REST* can regulate glioblastoma cells proliferation and migration, partly through regulating the cell cycle. Therefore, we can strongly suggest that *REST* was a gene significantly associated with gliomas tumor size and WHO grades, based on its effects on cell proliferation and cycle arrest. Furthermore, this study also demonstrated that high *REST* glioma patients had short OS and bad outcomes.

Temozolomide is an orally alkylating agent which can cross the brain–blood barrier and exhibit antitumor activity by transforming to its bioactive metabolite, MTIC.[31] RT plus concomitant TMZ chemotherapy is a common and recommended therapy which can significantly improve longevity and quality of life for patients with glioma.[32] MGMT promoter methylation is a strong prognostic biomarker for glioblastoma patients treated with TMZ.[33] In this study, we found that low *REST* glioma patients can get better therapeutic efficacy and less adverse reactions after treatment with RT plus concomitant TMZ chemotherapy. Data from the TCGA datasets also showed that low *REST* patients had better OS after RT or chemotherapy. The levels of MGMT promoter methylation were not associated with *REST* expression in our samples. For these circumstances, we can draw a speculation that *REST* may be a novel therapeutic pathway different from MGMT promoter methylation to influence

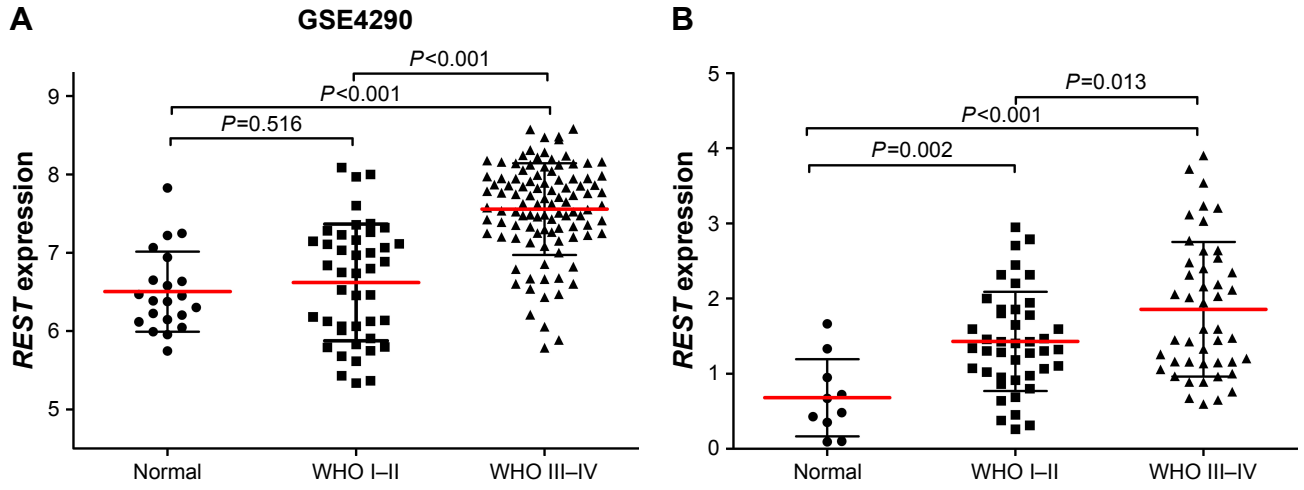

Figure 3 The scatterplot of *REST* mRNA expression level in non-tumor brain tissues, low grade gliomas, and high grade gliomas in (**A**) the GEO dataset and (**B**) validated tissues.
Note: Data were analyzed by Student's *t*-test.
Abbreviations: mRNA, messenger RNA; GEO, Gene Expression Omnibus; WHO, World Health Organization.

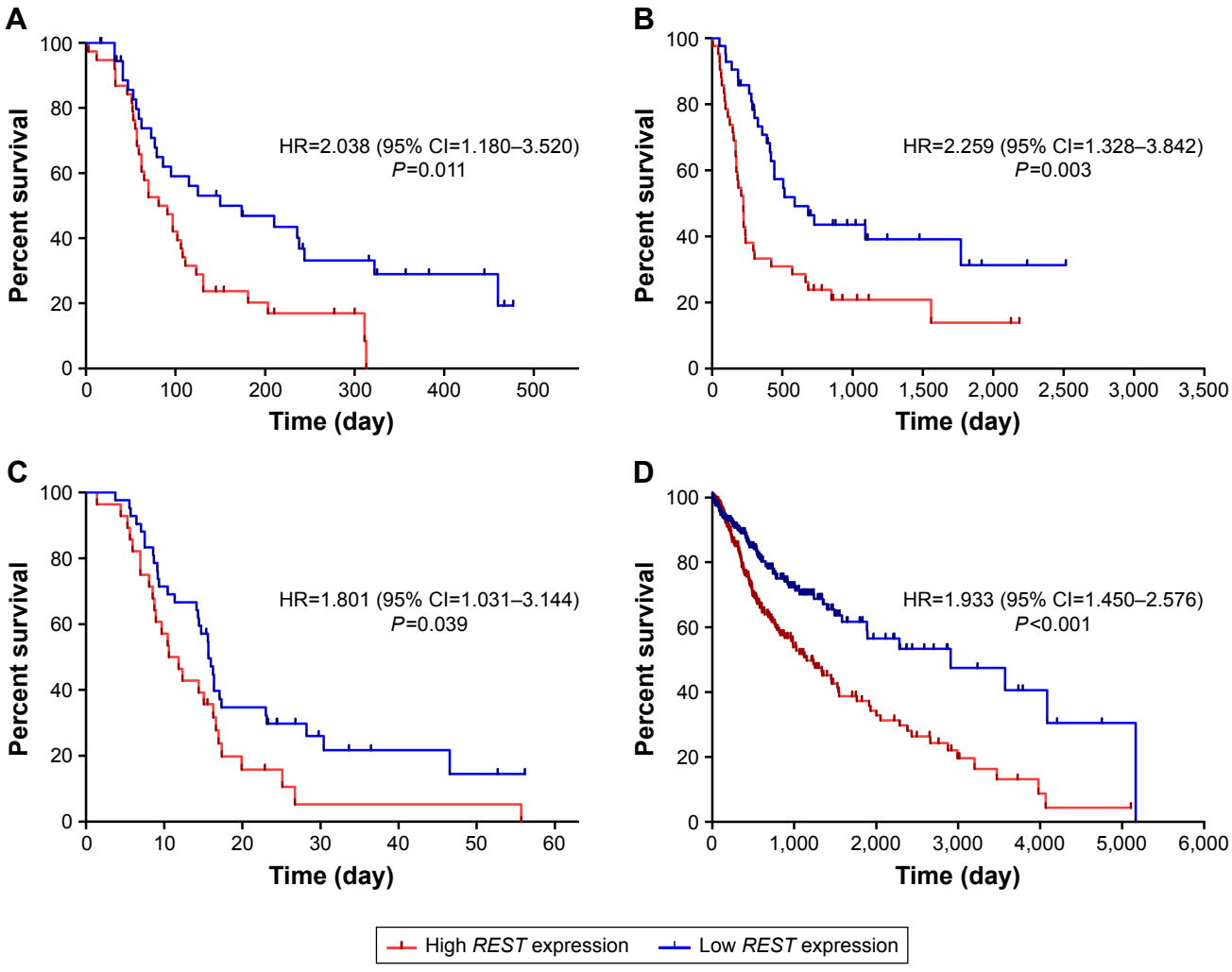

Figure 4 Kaplan–Meier curves of OS of gliomas patients grouped by *REST* expression level in GEO (**A**) GSE4271, (**B**) GSE4412, and (**C**) GSE7696) and (**D**) TCGA datasets.
Note: Data were analyzed by log-rank *t*-test.
Abbreviations: OS, overall survival; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas.

Table 3 Treatment, response, adverse reactions, and outcomes of glioma patients after RT+TMZ

Characteristics	n	Low *REST* expression (n=19)	High *REST* expression (n=21)	*P*-value
Duration of RT (days)*	40	27.84±3.69	27.81±3.96	0.979
RT dose*	40	56.86±3.60	56.65±4.10	0.867
TMZ dose*	40	131.05±11.97	133.95±16.06	0.525
Response after RT+TMZ				
CR+PR	17	12 (70.59%)	5 (29.41%)	0.031[a]
SD	10	4 (40.00%)	6 (60.00%)	
PD	13	3 (23.08%)	10 (76.02%)	
Adverse reaction after RT+TMZ				
Gastrointestinal reaction				
Negative	23	15 (65.22%)	8 (34.78%)	0.020[a]
Positive	15	4 (26.67%)	11 (73.33%)	
Head discomfort				
Negative	28	15 (53.57%)	13 (46.43%)	0.461
Positive	10	4 (40.00%)	6 (60.00%)	
Mucocutaneous lesion				
Negative	32	18 (56.25%)	14 (43.75%)	0.075
Positive	6	1 (16.67%)	5 (83.33%)	

Notes: Data were analyzed by chi-square (χ^2) test and *by Student *t*-test, given as mean±standard deviation. [a]Statistically significant values.
Abbreviations: RT, radiotherapy; TMZ, temozolomide; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

the therapeutic efficacy of TMZ in glioma. However, the patients involved in this study are limited, especially those treated with RT plus TMZ, and further investigations should enroll more glioma patients so as to eliminate the possibility of false positives.

REST4, the splicing variant of *REST*, was reported to silence the repression activity of *REST* in neuronal cells.[34,35] In this study, there was no significant correlation between the expression of *REST* and *REST4*. Also, *REST4* was not related to glioma patients' clinical characteristics. For the relationship between *REST* and *REST4*, different studies raise different opinions.[36] Some reported that *REST4* can bind to the *REST* DNA recognition sequence, NRSE/RE-1, so as to inhibit *REST* biological function.[37] Some hold the view that *REST4* impairs *REST*'s repression ability by binding to *REST* itself.[35,38] Regardless of which ways *REST4* binds to *REST*, it is confirmed that *REST4* can influence the biological functions of *REST*, but not influence its expression level in glioma. In other words, it is not the way that *REST4* silences *REST* repression ability by affecting *REST* expression level, but by changing *REST* protein structure or affecting the *REST* binding domain. Further, *REST4* was not recommended as a prognostic biomarker in glioma in this study because of its low positive expression rate. Moreover, *REST4* was not significantly associated with gliomas patients' clinical characteristics. Although there was no direct relationship between *REST* and *REST4* expression in this study, the molecular mechanism that *REST4* regulates *REST* still needs further investigation.

In brief, this study provided the evidence that *REST* was a therapeutic predictor related to glioma patients' outcome, but *REST4* was not. Further, we found that *REST* expression level was related to the short-term therapeutic efficacy and adverse reactions of glioma patients after treatment with RT plus concomitant TMZ chemotherapy. Further studies should focus on the mechanism that *REST* influences glioma outcome and further validate the relationship between *REST* expression level and RT+TMZ therapeutic efficacy.

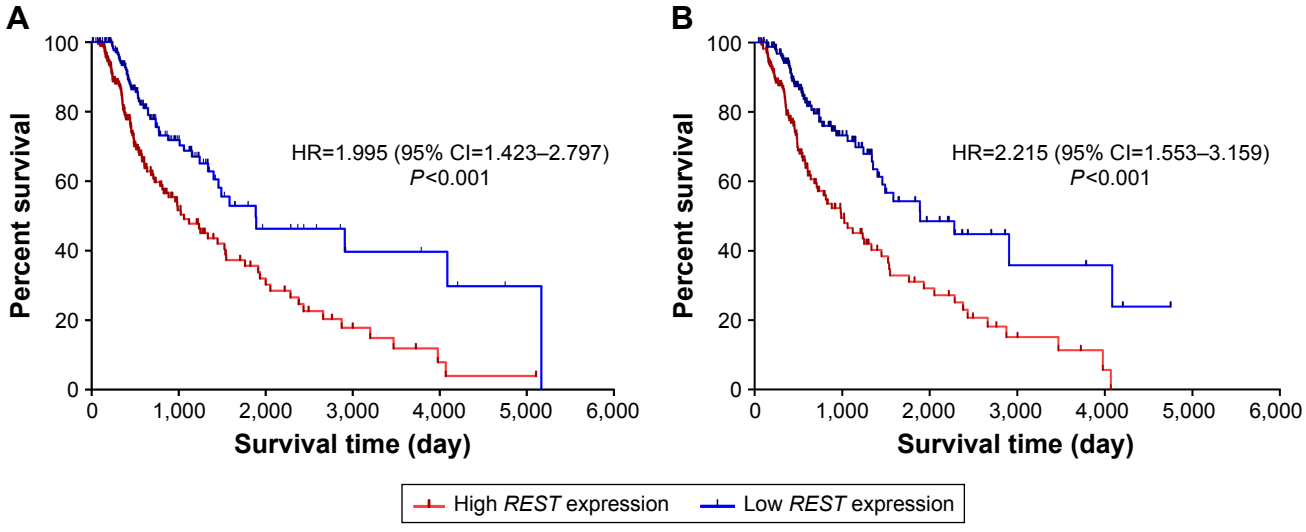

Figure 5 Kaplan–Meier curve of OS of glioma patients treated with (**A**) radiotherapy or (**B**) chemotherapy grouped by *REST* expression level in the TCGA dataset.
Note: Data were analyzed by log-rank *t*-test.
Abbreviations: OS, overall survival; TCGA, The Cancer Genome Atlas.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Relationship between *REST4* expression and glioma clinical characteristics in 40 patients

Characteristics	n	*REST4* expression		*P*-value
		Negative (n=77)	Positive (n=12)	
Age (years)*	89	39.38±19.69	44.67±16.10	0.379
Gender				
Male	46	40 (87.0%)	6 (13.0%)	0.900
Female	43	37 (86.0%)	6 (14.0%)	
Preoperative KPS score				
<80	4	3 (75.0%)	1 (25.0%)	0.788
≥80	77	67 (87.0%)	10 (13.0%)	
WHO grade				
I–II	43	39 (90.7%)	4 (9.3%)	0.264
III–IV	46	38 (82.6%)	8 (17.4%)	
Tumor size (cm)				
<3	13	11 (84.6%)	2 (15.4%)	0.413
3–5	31	25 (80.6%)	6 (19.4%)	
≥5	45	41 (91.1%)	4 (8.9%)	
GFAP				
Positive	86	74 (86.05%)	12 (13.95%)	0.487
Negative	3	3 (100%)	0 (0)	
Ki67				
<15%	46	41 (89.1%)	5 (10.9%)	0.455
≥15%	43	36 (83.7%)	7 (16.3%)	
P53				
Positive	57	49 (86.0%)	3 (14.0%)	0.839
Negative	32	28 (87.5%)	4 (12.5%)	
IDH1 mutation				
Mutation	36	30 (83.3%)	6 (16.7%)	0.469
Wild type	53	47 (88.7%)	6 (11.3%)	
MGMT promoter methylation				
Methylated	9	9 (100%)	0 (0)	0.212
Not methylated	80	68 (85.0%)	12 (15.0%)	
REST expression				
Low	44	39 (88.6%)	5 (11.4%)	0.583
High	45	38 (84.4%)	7 (15.6%)	

Note: Data were analyzed by chi-square (χ^2) test and *by Student *t*-test, given as mean±SD.
Abbreviations: KPS, Karnofsky Performance Score; WHO, World Health Organization; GFAP, Glial fibrillary acidic protein; MGMT, O-6-methylguanine-DNA methyltransferase; SD, standard deviation.