Biochemical and Biophysical Research Communications 463 (2015) 504–509

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Expression of REST4 in human gliomas in vivo and influence of pioglitazone on REST in vitro

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ARTICLE INFO

Article history:
Received 6 May 2015
Available online 21 May 2015

Keywords:
REST
REST4
Glioma
PPARγ agonist
Pioglitazon

ABSTRACT

The repressor element-1 (RE1) silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) has an irreplaceable role during the differentiation of neurons. REST has multiple splice variants which link to various types of cancer. Previous work had highlighted the role of REST in glioma, where the expression of REST is enhanced. But whether alternative splicing of REST is expressed in glioma has not been described. Here, we show that a specific isoform REST4 is expressed in glioma specimens, and will influence the mRNA level of REST in vivo. Peroxisome proliferator-activated receptor-γ (PPARγ) agonists have a role of antineoplastic in various tumor cells, which including glioma cells. Moreover, study indicated that PPARγ agonist pioglitazone can promote alternative splicing of REST pre-mRNA. In this study, we selected pioglitazone as a tool drug to explore whether the role of pioglitazone in anti-glioma is mediated by regulating REST expression or promoting alternative splicing of REST in glioma cells. Results show that pioglitazone can inhibit proliferation and induce apoptosis of glioma cell in vitro, which may be mediated by down-regulating REST mRNA level but not by inducing alternative splicing of REST pre-mRNA. Our study firstly reports the expression of REST4 in glioma tissue samples. And we recommend that pioglitazone, which can reduce the expression level of REST, represents a promising drug for therapy of glioma.

1. Introduction

Glioma represents the most frequently primary malignant brain tumor in adult and accounts for approximately 40%~50% of all intracranial tumor. The World Health Organization (WHO) classification system groups gliomas into 4 histological grades defined by increasing degrees of undifferentiation, anaplasia, and aggressiveness. The malignant gliomas, including WHO grade IV tumors and grade III tumors, account for 77.5% cases of glioma [1]. The survival rate and prognosis of patients have improved significantly, but the invading tumor cells still survive usually and cause tumor relapse. The overall survival rate of glioma patients still keeps very low, especially the high-grade gliomas. Thus, our work focuses on finding new biomarkers and therapeutic targets for glioma.

The repressor element-1 (RE1) silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) has been characterized as a transcriptional repressor that regulates the expression of neuronal genes in neural and non-neural cells [2–4]. REST, which is over-expressed in stem cells and low expressed in mature neurons, identified as an important gene which has an irreplaceable role during the differentiation of neurons initially [5,6]. In neural tissues, dysfunction of REST has been associated with neurological disorders, such as alzheimer's disease, cerebral ischemia, epilepsy, and brain tumors [7–9]. In brain tumors, the oncogenic role of REST has been proposed for medulloblastoma [10] and neuroblastoma [11]. Recently, the function of REST is also suggested in another brain tumor, glioma, which has been found to over-express REST [12].

Alternative splicing (AS) is an important mechanism for transcriptomic and proteomic diversity [13]. Studies indicate that REST undergoes extensive alternative splicing and has multiple splice variants which link to various types of cancer. REST4, an

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http://dx.doi.org/10.1016/j.bbrc.2015.05.058
0006-291X/

alternatively spliced isoform of REST which has an alternative exon N insertion between the third and fourth exons in REST mRNA, is limited to neuronal cells. Recently, REST4 is found to express in some tumor tissues and cell lines, and plays a role in pathogenesis [14,15]. Previous work has highlighted the role of REST in glioma but whether alternative splicing of REST is expressed in this disease has not been described. Thus, our works concentrated on exploring the relationship between REST and its splicing variants and glioma.

Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the nuclear hormone receptor family. The thiazolidinedione (TZD) family of PPARγ agonists, such as pioglitazone, troglitazone and ciglitazone, has a role of antineoplastic in various tumor cells [16–18]. Moreover, study recently showed that PPARγ agonist pioglitazone can promote alternative splicing of REST pre-mRNA and produce a truncated protein lose a domain essential for nuclear translocation [15]. Thus, we speculated that the effect of pioglitazone on glioma cells, including proliferation inhibition and apoptosis-inducing, was mediated by regulating the expression level of REST and promoting alternative splicing of REST.

2. Materials and methods

2.1. Glioma tissue and normal brain tissue samples

Tumor tissue samples from 25 patients, diagnosed with primary glioma at the Department of Neurosurgery, Second Xiangya Hospital of Central South University, were used as the material for this study. Archived 3 normal brain tissue samples were procured from the Department of Neurosurgery, Third Xiangya Hospital of Central South University. All samples were obtained in accordance with the ethics committee of the Central South University. Tissue samples got from surgery and stored at liquid nitrogen immediately until further analysis.

2.2. Extraction of mRNA from cells and tissues

Total RNA from cells was extracted using TRIzol reagent (Takara). Frozen tissues were prepared in liquid nitrogen and pestled, then, resuspended in TRIzol reagent (Takara) immediately. Operations of extracting total RNA were according to the manufacturer's instructions.

2.3. Reverse transcription

The integrity and quality of all RNAs were determined by comparing 28S/18S rRNA ratio. The concentration was assessed by Nanodrop spectrophotometer (Shimadzu biotech). Total RNA (500 ng) was transcribed to complementary DNA by using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara).

2.4. Real-time polymerase chain reaction (QPCR)

The abundance of transcripts was assessed by real-time PCR on LightCycle@480 ll。SYBR® Premix Ex Taq™ II (Takara) was used in a total volume of 20ul suggested in the manufacturers' instructions. The cycling profiles were carried out at 95 °C for 30 s for 1 cycle, and 95 °C for 5 s, 60 °C 30 s for 40 cycles. The values were normalized to the endogenous gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and each run was evaluated in triplicate for both target gene and GAPDH.

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

To avoid unintentional amplification of potential genomic DNA, the RT-PCR primers were selected to bind in different exons contamination. As described previously, REST primers and REST4 primers [19] were used for RT-PCR. All of the RT-PCR products were sequenced bySanger sequencing commercially. The experiments were performed at least three times with consistent results.

2.6. Immunohistochemistry (IHC)

Immunohistochemical analyses of REST were conducted using paraffin section specimens of glioma and normal tissues from 28 patients. After a standard deparaffinization procedure, epitope retrieval was performed. These sections were incubated overnight at 4 °C with the prediluted (1:200) anti-REST antibody (ab70300, abcam). After addition of a universal secondary antibody, staining was performed using a diaminobenzidine staining kit (K5007, DAKO). To determinate the REST index, all photographs were analyzed by Image Pro-plus 6.0 software.

2.7. Glioma cell lines and culture

Human glioma cell lines U87 and U251 were purchased from the ATCC and cultured according to the guidelines recommended by the ATCC. Cells were cultured in dulbecco modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cells were maintained at 37 °C with 5% CO_2.

2.8. Cell proliferation and apoptosis assays

For MTT-based cell viability assay, cells were seeded and incubated in 96 well at a density of 5000 cells/well for 24 h and then exposed to various concentrations of pioglitazone dissolved in DMSO in DMEM. Controls exposed to DMSO at a concentration equal to that in treatment groups. At each time point considered, medium was replaced by 100ul 0.5 mg/mL MTS which diluted in DMEM supplemented 10% FBS and incubated for 1 h at 37 °C and formazan release was quantified at 490 nm using a Microplate Reader.

Apoptosis assay was assessed by Hoechst 33342 staining (Beibo, shanghai). Cells were plated in 6 well for 24 h and exposed to various concentrations of pioglitazone as described previously. 48 h later, degree of cell apoptosis was observed by fluorescence microscopy.

3. Results

3.1. REST expression level is elevated in human glioma tissues

To investigate the role of REST in gliomas, we analyzed the expression of REST in 25 primary glioma patient samples, including 12 grade I ~ II and 13 grade III ~ IV glioma tissue samples and 3 normal brain tissue samples. Results showed that REST expressed in both glioma tissues and normal brain tissues. Compared to normal brain tissues, a majority of glioma tissue samples showed up-regulation in REST mRNA expression level by ~2 fold (Fig. 1A). While other research indicated that REST is up-regulated from two- to five-fold in all samples as compared to control [20]. Compared with grade I ~ II gliomas, the REST mRNA level significantly increased in grade III ~ IV gliomas (Fig. 1B). The expression of REST in glioma and normal tissues was further confirmed by immunohistochemistry and the results were basically consistent with QPCR. Positive signals were detectable in glioma tissue sample, whereas REST expression was negligible in normal tissue samples (Fig. 1C). In summary, our date indicated that the REST expression level, including mRNA and protein, was up-regulated in glioma tissue samples comparaed with normal brain tissue samples.

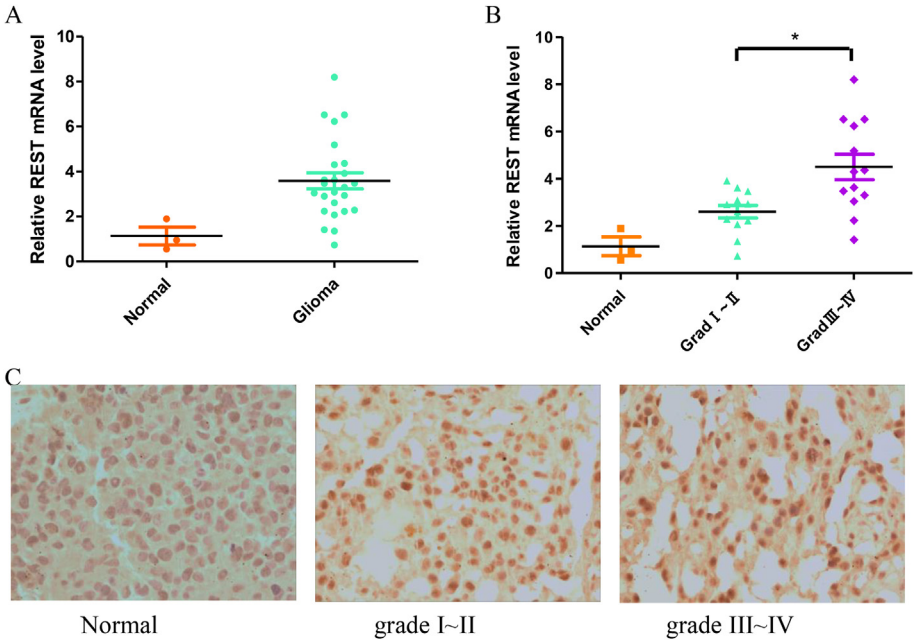

Fig. 1. REST expression level was upregulated in glioma tissues. (A) Real-time PCR analysis of REST in 25 glioma tissues and 3 normal brain tissues. (B) Expression level of REST in normal brain tissues and grade I ~ II glioma tissues and grade III ~ IV glioma tissues. (C) Immunohistochemical staining of REST in glioma (Grade I ~ II, Grade III ~ IV) and normal brain tissues using anti-human REST antibodies.

3.2. A specific isoform of REST is present in glioma tissue samples and influences the expression level of REST

Extensive Alternative Splicing of REST pre-mRNA has been identified [15,21,22]. REST4, which has an alternative exon N insertion between its third and fourth exons in REST mRNA and contains only 5 of the 9 zinc finger domains of REST C-terminal (Fig. 2A), is one of these truncated isoform. At first, RT-PCR analysis of REST was performed to measure the expression of REST. Oligonucleotide primers were designed based on the mREST cDNA sequences of hREST, which cross two different exons contiguous in the cDNA (Fig. 2A). Results of RT-PCR revealed that another product was presence unexpectedly except for the anticipated strap in some of glioma tissues samples (Fig. 2B). We supposed that it was REST4 according to our knowledge [23]. To verify the special product was REST4, a specific PCR primer pairs were synthesized to amplify REST4 uniquely. All of the RT-PCR products were sequenced by Sanger sequencing commercially. As showed in Fig. 2B, REST and REST4 shared the same exon 3, whereas REST4 had an alternative exon N insertion after exon 3. Results showed that REST4 expressed in 8 glioma tissues and was not found in the other 17 glioma tissues or normal brain tissues. To our knowledge, this is the first research that focuses on the expression of REST and its splice variants in glioma tissues.

To elucidate the relationship between REST and REST4 expression, we divided glioma samples into two groups, samples with REST and samples without REST4, and compared the mRNA expression level between the two groups. Results showed samples with REST4 expression had a significantly ($P < 0.05$) lower expression quality of REST as compared with samples without REST4 expression (Fig. 2C), Suggesting that the exist of REST4 might influence the expression level of REST in vivo. Then, we analysed the expression rate of REST4 in samples with different pathology classification. Results indicated that the positive rate of REST4 expression was higher in grade I ~ II gliomas than grade III ~ IV gliomas (Fig. 2D), suggested that REST4 was majorly expressed in early stage of glioma. As far as we know, it is the first report to demonstrate the expression of the REST4 in glioma tissue samples.

REST is a prognostic factor and perspectively therapeutic target for glioma. And alternative splicing of REST provides a new strategy for the treatment of glioma. Thus, we focus on REST and its splice variants to explore new drugs for glioma therapy. As stated above, PPARγ agonist pioglitazone has been confirmed as potent anti-neoplastic agent in vitro and vivo glioma models. And Chen GL proved that pioglitazone can promote alternative splicing of REST pre-mRNA and produce a truncated protein [15]. Thus, we selected pioglitazone as a tool drug to explore whether the role of PPARγ agonists in anti-glioma is mediated by regulating REST expression or promoting alternative splicing of REST in glioma cells.

3.3. Pioglitazone reduced cellular viability and induced apoptosis of human glioma cells

U87 and U251 cells were treated with pioglitazone at various doses of 0, 5, 10, 20, 50, 100uM for 24, 48, 72 h. MTT analysis showed that pioglitazone reduced the cellular viability of glioma cells in a concentration- and time-dependent manner (Fig. 3A and B). The cellular viability of U87 cells was reduced by 49.56% after 48 h treatment with 100uM pioglitazone. And the cellular viability of U251 cells was reduced by 48.26% after incubation with pioglitazone 72 h at 100uM pioglitazone. The dates showed that U87 cells seemed to be more sensitive to pioglitazone than U251 cells, which was not accordant with previous finding showed that U251 cells were more sensitive than U87 cells in their response to pioglitazone [24]. And, U87 cells were used for remaining experiments.

U87 cells were treated with pioglitazone at doses of 0, 50, 100uM for 48 h. We observed obviously morphological changes in U87 cells after incubated with pioglitazone at doses of 50, 100uM (Fig. 3C), including smaller volume, reduced amount and changed shape of cells. Apoptosis caused by pioglitazone was monitored by Hoechst 33342 staining. We detected apoptotic nuclei with intense

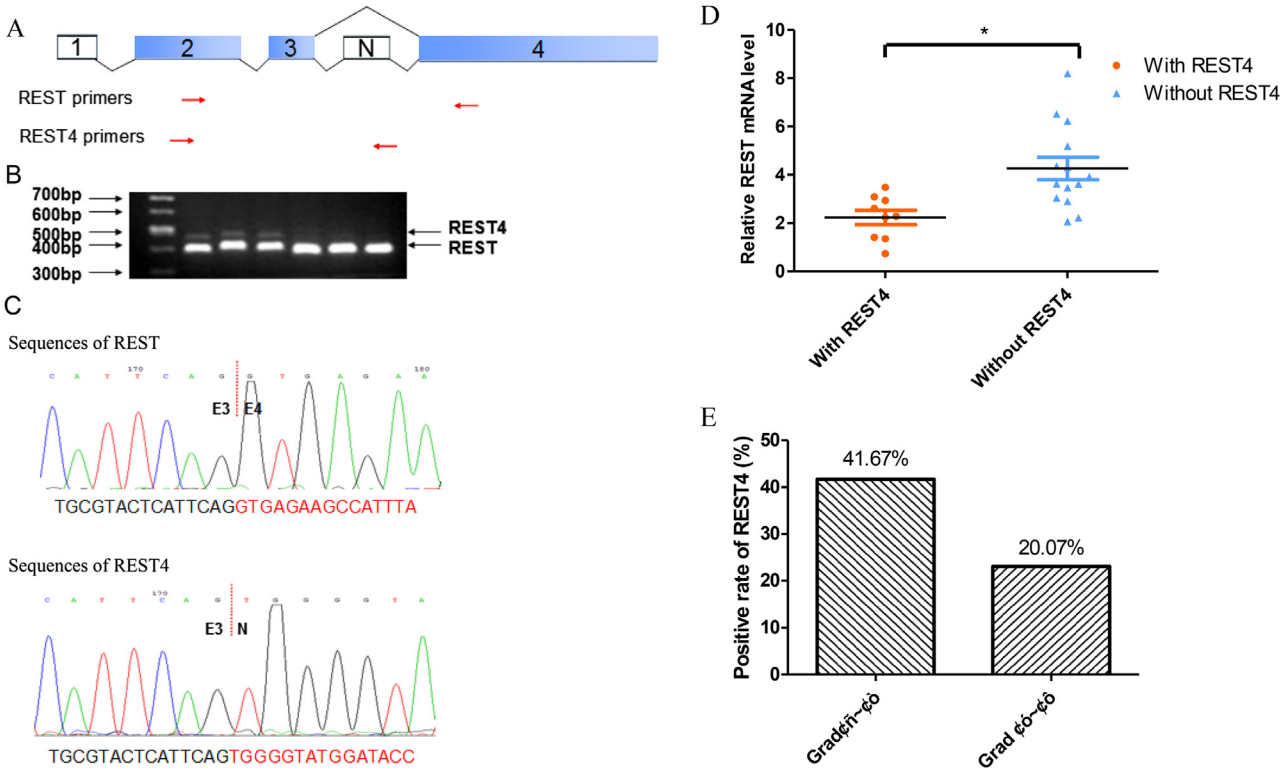

Fig. 2. A specific isoform of REST, REST4, was expressed in glioma tissues. (A) The structure of the REST and REST4 exons and the locations of primers employed for the identification of REST splice variants. (B) RT-PCR analysis of REST with F_1/R_1 primers in glioma tissues. RT-PCR products were separated in 2.5% resolution agarose. (C) Results of Sequencing analysis revealed the exon–exon junctions of REST and REST4. (D) REST mRNA expression level between Samples with REST4 and samples without REST4. *P < 0.05 (E) The positive rate of REST4 expression in grade I ~ II gliomas and grade III ~ IV gliomas.

fluorescence corresponding to chromatin condensation in U87 cells after pioglitazone treatment (50, 100uM).

3.4. The role of pioglitazone in anti-glioma is mediated by regulating REST expression but not by inducing alternative splicing of REST

To investigate the effect of pioglitazone on REST and its splice variant in glioma, human glioma cell lines U87 were incubated with pioglitazone at various doses of 0, 5, 10, 20, 50, 100uM for 48 h. REST mRNA contains 4 exons, and the sequence for protein coding is from exon 2 to exon 4. REST primers used in RT-PCR are located in exon 2 and exon 4 (Fig. 2A). Multiple RT-PCR products will be amplified if REST undergoes alternative splicing. And multiple strips will be present in high resolution agarose. In contrast to our expectation, multiple strips were not appeared in 3.0% resolution agarose after treated with pioglitazone at different concentration (Fig. 4A). The exclusive RT-PCR product was confirmed to be REST by sequencing analysis. This result indicated that pioglitazone can not make REST undergo alternative splicing in U87 cells.

Then, we sequentially detected whether pioglitazone regulate the mRNA expression level of REST. Results showed that the relative expression level of REST was remarkably decreased in U87 cells ($P < 0.05$) when the concentration of pioglitazone increased to 50 and 100uM (Fig. 4B). Relative expression levels normalized to GAPDH levels. Our experiment had indicated that pioglitazone can significantly inhibit growth of U87 in a concentration of 50, 100uM for 48 h compared with untreated control. These date suggested that the effect of pioglitazone on glioma cells, including proliferation inhibition and apoptosis-inducing, might be mediated by reducing the mRNA expression level of REST rather than by promoting alternative splicing of REST.

4. Discussion

Transcription factor REST represents a transcriptional and epigenetic regulator that regulates the expression of more than 2000 neuronal genes in both neuronal and non-neuronal cells [2–4,25,26]. REST is initially identified as an important gene which has an irreplaceable role during the differentiation of neurons [5,6]. Now, REST also functions as a suppressor or an oncogene of various types of cancer [27,28]. REST appears to function as a suppressor in some human mammary epithelial cells, such as human non-small cell lung carcinoma cells [29] and colorectal cancer cells [30]. In contrast, in neural cells REST was identified as a tumor promoter that prevents differentiation and assures self-renewal. Recently, the function of REST was suggested in another brain tumor, glioma, which has been found to overexpress REST [12]. Up-regulating of REST is able to drive cell proliferation and suppress differentiation. And knockdown of REST results in decreased self-renewal, increased survival in GSC-transplanted mice and slower growing, lower invasive and higher apoptotic in glioma cells [31–33]. These indicate that REST is a potential biomarker and therapeutic target for glioma therapy. The objective of this study was to analyze the role of REST in glioma and explore new drugs for glioma therapy based on REST.

Our results showed that the REST expression level, including mRNA and protein, was up-regulated in glioma tissue samples, which was consistent with previous findings. Our date clarified the elevated levels of REST in higher grad glioma samples, suggesting that heightened REST function may be associated with more aggressive glioma. Intriguingly, we showed that a splicing variant of REST, named REST4, was expressed in some glioma specimens. REST markedly represses target neural gene, whereas REST4 has a weak function [34]. REST4 protein, which reserves the DNA-binding domain (DBD), can competitively inhibit binding of NRSF

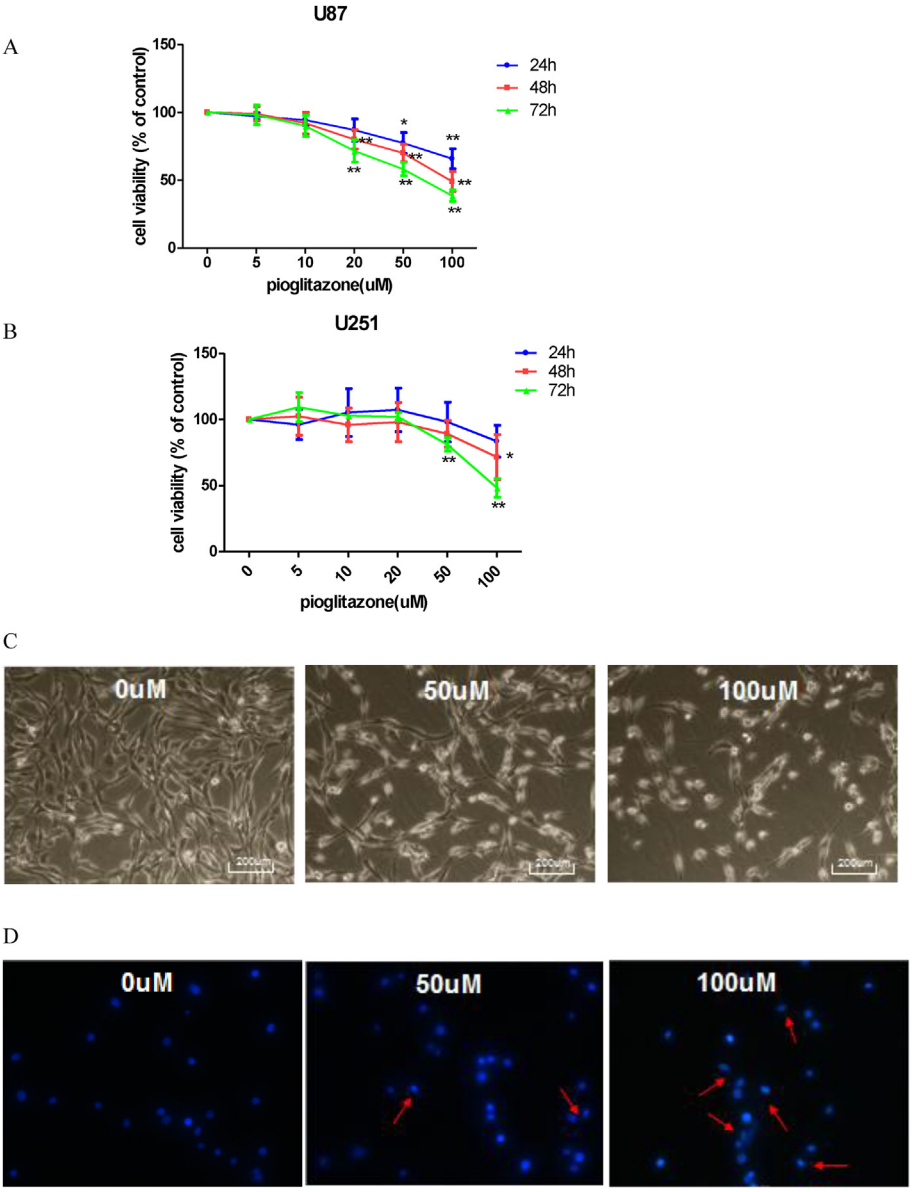

Fig. 3. Pioglitazone reduced cellular viability and induced apoptosis of human glioma cells. (A) and (B) Pioglitazone reduced cellular viability of U87 and U251 respectively. Points, mean; bars, SD (n = 3). Data was expressed as a percentage of untreated control. (*p < 0.05, **p < 0.01). (C) Morphologic analysis of pioglitazone effects on u87 cells. Images were collected by inverted microscope. (D) Apoptotic analysis of pioglitazone effects on u87 cells. Images were collected by inverted fluorescence microscope.

to RE-1 and restrains the silencing effect of REST on target gene [35]. But seldom research had indicated the relationship between REST and REST4 expression level in vivo. We divided glioma samples into two groups, samples with REST and samples without REST4. Our dates showed that samples with REST4 expression had a significantly ($P < 0.05$) lower expression quality of REST compared with samples without REST4 expression. As far as we know, this is the first research that focuses on the expression of REST and its splice variants in glioma tissues.

REST is a prognostic factor and perspectively therapeutic target for glioma. And alternative splicing of REST provides a new strategy for the treatment of glioma. So, we focused on REST and its splice variants to explore new drugs for glioma therapy. According to literatures we had read, PPARγ agonist pioglitazone was selected. PPARγ is implicated in many biological processes, including type II diabetes, atherosclerosis, inflammation, hypertension, and tumorigenesis [36]. PPARγ can be activated by synthetic ligands which belonged to the thiazolidinedione (TZD) class, such as pioglitazone, troglitazone, ciglitazone [37,38]. It has been suggested that TZDs have a role of anti-glioma and can suppress the growth of glioma cells in vitro and in vivo glioma models [24,39,40]. In addition, a research conducted by Chen GL prove that pioglitazone can promote alternative splicing of REST pre-mRNA and produce a truncated protein which lose a domain essential for nuclear translocation [15]. We detected whether the role of pioglitazone in antiglioma was mediated by regulating REST. In contrast to our expectation, splice variants of REST did not appear after treated with pioglitazone at different concentrations. Intriguingly, our dates indicated that pioglitazone could remarkably decrease REST mRNA level in U87 cells at high concentrations (50, 100uM).

In summary, our study revealed the expression of REST4 in glioma tissue samples and the link between REST4 and REST expression in vivo. REST and its splice variants provided a promising target for glioma therapy. We also uncovered a novel mechanism supporting the antiglioma potency of pioglitazone. We concluded that the effect of pioglitazone on glioma cells, including

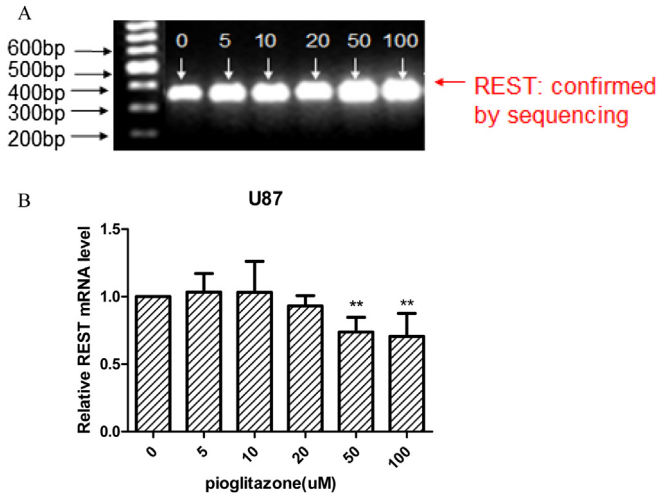

Fig. 4. Pioglitazone decreased the expression level of REST mRNA but could not induce alternative splicing of REST. (A) The effect of pioglitazone (5, 10, 20, 50 and 100uM) on inducing alternative splicing of REST in U87. The RT-PCR products were separated in 3.0% resolution agarose. (B) Relative expression of REST in U87 cells after treated with different concentrations pioglitazone.

proliferation inhibition and apoptosis-inducing, might be mediated by reducing the mRNA expression level of REST rather than by inducing alternative splicing of REST.

Acknowledgments

This work is supported by National Science and Technology Major Project (2012ZX09303013) and the Fundamental Research Funds for the Central Universities of Central South University (2013zzts333 and 2013zzts298).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.058.

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