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LABORATORY INVESTIGATION

A novel antisense oligonucleotide anchored on the intronic splicing enhancer of hTERT pre-mRNA inhibits telomerase activity and induces apoptosis in glioma cells

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Abstract

Introduction Alternative splicing of hTERT pre-mRNA is an important step in the regulation of telomerase activity, but the regulation mechanisms and functions remain unclear.

Methods RT-PCR analysis was used to detect hTERT splicing in glioma cell lines and brain tissues. TRAP assay was used to detect the telomerase activity. Then, we designed and synthesized 2′-O-methyl-RNA phosphorothioate AONs and transfected them into glioma cells to detect the changes in telomerase activity. MTT assay, plate colony formation assay, western blotting and Annexin V/PI assay were used to detect cell proliferation and apoptosis. At last, bioinformatics analyses were used to predict the expression and function of splicing protein SRSF2 in gliomas.

Results hTERT splicing occurs both in glioma cell lines and glioma patients' tissues. The telomerase activity was related to the expression level of the full-length hTERT, rather than the total hTERT transcript level. AON-Ex726 was complementary to the sequence of the intronic splicing enhancer (ISE) in intron six, and significantly altered the splicing pattern of hTERT pre-mRNA, reducing the expression level of the full-length hTERT mRNA and increasing the expression level of the -β hTERT mRNA. After transfection with AON-Ex726, the level of apoptosis was increased, while telomerase activity and cell proliferation were significantly decreased. By bioinformatic predictions, we found the AON-Ex726 anchoring sequence in ISE overlaps the binding site of SRSF2 protein, which is up-regulated during the development of gliomas.

Conclusions Our findings provided new targets and important clues for the gene therapy of gliomas by regulating the alternative splicing pattern of hTERT pre-mRNA.

Keywords Telomerase · Telomere · Alternative splicing · Exon skipping · Glioma

Introduction

In humans and other vertebrates, telomeres contain multiple copies of the tandem repeat sequence TTAGGG [1, 2]. During cell division, telomeres lose approximately 50 to 200 bps per generation [3, 4]. Telomerase is a ribonucleoprotein complex composed of RNA and proteins, which has the function of RNA-dependent reverse-transcription DNA polymerase [5]. It can use its own RNA as a template to synthesize telomere DNA repeats by reverse transcription, and maintain a relatively constant length of chromosome ends, thus solving the "terminal replication problem" [6]. The differentiated mature normal human cells do not have telomerase activity, while over 90% of cancers have telomerase activation, which is closely related to the malignant transformation of cells [7]. Telomerase mainly contains two subunits: telomerase RNA component (TERC) and

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catalytic reverse-transcriptase (TERT) [8]. TERC contains a sequence complementary to telomeres and is a template for the replication of telomere repeats. Telomere extension is accomplished by its catalytic component TERT and other telomere/telomerase-associated proteins [9, 10]. The telomerase inhibitor GRN163L, which inhibits telomerase activity by binding specifically to the template RNA in telomerase active site, is now in Phase I/II clinical trials [11]. Although the therapeutic effect of GRN163L is acceptable in certain cancers, it is not yet effective in some other cancers, which requires further enhancement of telomerase inhibition [12]. Therefore, it is necessary and urgent to find more effective ways to inhibit telomerase activity and tumour growth.

Alternative splicing refers to the formation of pre-mRNAs transcribed from genes in eukaryotic cells that give rise to different alternative splicing variants (ASVs) through different splicing patterns [13]. Alternative splicing is one of the main mechanisms for not only the production of protein diversity, but also for the gene expression control at the post-transcriptional level [14] (Supplementary Fig. 1A). The human TERT (hTERT) gene contains 16 exons and 15 introns. The hTERT mRNA has approximately 4.0 kbs in length. The hTERT protein contains a telomerase-specific motif and seven reverse-transcriptase motifs. The presence and strength of the hTERT protein activity depend on these motifs [15]. It has been reported that the hTERT pre-mRNA has at least three deletion sites (α, β, γ) and four insertion sites, resulting in a variety of different ASVs [16]. Thirty-six nucleotides in exon six are deleted in α deletion (−α) hTERT mRNA ASV, 182 nucleotides of the entire exon seven and exon eight are deleted in β deletion (−β) and 189 nucleotides in exon 11 are deleted in γ deletion (−γ) [17]. The two deletion sites, α and β, are the most-frequently studied sites for the regulation of hTERT pre-mRNA splicing. The γ deletion ASV and the combination ASVs of the αγ, βγ, and αβγ deletion were of very low intensities [18]. So in our present study, we studied the two main deletion sites, α and β, like most other studies. Until now, the functions of various hTERT ASVs and the mechanisms of regulating alternative splicing of hTERT pre-mRNA have remained unclear. Our present study attempts to inhibit telomerase activity and induce apoptosis in glioma cells by modulating alternative splicing of hTERT pre-mRNA.

The gene exon skipping can be mediated by antisense oligonucleotides (AONs) binding to the 3′ or 5′ splice site in pre-mRNA or splicing enhancer, and the pattern of alternative splicing of pre-mRNA can be altered to produce different ASVs [19, 20]. It has been reported that the AON-mediated exon skipping technique transforms the Bcl-X and WT1 mRNAs from translating into anti-apoptotic protein variants to the pro-apoptotic protein variants [21, 22]. Blocking the 3′splice site or the ESEs (exonic splicing enhancers) by AONs was reported to being effective in restoring normal splicing of minigenes and endogenous methionine synthase reductase transcripts in patient cells [23]. This method was applied to the treatment of breast cancer, prostate cancer and leukaemia, and used in clinical trials for Duchenne muscular dystrophy [24–27]. Gliomas are the most common intracranial primary tumours. The radiotherapy and chemotherapy effects are also not ideal for the treatment of gliomas [28]. Currently, there are still no effective treatment for gliomas, and the prognosis is generally poor [29, 30]. In the present study, we designed a novel AON, named AON-Ex726, which binds to the specific intronic splicing enhancer (ISE) in intron six of hTERT pre-mRNA. It could regulate the hTERT pre-mRNA splicing pattern, reduce the expression level of the full-length hTERT mRNA, inhibit the telomerase activity and cell proliferation, and induce apoptosis in glioma cells. Our findings explored the role of alternative splicing of hTERT pre-mRNA in regulating telomerase activity, providing an experimental basis for the development of the next generation of telomerase inhibitors.

Materials and methods

Cell culture and tissue specimens of human gliomas

Human glioblastoma cells U251, LN229, SF188, U118, U87MG and LN18 were cultured in DMEM/F12 (Hyclone, USA) medium supplemented with 10% foetal calf serum (Hyclone) at 37 °C with 5% CO_2. Nineteen cases of human glioma tissue specimens, including two cases of WHO Grade I (pilocytic astrocytoma), six cases of Grade II (two case of fibrillary astrocytoma, two cases of oligodendroglioma, two cases of oligoastrocytoma), six cases of Grade III (three cases of anaplastic oligodendroglioma, three cases of anaplastic oligoastrocytoma) and five cases of Grade IV (glioblastoma) were taken from the General Hospital of Tianjin Medical University. Pathological diagnosis was based on the classification and grade of gliomas according to the WHO criteria. All experiments were guided by the ethics committees of Tianjin Medical University, and all patients gave informed consent. The specimens were divided into two portions; one was quickly frozen in liquid nitrogen for DNA/protein analyses, and the other was fixed with 3.7% neutral formaldehyde, embedded in paraffin, and cut into 5-μm-thick tissue sections for HE staining.

Analysis of hTERT ASVs by RT-PCR

In the reverse-transcription reaction, we applied the hTERT-specific primer sequences to improve the detection sensitivity of hTERT ASVs. The sequence of the specific primer 2565 was: 5′-CGC AAA CAG CTT GTT CTC CAT GTC-3′ and was synthesized by Integrated DNA Technologies. The

sequence of the PCR sense primer Tert 2109F was 5′GCC TGA GCT CTT TGT CAA-3′ and the antisense primer Tert 2531R was 5′-AGG CTG CAG AGC AGC GTG GAG A GG-3′ (Supplementary Fig. 1B). The fragment lengths of the four ASVs (full-length, −α,−β and, −α−β) of hTERT mRNAs were 423 bp, 387 bp, 239 bp, 203 bp, respectively. The conditions of PCR amplification were: 94 °C for 20 s, 60 °C for 20 s, 72 °C for 40 s, for 30 cycles. The expression levels of hTERT mRNA ASVs were compared with that of β-actin, and analysed using the ImageQuant software.

Prediction of ISE motifs in hTERT pre-mRNA

ESEfinder, an online software developed by Krainer and Zhang's lab at Cold Spring Harbor Laboratory, is able to predict the site of the splicing enhancer through the site of binding to the serine/arginine-rich (SR) protein (Website address: http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home).The predicted SR proteins include SRSF1 (SF2/ASF), SRSF1 (SF2/ASF, IgM-BRCA1), SRSF2 (SC35), SRSF5 (SRp40), and SRSF6 (SRp55). The nucleic acid sequence of hTERT was retrieved from NCBI and stored in the FASTA format. We focused on predicting the ISEs in intron six of hTERT pre-mRNA, which might change the pattern of hTERT alternative splicing.

Design and synthesis of AONs

Previous studies on alternative splicing of hTERT pre-mRNA mostly designed AONs binding to the ESEs or splicing sites, but there were few reports on AONs binding to the ISEs. In our present study, we predicted the positions of the ISE motifs according to the sequence of intron six of hTERT pre-mRNA, then designed and synthesized several AONs based on these ISEs. The position where AON-Ex726 binds to intron six of hTERT pre-mRNA and the ISE motif to which it binds are shown in Supplementary Fig. 1C and Supplementary Table 1. Based on the prediction by ESEfinder software, the AONs with two different kinds of chemical structures, 2′-O-methyl-RNA phosphodiester and 2′-O-methyl-RNA phosphorothioate oligonucleotides, were synthesized. Also, we synthesized these AONs with different phosphorothioate ratios (sulfur-free, 50%-thio and 100%-thio) (Supplementary Fig. 1D and 1E). The random AONs as controls were synthesized contained the same amounts of thio with the experimental AONs (sulfur-free, 50%-thio and 100%-thio).

Transfection of AONs into glioma cells

AONs were labelled with 6-FAM, and BLOCK-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen) was used as a positive control for the evaluation of transfection efficiency. The glioma cells were transfected with AONs using Lipofectamine RNAiMAX (Invitrogen). Cells were incubated for 72 h in a 37 °C CO2 incubator. Transfection efficiency was analysed using a BD FACSCalibur flow cytometer. The effects on the cell morphology and the expression levels of hTERT ASVs in the glioma cells transfected with AONs with different phosphorothioate ratios (including sulfur-free, 50% and 100% phosphorothioate), and different concentrations of AONs were detected.

Telomeric repeat amplification protocol (TRAP) assay for telomerase activity analysis

Telomerase activity was analyzed by TRAP assay as previously described [31]. Briefly, cultured cells or patient tissues were collected and lysed with lysis buffer. 1 μl of sample was added to 49 μl TRAP mixture (containing TRAP buffer, dNTP, TS primer, primer mix, BSA, Taq polymerase). PCR amplification was performed under the following conditions: 95 °C for 5 min, then 95 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s, for 30 cycles. The PCR products were electrophoresed in a 12.5% non-denaturing polyacrylamide gel and the electrophoresis was performed at 300 V for 2 h. The ImageQuant software was used to analyse the amount of telomerase activity. Signals of telomerase ladders in TRAP images were summed for a net TRAP activity per sample. The data were normalized to the signal of the internal standard (IC), and then the ratio was calculated as relative to the Hela TRAP standard which was defined as 100%.

MTT assay for cell proliferation

Cell proliferation by MTT assay was detected after a long-term AON treatment for 12 weeks. The surviving cells can metabolize MTT (thiazolyl blue tetrazolium bromide) and produce formazan (FM), which is soluble in DMSO and appears blue. The colour degree can reflect the rates of cell survival and proliferation. Each group of glioma cells in the logarithmic growth phase was dissociated into a single-cell suspension by conventional digestion with 0.05% trypsin, seeded at 4000 cells per well in a 96-well plate. MTT at a concentration of 5 mg/ml was added to the 96-well plates to be tested. The supernatant was discarded, 100 μl of DMSO was added to each well and shaken for 15 min. Finally, the absorbance values (OD values) of each well were measured by a BIO-RAD Model 680 microplate reader at 570 nm and 655 nm, and cell growth curves were plotted based on the OD values.

Plate colony formation assay

Glioma cells of each group in the logarithmic growth phase were dissociated into a single-cell suspension by

conventional digestion with 0.05% trypsin, seeded in a 6-well plate and at 400 cells per well. The plate was shaken gently to allow the cells to disperse evenly. Each group was tested three times in parallel. After 2 weeks, the cell culture was terminated when a macroscopic clone appeared. The culture medium was discarded, and the plates were carefully washed twice with PBS (0.01 mol/l, pH 7.4), fixed and stained (6% glutaraldehyde + 0.5% Crystal Violet) for 30 min, then was washed with water and slowly dried. The numbers of colonies were counted, and the colony formation rates were calculated.

Western blot for analysis of protein expression levels

Cells were lysed with RIPA lysis buffer and the protein concentration was determined by the bicinchoninic acid method. Proteins were separated by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes overnight at 4 °C and 30 mA constant current conditions. Membranes were incubated in a 5% skim milk solution for 2 h at room temperature to block non-specific binding to the membrane. Bax and Bcl-2 antibodies were added and incubated at room temperature for 1.5 h. HRP-labelled secondary antibodies were added and incubated at room temperature for 1 h. The X-ray film was exposed with chemiluminescence detection, and the images were scanned. The protein actin was used as an internal reference for analysis of the relative expression levels of Bax and Bcl-2.

Annexin V/PI assay for apoptosis analysis by flow cytometry

The Annexin V/PI kit was used for apoptosis analysis detected by flow cytometry according to the manufacturer's instruction. Briefly, cells 1×10^6 cells treated with AON-Ex726 or random AON were collected, respectively, washed three times with PBS, and then resuspended in binding buffer containing annexin V-FITC and PI. After staining, cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, USA).

Bioinformatics data mining

To further understand the role of the SRSF2 gene in the development of glioma, we used the Oncomine database (https://www.oncomine.org/resource/login.html) for analysis. Oncomine is the largest cancer DNA database in the world and an integrated data-mining platform. It collected 715 sample data sets and gene expression data sets, 86,733 cancer tissues and normal tissue sample data. It has the most-comprehensive array of cancer mutations, gene expression data, and related clinical data to discover new biomarkers or therapeutic targets. The cBioPortal database (http://www.cbioportal.org/) was used to analyse the interaction networks between SRSF2 and TERT gene.

Statistical analysis

The measurement data were all presented as the mean ± standard deviation ($\bar{x} \pm s$). The SPSS 19.0 statistical software was used for statistical analysis. The means of multiple groups were compared by the one-way analysis of variance, and the q-test was used to compare the means of each group. $p < 0.05$ indicated the statistical difference.

Results

The proportions of hTERT ASVs differ in the various glioma cell lines

The full-length (FL) hTERT mRNA and three truncated hTERT ASVs were detected in the six glioma cell lines U251, LN229, SF188, U118, U87MG and LN18. The three truncated hTERT ASVs were the α deletion (−α) hTERT mRNA, the β deletion (−β) hTERT mRNA, and the αβ deletion (−α−β) hTERT mRNA. The proportions of hTERT ASVs differ in various glioma cell lines (Fig. 1a, b). Different degrees of telomerase activity were detected in the six glioma cell lines by using the TRAP assay (Fig. 1c, d).

Correlation analysis of the expression levels of hTERT ASVs and telomerase activity in glioma tissues

To analyse whether the telomerase activity in glioma tissues was related to the splicing patterns of the hTERT pre-mRNA, we examined the telomerase activity and the relative expression levels of various hTERT ASVs in all glioma tissues. Correlation analysis confirmed that the relative expression of full-length hTERT mRNA was positively correlated with the telomerase activity ($r = 0.915$, $p < 0.01$, Supplementary Fig. 2E). There was no correlation between the relative expression of the total hTERT transcript and telomerase activity ($r = 0.121$, $p = 0.623$, Supplementary Fig. 2F). This indicated that the strength of telomerase activity in glioma cells was related to the alternative splicing of hTERT pre-mRNA, and regulation of the post-transcriptional level of hTERT gene was one of the critical factors in the regulation of telomerase activity.

Efficiency evaluation of AON transfection to change hTERT splicing

AON-Ex726 was labelled with 6-FAM fluorescent moiety, and Alexa Red Fluorescent Oligo was a positive control. The

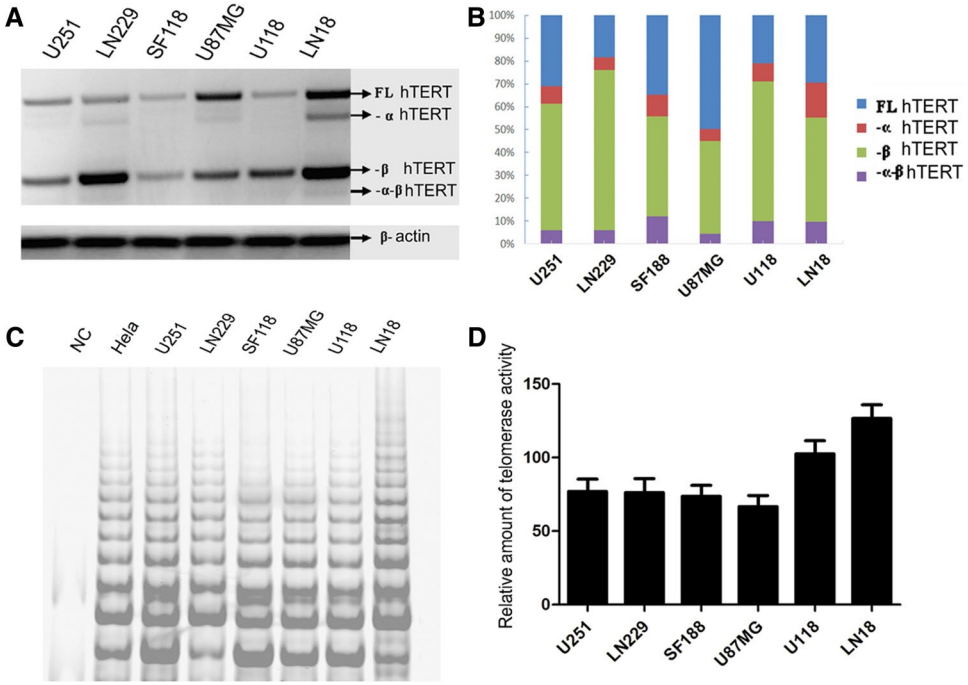

Fig. 1 Comparison of hTERT and telomerase activity in different glioma cell lines. **a** Expression of hTERT ASVs in six glioma cell lines was detected by RT-PCR. **b** The ratios of the hTERT ASVs in six different glioma cell lines. **c**, **d** Detection of telomerase activity in six glioma cell lines by the TRAP method

transfection efficiency of the Alexa Red Fluorescent Oligo positive control was 96.49% (Fig. 2b). The transfection efficiency of AON-Ex726 was 98.25% (Fig. 2c). Therefore, the transfection efficiency was high enough to carry out the following experiments on detection of hTERT ASVs, telomerase activity, cell growth, proliferation and apoptosis.

Effect of different ratios of thiophosphate and concentration of AON-Ex726 on cytotoxicity

Compared with untransfected U251 glioma cells (Supplementary Fig. 3A), the morphology of U251 cells transfected with a random oligonucleotide (as a negative control) did not significantly change (Supplementary Fig. 3B). When 100%-thio AON-Ex726 was transfected to U251 cells, it showed cytotoxicity at final concentrations of 250 nM, 125 nM, 62.5 nM, and 31.25 nM (Supplementary Fig. 3C and 3D) (data for 250 nM and 125 nM are not shown). After transfection with thio-free AON-Ex726, the cell morphology did not significantly change at concentrations of 250 nM, 125 nM, 62.5 nM and 31.25 nM, indicating the absence of cytotoxicity (Supplementary Fig. 3E, 3F, 3G and 3H). After transfection with 50%-thio AON-Ex726, some cells were necrotic at concentrations of 250 nM and 125 nM (Supplementary Fig. 3I and 3J), but there was virtually no cytotoxicity at 62.5 nM and 31.25 nM (Supplementary Fig. 3K and 3L). However, the random AON with 50%-thio at any concentration did not show cytotoxicity. These results indicated that the thio-free AON-Ex726 was less toxic. The 100%-thio AON-Ex726 had strong cytotoxicity, while the 50%-thio AON-Ex726 had an intermediate effect.

The 50%-thio AON-Ex726 changes hTERT pre-mRNA splicing and inhibits telomerase activity

We found that thio-free AON-Ex726 did not reduce the expression level of full-length hTERT mRNA. The 100%-thio AON-Ex726 significantly reduced the expression level of full-length hTERT mRNA but was more cytotoxic. The 50%-thio AON-Ex726 significantly reduced the expression of full-length hTERT mRNA at concentrations of 250 nM, 125 nM, and 62.5 nM, but did not work at 31.25 nM concentration (Fig. 2d). After transfection with 50%-thio AON-Ex726 at 62.5 nM concentration, the telomerase activity was significantly reduced compared with the control and random oligonucleotide groups not only in U251 but also in U87MG cells (Fig. 2e, f, supplementary 4A, $p < 0.05$). However, the random AON with 50%-thio at any concentration did not work. According to these findings, we used the 50%-thio AON-Ex726 at 62.5 nM concentration in the following experiments to detect cell proliferation and apoptosis after 12-week long-term treatment in U251 and U87MG glioma cells.

Inhibition of cell colony formation after transfection with 50%-thio AON-Ex726

The cell colonies in the control and random oligonucleotide groups appeared early, the numbers and diameters were large, and the morphology was regular. The cell colonies in the AON group appeared later, with the number and diameter decreased. After 2 weeks of culture, the rate of colony formation in the AON group was significantly lower than in

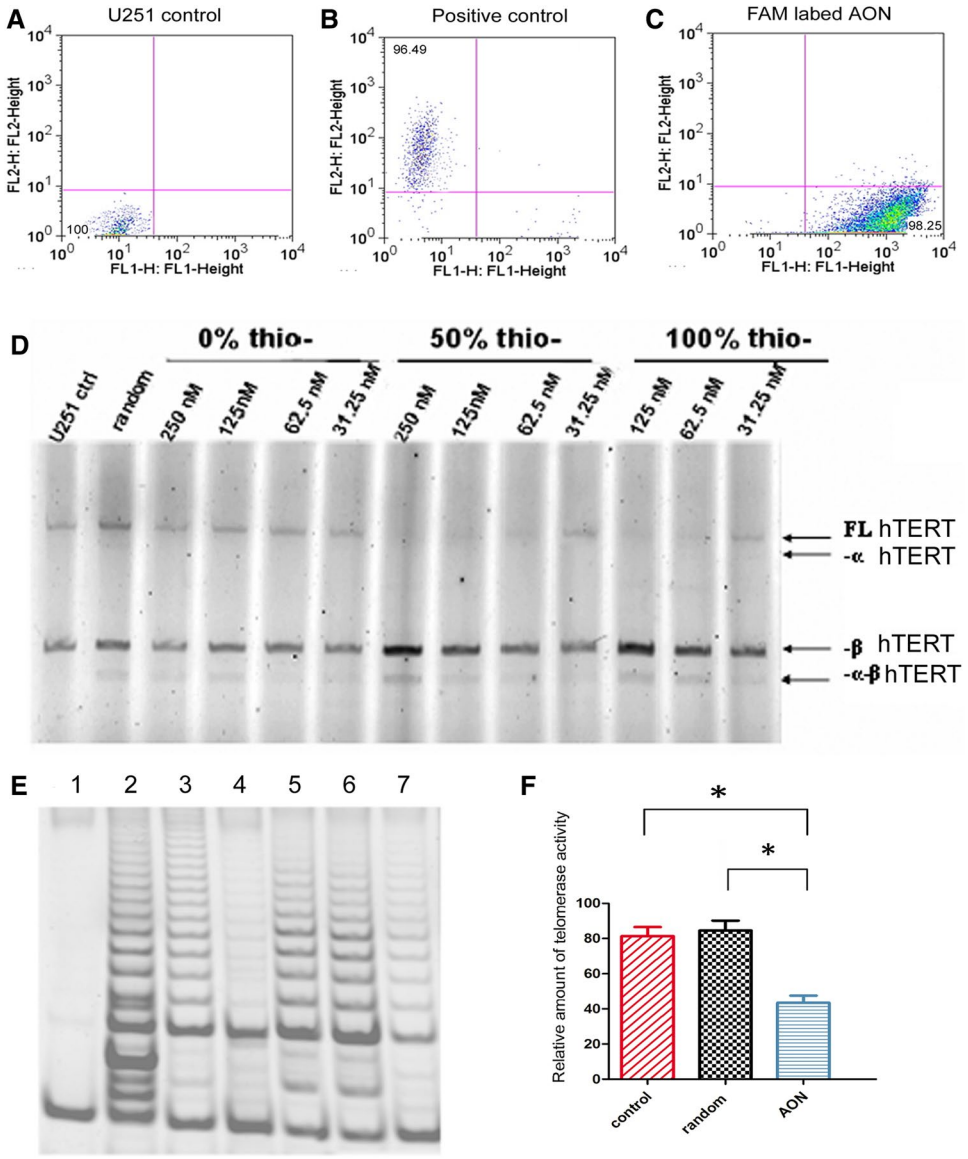

Fig. 2 The 50%-thio AON-Ex726 changes the alternative splicing of hTERT pre-mRNA and inhibits telomerase activity. **a**–**c**, the efficiency evaluation of AON transfection. **a** U251 cells as a negative control; **b** transfection of U251 cells with lipofectamine RNAiMAX resulted in the transfection efficiency for the AlexaRed fluorescent oligo positive control; **c** the transfection efficiency of AON-Ex726. **d** the effects of AON-Ex726 with different chemical modifications and different concentrations on alternative splicing of hTERT pre-mRNA; **e**, **f** 50%-thio AON-Ex726 inhibits telomerase activity. (1) Lysis buffer as negative control; (2) 2500 Hela cells; (3) 250 Hela cells; (4) 25 Hela cells; (5) U251 control; (6) random oligonucleotide; (7) AON-Ex276

the control and random oligonucleotide groups ($p < 0.05$). These results indicated that the colony formation of U251 and U87MG glioma cells was inhibited after transfection with 50%-thio AON-Ex726 at 62.5 nM concentration (Fig. 3a, b, Supplementary Fig. 4B).

The 50%-thio AON-Ex726 reduces cell proliferation and induces apoptosis

The results of cell proliferation detected by MTT assay showed that there was no significant difference in cell proliferation between the control and random oligonucleotide groups, but cell proliferation in the AON group was significantly inhibited 24 h after transfection, and the inhibition was persistent. It was demonstrated that the transfection with 50% thio AON-Ex726 significantly decreased the proliferation activity of U251 and U87MG cells (Fig. 3c, Supplementary Fig. 4C). The western blot results showed that the expression level of anti-apoptotic protein Bcl-2 was significantly decreased, and the expression of apoptotic protein Bax was significantly increased after transfection with 50%-thio AON-Ex726 (Fig. 3d, e, Supplementary Fig. 4E). The Annexin V/PI assay for apoptosis analysis by flow cytometry showed that apoptosis rate was increased after transfection with 50%-thio AON-Ex726 (Fig. 3f, g, Supplementary Fig. 4D). These results indicated that the 50%-thio AON-Ex726 reduced cell proliferation and induced apoptosis.

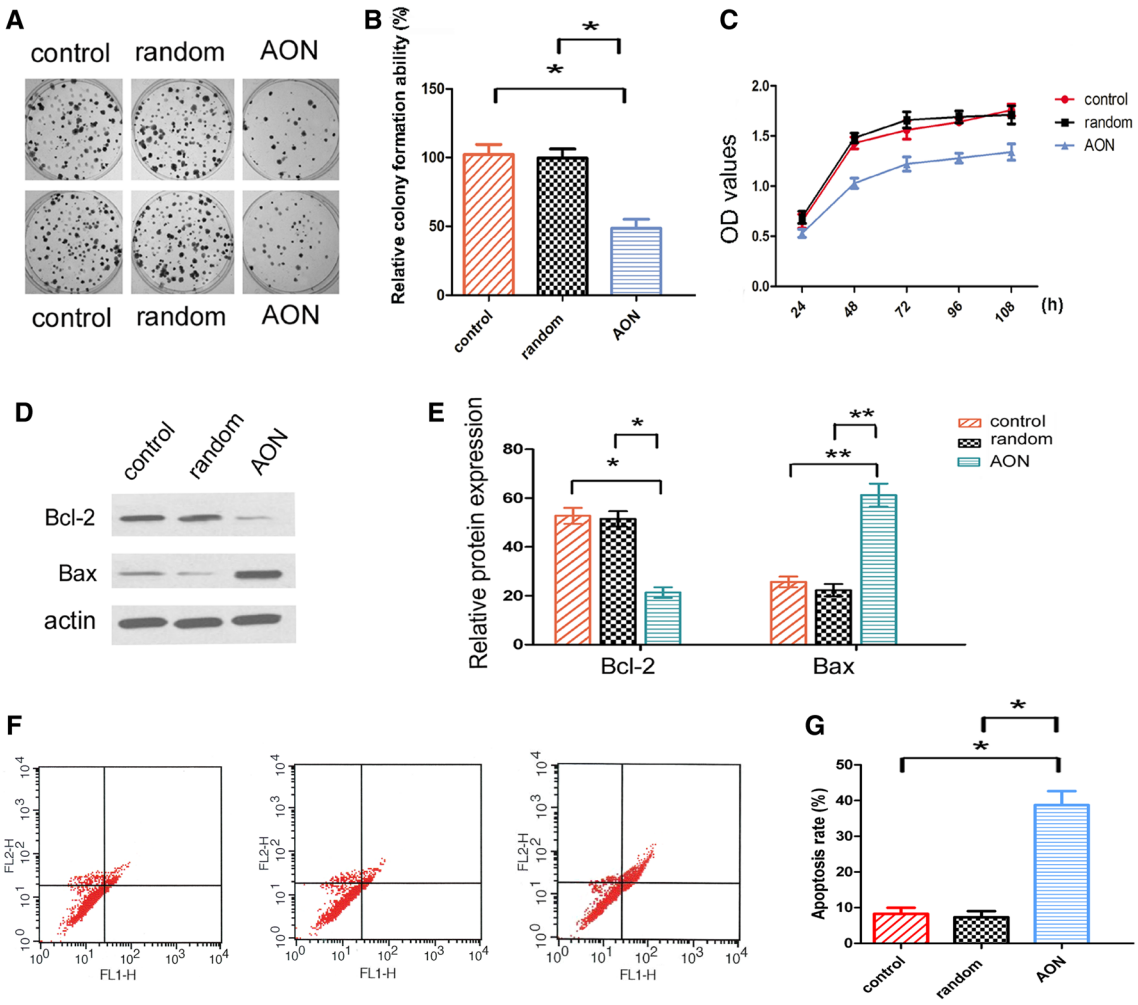

Fig. 3 Effects of 50%-thio AON-Ex276 on the cell proliferation and apoptosis in U251 glioma cells. **a**, **b** Numbers of colony-forming units in every group; **c** the MTT assay absorbance values at different time points; **d**, **e** protein expression levels of pro-apoptosis and anti-apoptosis proteins determined by western blotting. **f**, **g** Apoptosis rate in the Annexin V /PI assay. $^{*}P<0.05$, $^{**}P<0.01$

Data mining by bioinformatics analysis

We found that the binding site of AON-Ex726 in the intron 6 of hTERT pre-mRNA was the binding motif of the splicing protein SRSF2 (SC35) (Supplementary Fig. 1C). Then we used the Oncomine and cBioPortal databases to further analyse the roles of SRSF2 in development of gliomas and the interaction network between SRSF2 and TERT. SRSF2 was highly expressed in various cancer types. SRSF2 and its related genes had high expression levels in brain and CNS cancer (Fig. 4a–d). The expression level of SRSF2 was higher in the patients who were dead within 5 years than in the patients who were alive after 5 years (Fig. 4e). The correlation between overall survival and SRSF2 expression was shown in Fig. 4f. We also analysed the interaction gene network of SRSF2 and TERT by using the cBioPortal database. The common genes in the SRSF2-TERT networks are shown in Fig.ure 4 g and Supplementary Table 2.

Discussion

hTERT mRNA can be detected in telomerase-positive tumour tissues, where it is positively correlated with the degree of tumour malignancy. Tumours with high expression level of hTERT mRNA had rapid recurrence and poor prognosis [32]. hTERT mRNA is specifically expressed in cancer cells and is not expressed in normal somatic cells. Selecting hTERT as a therapeutic gene can specifically inhibit the growth of telomerase-positive cancer cells, but does not produce obvious side-effects on normal cells, greatly improving the safety of gene therapy for cancers. The expression level of hTERT mRNA can be used as a prognostic indicator for glioma diagnosis. In addition, inhibition of hTERT expression can effectively inhibit cancer cell growth and induce apoptosis, making it an ideal target for gene therapy of glioma [33].

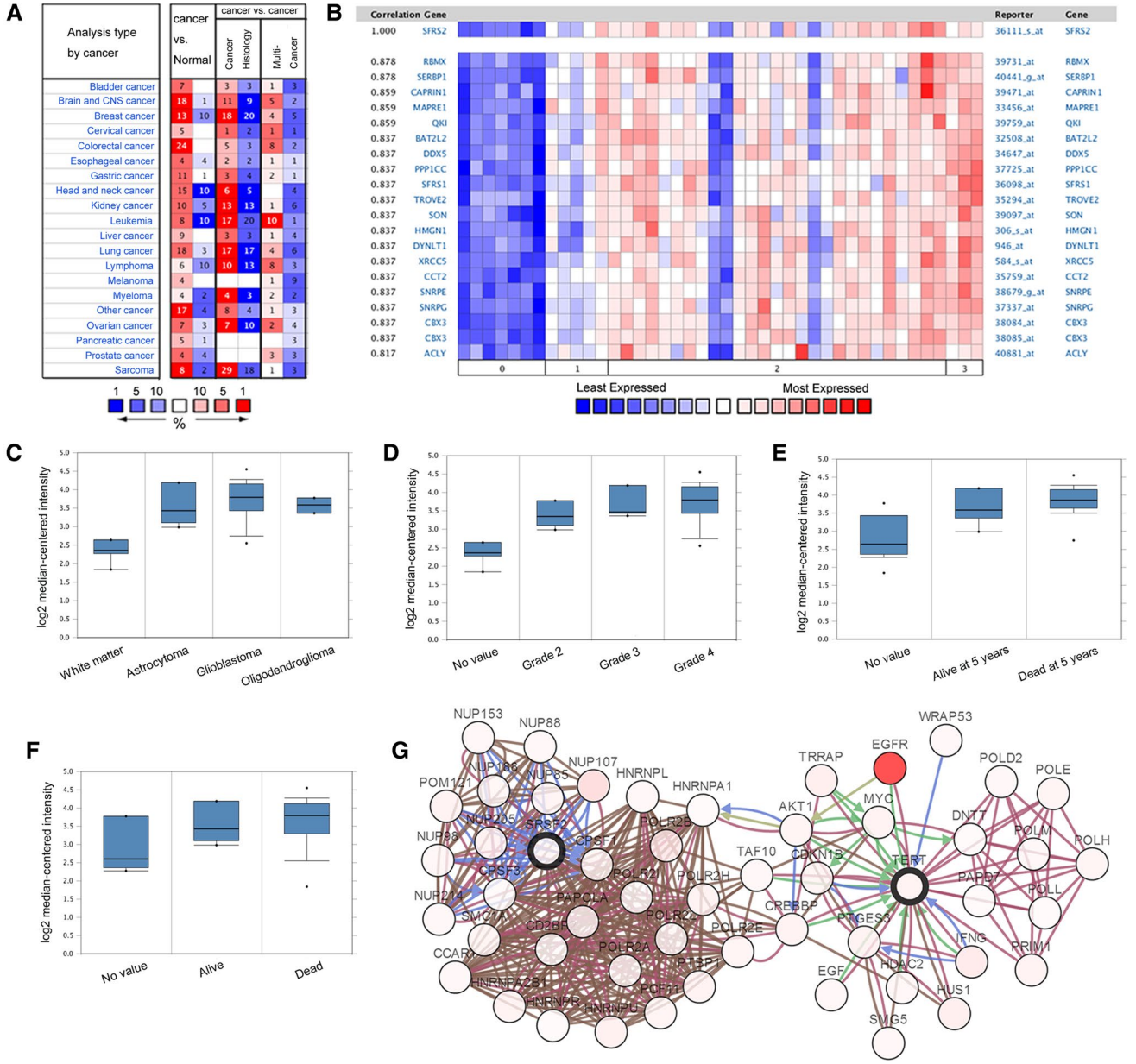

Fig. 4 The bioinformatics analysis of the SRSF2 role in gliomas and the interaction networks involving SRSF2 and TERT. **a** Expression of SRSF2 in various types of cancers; **b** expression of SRSF2 and its related genes in the brain and CNS cancer; **c** expression of SRSF2 in different types of gliomas; **d** expression of SRSF2 in different grades of gliomas; **e** expression of SRSF2 in patients surviving 5 years and died within 5 years; **f** the correlation between overall survival and SRSF2 expression; **g** interaction networks of SRSF2 and TERT

Alternative splicing plays important roles in the formation of biological properties of proteins, including protein-binding ability, intracellular localisation, enzyme activity, stability and post-translational modification. Modulating alternative splicing can effectively degrade mRNA variants by introducing premature stop codons, as well as altering the structures of transcripts [34]. Some tumour-associated genes can be regulated by alternative splicing. The changes in the splicing process and the conversion of the particular splicing patterns may occur during tumour progression and represent one of the major factors contributing to carcinogenesis. Previous studies have shown that alternative splicing products of CD44, WT1, Bcl-2, BRCA1, E-cadherin, and caspase are associated with cancers. The ASVs have diagnostic values and may become potential targets for cancer therapy drugs. The identification and analysis of ASVs contributes to understanding the process of malignant transformation of cancer cells [35–40].

Alternative splicing of hTERT pre-mRNA regulates telomerase activity at the post-transcriptional level.

Woodring Wright et al. studied the relationship between the expression ratio of hTERT ASVs and telomerase activity in normal cells and cancer cell lines. They found that the full-length hTERT ASVs accounted for 5% of the total hTERT transcript, the β-deletion ASV accounted for about 80%–90%, the αβ-deletion ASV accounted for 5%–15%, and the α-deletion ASV only accounted for < 1% in various types of telomerase-positive cancer cells. They cloned the cDNAs of the α-deletion ASV, β-deletion ASV and αβ-deletion ASV into expression vectors and transfected into human fibroblasts and several telomerase-positive immortalized cells. None of the three ASVs could reconstitute telomerase activity in human fibroblasts. Overexpression of the α-deletion ASV in immortalized or cancer cell lines inhibited telomerase activity and gradually shortened telomere length, ultimately leading to apoptosis. The β-deletion ASV and αβ-deletion ASV did not affect telomerase activity. Therefore, the α-deletion ASV had a significant negative regulatory effect on telomerase activity and telomere maintenance [16, 41]. Colgin et al. also demonstrated that the α-deletion ASV inhibited telomerase activity, leading to shortening of telomeres and apoptosis of jejunal fibroblast cells. Upregulation of the α-deletion of ASV by changing the alterative splicing of hTERT pre-mRNA, thereby inhibiting telomerase activity, may become a novel strategy for anti-tumour therapy [42]. Our previous studies showed that the hTERT alternative splicing pattern was different during the multi-stage evolution of gastric cancer. The expression level of the full-length hTERT was increased, and the β-deletion ASV was widely expressed in the multi-stage evolution of gastric cancer. The expression level of the β-site retained ASV gradually increased during the multi-stage evolution of gastric cancer, suggesting that the detection of the β-site retained ASV may provide the diagnostic value for gastric cancer and precancerous lesions [43]. In the present study, the results for glioma tissue and six glioma cell lines showed that the relative amounts of telomerase activity were correlated with the expression of the full-length hTERT mRNA with neither deletion nor insertion of splicing, rather than all of the hTERT ASVs (the sum of −α, full-length, −β,−α−β hTERT mRNA). Therefore, it was suggested that alternative splicing of hTERT pre-mRNA plays a crucial role in the regulation of telomerase activity. There is no one-to-one correspondence between the expression level of total hTERT transcript and telomerase activity, because the total hTERT transcript contains four types of hTERT splicing variants: the full-length, −α,−β and −α−β hTERT mRNA. Only the protein product translated from the full-length hTERT mRNA has telomerase activity and is associated with telomerase activity. These findings will provide more experimental evidence for the study of inhibition of telomerase activity by changing the alternative splicing of hTERT pre-mRNA.

AONs with different chemical structures have certain effects on the exon skipping. Previous studies reported AONs that have been designed with different chemical structures to minimize the cytotoxicity and maximize the cell penetration [44]. In the present study, we applied the 2′-O-methyl-RNA phosphorothioate AONs and explored the effects of different thio-proportions (sulphur-free, 50%-thio, 100%-thio) on cytotoxicity, cell penetration and exon skipping. Our results indicated that sulphur-free AON-Ex726 was less cytotoxic but had poor stability; 100%-thio AON-Ex726 was stable, but more cytotoxic, while 50%-thio AON-Ex726 fell in between. AONs interfere with alternative splicing by blocking certain domains of pre-mRNA and inducing specific exon resection, such as resection of exons containing a stop codon or induction of one or more exon-skipping thus leading to frameshift mutations. The use of AONs to modulate pre-mRNA alternative splicing to alter gene expression has potential applications for the treatment of certain diseases. Studies by Bauman et al. showed that alteration of alternative splicing of Bcl-x pre-mRNA from the Bcl-xL to Bcl-xS isoform induced apoptosis in breast and prostate cancer cells. They applied lipid nanoparticles to carry AONs into melanoma B16F10-transplanted mice, which regulated the splicing of Bcl-x pre-mRNA and reduced the size of tumour formation [25]. Zaffaroni et al. found that 2′-O-methyl-RNA phosphorothioate oligonucleotide binding to the splice sites between intron five and exon six in the hTERT pre-mRNA could regulate the alternative splicing pattern of hTERT pre-mRNA in prostate cancer DU145 cells, reduce the full-length hTERT and correspondingly increase other ASVs. That AON could inhibit telomerase activity, slow the proliferation activity and induce apoptosis [45]. A very recent study reported that the G-quadruplex stabilizer CX-5461 altered the patterns of hTERT splicing, leading to an increase in −β hTERT ASV and a decrease in+β hTERT transcript expression, which inhibits telomerase activity. In addition, CX-5461 had a cytotoxic effect on glioma cells and induced telomere DNA damage response, G2/M arrest and apoptosis [30]. In our present study, we designed the AON-Ex726 according to the ISE site in intron six of hTERT pre-mRNA, which caused hTERT exon skipping, reducing the expression level of the full-length hTERT and increasing the expression of the β-deletion hTERT mRNA. Moreover, AON-Ex726 reduced the telomerase activity, cell proliferation activity and plate colony formation of glioma U251 cells. We evaluated the findings on a second GBM cell line U87MG and we found that AON-Ex726 also reduced the telomerase activity, cell proliferation activity, plate colony formation and increased the apoptosis of U87MG cells.

Using bioinformatic analysis, we predicted that the binding site of AON-Ex726 on the hTERT pre-mRNA shared a motif with the splicing protein SRSF2 (SC35). SRSF2 is a member of the classical SR protein family that not only regulates alternative splicing of genes, but also plays an activating role during transcription. As a splicing regulator, SRSF2 down-regulated E-cadherin expression by increasing its false splicing, which was found to be highly abundant in head and neck cancer cell lines and tissues [46]. Recent studies found that SRSF2 promotes the development of liver cancer by regulating alternative splicing of tumour-associated genes. SRSF2 showed a high expression trend in liver cancer patient samples, and there was a significant correlation between the protein expression level and the pathological grade of liver cancer and the poor prognosis of patients. Furthermore, SRSF2 played a significant role in promoting the growth of liver cancer cells, and its function depended largely on its splicing regulation of multiple downstream genes. SRSF2 regulated alternative splicing of the GCH1 and STK39 target genes, resulting in increasing expression levels of splice isoforms, which promote growth of liver cancer cells [47, 48]. However, until now, the role of SRSF2 in the development of glioma remained unclear. By using the bioinformatics database mining methods, we found that SRSF2 was highly expressed in various cancer types. SRSF2 and its related genes were highly expressed in brain and CNS cancer. The expression levels of SRSF2 were higher in died patients than in survived patients within 5 years. Blackburn et al. reported that hTERT splicing was controlled by the splice regulators SRSF11, HNRNPH2, and HNRNPL in a breast cancer cell panel [49]. A very recent study identified neuro-oncological ventral antigen 1 (NOVA1) that regulates the hTERT splicing in non-small cell lung cancer cells. The stable reduction in NOVA1 levels shifted hTERT splicing from FL hTERT to the inactive ASVs and reduced telomerase activity, thus leading to progressively shortened telomeres [50]. Knowledge about the splicing factors that regulate hTERT splicing is lacking. Based on our present findings, we speculated that the regulatory mechanisms of hTERT splicing may be different in various cancer types. SRSF2 may upregulate the telomerase activity by shifting the alternative splicing of hTERT pre-mRNA in glioma. The specific role of SRSF2 in gliomas and the mechanism of regulation of telomerase activity remain to be elucidated in future studies.

Taken together, our present study indicated that the alternative splicing of hTERT pre-mRNA was present in both glioma cells and patient tissues. There was a correlation between the expression level of the full-length hTERT mRNA, rather than the total hTERT transcript, with the telomerase activity. After transfection with antisense oligonucleotide AON-Ex726, the splicing of hTERT pre-mRNA was changed in glioma cells, increasing the loss of exon seven and exon eight. The expression of full-length hTERT mRNA was reduced, while expression of the β-deletion hTERT mRNA was increased. The telomerase activity of glioma cells decreased, which may be due to the decrease in expression of the splicing factor SRSF2, thereby inhibiting cell proliferation and cell colony formation and increasing apoptosis. The alternative splicing of hTERT pre-mRNA, as the regulation mechanism at the post-transcriptional level, plays a crucial role in the regulation of telomerase activity and may become a potential target for gene therapy of glioma.

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Compliance with ethical standards

Conflict of interest All authors declared that they have no conflict of interest.

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