RNA interference mediated downregulation of human telomerase reverse transcriptase (hTERT) in LN18 cells

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Abstract Human telomerase reverse transcriptase (*hTERT*) gene is a biomarker for the targeted therapy in various cancers. Presence of increased telomerase activity is a common feature of all cancers including glioblastoma. Both RNA and catalytic subunits of hTERT are the target sites for blocking its activity. The current study focuses on the expression of hTERT in glioblastoma and its regulation using two different novel siRNAs (small interfering RNA). Our patient data demonstrated increased expression of hTERT, which could be correlated with carcinogenesis in glioma. In vitro studies in siRNA transfected LN18 cells confirmed significant cell death ($p < 0.05$) as evidenced

by MTT and trypan blue exclusion assay. These results were further supported by flow cytometry data, which showed significant increase in early and late apoptosis. The hTERT mRNA expression was effectively down-regulated by 45 and 39 % with siRNA1 and siRNA2, respectively. These results were further confirmed by immunoblotting analysis ($p < 0.05$). Our results suggest that both the siRNAs effectively down regulated the expression of *hTERT* at mRNA and protein levels, thereby decreasing cell viability and proliferation rate. Hence siRNA mediated downregulation of *hTERT* could be a potential therapeutic avenue in glioblastoma.

Keywords hTERT · LN18 cell line · Glioblastoma · Small interfering RNA · Apoptosis

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Introduction

Gliomas are the most common primary malignant neoplasms of the central nervous system. Almost 80 % of gliomas are astrocytic tumors including glioblastoma (Goodenberger and Jenkins 2012). Although significant advances have been made in the treatment strategies, the prognosis of glioblastoma patient's remains dismal (George et al. 2010). A number of biomarkers have been identified and are currently used in the diagnosis and prognosis of glioblastoma. The most common biomarkers are *MGMT*, *IDH*, *EGFR*, *VEGF*, *P53*, and *hTERT* (McNamara et al. 2013;

Westphal and Lamszus 2015). Among these, it has been reported that increased telomerase activity is considered as a marker of malignancy in majority of brain tumors including gliomas (Kotoula et al. 2004).

Telomeres are DNA protein complexes present at the ends of eukaryotic chromosomes and are comprised of TTAGGG repeats and associated proteins (Blackburn 2001; Cong et al. 2002). They protect the chromosomes from degradation and end to end fusions, maintain chromosomal stability and act as a mitotic clock of a cell. Telomerase is a ribonucleoprotein DNA polymerase that maintains the telomeres (Blackburn et al. 1989; Greider 1991; Cong et al. 2002). Telomerase consists of telomerase RNA (hTR) and the catalytic component human telomerase reverse transcriptase (hTERT) for telomeric DNA synthesis. Evidence from the previous studies shows that, beyond the telomeric DNA synthesis activity, TERT is involved in several other functions including tumor development, cell proliferation, gene expression regulation and mitochondrial functionality (Chiodi and Mondello 2011; Maida and Masutomi 2015). It also plays a major role in stem cell maintenance and cell reprogramming processes. Furthermore, TERT RNA-dependent RNA polymerase activity (RdRP) is involved in gene regulation and heterochromatic transcription (Maida and Masutomi 2015). Telomerase is usually inactive in somatic cells but remains active in germ cells, embryonic stem cells and tumor cells (Kim et al. 1994; Shay and Bacchetti et al. Shay and Bacchetti 1997; Cowell 1999). Almost 80–90 % of human cancers display telomerase activity. Several studies showed that more than 50 % of gliomas display telomerase activity and its detection rates increases with the grades of malignancy (Langford et al. 1995; Nakatani et al. 1997; Sano et al. 1998; Tchirkov et al. 2003). Much attention has been given to telomerase, however, telomerase independent mechanism like alternative lengthening of telomeres (ALT) can also maintain telomere lengths in cancer cells by homologous recombination between telomere sister chromatids (Cesare and Reddel 2010). hTERT is the rate-limiting subunit of telomerase holoenzyme, therefore, knockdown of hTERT expression may contribute to evolve novel therapeutic strategies. Several studies demonstrate that small-interfering RNA (siRNA) mediated knockdown of *hTERT* expression both in vitro and in vivo is considered as a promising approach for targeted cancer therapy (Kurvinen et al. 2006; George et al. 2009; Zhang and Xing 2013; Shi et al. 2014). However, this approach has not been exhaustively

tested in glioblastoma and the mechanism is unclear. Towards this objective, we have in this study, investigated the effect of two novel siRNAs on hTERT expression in glioblastoma.

Materials and methods

Human tumor samples

High grade glioma tumor samples were collected from the 50 patients, enrolled at the Department of Neurosurgery during 2012–13 and pathological grading was done at Department of Neuropathology, National Institute of Mental Health and Neurosciences (NIMHANS, Bangalore, India). Informed consent was obtained from all patients as approved by the Institute human ethics committee. Control brain tissues were obtained from five patients who underwent epilepsy surgery. Patient follow up was done and their survival rate, progression free survival (PFS) and overall survival (OS) was monitored up to 41 months from the date of surgery. hTERT mRNA expression within patient samples were analysed by quantitative PCR.

Cell culture

The LN18 (human glioblastoma) cell line used in this study was purchased from the National Center for Cell Science (NCCS), Pune, India. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) containing 10 % fetal bovine serum (Invitrogen, Life Technologies, Carlsbad, CA, USA) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies)) in a humidified incubator containing 5 % CO₂ and 95 % air at 37 °C.

siRNA transfection

The *hTERT* specific siRNAs were obtained from Invitrogen. Two different hTERT specific siRNAs: siRNA1, siRNA2 (Table 1) and scrambled siRNA (negative control) were transfected into cells using Lipofectamine[®] 2000 reagent (Invitrogen, Life Technologies). Briefly, LN18 cells (2×10^5 cells/well) were seeded into six-well plates containing an antibiotic-free medium and were incubated overnight at 37 °C. For transfection, siRNA and Lipofectamine[®] 2000 mix (1:3) was prepared in serum free medium (Opti-MEM). The mixture was

Table 1 Sequences of hTERT specific siRNA

	siRNA	Sequence	hTERT mRNA target region
The two selected siRNA sequences and their target regions on hTERT mRNA	siRNA1-sense	5'-GGCCGAUUGUGAACAUGGAtt-3'	Exon 4
	Antisense	5'-UCCAUGUUCACAAUCGGCCgc-3'	
	siRNA2-sense	5'-CGGAGACCACGUUUCAAAAtt-3'	Exon 3
	Antisense	5'-UUUUGAAACGUGGUCUCCGtg-3'	

incubated at room temperature for 20 min to facilitate the formation of siRNA-Lipofectamine complex. The mixture was added to the cells in an appropriate volume of Opti-MEM to achieve a final concentration of 100 nm for each siRNA. After incubation for 6 h at 37 °C, DMEM supplemented with serum was added and cells were cultured for 24 h before analysis.

Trypan blue exclusion assay

LN18 cells were cultured in 6-well plate at a density of 1×10^5 cells/well. After transfection, the cells were trypsinized at regular time intervals and viable cells were counted using trypan blue dye (0.4 %) staining. The average number of cells from all wells was compared between control and treated groups from three independent experiments.

MTT assay

Cell viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma Aldrich) assay. The principle of the assay is based on the cleavage of tetrazolium salts by mitochondrial succinate reductase in viable cells to form formazan dye. The LN18 cells were seeded in 96-well plates at a density of 5×10^3 cells/well and grown for 24 h and then the cells were transfected with different siRNAs. After transfection, MTT (5 mg/ml) was added to each well and the cells were incubated for 2 h at 37 °C and the formed formazan crystals were dissolved in DMSO. The absorbance was then measured at 570 nm using a plate reader (Tecan, infinite M200 Pro, Grödig, Austria). Cell viability was expressed as percentage against the control group.

RNA isolation and qRT-PCR

Total cellular RNA was isolated using Pure LinkTM RNA Mini Kit (Ambion, Life technologies) as per the manufacturer's instructions. One microgram of total RNA was

reverse transcribed into cDNA using MMLV-reverse transcriptase, oligo-dT, dNTPs and buffer, following the manufacturer's instructions (Life Technologies). The primers with the following sequences were used for PCR amplification: (1) hTERT sense: 5'-GGAGCAA GTTGCAAAGCATTG-3'; hTERT antisense: 5'-TCCC ACGACGTAGTCCATGTT-3' (2) GAPDH sense: 5'-G AAGGTGAAGGTCGGAGTCAAC-3', GAPDH antisense: 5'-CAGAGTTAAAAGCAGCCCTGGT-3'. Quantitative PCR amplification was performed on the Applied Biosystems 7500 Fast (Applied Biosystems, Foster City, CA, USA) using Eva green master mix (2X) (Life Expression, abm, Richmond, BC, Canada) with a total reaction volume of 10 µl and the following cycling conditions: polymerase activation at 95 °C for 10 min, followed by 40 cycles of PCR at 95 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 7 min. Comparative analysis by $2^{(-\Delta\Delta CT)}$ was used to compare the expression of hTERT.

Western blotting

The LN18 cells were seeded in 6 well plates of 2×10^5 cells/well and grown for 24 h and then subjected to transfection. After transfection, the cells were lysed with cold RIPA lysis buffer [50 mM Tris, 150mM NaCl, 1 mol/L EDTA, 0.1 % sodium dodecyl sulphate, 1 % Triton X-100, 1 mol/L phenyl methyl sulfonyl fluoride (pH 8.0): SRL Ltd, Bangalore, India]. The lysate was centrifuged at $8000 \times g$ for 20 min at 4 °C. The protein content in the cell supernatant was measured by Bradford assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). The proteins separated on 12 % SDS-PAGE were transferred onto a polyvinyl difluoride (PVDF) membrane (Bio-Rad Laboratories Inc.). The membrane was blocked with 5 % bovine serum albumin in TBST (1 % Tween-20 in 20 mmol/l Tris-buffered saline (pH 7.6) (SRL Ltd.). The blots were probed with monoclonal anti-hTERT (#sc-377511) or anti-β-tubulin (#sc-58882) antibodies at a dilution of 1:1000 and anti-mouse horseradish

peroxidase-conjugated secondary antibody (#sc-358914) at a dilution of 1:5000 (antibodies were purchased from Santa Cruz Biotech Inc, Dallas, TX, USA) and detected by enhanced chemiluminescence detection kit (Bio-Rad Laboratories Inc.). The band intensity was measured using NIH Image-J software (National Institutes of Health, Bethesda, MD, USA).

Flow cytometry

Flow cytometry assay was performed using FITC-Annexin V apoptosis detection kit (BD Pharmingen, Franklin Lakes, NJ, USA). Cells were harvested 48 h after transfection; harvested cells were washed with 1X PBS followed by 1X binding buffer. The cells were incubated with 5 μ l of fluorochrome-conjugated Annexin V for 15 min at room temperature in the dark. After incubation cells were washed and stained with propidium iodide staining solution and analyzed by flow cytometry in the dark. Diverse labeling patterns allowed us to identify different cell populations: early apoptotic cells ($PI^-/Annexin V^+$); apoptosis/necrotic cells ($PI^+/Annexin V^+$) and viable cells ($PI^-/Annexin V^-$). Apoptotic cells are expressed as the percentage of total cells.

Telomeric repeat amplification protocol (TRAP) assay

To investigate whether siRNA transfection reduces telomerase activation, the enzymatic activity was measured by Telo TAGGG telomerase PCR ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. In brief, LN18 cells were collected 48 h after siRNA transfection. The cells were washed with cold PBS, lysed in 200 μ l lysis buffer and incubated on ice for 30 min. The lysate was centrifuged at $16,000\times g$ at 4 °C for 20 min. The supernatant was collected and the protein extracts were subjected to telomeric repeat amplification protocol (TRAP) assay. For the TRAP reaction, 25 μ l of reaction mixture, 10 μ g of sample, and nuclease free water to make a final volume of 50 μ l. PCR was performed; hybridization and ELISA were carried out following the manufacturer's instructions. A total of 5 μ l of PCR amplified telomerase products was added to a streptavidin-coated 96-well plate and hybridized to a digoxigenin (DIG)-labeled telo-meric repeat-specific detection probe. The immobilized PCR products were detected with

peroxidase-conjugated anti-DIG antibody. After addition of the stop reagent, the plate was assessed on a plate reader at a wavelength of 450 nm within 30 min. The heat inactivated cell lysates were used as negative controls.

Statistical analysis

Statistical analysis for patient samples and cell culture was conducted using R-3.0.1 and Graph pad prism version 5, respectively. The data are expressed as mean $\pm$ standard deviation (SD) of three independent experiments. Comparison between hTERT mRNA expression levels in different groups was performed using Kruskal–Wallis test. Survival analysis was done using Kaplan–Meier survival analysis. The data were statistically evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc *test*. The least significant difference method was used to compare the mean values of different experimental groups. In all experiments, $p < 0.05$ was considered to be statistically significant.

Results

hTERT mRNA expression in tumor samples

To determine the importance of hTERT in glioma, we first quantitated its expression in fifty patient samples with high grade gliomas (Grade III and IV). Quantitation of hTERT mRNA showed that thirty-six samples (72 %) had high expression, compared to control samples ($p < 0.05$). The median fold change of expression was 3.19 ranging from 0.04 to 59.54-fold (Fig. 1).

Fig. 1 Box plot showing hTERT increased mRNA expression in high grade glioma compared to non-glioma tissues

There was no correlation between hTERT mRNA expression and other clinical variables (age, grade and tumor type) except gender. Males showed higher expression of hTERT mRNA when compared to females (Table 2). hTERT mRNA expression could predict the patient survival in both PFS ($p = 0.0068$) and OS ($p = 0.0282$). Patients with low hTERT mRNA

Table 2 Comparison of hTERT fold change among different clinical parameters

Variable	Fold change above 1.5	<i>p</i> value
Age		
<40	19/29	0.2302
≥40	17/21	
Gender		
Male	30/37	0.01583
Female	6/13	
Grade		
Type 3	26/39	0.1138
Type 4	10/11	
Tumor type		
AA	4/5	0.1705
AOA	5/6	
AOD	16/27	
GBM	11/12	

AA Anaplastic astrocytoma, AOA Anaplastic oligoastrocytoma, AOD Anaplastic oligodendroglioma, GBM Glioblastoma multiforme

levels had longer survival rate with a median PFS of 24 months (95 % CI 18–39) and OS (Fig. 2). In contrast patients with high hTERT mRNA levels had a poor survival rate with a median PFS of 11 months (95 % CI 7–17) and OS of 24 months (95 % CI 24–30). These data suggest that, elevated hTERT expression could be a factor that may lead to carcinogenesis in glioma.

hTERT mRNA and protein levels in LN18 cells after siRNA transfection

hTERT expression was down-regulated via siRNA mediated *hTERT* knockdown in the LN18 glioblastoma cell line. The LN18 cells were transiently transfected with two different double stranded siRNAs directed against target regions of hTERT mRNA. siRNA1 was directed against a region on exon 4 whereas siRNA2 targets a region on exon 3. 48 h after transfection with two siRNA constructs the level of hTERT mRNA expression was decreased by ~45 % and ~39 % with siRNA1 and siRNA2, respectively ($p < 0.05$), compared to control. The scrambled siRNA had no effect on the expression of hTERT mRNA (Fig. 3). Among the two siRNAs used, we observed that siRNA1 was more effective in down-regulating hTERT expression than siRNA2. These results were further confirmed by immunoblotting (Fig. 4a) studies. The expression of *hTERT* protein was

Fig. 2 Kaplan-Meier survival curve showing prognostic role of hTERT mRNA expression. Patients with decreased hTERT mRNA expression showing favorable prognosis compared to increased expression in PFS and OS. Survival time shown is in months

Fig. 3 The effect of hTERT specific siRNAs on the expression of hTERT mRNA level in LN18 cells was assessed using Real-time RT-PCR. The relative mRNA expression was calculated using $2^{(-\Delta\Delta CT)}$ method, with GAPDH as internal control. The values indicated the mean $\pm$ standard deviation of 3 independent experiments in each group. ** $p < 0.01$ between scr siRNA and siRNA1. * $p < 0.05$ (siRNA2) and *** $p < 0.005$ (siRNA1) versus the respective control group

decreased by 49 and 38 % by siRNA1 and siRNA2, respectively, compared to controls ($p < 0.05$, Fig. 4b).

Effect of siRNA on cell viability and proliferation in LN18 cells

The effect of downregulation of hTERT on the cell viability and proliferation status was evaluated by MTT and trypan blue exclusion assays. There was a

significant decrease in the cell viability with siRNA1 and siRNA2 transfection ($p < 0.05$). Cell viability decreased with 47.7 % and 53.3 % after 24 and 48 h of siRNA1 transfection, whereas in case of siRNA2 transfection the cell viability was 74.8 and 83.3 % after 24 and 48 h, respectively (Fig. 5). The effect was minimal with both siRNAs 72 h after transfection indicating that the optimal effect was at 24 h. Trypan blue assay was performed to observe the proliferation potential of LN18 cells after siRNA transfection. There was a significant decrease ($p < 0.05$) in the proliferation response in cells treated with siRNA1 compared to siRNA2, scrambled siRNA and control groups (Fig. 6).

Effect of siRNA on apoptosis in LN18 cells

Analysis of hTERT knockdown induced apoptosis was carried out by Annexin-V/PI double staining method in LN18 cells after transfection (Fig. 7). 48 h after transfection 17.5 % (4.5 % early and 13 % late apoptotic cells), 10.8 % (3.6 % early and 7.2 % late apoptotic cells) and 6.65 % of total apoptotic cells were observed in siRNA1, siRNA2 and scramble siRNA treated cells, respectively, (Annexin-V-FITC and/or PI positive, Fig. 8). The FACS data showed a significant increase in LN18 cells entering early ($p < 0.05$) and late ($p < 0.05$) apoptosis following transfection with siRNA1 and siRNA2 compared with scramble siRNA.

Fig. 4 a Western blot image of hTERT protein levels in different groups. β -tubulin was used as an internal loading control. The band intensity was calculated by Image-J software. **b** The ratio of hTERT versus β -tubulin expression was used as a

measurement of the hTERT levels. The data presented are mean $\pm$ SD of three independent experiments. *** $p < 0.001$ versus control group and ### $p < 0.001$ versus scr siRNA treated group

Fig. 5 Cell viability was measured by MTT assay at different time points in LN18 cells after transfection with hTERT specific siRNAs. Cell viability was decreased in the transfected groups. There was no significant difference in the control and scrambled siRNA group. The data represent mean $\pm$ SD from three independent experiments. ** $p < 0.001$ and * $p < 0.05$ versus control group

Fig. 6 A total of 1×10^5 cells were seeded in each well of a 6-well plate on Day 0. Day t0 represents the treatment day. Replicative potential of LN18 cells was decreased after treatment with siRNA1 and siRNA2. The data are presented as mean $\pm$ SD of three independent experiments. There was a significant difference in the proliferation response in siRNA1 and siRNA2 treated groups at day 4 and 5. * $p < 0.05$ with reference to the respective control group

Effect of siRNA on telomerase activity

Telomerase activity was detected by Telo TAGGG telomerase PCR ELISA kit. The level of telomerase activity in LN18 cells was correlated with the hTERT mRNA expression. The siRNA1 group showed

stronger inhibition of telomerase activity than siRNA2. A 61.6 % reduction in telomerase activity in siRNA1 and a 32.7 % reduction in siRNA2 was observed. There were significant differences in the telomerase activity among the siRNA1 and siRNA2, and scrambled siRNA groups ($p < 0.05$) (Fig. 9).

Discussion

Telomerase plays a key role in cellular immortality and tumorigenesis. Reactivation of telomerase is an essential step during malignant tumor progression (Counter et al. 1994). In the majority of human cancers telomerase activity is regulated by its catalytic subunit hTERT, which is highly expressed in tumor cells. This makes *hTERT* as an excellent candidate gene to target in anticancer therapy (Shay et al. 2001; Cong et al. 2002). It has been reported that, more than 50 % of gliomas have increased telomerase activity (Langford et al. 1995; Nakatani et al. 1997; Le et al. 1998; Sugita et al. 2000; Tchirkov et al. 2003). Several studies showed that, silencing of hTERT resulted in progressive shortening of telomeres and thereby inhibiting cellular proliferation and induce apoptosis in various cancer cells (Natarajan et al. 2004; Zhao et al. 2005; Wang et al. 2007; Shi et al. 2014). Progression of cancer largely depends on the equilibrium between pro and anti-apoptotic factors such as Bax and Bcl-2. Imbalance between the expression of Bax and Bcl-2 increases P53 which induces cell cycle arrest, DNA repair mechanisms and apoptosis. Apoptosis is also mediated through non-canonical activity of hTERT. Therefore targeting hTERT may directly or indirectly modulate the expression of genes involved in apoptosis.

Currently there are several methods to inhibit telomerase activity in human cells either by using drugs, antisense molecules, inhibitors, ribozymes and mutant expression (Saretzki 2003; Guittat et al. 2004; de Souza Nascimento et al. 2006; Cunningham et al. 2006). hTERT expression can also be down-regulated by epigenetic silencing of its gene target (Shervington and Patel 2008). RNA interference mediated gene suppression has become one of the potential techniques in the treatment of a number of diseases including cancer (Cunningham et al. 2006; Andrews and Tollefsbol 2007). siRNA silencing hTERT has been performed in various cancer cell lines including

Fig. 7 48 h after transfection hTERT specific siRNAs induced apoptosis in LN18 cells was detected using Annexin-V/PI double staining by Flowcytometry (*X axis*, Annexin V and *Y axis* PI). The Annexin V+/PI− cells represent apoptotic cells (Q4), and the Annexin V−/PI+ represent necrotic cells (Q1). Q3 represents viable cells and Q2 represents late apoptotic cells (Annexin V±/PI± cells). **a, b**) In the control and scrambled siRNA, the cells were not stained for Annexin V indicating no early apoptosis. **c, d**) siRNA1 and siRNA2 treated groups cells have stained for Annexin V showing increased early apoptosis

Fig. 8 siRNA targeted hTERT induced apoptosis. 48 h post-transfection the percentage of early apoptosis in siRNA1 and siRNA2 treated groups were 4.55 ± 0.78 and 3.65 ± 0.354 . The mean $\pm$ SD are taken from three independent experiments

glioblastoma cell lines. There are a few studies on glioblastoma cell lines using combination of siRNA with drugs and/or radiation. George et al. (2009) showed that, in glioblastoma cells treated with hTERT siRNA and IFN gamma effectively inhibits

Fig. 9 Telomerase activity levels were measured by TRAP assay in LN18 cells after 48 h post transfection with siRNA1, siRNA2 and scrambled siRNA. The data presented are mean $\pm$ SD and are representative of three independent experiments. ** $p < 0.005$ and * $p < 0.05$ versus control group. No significant difference was observed between control and scrambled siRNA group

angiogenesis and tumor progression through down-regulation of molecules involved in apoptosis (George et al. 2009). Down-regulation of hTERT significantly reduces telomerase activity and cell proliferation by decreasing the number of cells in S phase and induces apoptosis through Bax/Bcl-2 in T98G glioma cells (Wang et al. 2012). Our study differs from the above studies as it aims to check the difference in efficiency of two siRNAs across two target regions.

siRNA approach for gene silencing is widely seen as a promising method in cancer studies. “However, several challenges have been encountered in the delivery of siRNA in solid tumors. A great variety of delivery systems have been studied for safe and effective delivery of siRNAs. Currently several methods of delivery systems are in clinical use. Among them the most commonly used delivery systems are cationic peptides, recombinant adenoviral vectors and lipid based nanoparticles. Lipid based nanovectors are excellent for delivering siRNA to solid tumors. siRNA delivered through lipid based nanoparticles are able to cross the BBB completely” (Burnett et al. 2011; Shen et al. 2012).

In the present study we have clearly shown the increased mRNA expression of hTERT (72 %) in high grade glioma patients. Patients with high levels of hTERT mRNA have poor survival status whereas patients with low levels of hTERT mRNA have increased survival rate; hence, prognosis of OS and PFS is correlated with the levels of hTERT mRNA. In addition to this we also found that hTERT expression levels were higher in male patients. These results are in support with the earlier studies where the investigators have demonstrated that siRNA-hTERT complex was effective in inhibition of telomerase activity in cervical cancer (Natarajan et al. 2004; Zhao et al. 2005). Knockdown of hTERT with hTERT specific siRNA plasmid–CPP complex also showed similar effect in hepatoma cells (Li et al. 2015). In addition to this siRNA targeting hTERT effectively regulate the telomerase activity and reduced the telomere length in Barrett’s adenocarcinoma cells, SGC-7901 human gastric cancer cells, lung cancer cells (Shammas et al. 2005; Qian et al. 2008; Ge et al. 2011). siRNA transferred by lentiviral delivery also significantly decreased telomerase activity. They also observed that the anti-hTERT siRNA also decreased the expression of specific genes such as integrin α V and Met oncogene protein that are involved in tumor growth,

angiogenesis, metastasis (Li et al. 2004, 2005). Knock-down of hTERT by siRNA significantly decreases hTERT expression and induces apoptosis through activation of multiple pathways viz., mitochondrial signal transduction pathway, PI3 K/AKT pathway, Bax/Bcl-2 (Wang et al. 2007, 2012; Shi et al. 2014). Both transient and stable transfection of hTERT siRNA in SW480 colon cancer cells inhibits hTERT expression, down regulates telomerase activity and suppresses cell proliferation (Liu et al. 2014).

In the current study we used chemically synthesized siRNAs having shorter half-life period inside the cells as they are susceptible to RNase degradation (Ge et al. 2011). We used two siRNAs, siRNA1 and siRNA2 targeting hTERT at exon 4 and exon 3, respectively. There was a significant reduction in the hTERT mRNA levels at 48 h post-transfection. The level of hTERT mRNA expression with siRNA1 and siRNA2 treated groups was decreased ~45 % and ~39 %, respectively. At 48 h post-transfection, the hTERT protein was decreased by ~49 % in siRNA1 and by ~38 % in siRNA 2 treated groups. The difference in the percentage of down-regulation of mRNA and protein levels with two different siRNAs explains that, the effect of RNA interference varied with the different regions in the hTERT mRNA. Furthermore, we also observed that siRNA directed to hTERT significantly decreased telomerase activity in LN18 cells. The telomerase activity with siRNA 1 and siRNA 2 was decreased by 61.6 and 32.7 %, respectively.

Our data also demonstrated that, siRNA mediated hTERT down-regulation significantly reduced cell proliferation and increased apoptosis in human glioblastoma LN18 cells. The results from MTT assay suggest rapid decrease in the growth rate at 24 h post-transfection with both siRNA1 and siRNA2. But, the inhibitory effect was only temporary due to transient transfection, and the cells started proliferating after 48 h. The cell viability was gradually increased at the 72 h time point indicating that the transiently transfected siRNAs were only expressed for a limited period of time and usually lasted for about 2–4 days (Kim and Eberwine 2010). Flow cytometry studies showed that, at 48 h post-transfection the number of total apoptotic cells was significantly increased. There are several pathways involved in hTERT mediated apoptosis; this can be telomerase dependent pathways such as Bax/Bcl-2 pathway, PI3 k/Akt pathway (Wang et al. 2012; Shi et al. 2014) or telomerase

independent pathways (non-canonical) like DNA-damage repair, WNT/ β -catenin signaling, NF- κ B signaling and regulation of mitochondrial activity (Chiodi and Mondello 2011; Maida and Masutomi 2015). However, our results cannot conclude whether the apoptosis is mediated through hTERT dependent or independent pathway.

In conclusion, our study clearly demonstrated that, the down-regulation of hTERT by siRNA significantly reduces hTERT mRNA and protein expression in glioblastoma cell lines. It could effectively decrease cell proliferation and viability mediated through apoptosis. Further studies are required for effective utilization of siRNAs in animal models and humans in treating high grade gliomas.

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Conflict of interest The authors declare that there are no conflicts of interest.

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