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Simultaneous siRNA Mediated Downregulation of hTERT and Treatment with Interferon-γ in Human Glioblastoma SNB-19 and LN-18 Cells Causes Cell Cycle Arrest and Inhibits Invasion, Angiogenesis, and Tumor Growth in Nude Mice

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ABSTRACT

Telomerase is highly upregulated in majority of the cancers including glioblastoma to provide immortality. Human telomerase reverse transcriptase (hTERT) is the catalytic component that regulates the telomerase activity. Interferon-â orchestrates several cellular activities including cell cycle and growth through transcriptional regulation. In the present investigation we downregulated hTERT using cognate siRNA in two highly invasive human glioblastoma cell lines SNB-19 and LN-18 and simultaneously treated them with interferon-â for 48 h. Matrigel invasion, spheroid migration, and cell proliferation studies demonstrated inhibition of cell invasion, migration, and proliferation in both glioblastoma cell lines after treatment with hTERT siRNA and interferon-â. In vitro (co-culture with endothelial cells) and in vivo angiogenesis (in immunocompromised mice) assays demonstrated inhibition of capillary-like structure and neovascularization, respectively, after treatment with both agents. Furthermore, the combination treatment showed remarkable reduction in tumor growth in the subcutaneous and intracerebrum of nude mice. Western blot analysis demonstrated significant decreases of PCNA, MMP-9, VEGF, c-Mye, CDK2, CDK4, and cyclin D1 and marked increases of p21Waf1 and p27Kip1 after treatment with both agents. Semiquantitative and real-time reverse transcription-polymerase chain reaction (RT-PCR) studies showed downregulation of PCNA, c-Myc and VEGF and increased expression of p21Waf1 and p27Kip1. Taken together, the results of the present study indicate that the combination treatment of hTERT siRNA and interferon-â in glioblastoma cells effectively prevents cell invasion, angiogenesis, and tumor growth through downregulation of molecules involved in angiogenesis and cell cycle. This unique combination of hTERT siRNA and interferon-â offers a potential therapeutic approach for treatment of glioblastomas. This work was supported by the R01 CA-91460 grant from the NCI.

INTRODUCTION

Glioblastomas are primary brain tumors characterized by their invasive infiltration and destruction of surrounding normal brain tissue, making complete surgical resection of these tumors virtually impossible. The prognosis for patients diagnosed with glioblastoma is very poor, with a mean survival rate of 9-12 months even after surgery, radiation, and chemotherapy. In the United States, there are more than 20,000 new cases of primary malignant brain tumors diagnosed every year that account for 1.4% of all cancers and 2.3% of all cancer deaths. Over the last 30 years, there has been little progress in the treatment of malignant brain tumors. Glioblastomas remain highly refractive to therapy, and current treatments produce no long-term survivors in patients with these tumors. The traditional means of cancer therapy are plagued with numerous side effects and subsequent poor quality of life during the course of treatment. So it is important to develop new effective therapeutic tools incorporating conventional chemotherapy and the emerging gene therapy.

Telomerase is responsible for the addition of telomeres to the 3' end of chromosomes, which is a crucial step in enabling tumor growth. Telomerase is highly upregulated in majority of the cancers including glioblastoma to provide immortality to tumor cells. Human telomerase reverse transcriptase (hTERT) is the catalytic component that regulates the telomerase activity. So knockdown of the expression of hTERT is an effective way to inhibit telomerase activity and thus prevent cell cycle and tumor growth.

Interferon- (IFN-â) orchestrates several cellular activities including cell cycle and growth through transcriptional regulation. IFN-â has antiviral and anti-tumour properties. It alters transcription of up to 30 genes producing a variety of physiological and cellular responses. The aim of our present study was to inhibit telomerase activity through knockdown of hTERT using a mammalian expression vector carrying hTERT siRNA cDNA in combination with IFN-â to inhibit glioblastoma cell invasion, angiogenesis, and tumor growth in nude mice. We studied two highly invasive glioblastoma SNB-19 and LN-18 cell lines.

Figure 1

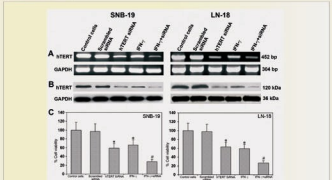

Figure 1. Expression of hTERT mRNA and protein levels in SNB-19 and LN-18 cells after transfection with a mammalian expression vector carrying hTERT siRNA cDNA (pRNAT-CMV3.2/Neo, GenScript, Piscataway, NJ) or treatment with 10 ng/ml interferon-γ (IFN-γ) or both together for 48 h. **(A) Semiquantitative RT-PCR.** Total RNA was isolated using BioRad Aurum kit. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was used as an internal control. **(B) Western blotting for hTERT.** The blots were reprobed for GAPDH content to demonstrate equal loading of protein in all lanes. **(C) MTT assay for cell viability and proliferation.** The MTT-(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was performed in SNB-19 and LN-18 cells after transfection with hTERT siRNA or treatment with 10 ng/ml IFN-γ or both agents together. Data are representative of 6 independent experiments in duplicate (*p<0.001 when compared to the control mean values and †p<0.001 when compared to hTERT siRNA or IFN-γ mean values).

Figure 1 demonstrates that transfection with hTERT siRNA resulted in 60% down regulation of hTERT mRNA as well as protein levels. The treatment with both agents together resulted in 85% knockdown of hTERT mRNA and protein levels in both cell lines.

Figure 2

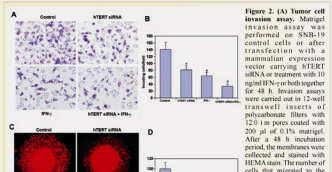

Figure 2. (A) Tumor cell invasion assay. Matrigel invasion assay was performed on SNB-19 control cells or after transfection with a mammalian expression vector carrying hTERT siRNA or treatment with 10 ng/ml IFN-γ or both together for 48 h. Invasion assays were carried out in 12-well transwell inserts of polycarbonate filters with 12.0 ì m pores coated with 200 µl of 0.1% matrigel. After a 48 h incubation period, the membranes were collected and stained with HEMA stain. The number of cells that migrated to the undersurface of the membrane were examined under a microscope, counted and photographed. A significant reduction in the number of invaded cells indicates the decreased invasive potency of SNB-19 cells after treatment with hTERT siRNA or IFN-γ or combination of both.

(B) Quantitative evaluation of matrigel invasion assay. The data represented are mean ± S.D. from 10 randomly selected microscopic fields from three independent wells (*p<0.001 when compared to the control mean values and #p<0.001 when compared to mean values). **(C) Inhibition of cell migration from spheroids.** Migration of SNB-19 cells from spheroids after transfection with a mammalian expression vector carrying hTERT siRNA or 10 ng/ml IFN-γ or both agents together. The spheroids were prepared from SNB-19 cells stably transfected with red fluorescent protein, transferred to a 24-well plate and allowed to migrate for 24 h. The cell migration from the spheroids were observed under a fluorescent microscope and photographed. **(D)** The percentage migration of cells from the center of the spheroids to the monolayers was measured using a microscope calibrated with a stage and ocular micrometer. The data are represented as mean value ± SD of the results of 4 independent experiments from each group (*p<0.001 when compared to the control mean values and #p<0.001 when compared to hTERT siRNA or IFN-γ mean values).

Figure 3

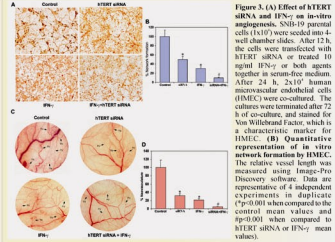

Figure 3. (A) Effect of hTERT siRNA and IFN-γ on in-vitro angiogenesis. SNB-19 parental cells (1x10⁵) were seeded into 4-well chamber slides. After 12 h, the cells were transfected with hTERT siRNA or treated 10 ng/ml IFN-γ or both agents together in serum-free medium. After 24 h, 2x10⁵ human microvascular endothelial cells (HMEC) were co-cultured. The cultures were terminated after 72 h of co-culture, and stained for Von Willebrand Factor, which is a characteristic marker for HMEC. **(B) Quantitative representation of in vitro network formation by HMEC.** The relative vessel length was measured using Image-Pro Discovery software. Data are representative of 4 independent experiments in duplicate (*p<0.001 when compared to the control mean values and #p<0.001 when compared to hTERT siRNA or IFN-γ mean values).

(C) In vivo angiogenesis assay. SNB-19 parental cells (2x10⁷) or cells after transfection with a mammalian expression vector carrying hTERT siRNA cDNA or treatment with 10 ng/ml IFN-γ or both agents together were suspended in 200 µL of serum-free medium, injected into a diffusion chamber and the opening was subsequently sealed with sterile bone wax. The diffusion chambers loaded with cells were surgically implanted under the dorsal skin of nude mice and left for 10 days. Strong micro-vessel development (as indicated by arrows, TN) with curved thin structures arising from pre-existing vessels was observed in SNB-19 parental cells. The formation of such microvasculature was considerably reduced and attenuated in both hTERT siRNA and IFN-γ treated cells and inhibited after treatment with both agents together. **(B) Quantitative representation of *in vivo* angiogenesis.** Tumor-induced neovasculature was measured in control, hTERT siRNA and IFN-γ treated cells (*p<0.001 when compared to the control mean values and #p<0.001 when compared to hTERT siRNA or IFN-γ mean values). *TN: tumor-induced neovasculature, PV: pre-existing vasculature.*

Figure 4

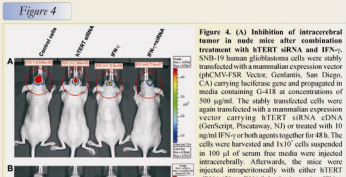

Figure 4. (A) Inhibition of intracerebral tumor in nude mice after combination treatment with hTERT siRNA and IFN-γ. SNB-19 human glioblastoma cells were stably transfected with a mammalian expression vector carrying hTERT siRNA cDNA (pRNAT-CMV3.2/Neo, GenScript, Piscataway, NJ) or treated with 10 ng/ml IFN-γ or both together in serum-free medium and propagated in media containing G-418 µt concentrations of 500 µg/ml. The stably transfected cells were again transfected with a mammalian expression vector carrying hTERT siRNA cDNA (GenScript, Piscataway, NJ) or treated with 10 ng/ml IFN-γ or both agents together for 48 h. The cells were harvested and 1x10⁷ cells suspended in 100 µl of serum free media were injected intracerebrally. Afterwards, the mice were injected intraperitoneally with either hTERT siRNA (50 µg DNA/injection/mouse) or IFN-γ (1 µg/injection/mouse) or both together for 20 days on alternate days. On day 21, the mice were injected with 100 µl of luciferin (Gentlantis, San Diego, CA) at a concentration of 50 mg/mL. After 10 min, the mice were visualized for luciferase activity using Xenogen IVIS-200m (Xenogen, Hopkinton, MA) imaging system. The combination treatment with hTERT siRNA and IFN-γ resulted in complete inhibition of intracerebral tumor formation in nude mice. The data are representative of 6 sets of animals in each group.

(B). Inhibition of subcutaneous solid tumor in nude mice after combination treatment with hTERT siRNA and IFN-γ. The cells were cultured and treated as mentioned above. About 1x10⁷ cells suspended in 100 µl of serum free media were injected subcutaneously. Afterwards, the mice were injected intraperitoneally with either hTERT siRNA (50 µg DNA/injection/mouse) or IFN-γ (1 µg/injection/mouse) or both agents together for 20 days on alternate days. On day 21, the mice were injected with luciferin and visualized for tumor inhibition using Xenogen IVIS-200 imaging system. The individual treatments with both hTERT siRNA and IFN-γ resulted in a marked decrease of subcutaneous tumor growth, and the combination treatment with both agents resulted in complete inhibition of tumor formation in nude mice. The data are representative of 6 sets of experiments in each group.

Figure 5

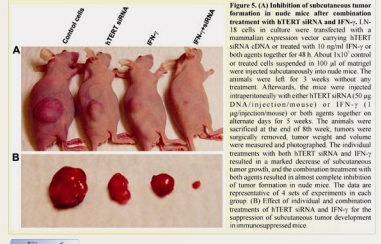

Figure 5. (A) Inhibition of subcutaneous tumor formation in nude mice after combination treatment with hTERT siRNA and IFN-γ. LN-18 cells in culture were transfected with a mammalian expression vector carrying hTERT siRNA cDNA or treated with 10 ng/ml IFN-γ or both agents together for 48 h. About 1x10⁷ control or treated cells suspended in 100 µl of matrigel were injected subcutaneously into nude mice. The animals were left for 3 weeks without any treatment. Afterwards, the mice were injected intraperitoneally with either hTERT siRNA (50 µg DNA/injection/mouse) or IFN-γ (1 µg/injection/mouse) or both agents together on alternate days for 5 weeks. The animals were sacrificed at the end of 8th week, tumors were surgically removed, tumor weight and volume were measured and photographed. The individual treatments with both hTERT siRNA and IFN-γ resulted in a marked decrease of subcutaneous tumor growth, and the combination treatment with both agents resulted in almost complete inhibition of tumor formation in nude mice. The data are representative of 4 sets of experiments in each group (B) Effect of individual and combination treatments of hTERT siRNA and IFN-γ for the suppression of subcutaneous tumor development in immunosuppressed mice.

Figure 6

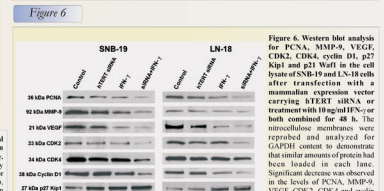

Figure 6. Western blot analysis for PCNA, MMP-9, VEGF, CDK2, CDK4, cyclin D1, p27 Kip1 and p21 Waf1 in the cell lysate of SNB-19 and LN-18 cells after transfection with a mammalian expression vector carrying hTERT siRNA or treatment with 10 ng/ml IFN-γ or both combined for 48 h. The nitrocellulose membranes were reprobed and analyzed for GAPDH content to demonstrate that similar amounts of protein had been loaded in each lane. Significant decrease was observed in the levels of PCNA, MMP-9, VEGF, CDK2, CDK4 and cyclin D1 indicating marked decrease in cell proliferation and cell cycle arrest. The levels of both p27 Kip1 and p21 Waf1 were notably increased. Data are representative of3 independent experiments.

CONCLUSIONS

- *Combination treatment with hTERT siRNA and IFN-γ resulted in about 85% downregulation of hTERT mRNA and protein levels in both SNB-19 and LN-18 cells.*
- *Combination treatment with hTERT siRNA and IFN-γ resulted in marked reduction of tumor cell invasion and complete inhibition of cell migration from spheroids.*
- *Combination treatment with hTERT siRNA and IFN-γ resulted in almost complete inhibition of both in vitro and in vivo angiogenesis.*
- *Simultaneous administration of hTERT siRNA and IFN-γ prevented both intracerebral and subcutaneous tumor growth in nude mice.*
- *Combination of hTERT siRNA and IFN-γ offers a novel therapeutic tool for controlling growth of glioblastomas.*

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