

## RESEARCH ARTICLE

# siRNA-mediated Inhibition of hTERT Enhances Radiosensitivity of Cervical Cancer

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### Abstract

**Background:** To investigate the influence of telomerase activity, apoptosis, radiosensitivity of cervical cancer after siRNA-mediated knockdown of telomerase RNA and evaluate *in vivo* growth with gene interference. **Methods:** We studied siRNA-targeting-telomerase RNA transfection into the HeLa cell line. Expression of hTERT mRNA was detected by RT-PCR and telomerase activity was measured by the TRAP assay. Growth inhibition was determined by MTT assay and radiosensitivity of the cervical cancer cells was examined by colony formation assay. In addition, effects of hTERT inhibition *in vivo* were studied by injection of siRNA-transfected HeLa cells into nude mice. **Results:** The hTERT siRNA effectively downregulated the expression of hTERT mRNA and also reduced the telomerase activity to 30% of the untreated control value. The viability of hTERT siRNA transfected HeLa cells was reduced by 44.7% after transfection. After radiation treatment, the radiosensitivity of HeLa cells with hTERT knockdown was increased. *In vivo*, the tumors developing from the hTERT siRNA-transfected cells were of reduced size, indicating that the hTERT siRNA also depressed the tumorigenic potential of the HeLa cells. **Conclusions:** Our results supported the concept of siRNA-mediated inhibition of telomerase mRNA which could inhibit the expression of hTERT and telomerase activity. Furthermore, radiosensitivity was upregulated after knockdown the hTERT *in vivo* and *in vitro*.

**Keywords:** siRNA-mediated inhibition - hTERT - cervical cancer

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### Introduction

Cervical cancer is one of the most common malignancies among females. Approximately, 250,000 women die each year from cervical cancer and 80% are from developing countries (Parkin et al., 2005; 2006). Ionizing radiation plays a key role in the treatment of cervical cancer but the frequent occurrence of radiation-resistant tumors is a common clinical problem.

Telomerases are nucleoprotein structures at the end of eukaryotic chromosomes that function to maintain genomic stability. In cells which are lack telomerase or with inadequate levels of telomerase activity, telomeres shorten with each cell division. This progressive eventually leads to a situation where cells stop dividing and enter into replicative senescence (Blackburn et al., 2005). As we know, telomerases is detected in approximately 90% of all malignant tumors (Heselmeyer et al., 2003), but not in normal cells. The special characteristic of telomerase has made it not only was used as a predictive parameter of radiation but also the target of gene therapy which is widely researched. Telomerase is comprised by telomerase RNA component, which is the template of telomere, the catalytic subunit hTERT, and telomerase-associated protein (TAP). In the past researches the human telomerase reverse transcriptase as the catalytic component of the telomerase

activity regulation was highlight. But more and more recent studies showed that both components are required for telomerase activity and telomere length *in vitro* (Feng et al., 1995; Cristofari et al., 2006). The telomerase RNA is also important for telomere maintenance.

Radiation therapy is an important approach in cervical cancer therapy. But the clinical utility of radiotherapy are limited by characteristic radiation resistant. Increasing the sensitivity of tumor cells to radiotherapy would improve the outcome in patients with cervical cancer. Several studies have shown that there is a close relationship between the telomerase activity and the cellular response to irradiation. Ionizing radiation induces a variety of base alterations and DNA strand breaks, of which the unrepaired double-strand breaks (DSB) are the most lethal lesions leading to cell death. Slijepcevic study highlights the fact that the DNA DSB repair-associated proteins can maintenance of chromosomal integrity by balancing repair activities and telomere maintenance, in which telomerase is also involved (Slijepcevic et al., 2005).

There are several approaches to be used for blocking the telomerase activity such as pharmacological drugs chemical agents, ribozymes and antisense molecules. And RNA interference is an important method of inhibiting the gene expression. RNAi is induced by double-stranded, 21- to 23-nucleotide RNAs that induced the sequence-

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specific degradation of homologous RNAs. The strong and specific suppression of gene expression by RNAi is currently being evaluated as a potentially useful method for developing gene-silencing therapies for cancer (Tong et al., 2005; Zhang et al., 2006).

In the present study, to clarify more closely possible connections between telomerase and relative intrinsic radioresistance, we designed siRNA targeting telomerase RNA and delivered plasmids siRNA into the human HeLa cell line. Those stably transfected HeLa cell showed a significant decreased of expression of the telomerase mRNA and enhancement in radiosensitivity.

## Materials and Methods

### Cell cultures

The HeLa cell line was kind gift from the Experiment Central of Second Affiliated Hospital of Harbin Medical University. The HeLa cell line was grown in DMEM-HIGH Glu medium (Gibco, Paisley, Scotland, UK) supplemented with 10% calf serum and at 37 °C with 5% in a humidified incubator.

### siRNA preparation

The siRNAs sequences for hTERT were designed by a commercial software based on the principle for siRNA. The siRNA-hTERT-I sense sequence is 5'-CGAAGGTGCCTTGGAAATAT-3'. The siRNA-hTERT-II sense sequence is 5'-TGTCCTCCATGGTCATAG A-3'. The siRNA-hTERT-III sense sequence is 5'-GGAGCATGTTGAGGTCTAT-3'. And a negative control siRNA sequence is 5'-TTCTCCGAACGTGTCACGT-3'. The three sequences for hTERT were submitted to BLAST to ensure that the sequences were targeted specifically and the negative control siRNA had no homology with any human gene in the gene library. All sequences include the negative control siRNA were confirmed by Shanghai Shengong Company, China.

### Cell Transfection

The HeLa cell in logarithmic growth phase were chosen and then were plated at  $2 \times 10^5$  in each well of six-well plates. Each well contained DMEM without antibiotics or FBS. After incubation for 24 h, the transfections were performed using Lipofectamine2000 transfection reagent (Invitrogen, USA) according to the manufacture's guidebook. Transfection efficiency was valued by a fluorescence microscope.

### Reverse Transcription and semiquantitative

Approximately  $1 \times 10^6$  different kinds of cells including untransfected cells, the cells transfected with parental vector and the cells transfected with siRNA vector were harvested, respectively. TRIzol agent was used for total RNA extraction and then synthesized cDNA by an RT-PCR kit. The cDNA was added to a final volume of 20  $\mu$ L PCR reaction. The cycling program was performed as follow: 94 °C for three minutes; 94 °C for 40 s, 60 °C for 40 s 72 °C for 90 s (35 cycles); and 72 °C for ten minutes. The primers sequence for hTERT are shown as follows:

forward: 5'-CTGGGAGGGGTGGTGGCCATTT-3', reverse: 5'-CGAACGGGCCAGCAGCTGACAT-3'. The  $\beta$ -actin was used as internal reference. The primer sequence were as follow: forward: 5'-CGGGAAACTGTGGCGTGAT-3', reverse: 5'-CAAAGGTGGAGGAGTGGGT-3'. The PCR products were visualized on agarose gel.

### MTT assay

Five groups were set up. Cells in each groups plated at 103 in 6 wells of 96-well seeded and then incubated overnight. Each well was added to 20  $\mu$ L of 5 mg/mL MTT and the absorption at 492 nm was detected on spectrometre. The figures were recorded every 24 h for 5 days.

### Telomerase activity assay

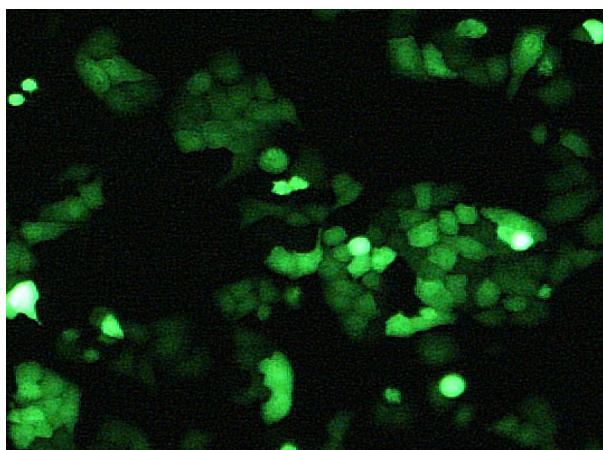
Telomerase activity was examined by telomeric repeat amplification protocol (TRAP). In brief, the transfected cells were harvested and adjusted to a density of  $2 \times 10^5$  cells and then were added to 200  $\mu$ L lysate. After incubated on ice for 30 minutes, the mixture was centrifuged at 12000 r/min and 4 °C for 25 minutes. The supernatant was transferred to a 1.5 mL DEPC-treated tube. For the TRAP reaction 2  $\mu$ L of cell extraction was added to 25  $\mu$ L of reaction mixture and DEPC-treated water was added to a final volume of 50  $\mu$ L. The extension reaction was performed at 30 °C for 30min and then denatured at 90 °C for 3 min. CX Primer was then added. The mixture was denatured at 94 °C for 5min, and underwent 30 Cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 90 s with a final extension reaction at 72 °C for 5 min. The A value of the product was measured after this reaction.

### Clonogenic survival assay.

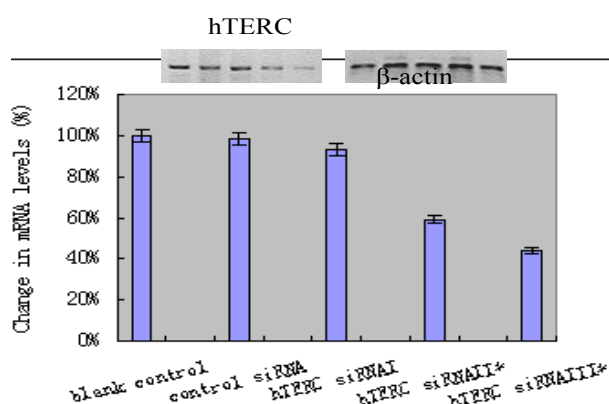
The cells were seeded in 60mm dishes for about 10 hours, then cells were treated with different dose (0Gy, 2Gy, 4Gy, 6Gy, 8Gy) of 6MV X-ray radiation by a 23EX accelerator. After cultured for 14 days, the clones were fixed and stained. The clones that more than 50 cells were counted. The plating efficiency and survival fraction were calculated. Sensitizer enhancement ratio was defined as the radiation does required to obtain a specific surviving fraction(SF).

### Xenograft mice study

BALB/c nude mice were maintained in pathogen-conditions within the Laboratory Animal Resource Center at the Harbin Medical University.  $5 \times 10^6$  HeLa cells were injected into the left flanks, respectively, of 18 mice of 8-10 weeks. After the tumors reached a mean volume of 50 mm<sup>3</sup>, the treatment was given. The 30 mice were divided into 6 groups, untreated, IR-alone, hTERT siRNAIII-alone, control siRNA-alone, IR+hTERT siRNAIII, IR+control siRNA. The mice in the IR-alone, IR+hTERT siRNAIII, IR+control siRNA groups were subjected to 6Gy irradiation. After received irradiation, the hTERT siRNAIII and control hTERT were injected in situ. The injection was repeated twice. The volume of the tumor sizes were subsequently measured every three days, the results represented the mean value of three independent



**Figure 1. Representative Photograph Showing the Uptake and Cellular Distribution Pattern of GFP-Conjugated siRNAs in HeLa Cells, as Detected by Fluorescence Microscopy (×400)**



**Figure 2. Levels of Expression of hTERT mRNA in HeLa Cells after Transfection with a Plasmid Encoding hTERT siRNA for 48 h.** (A) Semiquantitative RT-PCR for hTERT. Expression of  $\beta$ -actin was used as an internal control. The data are representative of 3 independent experiments. (B) Determination of hTERT mRNA levels after the treatments with hTERT siRNA. Data are shown as mean  $\pm$  SD from three independent experiments (\* $p$ <0.05 compared with the control mean values)

experiments.

#### Statistics analysis

With SPSS software, the results were presented as means $\pm$ SD. Statistical analysis was done using one way analysis of variance (ANOVA) for multiple samples and Student's t-test for comparing paired sample sets. Statistical significance was considered at P value less than 0.05.

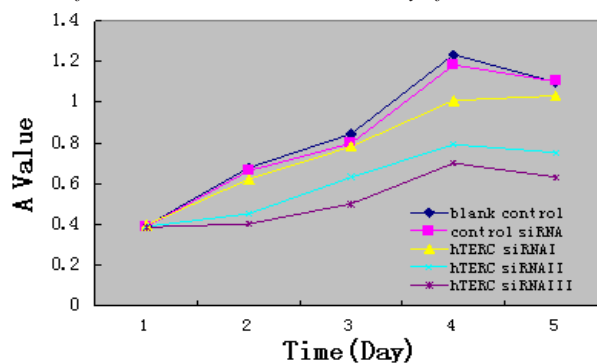
## Results

#### *siRNA are efficiently internalized.*

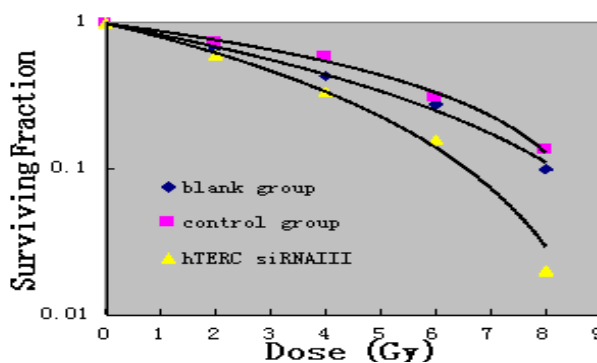
After transfection for 24 hours, the transfected HeLa cells were observed by fluorescence microscopy (Figure 1). The transfection efficiency was more than 65%.

#### *The expression of hTERT mRNA were downregulation in HeLa cells*

We examined the changes in expression of hTERT after the treatments (Figure 2). Transfection with a plasmid



**Figure 3. After Transfected with a Plasmid Encoding hTERT siRNA Cell Viability was Measured by the MTT Assay Every Day for 5 Days.** The cell proliferation of hTERT siRNAII and siRNAIII was inhibited notably. Data are shown as mean  $\pm$  SD from three independent experiments



**Figure 4. Radio-sensitivity of HeLa Cells with or Without Treatment.** After incubation, cells were treated by radiation with various doses (0, 2, 4, 6, 8, Gy). Data are shown as mean  $\pm$  SD from three independent experiments

encoding hTERT siRNAII and hTERT siRNAIII resulted in marked downregulation of cognate mRNA in HeLa cell. Compared with blank group, the expression of HeLa cell transfected with siRNAII and siRNAIII was inhibited which was 59.1% and 44.6% respectively ( $P$ <0.05). But hTERT mRNA levels were not altered after transfection with hTERT scrambled siRNA and hTERT siRNAI. The expression of  $\beta$ -actin was used as an internal control for the mRNA analyses. The siRNAT-hTERT-III vector had the strongest suppressive effect on hTERT mRNA expression.

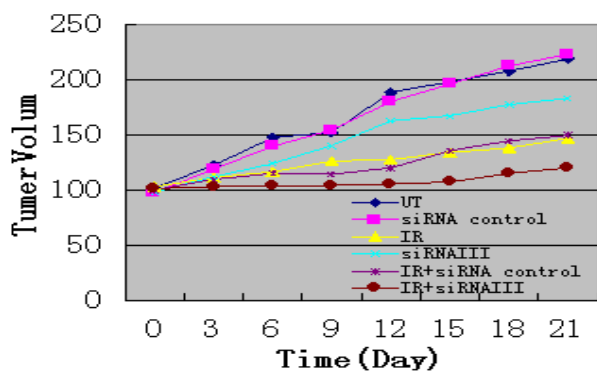
#### *Marked reduction in cell viability*

We observed that the cell viability had different degrees of reduction in the HeLa cell which were treated with the hTERT siRNAII and siRNAIII, while the viability of transfection with hTERT siRNAI had not change compared with the control group. And the hTERT siRNAIII vector had the strongest suppressive.

#### *The telomerase activity of HeLa cells with hTERT siRNA are significant decrease*

Telomerase activity was detected by TRAP-PCR kit and the A value was 1.907 0.065, 1.917 0.032, 0.87 0.074, respectively. The ratio of inhibition of telomerase activity in HeLa cells exposed to the hTERT siRNAIII was 54.4% ( $p$ <0.05). And telomerase activity of HeLa cell with hTERT siRNAIII treatment was marked declined.





**Figure 5.**  $2 \times 10^6$  HeLa Cells that were Untreated or Transfected with Control siRNA or hTERT siRNA were Injected Subcutaneously into Each Flank of Athymic Nude Mice. The tumor dimensions were measured every 3 days. The mean tumor volume (mm<sup>3</sup>) was calculated according to the formula:  $(d^2 \times D)/2$ , where d and D are the shortest and longest diameters of the tumor, respectively. All measurements were performed in a coded, blinded fashion. Data are shown as mean  $\pm$  SD with 5 mice per treatment group

#### *The treatment with hTERT siRNA synergistically enhance the radiation sensitivity*

Compared with untransfected HeLa cells, colony-forming efficiency declined notably in hTERT-siRNAIII at the same dose of radiation ( $P < 0.05$ ) and the radiation enhancement ratios was 2.4 ( $P < 0.05$ ). There were no obvious differences in colony-forming efficiency among blank group and control group ( $P > 0.05$ ), their  $\alpha$  and  $\beta$  were similar, and the radiation enhancement ratios were close to 1.

#### *The hTERT siRNA combined with ionizing radiation could additively inhibit the growth of the xenografts*

To determine whether the hTERT siRNA can sensitize HeLa to irradiation in vitro, we used a xenograft mouse model. We found that the growth of xenograft treated with hTERT siRNA and the ionizing radiation significant decline compared with the IR-alone and hTERT siRNA-alone ( $P < 0.05$ ). And the xenograft treated with hTERT siRNAIII alone also had different size comparing with untreated group and transfection with scrambled siRNA ( $P < 0.05$ ).

## Discussion

Our study demonstrated that the knockdown the hTERT via siRNA effectively inhibited the expression of telomerase mRNA and the HeLa cell proliferation, and the cell cycle arrest. Furthermore the hTERT siRNA also resulted in marked upregulation of radiosensitivity of the cell. In the present study, we observed that in vitro after treatment with the combination of hTERT siRNA and ionizing radiation, the volume of the xenograft was more significant reduced than the treatment with the ionizing radiation only.

Telomerase consist of two important compents, the catalytic subunit hTERT and the RNA subunit hTERC. In the past, the research of telomerase activity regulation was focussed on hTERT and also get many significant outcome. Many researches suggested that

telomerase activity closely correlated with level of hTERT transcription in most malignancy include breast cancer, malignant glioma, meningioma, cervical cancer. There have been reports which demonstrated that RNAi against telomerase reverse transcriptase (hTERT), could successfully inhibit telomerase activity in several cancer cell lines (Mo et al., 2003; Natarajan et al., 2004; Nakamura et al., 2005; Souza et al., 2006; Zhang et al., 2006; Gandellini et al., 2007) and can enhance these radiosensitivity and chemosensitivity (Dong et al., 2009; George et al., 2010; Meng et al., 2012). However, the role of hTERT was not attracted enough attention as other target for telomerase activity regulation. hTERT gene encodes for an RNA unit of telomerase that maintains the length of telomeres through cellular divisions. When this gene is overexpressed, the cells avoid undergoing apoptosis, potentially leading to tumorigenesis. Several studies over years from biochemistry, promoter studies and mouse models has proved that the hTERT has essential contribution in telomere maintain and telomerase activity (Ren et al., 2003; Caimey et al., 2008). It is well known that a high number of chromosomal alterations have been described related to dysplastic lesions of the uterine cervix. Among them, the most frequent are 3q gains, specifically those involving 3q26 chromosome bands, where human telomerase RNA gene (hTERT) is located (Alameda et al., 2009; Zheng et al., 2010; Gao et al., 2011). In the present study, we designed three siRNAs target at hTERT, after transfecting HeLa cells respectively, we detected that the expression of telomerase RNA reduced more or less, and the most powerful siRNA directed against hTERT resulted in more than 70% suppression at the mRNA levels. The decrease in the cell viability and proliferation could be responsible for the downregulation of the hTERT. Furthermore, we noticed that the various siRNAs has different suppression efficiency on mRNA expression. This data show that effectiveness of siRNA inhibition may be highly sequence-dependent.

Cervical cancer is the second most common female cancer worldwide and its main therapeutic method is radiotherapy. Increasing the sensitivity of tumor cells to radiotherapy would improve outcome in patients with cervical cancer. Ionizing radiation can induce many base alteration and the most lethal lesions leading to cell death is the unrepaired double-strand breaks. The effectiveness of ionizing radiation is closely related to the dose dependent capacity of the ionizing radiation to cause DNA damages and the capability of cells to repair the damages. Many recently studies show that the telomerase involved in the progress of the DNA DSB repair. And several studies also supports that the telomerase activity and the telomere status have impacts on the response to ionizing radiation (Aravindan et al., 2011). Current literature indicates that telomerase has a radiation-inducible function lead to resistance of cells to irradiation. Ogawa et al. (1998) reported that the advanced cancers of oral cavity and oropharynx with higher telomerase activity were not sensitive to radiation therapy but the other cancers with lower telomerase activity were sensitive to radiation therapy. Rubio and colleagues (Rubio et al., 2004) reported that high telomerase activity provided

resistance to irradiation in human fibroblasts with short telomeres, and this resistance was dependent on the ability of telomerase to maintain telomere functionality. In our study the HeLa cells transfected by siRNA, the telomerase activity was significantly reduced while the radiosensitivity was upregulation. This is consistent with previous studies. The in vivo tumor growth experiment also demonstrated impaired growth of the tumors formed by the hTERT siRNA transfected HeLa cells.

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