Mechanism of Induction of Apoptosis by siRNA Targeting hTERT in HeLa Cells

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ABSTRACT

Objective: To investigate the molecular mechanism of induction of apoptosis by siRNA targeting human telomerase reverse transcriptase (hTERT) in HeLa Cells. **Methods:** HeLa cells were transfected with siRNAs by liposome method. RT-PCR was used to examine mRNA levels of hTERT in HeLa cells. Microarray assay was adopted to explore the transcriptional profiling of apoptosis associated genes. The protein levels of hTERT, TRAIL, Bcl-2, and cytoplasm Cyt c were detected by Western Blotting. The apoptosis rate was determined by flow cytometry using PI staining. Relative activity of Caspase-3 and Caspase-8 was measured by colorimetric assay. **Results:** The siRNA targeting hTERT suppressed the expression of hTERT gene significantly. Forty-eight hours after transfection, the expression level of TRAIL was increased, the expression level of Bcl-2 was decreased, the releasing of Cyt c was enhanced, the activation of Caspase-3 was increased and the apoptosis rate was increased. **Conclusion:** hTERT-siRNA induces apoptosis of HeLa cells via activating mitochondrial signal transduction pathway.

Key words: hTERT, RNAi, Apoptosis, Mitochondrial pathway

The human telomerase reverse transcriptase (hTERT) is the catalytically active component of the telomerase complex. hTERT catalyzes the telomere elongation by reverse transcription, using RNA component of telomerase as template. Its expression correlates with telomerase activity and is restricted to germ cells, stem cells and to more than 90% of human cancers, whereas most normal human somatic cells have no hTERT expression[1]. Because of their close relations with the development and progression of many malignant tumors, telomerase and hTERT were in the spotlight of research. With research going deeply, some new phenomena and results were revealed and indicated that telomerase has important functions in anti-apoptosis, and inhibiting hTERT could induce tumor apoptosis via independent-telomeres pathway in cells[2,3]. This research was aimed at the exploring of mechanisms of induction of apoptosis by siRNA targeting hTERT in HeLa cells.

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MATERIALS AND METHODS

Materials

HeLa cell strain was purchased from Shanghai Cell Bank, Chinese Academy of Medical Sciences. New-born calf serum was purchased from Gibco. siRNAs, RNAi-Mate and PCR primer were purchased from Shanghai GenePharma. Antibodies against hTERT, bcl-2, TRAIL and Cyt c were purchased from Santa Cruz. AMV-one step RT-PCR amplifying kit was purchased from Shanghai Sangon. IntelliGene Human Cancer CHIP Ver. Four0 was purchased from Takara. Trizol, Cy3-dUTP and Cy5-dUTP were

purchased from Invitrogen Co.. Activity detection kit of Caspase-3 and Caspase-8 was purchased from R&D Co..

Cell Transfection

HeLa cells were cultured and divided into 5 groups: untreated control group(A), blank vector group transfected with blank vector(B), non-specific siRNA group transfected with unrelated siRNA(C), and 2 specific groups transfected with siRNA-hTERT (S_1, S_2). The day before transfection, incubate 2×10^5 cells were inoculated into 6-well plate. On the day of transfection, 5μg siRNA in 400μl serum-free medium was mixed with 15μg RNAi-Mate reagent and incubate at room temperature for 30 min in order to from siRNA/RNAi-Mate complex. siRNA/RNAi-Mate complex was added into culture medium and mix up gently. After incubating cells at 37℃ for 36-48h, other steps were continued for transfection.

RT-PCR

The total cellular RNA of the cells treated by transfection for 36h was extracted using Trizol reagent according to the manufacturer's instructions. Aliquots of total RNA were separated in agarose gel(1%) to detect its integrity. The RT-PCR amplification was performed using the AMV-one step RT-PCR amplifying kit, with β_2-microglobulin as the internal-control. The sense primer used to amplify hTERT was 5'-TCTACCGGAAGAGTGTCTGGAG CAA-3' and the antisense primer was 5'-GCTCCC ACGACGTAGTCCATGTTCA-3'. The sense primer used to amplify β_2-microglobulin was 5'-TTCAGG TTTACTCACGTCATCC-3, and the antisense primer was 5'-CCAAATGCGGCATCTTCAAACCC-3'. RT-PCR conditions were as follows: 50℃ for 30min; 95℃ for 15min; followed by 30 cycles of 94℃ for 60s, 65℃ for 60s, 72℃ for 60s, at last, final elongation at 72℃ for 10min. The RT-PCR products were run on 1.2% agarose gels containing ethidium bromide and visualized under a UV transilluminator.

Chip Hybridization and Analysis

The total RNA was isolated from untreated control group and RNAi group with an improved 1-step method. The fluorescent cDNA probes were prepared through reverse transcription. The RNA samples from untreated control group were labelled with Cy3-dUTP and those from RNAi group with Cy5-dUTP. The two color probes were then mixed, precipitated with ethanol and dissolved in 20μl of hybridization solution. Fluorescent probe mixtures and chips were denatured at 95℃ for 5min, and the denatured probe mixtures were applied onto the pre-hybridized chip under a cover glass. Chips were hybridized at 60℃ for 16h.The hybridized chips were then washed at 60℃ for 10min each in solutions of 2×SSC and 0.2% SDS, 0.1×SSC and 0.2% SDS, and 0.1×SSC, then dried at room temperature. The chip was scanned with a ScanArray 4000 type Scanner. The acquired images were analyzed by ImaGene3.0 software with a digital computer to determine the intensities of fluorescent signals and the Cy5/Cy3 ratio. The differentially expressed genes were defined as follows: (1)The signal values of Cy3 and Cy5 were all more than 200; (2)The signal Cy5/Cy3 ratio was more than 2.0 or less than 0.5.

Western-blot

After transfection for 48h, total proteins from cultured HeLa cells were extracted and the protein concentration determined by the Lowry method. Particularly, the protein extraction for cytochrome c(Cyt c) was only from cytoplasm. Equal amounts of protein were resolved in sample buffer, separated on SDS-PAGE, transferred to a nitro-cellulose membrane and blocked under room temperature over night. The membranes were then incubated with specific antibodies for 2h, TBST to remove the remained antibodies, incubated with the second antibodies labelled with alkaline phosphatise for 2h and coloured with alkaline phosphatase coloration liquid. The results were analyzed by the gel imagination apparatus.

Flow Cytometry Analysis

After transfection for 48h, cells were collected, washed with PBS and resuspended in PBS. Cell apoptosis rate was identified by double supravital staining with FITC-conjugated Annexin V and PI, using the Annexin V-FITC Apoptosis Detection kit according to manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a LAB-FACS flow cytometer using CellQuest software.