Manipulation of Human Telomerase Activity in Cancer and Stem Cells: Application of siRNA-induced Inhibition of Human Telomerase RNA (hTR)

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Background: We have determined the effects of human telomerase RNA inhibiton using siRNA in tumor cells and human embryonic and mesenchymal stem cells.

Methods: We selected the sequences against the predicted loop; these sequneces were comprised of nucleotides from 76 to 94 residues and from 143 to 163 residues as the target sequences, and we cloned these sequences into pU6sh75 and pU6sh143 cells. Three different kinds of cell lines were used: HeLa, SNUhES3, and human mesenchymal stem cells. The degree of inhibition of telomerase activity was assessed by TRAP assay and RT-PCR.

Results: The telomerase activity of the HeLa and SNUhES3 cells were 135.3±14.5 and 109.0±18.2; these cells showed higher activity than human mesenchymal stem cells and Wi38 cells (46.3±5.0 and 26.0±12.0), which were control cells. When each of the types of cells was treated with siRNA-hTR, the transfection efficiency of pU6sh75 for the HeLa, SNUhES3, and human mesenchymal stem cells was 91.0±8.4%, 83.3±16.0% and 81.9±12.3%, respectively. In the case of pU6sh143, its transfection efficiency was similar to pU6sh75; the HeLa, SNUhES3 and human mesenchymal stem cells tranfection efficiency was 90.1±9.0%, 79.9±18.2% and 79.4±15.1%, respectively. After two days of transfection, the level of telomerase activity in the pU6sh75 transfected cells decreased to 64.3±10.1% and 56.0±11.0% in the HeLa and SNUhES3 cells, respectively. When the cells were transfected with pU6sh143, the telomerase activity also decreased in the HeLa and SNUhES3 cells (71.3±9.1% and 61.6±8.3%, respectively). However, the difference of telomerase activity was not significant in the human mesenchymal stem cells: 43.0±7.2% with pU6sh75 and 46.0±9.0% with pU6sh143.

Conclusion: Telomerase RNA inhibiton with siRNA may be a feasible way to inhibit the telomerase activity of human tumor and embryonic stem cells. ***(Korean J Hematol 2006;41:179-185.)***

Key Words: Telomerase, Tumor, Stem cell, siRNA

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INTRODUCTION

Telomerase is a ribonucleoprotein involving the RNA component (hTR) and the telomerase catalytic subunit (hTERT).[1-4] It is known that the activity of telomerase is higher in both tumor and stem cells than other somatic cells. That is why tumor and stem cells can self-renew and proliferate continuously. Among the components of telomerase, the hTR acts as a template for telomere synthesis.[5] This hTR expression and telomerase activation may occur early in the process of human tumor development. Thus, the inhibition of telomerase activity has been expected to play a role in the management of tumor as an anti-tumor mechanism. Increased activity of telomerase is also reported in stem cells, thus high telomerase activity is known to be essential for the preservation of stem cells' characteristics.[6] The basic characteristics of stem cells are self-renewal and differentiation to multi-lineage cells. For the clinical application of stem cells into cell-based therapy or regenerative medicine, the manipulation of this peculiar characteristics of stem cells is required. Because the manipulation of telomerase activity may produce significant changes in biological characteristics of stem cells, several approaches targeting telomerase activity of stem cells have been tried to modulate the proliferation and differentiation of stem cells using various kinds of agents such as inhibitors of retroviral reverse transcriptase, peptide nucleic acid, cisplatin, hammerhead ribozyme, hTR antisense RNA, and hTR gene deletion.[1-4] Recently, it has been demonstrated that synthesized antisense oligonucleotide against open part of hTR (2-5A-anti-hTR) showed cytotoxic effects on tumor cells.[7] This effect of 2-5A-anti-hTR was supposed to be developed by an active induction of caspase-dependent apoptosis, which is independent of telomere length.[8] RNA interference (RNAi) is a sequence-specific posttranscriptional gene silencing (PTGS) process by the siRNA (short interfering RNA) in animals and plants.[2] The dsRNA consisting of a sense and antisense strand of an endogenous mRNA, is rapidly processed by the RNase III type Dicer enzymes and assembled into RNA-induced silencing complex (RISC). The RISC results in the sequence-specific degradation of homologous target sequence. Chemically synthesized siRNA has been routinely used for gene knock-down, but the high cost, temporary and low efficiency gene silencing of synthetic siRNA, hinder the use of this strategy. There have been some studies for the application of RNAi to inhibit telomerase activity in tumor cells.[1,2] However, the application of RNAi to modulate telomerase activity in stem cells has never been reported. In this study, we have tried to inhibit the human telomerase RNA (hTR) using small hairpin siRNAs, and determined the effects of telomerase inhibition on the biology of tumor and stem cells.

MATERIALS AND METHODS

1. Production of telomerase antisense by siRNA

To knock-down a target gene, the target sites in the mRNA should not have mononucleotide repeats of more than 3 bases. Its GC contents should be 30～70%, and it should not be located at exon-intron boundaries or within the first 100 bases of the coding sequence that may have regulatory protein binding sites.[9] To determine an optimal part fulfilling these criteria, the telomerase RNA (hTR) structure was analyzed using synthetic siRNA design softwares of internet sites of invivogen, oligoengine, genescript, wister, ambion. The target part of the telomerase RNA (hTR) structure between residues 143 and 163 of the telomerase template sequence was selected. To maximize homologous binding, we also find the most 'open' part of the RNA molecule, thus we selected the hTR was between residues 94 and 76 of the telomerase template sequence. Because the previous analysis of telomerase RNA (hTR) structure by Kondo *et al* has shown that the most 'open'

Fig. 1. Sequences and predicted secondary structure of hairpin RNAs derived from the U6 promoter-driven expression constructs. Mutant versions of each siRNA with 3 points mutation at the sequences of the target were also shown.

part of the hTR was between residues 94 and 76 of the telomerase template sequence.[10] Therefore, we used the sequence against the predicted loop comprising nucleotides from 76 to 94 residues and from 143 to 163 residues in this study. Each pairs of oligonucleotides were synthesized, heated at 90°C for 10 minutes, and annealed at 55°C for 4 hours in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The resulting fragments included restriction endonuclease sites of *Eco*RI and *Xba*I at the 5' end and 3' end, respectively, and cloned into pU6shX (VCA-shPlasmid small hairpin RNA expression vector, VectorCoreA, Korea) and named as pU6sh75 and pU6sh143 (Fig. 1). The mutant versions with 3 point mutations at the sequences, pU6sh75m and pU6sh143m were constructed the same way as above.

2. Transfection of telomerase antisense

We have used three different kinds of cells in this study: HeLa cell line (a human cervical adenocarcinoma cell line), human mesenchymal stem cells (MSCs) from human bone marrow aspirates, and SNUhES3 cell line (a human embryonic stem cells (ESCs)). A human normal fibroblast cell line, Wi38 cell line was used as normal control. The constructed plasmid vectors were transfected using FuGENE^{TM}6 (Roche, Germany) according to the manufacturer's protocol. Briefly, 10^5 cells seeded in a 35-mm tissue culture dish were exposed to transfection mixture containing 2 μg of siRNA expressing plasmids and 0.5 μg of pSV-β-galactosidase control plasmid vector (Promega, USA) for 5 hr at 37°C. Then, 3mL of growth media was added to the cells, followed by incubation for an additional 16hr.

3. Measurement of transfection efficiency

Transfection efficiency was examined by galactosidase assay, thus, these vectors containing the galactosidase gene were tested after 24 hours of transfection. The percentage of transfected cells was calculated under an optic microscope (100X) and determined by the ratio between the number of galactosidease expressing cells (blue cells), and the number of total cells at the observed field. Five distinct fields were recorded for each sample. All experiments were performed in triplicate.

4. Measurement of telomerase activity

The cells were harvested 48 hr after the transfection to measure telomerase activity. The telomerase activity was examined by a telomere repeat amplification protocol (TRAP) assay with 2,000 cells using a TeloTAGGG Telomerase PCR ELISA Kit in accordance with the manufacturer's instructions (Roche, Germany). After Primers TS (5' AATCCGTCGAGCAGAGTT) and CX (5' CCCTTACCCTTACCCTTACCCTAA) were designed, thirty cycles of PCR were performed at 25°C for 30 min, at 94°C for 5 min, at 94°C for 30s, at 50°C for 30s, at 72°C for 90s, and a final extension at 72°C for 10 min. The PCR products were analyzed and defined positive when $A > 0.2$ on the reading of the microplate reader. Telomerase activity of studied cells was relatively expressed on the basis of human epithelial U293 cell line's telomerase activity as a value of 100.

5. Evaluation

Telomerase activity of each cells were compared between the pretreatment and post treatment of plasmid, respectively. And transfection degree and telomerase suppression effect of pU6sh75 and pU6sh143 were evaluated in each cell lines. All experiments were repeated nine times and the data were presented as mean±SEM. Statistical significance was determined using a one way ANOVA test. Results were considered significant when the *P* value was less than 0.05.

RESULTS

Telomerase activity of each cell line was documented before each cell line were treated with siRNA-hTR as shown in Fig. 2. HeLa and SNUhES3 showed significantly higher telomerase activity than the Wi38, a control cell line. Thus, the telomerase activity of HeLa and SNUhES3 were 135.3±14.5 and 109.0±18.2, respectively. However, human mesenchymal stem cells and Wi38 showed lower telomerase activity: 46.3±5.0 and 26.0±12.0, respectively (Fig. 2). When each cells were treated with siRNA-hTR, the transfection efficiency of pU6sh75 for HeLa, SNUhES3, and human mesenchymal stem cells was 91.0±8.4%, 83.3±16.0% and 81.9±12.3%, respectively. In the case of pU6sh143, its transfection efficiency was similar to pU6sh75; HeLa, SNUhES3, and human mesenchymal stem cells showed 90.1±9.0%, 79.9±18.2% and 79.4±15.1%, respectively. After two days of transfection, the TRAP assays were done with 2,000 cells. Compared to the telomerase activity of the mutant version transfected cells, the level of telomerase activity in the pU6sh75 transfected cells decreased to 64.3±10.1% and 56.0±11.0% in HeLa and SNUhES3. When the cells were transfected with pU6sh143, the telomerase activity also decreased in HeLa and SNUhES3: 71.3±9.1%, 61.6±8.3%, respectively. However, the difference of telomerase activity was not significant in the human mesenchymal stem cells compared to the baseline telomerase activity: 43.0±7.2% with pU6sh75, 46.0±9.0% with pU6sh143 (Fig. 3). There was no significant difference of telomerase inhibition between pU6sh75 and pU6sh143 even though those of pU6sh143 transfected cells seemed to be higher than pU6sh75 transfected cells. To estimate the direct effect of siRNA, the transcriptional activity of target gene, hTR was measured by RT-PCR. In the SNUhES3 transfected with pU6sh75 and

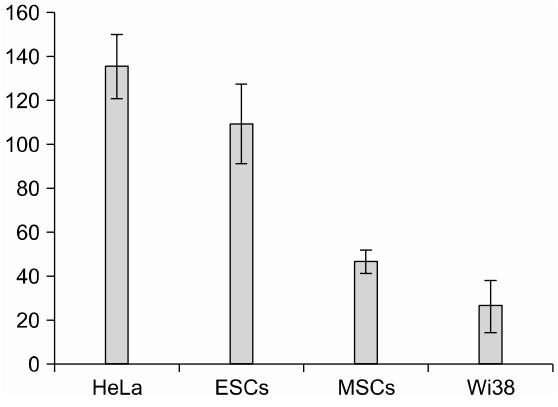

Fig. 2. Telomerase activity in experimental cell lines. The telomerase activity was assayed by a telomere repeat amplification protocol (TRAP) assay using 2,000 cells. The telomerase activity of the U293 cell line was considered as 100, with the telomerase activity of each cell line normalized to this. The means from at least three independent experiments are shown.

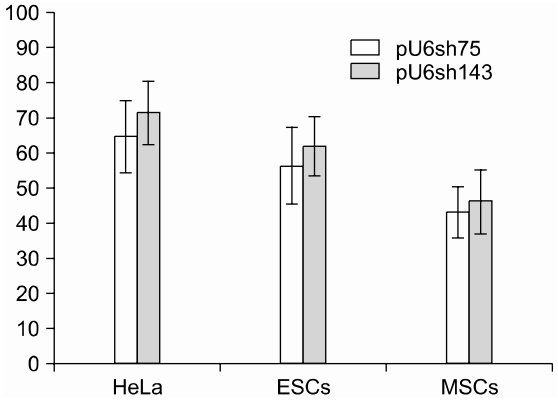

Fig. 3. Inhibition of Telomerase activity by siRNA. The effects of siRNAs are expressed as the percentage of the telomerase activity compared with mutant versions (hTR75 m, hTR143m). The means from at least three independent experiments are shown.

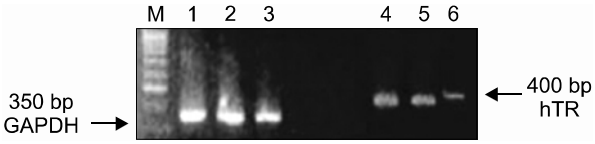

Fig. 4. Inhibition of mRNA expression of hTR. In the siRNA-hTR143 transfected ES, mRNA expression of hTR was decreased compared to control ES and siRNA-hTR143m transfected ES.
M, marker for molecular weight; lane 1 and 4, control ES; lane 2 and 5, siRNA hTR143m transfected ES; lane 3 and 6, siRNA hTR143 transfected ES.

pU6sh143, mRNA expression of hTR was decreased compared to that of untransfected SNUhES3 and SNUhES3 transfected with pU6sh75m and pU6sh143m (Fig. 4).

DISCUSSION

Telomerase activity is usually considered to be low in somatic cells, however it is higher in cells replicating continuously especially, tumor cells. Recently, the elevated telomerase activity of human stem cells have been highlighted because the role of telomerase have been suggested in the proliferation and differentiation of stem cells.[3,4] Considering the role of telomerase for tumorigenesis and its exclusive expression in most tumor cells, telomerase can be a promising candidate for a targeted therapy against tumor. For the clinical application of human stem cells as a tool of regenerative medicine, the manipulation of telomerase activity may be a feasible way to modulate the function of human stem cells. Telomerase mainly consists of hTR and hTERT, and the expression of hTERT is closely associated with telomerase activity.[11] Therefore, the inhibition of hTR might be a possible target for the strategy to manipulate telomerase activity. Up to now, there have been several attempts to inhibit telomerase activity, for example, inhibitors of retroviral reverse transcriptase, peptide nucleic acid, hammerhead ribozyme, and hTR antisense RNA, and hTR gene deletion.[12-14]

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing mechanism, causing degradation of mRNAs homologous in sequence to the dsRNA and inhibiting specific gene expression effectively which is triggered by double-stranded RNA (dsRNA). RNAi technology has been a useful tools for investigating the function of genes and proteins, application of gene therapy against tumor, virus protection, drug target validation, or discovery screening.[1,2] Gene therapy using a siRNA sequence could be an attractive approach for the treatment of viral infections, cancers, and genetic disorders. One of the major advantages of this approach is that it can inhibit the expression of the disease-associated gene in a sequence specific-manner. Ever since the discovery that short siRNA could induce specific gene silencing, there have been numerous studies demonstrating the use of siRNA to modulate gene expression.[12-14] There are two kinds of siRNAs, synthetic siRNA (*in vitro* preparation of siRNA) and expressed siRNA (*in vivo* expression of siRNA). For drug development, synthetic siRNA have been more commonly used due to the ease of dose control and more convenience in clinical trials. However, synthetic siRNA has several disadvantages; it is unstable under the influence of nuclease, or toxin, and is not effective for the infusion into cells. Because such an expressed siRNA has various advantages over synthetic siRNA, it can be easily delivered by a viral vector and has a low toxicity. There have been several approaches that use expressed siRNA by siRNA expressing plasmid, knockdown of p53 or GαS, one of the ligand-dependent, G-protein-coupled signaling by siRNA expressing adenovirus.[12-14]

In this study, we have tried to inhibit the human telomerase RNA (hTR) using a siRNA. The selection of the target sequences was performed using synthetic siRNA design softwares of internet sites and previous studies.[15] The selection criteria described by the Tuschl lab[15] was used in this study: preferred GC contents $<50\%$,

>50～100 nucleotide downstream of the start cordon, etc. The most probable part of hTR for siRNA knock-down was between residues 143 and 163, 20 nucleotides 3' of the telomerase template RNA. BLAST searches of available databases for this region revealed that this region is homologous only to the human hTR sequence in human genome. Therefore, the sequence of 143～163 region of hTR was selected for the design of siRNA expressing vector. In this study, the human U6 promoter which is a strong, ubiquitously active, and lacks essential promoter elements within transcribed region, was used. In this study, the telomerase activity was higher in the human embryonic stem cell line, SNUhES3 than human mesencyemal stem cells extracted from human bone marrow aspirates. Because its baseline telomerase activity was low, the difference between before and after siRNA treatment targeting hTRwas not significant in human mesenchymal stem cells compared to SNUhES3. It is still not clear the reason why the telomerase activity of human mesenchymal stem cells is relatively low compared to human embryonic stem cells. However, the fact that mesenchymal stem cells are the group of heterogeneous cells including fibroblast, etc might be related with this difference from human embryonic stem cells. Thus, the strategy using siRNA targeting telomerase activity can be more useful in the manipulation of human embryonic stem cells than human mesenchymalo stem cells. Further study should be warranted to find the underlying mechanism of this difference in the future.

In conclusion, this study shows that the siRNA could inhibit telomerase activity of tumor as well as human embryonic stem cells. Therefore, the strategy using siRNA targeting telomerase activity might be a feasible way to modulate the activity of human stem cells and suppress the growth of tumor cells.

요 약

배경: 종양 세포와 줄기세포에서 텔로머라제 활성도의 변화가 종양세포와 줄기세포의 생물학적 특성을 변화시킬 수 있다. 본 연구에서는 siRNA를 이용하여 텔로머라제 활성도를 억제하고자 하였다.

방법: 대상이 된 줄기 세포는 인간 중간엽 세포와 인간 배아줄기세포주(SNUhES3)를 시용하였고, 종양세포주는 Hela 세포주를 이용하였다. 인간 텔로머라제 억제를 위해 염기 서열 76에서 94까지의 뉴클레오타이드와 143에서 163까지를 표적으로 하여 두 가지 anti-sense를 제조하였다(pU6sh75, pU6sh143).

결과: HeLa 세포주와 인간 배아줄기세포주에서의 텔로머라제 활성도는 인간 중간엽 세포에 비해 의미있게 높았다(135.3±14.5, 109.0±18.2 vs. 46.3±5.0). Transfection 효율은 pU6sh75의 경우, HeLa 세포에서는 90.1±9.0%, 인간 배아줄기세포주에서는 79.9±18.2%, 그리고 인간 중간엽 세포에서는 79.4±15.1%였다. siRNA 처리 후 텔로머라제 억제 정도는 pU6sh75 transfected 세포에서는 HeLa 세포주, 64.3±10.1, 인간 배아줄기세포주, 56.0±11.0, 그리고 인간 중간엽 세포, 43.0±7.2%였다. 그리고 pU6sh143 transfected 세포에서는 HeLa 세포주, 71.3±9.1, 인간 배아줄기 세포주, 61.6±8.3, 그리고 이간 중간엽 세포, 46.0±9.0%였다.

결론: siRNA를 이용한 인간 텔로머라제 활성도의 억제는 종양세포주와 줄기세포주에서 효과적인 텔로머라제 억제 방법으로 이용될 수 있겠다.

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