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Attenuation of Telomerase Activity by Hammerhead Ribozymes Targeting Human Telomerase RNA and Telomerase Reverse Transcriptase in Pancreatic Carcinoma Cells

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ABSTRACT

In cancer gene therapies, it is ideal to target tumor-specific genes. Since telomerase is activated in almost all cancer cells but not most somatic cells, it is considered as one of the favorite targets for cancer gene therapies. Ribozymes are catalytic RNA molecules with site-specific endoribonuclease activity. In the present study, we designed hammerhead ribozymes against human telomerase RNA (hTR) and human reverse transcriptase subunit (hTERT) to evaluate their effect on the attenuation of telomerase in the pancreas cancer cell line, PK-8. Hammerhead ribozyme targeting hTR depressed the level of telomerase activity in PK-8 cells. pRc-hTR vector with ribozyme targeting hTR and pRc-hTERT vector with ribozyme targeting *hTERT* mRNA were transfected into PK-8 cells and depressed telomerase activity and target RNA, but in pRc-hTR transfectant, *hTERT* mRNA expression was slightly upregulated and in pRc-hTERT transfectant hTR expression was also slightly upregulated. These findings indicate the co-regulation of hTR and *hTERT* mRNA expression in cancer cells. Extrachromosomal replicational vector, pCEP4, containing ribozyme targeting *hTERT* mRNA showed the most effective inhibition of telomerase activity, suggesting that the continuous effect of ribozyme is necessary to inhibit telomerase activity. Since the level of *hTERT* mRNA expression is less than that of hTR expression in cancer cells, ribozyme targeting *hTERT* mRNA might be a more useful therapeutic strategy for cancer gene therapy. Moreover, the co-regulation of hTR and *hTERT* mRNA expression in cancer cells to maintain the levels of telomerase activity suggested that the strategy of inhibiting *hTERT* mRNA and hTR simultaneously has a powerful potential as a gene therapy for targeting human telomerase.

Key words: *Hammerhead ribozyme, Telomerase, Pancreas cancer*

Generally, normal somatic cells lose telomeric repeats (5'-TTAGGG-3') at a rate of 50–200 bp at each cell division, and when telomeres become critically short, the cells become senescent. In almost all cancer and germ cells, maintenance of telomere length has been acquired with the activation of telomerase, which maintains telomere length by *de novo* synthesis of tandem telomeric repeats. This function of telomerase for the maintenance of telomeres has been identified as one of the most important requirements for immortality and infinite growth of cancer cells. Telomerase is a large ribonucleoprotein enzyme complex, composed of human reverse transcriptase (hTERT), human telomarase RNA (hTR), that serves as a template of telomeric repeats, telomerase-associated protein 1 (TEP1), hsp 90 (heat shock protein 90), and p23. In theory, inhibition of any of these components might be useful to attenuate telomerase activity and cause telomere shortening and senescence in cancer cells, as is the case in normal somatic cells.

Infiltrating ductal carcinoma of the pancreas remains one of the most aggressive and lethal cancers[3,14,15)]. We reported a large up-regulation of telomerase activity in 95% of resected pancreatic cancer specimens, but we found no such up-regulation in benign tumors[2)]. Suehara et al also reported that telomerase activity was detectable in 80% of specimens from surgical resected tissues of pancreatic cancer and in 75% of pancreatic juice samples of patients with infiltrating ductal carcinoma[20)]. On the basis of these findings, telomerase has been proposed as a new target for anticancer therapies of pancreatic cancer. Reverse transcriptase inhibitors, such as azydothymidine[6)]

and dideoxyinosine[1,16,19], antisense oligodeoxynucleotides[10,13], ribozyme[8,12] and short-interfering RNA[11] have been shown to be effective agents for the attenuation of telomerase activities in tumor cells *in vivo* and *in vitro*.

In this study, we set out to inhibit telomerase activity in human pancreatic cancer cells using hammerhead ribozymes. The hammerhead ribozyme, one of the smallest types of ribozyme, is a catalytic RNA molecule with site-specific endoribonuclease activity. Yokoyama reported that hammerhead ribozyme targeting of hTR and *hTERT* mRNAs could attenuate telomerase activity in human endometrial carcinoma cells[22,23], and Folini also demonstrated inhibition of telomerase activity by a hammerhead ribozyme targeting hTR in human melanoma cells[4]. We transfected human pancreatic cancer cells with hammerhead ribozymes targeting either hTR or *hTERT* mRNA. Following expansion of the transfected cells, we measured their levels of telomerase activity and quantified their levels of hTR and *hTERT* mRNA.

MATERIALS AND METHODS

Cell line

The PK-8 human pancreatic ductal cancer cell line was kindly provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, and was maintained in the logarithmic growth phase at 37°C in 5% CO_2 humidified atmosphere in RPMI 1640 with 10% fetal calf serum.

Design of ribozyme targeting hTR

A hammerhead ribozyme, which recognizes a GUC sequence located 34–36 bp from the 5' end of hTR, was designed using the following DNA templates, which contain the sequences of the hammerhead ribozyme to target hTR and the T7 RNA polymerase promoter:

5'-ATTTTTTGTTTCGTCCTCACGGACTCATCAGTAACCCTAACCTAT
AGTGAGTCGTATTAGGATCC-3'

5'-GGATCCTAATACGACTCACTATAGGTTAGGGTTACTGATGAGTC
CGTGAGGACGAAACAAAAAAT-3'

Using T7-MEGAshortscript kit (Ambion Inc., Austin, Texas, USA), 500 ng of annealed DNA template was mixed with 20 μl of the transcription reaction mixture provided with the kit, and was incubated overnight at 37°C. The transcriptional products were treated by phenol-chloroform extraction followed by ammonium acetate ethanol precipitation.

Transfection of PK-8 cells with ribozyme targeting hTR

Prior to transfection, the ribozyme was complexed with DOTAP by mixing 20 μg of ribozyme with 15 μl N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethyl-ammonium methylsulfate (DOTAP, Roche Molecular Biochemicals, Mannheim, Germany) and enough HBS buffer (20 mM HEPES, cell culture grade, 150 mM NaCl, pH 7.4) to make a final volume of 75 μl. PK-8 cells were seeded in 6-well plates (5×10^5 cells / well). 75 μl of the ribozyme–DOTAP complex was added to each well at 0, 12, 24 and 36 hours after cell seeding. The cells were harvested 48 hours after seeding. For negative controls, DOTAP in HBS buffer, and HBS buffer only, was added in place of the ribozyme–DOTAP complex.

Radiolabelled assay for Telomerase activity detection

The telomerase activity of cells infected with ribozyme targeting hTR was measured by the telomeric repeat amplification protocol (TRAPeze®, Intergen Company, NY, USA). According to the manufacturer's protocol, 10^5 cells were resuspended in 200 μl CHAPS lysis buffer and kept on ice for 30 min. After centrifugation at 12000 g at 4°C for 20 min, the supernatants were immediately frozen on dry ice and stored at –80°C. 2 μl of the extract was mixed with 48 μl of TRAPeze reaction mixture provided with the kit and 2 μl γ-^{32}P-ATP and Taq polymerase. After incubation at 30°C for 30 min, 2-step PCR was performed for 27 cycles (30 sec at 94°C; 30 sec at 59°C). The PCR products were electrophoresed in a 10% acrylamide gel and autoradiographed.

Construction of pRc/CMV2 vectors expressing ribozyme targeting hTR and ribozyme targeting hTERT mRNA

The oligonucleotide sequences encoding the ribozyme to target hTR (Fig. 1) were designed as follows:

5'-AGCTGTTAGGGTTACTGATGAGTCCGTGAGGACGAAACAAAAAATG-3'

5'-GGCCCATTTTTTGTTTCGTCCTCACGGACTCATCAGTAACCCTAAC-3'

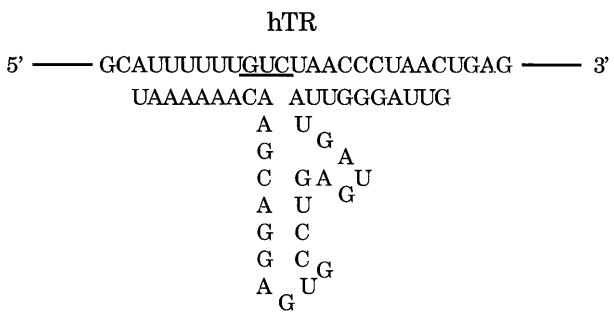

Fig. 1. Structure of hammerhead ribozyme targeting human telomerase RNA
The specific cleavage site localized in the RNA template is the underlined GUC sequence.

The oligonucleotide sequences encoding the ribozyme to target *hTERT* mRNA (Fig. 2) were designed as follows:

5'-AGCTTGCGCAGCAGCTGATGAGTCCGT-GAGGACGAAACGCAGCGCT-3'

5'-GGCCAGCGCTGCGTTTCGTCCTCACG-GACTCATCAGCTGCTGCGCA-3'

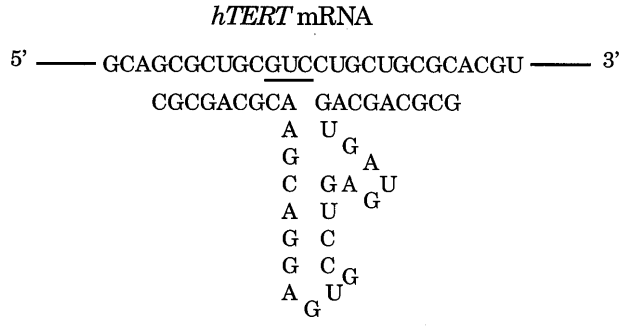

Fig. 2. Structure of hammerhead ribozyme targeting *hTERT* mRNA
As with hTR, the specific cleavage site localized in hTERT mRNA is the underlined GUC sequence.

The annealed oligonucleotides, with *Hind*III and *Not*I protruding ends, were inserted into pRc/CMV2 vectors (Invitrogen Corp., Carlsbad, CA, USA), previously digested with *Hind*III and *Not*I restriction enzymes and dephosphorylated of 5'-terminal phosphate groups. The presence and the correct orientation of ribozyme sequence was verified by DNA sequencing, and the resulting vectors were named pRc-hTR and pRc-hTERT.

Transfection of pRc/CMV2 vectors into PK-8 cells

To complex pRc-hTR and pRc-hTERT with DOTAP, 5 μg of vector was mixed with 25 μl N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium methylsulfate (DOTAP, Roche Molecular Biochemicals, Mannheim, Germany) and enough HBS buffer (20 mM HEPES, cell culture grade, 150 mM NaCl, pH 7.4) to make a final volume of 100 μl. PK-8 cells were seeded in Petri dishes at a density of 30% confluency. At four days after seeding, 25 μl of vector–DOTAP complex was added to the medium and cultured for 2 days.

Culture of transfectants

The transfectants were cultured in a selection medium containing G418. G418, when added to the medium, killed normal PK-8 cells, but not when the PK-8 cells were transfected with pRc/CMV2. Because PK-8 cells died in the medium with 0.4 mg/ml G418, we selected the pRc-hTR transfectants in the medium by adding 0.6 mg/ml G418 to the medium. The cells were cultured for 2 weeks, transfected with the vector once again and then cultured for 2 more weeks. Finally, pRc-hTR transfectant cells were harvested and telomerase activity was measured by fluorometry- based TRAP (telomeric repeat amplification protocol) assay, and hTR and *hTERT* mRNA expression was assessed. For comparison, normal PK-8 cells cultured without G418, and PK-8 cells transfected with pRc/CMV2 vector not expressing a ribozyme, were evaluated.

Quantitative detection of hTR and hTERT mRNA

Total RNA was extracted using TRIZOL® Reagent (Invitrogen Corp., Carlsbad, CA, USA). Quantitative detections of hTR and *hTERT* mRNA were performed with a LightCycler Telo TAGGG hTR Quantification Kit® (Roche Molecular Biochemicals, Mannheim, Germany) and a LightCycler Telo TAGGG hTERT Quantification Kit® (Roche Molecular Biochemicals, Mannheim, Germany) using LightCycler® instruments (Roche Molecular Biochemicals, Mannheim, Germany) for real-time RT-PCR and all subsequent quantification steps according to the manufacturer's instructions. Total RNA (200 ng) was mixed with 20 μl of RT-PCR reaction mixture provided with the kit, and was reverse-transcribed for 10 min at 60°C. PCR amplifications of hTR were performed for 45 cycles (0.5 sec at 95°C; 10 sec at 60°C; 10 sec at 72°C) and PCR amplifications of *hTERT* mRNA were performed for 40 cycles (0.5 sec at 95°C; 10 sec at 60°C; 10 sec at 72°C) using manufacturer-supplied reaction mixtures specific for hTR and *hTERT* mRNA or the housekeeping gene PBGD, respectively. The PBGD reaction product served as a reference for relative quantification of hTR and *hTERT* mRNA. Each sample was normalized on the basis of PBGD content according to the formula hTR ratio = (hTR copies sample / *PBGD* mRNA copies sample) × 1000 and the formula *hTERT* mRNA ratio = (*hTERT* mRNA copies sample / *PBGD* mRNA copies sample) × 100000.

Construction and transfection of extrachromosomal replicative vector expressing ribozyme targeting hTERT mRNA

pCEP4 (Invitrogen Corp., Carlsbad, CA, USA), an extrachromosomal replicative vector containing CMV promoter and *Hind*III and *Not*I protruding ends, was combined with the ribozyme targeting *hTERT* mRNA using the same oligonucleotide sequences as for pRc-hTERT. The new vector was named pCEP-hTERT. pCEP-hTERT was complexed with DOTAP and transfected into PK-8 cells in the same way as were the pRc/CMV2 vectors. The transfected cells were subsequently exposed to a selection medium containing hygromycin B for 4 weeks. Because the minimum concentration of hygromycin B required to kill PK-8 cells was 0.4 mg/ml, the pCEP-hTERT transfectant was selected in 0.6 mg/ml of hygromycin B.

Finally telomerase activity was measured by fluorescence-based TRAP assay.

Fluorometric detection TRAP assay

TRAPeze® XL (Intergen Company, NY, USA) is a non-radioactive, fluorescent method of determining telomerase activity, adapted from the original TRAP assay. According to manufacturer's protocol, 10^5 cells were resuspended in 200 μl CHAPS lysis buffer and kept on ice for 30 min. After centrifugation at 12000 g at 4°C for 20 min, the supernatants were immediately frozen on dry ice and stored at –80°C. 2 μl of the extract was mixed with 48 μl of TRAPeze® XL reaction mixture provided with the kit and 2 μl Taq polymerase. After 30 min of incubation at 30°C, PCR amplifications of the reaction mixture were performed for 36 cycles (30 sec at 94°C; 30 sec at 59°C; 1 min at 72°C) followed by an extension step for 3 min at 72°C. The PCR products were determined by Fluorescence Plate Readers (ARVO multilabel counter, Wallac Oy, Turku, Finland).

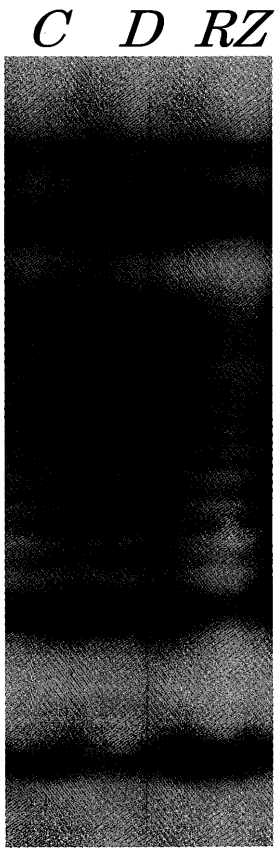

Fig. 3. Inhibition of telomerase activity by ribozyme targeting hTR delivered to PK-8 cells through cationic liposome-meditated transfer
Control and DOTAP showed a high level of telomerase activity, but the ribozyme transfectant showed less activity of telomerase. C, control; D, exposed DOTAP only; RZ, introduced ribozyme.

RESULTS

A hammerhead ribozyme, which recognizes a GUC sequence located 34–36 bp from the 5' end of hTR, was designed. We introduced the ribozyme, considered to be unstable in medium supplemented with fetal calf serum, into PK-8 cells using a liposomal transfection method. For negative controls, the ribozyme-DOTAP complex was replaced with DOTAP in HBS buffer, and HBS alone. 48 hrs after initial exposure to the ribozyme, telomerase activity was significantly reduced in the ribozyme transfected cells (Fig. 3), but not in the cells exposed to DOTAP and HBS buffer or HBS buffer alone.

PK-8 cells were transfected with vectors expressing ribozymes targeting hTR or *hTERT* mRNA. Subsequent to transfection, the cells were cultured in a selection medium containing G418. pRc-hTR and pRc-hTERT transfectants were harvested and telomerase activity was detected by fluorometry, and hTR and *hTERT* mRNA levels were assessed. pRc-hTR transfectant cells expressed less telomerase activity than control pRc/CMV2 transfectant cells (Fig. 4). Subsequently, we changed the target site of ribozyme from hTR to *hTERT* mRNA. pRc-hTERT transfectant cells showed a greater inhibition of telomerase activity than control pRc/CMV2 transfectant cells (Fig. 5).

hTR expression, analyzed by real time RT-PCR, was low in the hTR transfected cells compared

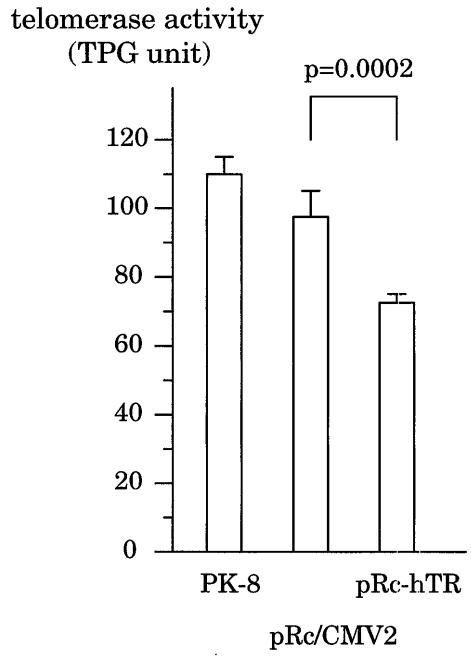

Fig. 4. Detection of telomerase activity of ribozyme targeting hTR transfectant by fluorometric method refined TRAP assay
pRc-hTR transfectant slightly depressed telomerase activity compared with original pRc/CMV2 transfectant. TPG; total product generated.

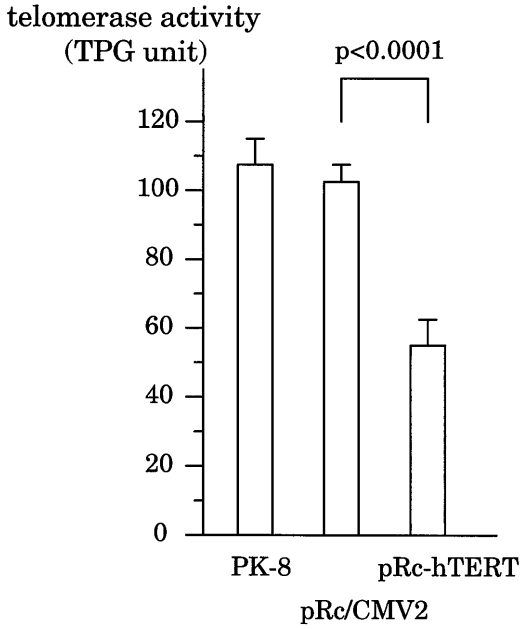

Fig. 5. Detection of telomerase activity of ribozyme targeting *hTERT* mRNA transfectant by fluorometric method refined TRAP assay
pRc-*hTERT* mRNA transfectant expressed less telomerase activity than pRc/CMV2 transfectant.

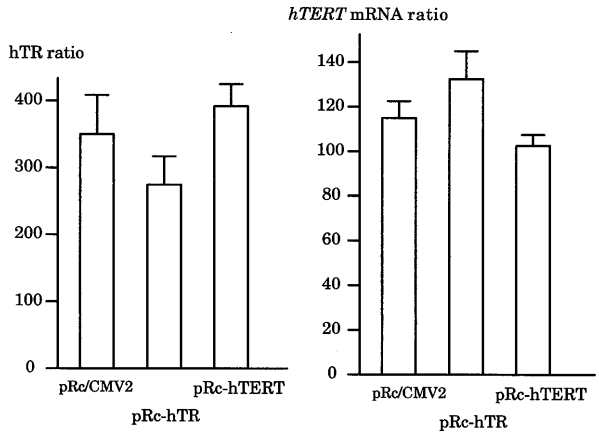

Fig. 6. Quantification of hTR and *hTERT* mRNA by real-time RT-PCR
hTR expression was low in the hTR transfected cells compared with the pRc/CMV2 transfectant cells, but in the pRc-hTERT transfectant was slightly increased. hTERT mRNA expression level in pRc-hTERT transfectant decreased in comparison with the control, but in pRc-hTR transfectant was slightly increased.
hTR ratio: (hTR expression / *PBGD* mRNA expression) × 100
hTERT mRNA ratio: (*hTERT* mRNA expression / *PBGD* mRNA expression) × 10000

with the pRc/CMV2 transfectant cells, but in the pRc-hTERT transfectant, interestingly, hTR expression was slightly increased compared with the control, and the *hTERT* mRNA expression

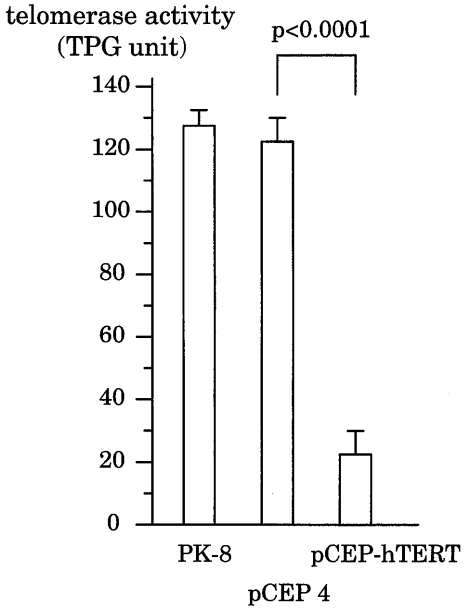

Fig. 7. Detection of telomerase activity of extrachromosomal replicative vector transfectant by fluorometric method refined TRAP assay.
pCEP-hTERT transfectant expressed significantly low telomerase activity.

level in pRc-hTERT transfectants decreased in comparison with the control, while in the pRc-hTR transfectants *hTERT* mRNA expression was slightly increased (Fig. 6). This was unexpected as the expression level of *hTERT* mRNA normally correlates with the level of telomerase activity.

Finally, the extrachromosomal replicational vector, pCEP4, was used to transfect a ribozyme targeting *hTERT* mRNA into PK-8 cells. pCEP-hTERT transfectant cells were selected in a medium containing hygromycin B. Four weeks after transfection, pCEP-hTERT transfectants were harvested and detected for telomerase activity. Strong inhibition of telomerase activity was shown at pCEP-hTERT transfectant cells (Fig. 7), exceeding the inhibition achieved by either pRc-hTR or pc-hTERT.

DISCUSSION

In cancer gene therapies, it is ideal to target tumor-specific genes. However, few cancer-specific genes which are activated in the majority of human cancers have been identified. Telomerase, which is neither oncogenic nor anti-oncogenic, is repressed in most human somatic cells, and generally upregulated in almost all cancer cells, including pancreatic cancer cells[7,17,18,21]. Thus, suppression of telomerase is considered as a new strategy for anticancer therapy. By taking advantage of understanding the composition and function of telomerase, a number of potential inhibitors have been developed, including antisense oligodeoxynucleotides against the template region of hTR and traditional reverse tran-

scriptase inhibitors. Telomerase consists of two essential components, the catalytic reverse transcriptase subunit (hTERT), and the template RNA of telomeric repeats (hTR). hTR and hTERT are considered suitable targets for anticancer therapies.

We tried to inhibit telomerase activity using ribozymes, catalytic RNA molecules, targeting the two RNA components associated with telomerase. A major advantage in using ribozymes compared with conventional antisense oligodeoxynucleotides and reverse transcriptase inhibitors-mediated strategies is considered to be its specific catalytic potential. One molecule of hammerhead ribozyme can cleave several of the chosen RNAs, which implies that, theoretically, very low concentrations are required to obtain a significant biological effect.

In the present study, we used pRc/CMV2 to transfect pancreatic cancer cells with ribozymes. PK-8 cells, which have a stable level of telomerase activity, are suitable for examination of the attenuation of telomerase. pRc-hTERT transfectant cells showed reduced activity of telomerase, but in pRc-hTR transfectant cells, small attenuation of telomerase was found. Usually, the level of *hTERT* mRNA expression is lower than that of hTR, which may account for the greater attenuation by the pRc-hTERT ribozyme. The proper three-dimensional structure of the ribozyme is required for the ribozyme to exert catalytic activity, and the target region must be sufficiently exposed on the outer surface of the three-dimensional structure of the molecules[24]. pRc-hTR and pRc-hTERT transfectants had low attenuation of telomerase activity, suggesting that the target region might be hidden within the telomerase molecule.

In the examination of expression of hTR and *hTERT* mRNA by real time RT-PCR, the expression level of the targeted RNA was attenuated, but the expression of the other RNA was upregulated. These phenomena suggested that two essential components of human telomerase, hTR and hTERT, work co-operatively to maintain telomerase activity. Thus, inhibition of the expression level of hTR or *hTERT* mRNA independently is limited to attenuating telomerase activity. For example, the transfection of the vector expressing the ribozyme to cleave both hTR and *hTERT* mRNA, to attenuate telomerase activity more effectively may require the inhibition of hTR and hTERT simultaneously.

These findings also indicate the co-regulation of hTR and *hTERT* mRNA expression in cancer cells. M. Folini reported a shortening of telomere length in ribozyme transfectants displaying reduced telomerase activity, but after 45 days in culture, they were still able to proliferate[5]. Yokoyama also reported that telomerase activity and hTR in the pooled clones of ribozyme transfectant were clearly diminished compared with that of the parental cells and the naked vector transfectant, but in some clones in which telomerase activity was almost undetectable, hTR was still expressed at a steady level[9]. One explanation for such findings is that in tumor cells subjected to the effective inhibition of telomerase associated RNA components, there would be a strong selection pressure for the emergence of resistant cells by the activation of alternative lengthening of telomere mechanisms.

There are two ways to deliver the ribozyme to its cellular target RNA. One is by endogenous delivery, the intracellular transcription of ribozyme coding sequence accomplished by transfection/infection of ribozyme-producing vectors into cells. This has been effective against several targets in *in vitro* and *in vivo* experimental models, even though no data are available on its therapeutic potential in the clinical setting. Another way is exogenous ribozyme delivery, in which ribozymes are introduced directly into cells with the aid of cationic liposome-mediated transfer, but this way is related to multiple factors, in particular, endogenous nuclease. The ribozyme transfectants grew more slowly than did the parental cells, and the doubling time of the transfectant became double that of the parental cells. We tried ribozyme expression vector in other pancreatic cancer cells but could not obtain the ribozyme transfectant. This may be explained by the toxity of the ribozyme in some cell lines.

In the present study, expressing the ribozyme from an episomal replicative vector showed more effective inhibition of telomerase activity, suggesting that high and continuous expression of ribozyme by the extrachromosomal replicative vector is required to inhibit telomerase activity. Sicne the inhibition of telomerase by pRc/CMV vector is transient, the continuous expression of ribozyme is more effective for inhibiting telomerase activity. Continuous inhibition by extrachromosomal replicative vector expressing the hammerhead ribozymes to affect both hTR and *hTERT* mRNA has therapeutic potential in cancer patients, especially in pancreatic cancer patients.

Targeting telomerase by siRNA may also be effective, so that the sites we designed for ribozyme transfection may be effective targets for siRNA transfectant. Like ribozyme treatment, the effect of siRNA may be also transient. It is necessary to make an additional method to maintain the effect of ribozyme or siRNA in cells.

This study revealed that the repression of one of the two essential components of human telomerase, hTR and hTERT, overexpressed the other component. Thus, the most effective strategy for attenuating human telomerase is to inhibit both components simulutaneously. Moreover, a continuous and high inhibitory effect such as the expres-

sion of ribozyme by the extrachromomal replicative vector is a favorite for attenuating telomerase activity in human cancer.

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