Cancer Cell Targeting with Mouse TERT-Specific Group I Intron of *Tetrahymena thermophila*

Ban, Guyee, Min-Sun Song, and Seong-Wook Lee*

Department of Molecular Biology, BK21 Graduate Program for RNA Biology, Institute of Nanosensor and Biotechnology, Dankook University, Yongin 448-701, Korea

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Telomerase reverse transcriptase (TERT), which prolongs the replicative life span of cells, is highly upregulated in 85–90% of human cancers, whereas most normal somatic tissues in humans express limited levels of the telomerase activity. Therefore, TERT has been a potential target for anticancer therapy. Recently, we described a new approach to human cancer gene therapy, which is based on the group I intron of *Tetrahymena thermophila*. This ribozyme can specifically mediate RNA replacement of human TERT (hTERT) transcript with a new transcript harboring anticancer activity through a *trans*-splicing reaction, resulting in selective regression of hTERT-positive cancer cells. However, to validate the therapeutic potential of the ribozyme in animal models, ribozymes targeting inherent transcripts of the animal should be developed. In this study, we developed a *Tetrahymena*-based *trans*-splicing ribozyme that can specifically target and replace the mouse TERT (mTERT) RNA. This ribozyme can trigger transgene activity not only also in mTERT-expressing cells but hTERT-positive cancer cells. Importantly, the ribozyme could selectively induce activity of the suicide gene, a herpes simplex virus thymidine kinase gene, in cancer cells expressing the TERT RNA and thereby specifically hamper the survival of these cells when treated with ganciclovir. The mTERT-targeting ribozyme will be useful for evaluation of the RNA replacement approach as a cancer gene therapeutic tool in the mouse model with syngeneic tumors.

**Keywords:** Cancer, gene therapy, group I intron, mTERT, RNA replacement, *trans*-splicing ribozyme

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Telomerase is a ribonucleoprotein enzyme that maintains the protective structures of telomeres of eukaryotic chromosomes, thereby extending the life span of replicating cells [1]. Most human normal somatic tissues possess undetectable activity of the telomerase [4, 26]. In contrast, the telomerase activity is highly upregulated in a vast majority of human cancers and the activity is mainly modulated by hTERT expression [11, 18, 21, 25, 29]. Moreover, telomerase upregulation could actively contribute to tumor growth [35]. Therefore, telomerase is an attractive target for the development of anticancer therapeutic ligands.

*Tetrahymena* group I intron has catalytic *trans*-splicing activity, which can mediate cleavage of a target RNA and *trans*-ligation of an exon tagged at the 3' end of the intron onto the cleaved substrate RNA in cells [8, 33]. Therefore, the *trans*-splicing ribozyme could target and reprogram a specific disease-associated or causative RNA with transgene transcript containing a therapeutic effect, selectively in cells that express the target RNA, representing an attractive gene therapy tool against diverse human diseases including genetic or infectious ones [3, 13, 23, 24, 27].

Previously, we suggested that *Tetrahymena*-based *trans*-splicing ribozyme could also be a potent anticancer agent by mediating RNA replacement of tumor-specific transcripts with new RNA exerting anticancer activity. We validated the potential of the ribozyme by developing a *trans*-splicing ribozyme specifically targeting and replacing hTERT RNA to induce transgene activity selectively in hTERT-positive cancer cells. This hTERT-specific ribozyme was shown to selectively mark tumor cells expressing hTERT, or specifically and efficiently retard the growth of tumors in tumor xenografts as well as in tissue cultures [6, 7, 12, 32].

A preclinical index that includes therapeutic efficacy, toxicities, pharmacokinetics, or pharmacodynamics should be predetermined in animals before evaluation of the therapeutic potential of the hTERT-specific ribozyme in a clinical setting. An established animal model with syngeneic tumors and normal immunological surveillance will be useful for evaluating the ribozyme [28]. To this end, we developed a *trans*-splicing ribozyme specifically targeting...
and reprogramming mouse TERT (mTERT) transcript and observed whether the ribozymes selectively induce transgene activity, and moreover, specifically retard cell survivability in TERT-expressing cancer cells.

MATERIALS AND METHODS

Ribozyme Design
A specific ribozyme targeting position 67 on the mTERT RNA, Rib67, was constructed by in vitro transcription of the cDNA template. The pT7L-21 plasmid, which encodes a slightly shortened version of the natural group I intron from *Tetrahymena*, was used for construction of the template by PCR. The 5' primer contained the T7 promoter and internal guide sequence (IGS) of the ribozyme, whereas the 3' primer was specific for the 3' exon sequence [8, 16, 33]. The IGS on the original L-21 trans-splicing ribozyme (5'-GGAGGG-3') was replaced with 5'-GGAGGAG-3' in Rib67.

To construct ribozyme-expressing vectors, Rib67 was modified by insertion of synthesized complementary oligonucleotides containing an extended P1 and a 6-nt-long P10 helix upstream to the IGS of the ribozyme and flip-fry luciferase (Fluc) cDNA in frame at the 3' exon of the ribozyme. The DNA fragment consisting of the Rib67 sequence with the extended IGS and Fluc cDNA was then inserted between the HindIII and NotI sites of AVR vector, which encodes the transgene under the regulation of the CMV promoter (Qbiogene). Rib67AS100-Fluc or Rib67AS300-Fluc was constructed by insertion of synthesized complementary oligonucleotides containing an extended P1 and a 6-nt-long P10 helix upstream to the IGS of the ribozyme on the template by PCR. The 5' primer contained the T7 promoter and internal guide sequence (IGS) of the ribozyme, whereas the 3' primer was specific for the 3' exon sequence [8, 16, 33]. The IGS on the original L-21 trans-splicing ribozyme (5'-GGAGGG-3') was replaced with 5'-GGAGGAG-3' in Rib67.

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Cell Cultures
Mouse fibroblast NIH3T3 cells and hepatocarcinoma Hepa1-6 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Human hepatocarcinoma Hep3B cells and human lung embryo fibroblast IMR90 cells were cultured in MEM (Invitrogen) supplemented with 10% FBS. All cells were purchased from the ATCC (American Type Culture Collection).

Ribozyme Assay in Cells
For the analysis of reporter gene activation, cells were seeded in 35-mm plates at a density of 3.0×10⁵ cells per plate, 24 h before transfection. Cells were cotransfected with 250 ng of CMV-Rluc, which encodes the renilla luciferase (Rluc) gene regulated by the CMV promoter, along with 2.5 μg of CMV-Fluc, Rib67AS100-Fluc, Rib67AS300-Fluc, or control empty vector using wellic M Gold transfection reagent (Welgen). Cell lysates were harvested 24 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega) [15] and a luminometer TD-20/20 (Turner Design Instrument). The reporter Fluc gene activities were detected and normalized to the Rluc gene activities.

For the assessment of suicide HSV-tk gene activity, cells were transfected with 250 ng of pEGFR-N1 (Clontech) along with 1 μg of CMV-TK, Rib67AS100-TK, Rib67AS300-TK, or CMV-Fluc. The next day after transfection, the cells were inoculated with 100 μM of ganciclovir (GCV; Cymevene, Roche). After 7 days of GCV treatment, surviving cells were analyzed by counting the number of GFP-expressing cells in 20 random fields of view in each transfected cell under a fluorescence microscope (Olympus Optical). Cell viability after GCV treatment was quantified as the percentage of GFP positive cells without GCV.

Statistical Analysis
Statistical analysis was performed using Statistical Analysis System (SAS) software (SAS Institute). The between-group differences were evaluated through analysis of variance (ANOVA). In the case of highly skewed distribution of measurements and small sample sizes, we employed nonparametric statistical tests (Kruskal–Wallis test for overall comparison and Wilcoxon’s rank-sum test for pair-wise comparison). All data were represented as the average±standard deviation. A P-value of 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Ribozyme Design
Although any uridine residues in a 5' exon can be theoretically targeted by *Tetrahymena* trans-splicing ribozyme through alteration of a nucleotide composition of 6-nt-long IGS on the ribozyme [17, 34], only a limited number of uridines on the target RNA can be accessible to the ribozyme owing to the complex tertiary structure of the
substrate RNA [14]. Previously, we employed an RNA mapping strategy that is based on RNA tagging [8] and a trans-splicing ribozyme library to identify which regions of the mTERT transcript were most accessible to the ribozymes [31]. Then, we found that the most accessible site was located in the uridine at position 67, 37 nt downstream of the AUG start codon, of the mTERT RNA. A specific ribozyme targeting position 67 on the mTERT RNA (Rib67) was constructed and shown to replace mTERT RNA with a 3' exon tagged at the 3' end of the ribozyme with high reliability in vitro via the targeted trans-splicing reaction [31].

To observe whether Rib67 could also be active in cells, we designed an enhanced ribozyme construct, because group I trans-splicing ribozymes with only a 6-nt-long IGS were inefficient in their activity and specificity when expressed in mammalian cells [8]. We modified the mTERT-targeting ribozyme, designated Rib67AS, to harbor a sequence of extended P1 helix, additional 6-nt-long P10 helix, and a 100- or 300-nt-long antisense sequence complementary to the downstream region of the targeted uridine of the target RNA, as previously described [9, 12, 24] (Fig. 1). We used the CMV promoter system for the intracellular expression of the modified ribozymes.

Induction of Transgene Activity in TERT-Expressing Cells by the Specific Ribozyme

To determine whether ribozymes could elicit 3' exon activity in cells expressing the TERT RNA, we transfected vectors encoding the ribozymes with the Fluc gene as a 3' exon into mTERT-positive NIH3T3 or Hepa1-6 cells or hTERT-expressing Hep3B cells (Fig. 2A). Because the ribozyme targeted the open reading frame region of the mTERT mRNA, we inserted Fluc cDNA as the 3' exon of the ribozyme in frame with the cleaved substrate RNA. We assessed the level to which ribozyme-mediated luciferase activity was stimulated by measuring activities of Fluc relative to those of Rluc.

To compare the ribozyme activity between the cells, the Fluc activities induced by the ribozymes were quantitated as a percentage of those expressed from the CMV promoter in each cell (Fig. 2B). The transfection of vector control exhibited no expression of Fluc in any cells. By sharp contrast, transfection of Rib67AS100-Fluc, the expression vector of mTERT-specific ribozyme with 100-nt antisense sequence, efficiently induced Fluc expression not only in mTERT+ but also in hTERT+ cells (Fig. 2B). Stimulation of transgene activity in hTERT-expressing human cells by the mTERT-specific ribozyme could be due to the presence of 80% homology between the two TERT mRNA sequences, which can render the ribozyme targeting and replacing the hTERT mRNA. Rib67AS300-Fluc, the expression vector of mTERT-specific ribozyme with 300-nt antisense sequence, poorly induced the Fluc expression, compared with Rib67AS100-Fluc, indicating that addition of too long an antisense sequence to the IGS of the ribozyme could impede the target accessibility and/or catalytic activity of the ribozyme. This result is in contrast with the previous report

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**Fig. 2.** Induction of transgene activity by the mTERT-specific trans-splicing ribozyme.

A. Vectors encoding the specific ribozymes with the firefly luciferase gene as 3'exon are represented. B. NIH3T3 cells (mTERT+), Hepa1-6 (mTERT+), or Hep3B cells (hTERT+) were cotransfected with the ribozyme-expressing vector (AS100; Rib67AS100-Fluc; AS300, Rib67AS300-Fluc) or control vector (V.C). Relative luciferase activity was quantitated as a percentage of the sample transfected with CMV-Fluc. Results represent the means±S.D. of three independent experiments.
showing that increased complementarities between the ribozyme and the target RNA improved biological activity. This contradiction could be dependent on the nature of the structure and sequence of the substrate RNA. In the case of mTERT-targeting ribozyme, highly lengthy antisense sequences will internally interact or bind nonspecifically to other cellular RNAs, resulting in the inhibition of base-pairs between the extended IGS of the ribozyme and the mTERT RNA. This phenomenon was also seen in the trans-splicing ribozyme targeting mouse cytoskeleton-associated protein 2 transcripts [10]. Moreover, the length of antisense–substrate interaction could be shortened to 5 bp without compromising trans-splicing efficacy in the case of the sickle β-globin-targeting trans-splicing ribozyme [2]. Taken together, mTERT-specific trans-splicing ribozymes could stimulate transgene activity efficiently in TERT-expressing cells.

Selective Induction of Suicide Gene Activity in TERT-Expressing Cancer Cells by the Specific Ribozyme

Based on the observation of transgene induction by the specific ribozyme, we next determined whether the ribozyme stimulated cytotoxicity activity selectively in the TERT RNA-expressing cancer cells. We transfected the expression vector encoding the mTERT-specific ribozymes with the suicide HSV-\(tk\) gene as a 3’ exon into mTERT-positive NIH3T3 or Hepa1-6 cells or hTERT-expressing Hep3B cells (Fig. 3A). In addition, we transfected the vector into TERT-negative IMR90 cells to assess the target specificity of the ribozyme. We then monitored their growth susceptibility to treatment with the prodrug GCV. HSV-\(tk\) can efficiently convert the prototrex nucleoside analog GCV into a toxic phosphorylated derivative that interferes with normal DNA replication [19].

For the suicide gene activity, we cotransfected with a GFP-expression vector, and the cytotoxicity was assessed by comparing relative numbers of GFP-positive cells in each transfectant (Figs. 3B–3E). No cytotoxicity was observed in cells transfected with CMV-Fluc irrespective of not only the cell type but also the GCV treatment. Efficient cytotoxicity was observed in both TERT+ and TERT- cells, when transfected with the positive control CMV-TK that encodes the HSV-\(tk\) gene under the CMV promoter and treated with GCV treatment. On the contrary, expression of Rib67AS100-TK conferred significant susceptibility to GCV only in TERT+ NIH3T3 cells (Fig. 3B, \(P=0.0002\), Hepa1-6 cells (Fig. 3C, \(P=0.0026\)), and hTERT+ Hep3B cells (Fig. 3D, \(P=0.0009\)), as compared with that of CMV-Fluc. These results concur with the observation that the mTERT-specific ribozyme mediated transgene activation in both mTERT- and hTERT-expressing cells, as shown in Fig. 2. However, the TERT-negative IMR90 cells were negligibly affected by the ribozyme, suggesting the target selectivity of the ribozyme (Fig. 3E, \(P=0.7430\)). In contrast to the transgene induction test, transfection of Rib67AS300-TK, which encodes the mTERT-specific ribozyme with a 300-nt long antisense sequence, could also efficiently and selectively induce cell cytotoxicity in a TERT RNA-dependent manner when treated with GCV. This suggests that the level of HSV-\(tk\) RNA spliced by the ribozyme could be sufficient to hamper the growth of TERT-expressing cells with GCV treatment. Moreover, a bystander effect can confer regression of not only the ribozyme-transfected cells but also neighboring others in the presence of GCV [20]. Importantly, the cytotoxicity in the TERT+ cells, which were transfected with the ribozyme-encoding vector and inoculated with GCV, was elicited with high efficiency, comparable to the cells transfected with CMV-TK. Transfection of ribozyme-vectors hardly affected cell survival even in the TERT+ cells in the absence of GCV, indicating that cytotoxicity could not be induced from trans-cleavage or from antisense effect, but from selective induction of HSV-\(tk\) gene activity. Taken together, the mTERT-specific trans-splicing ribozyme could induce suicide gene activity selectively and efficiently in TERT-expressing cells, regressing TERT+ cancer cells with prodrug treatment.

In this study, we developed a \(Tetrahymena\) group I intron-based trans-splicing ribozyme specific to mTERT RNA. The ribozyme efficiently elicited functional transgene activity in TERT-expressing cells. Of note, this ribozyme conferred anticancer activity specifically and efficiently in cancer cells expressing TERT RNA, which could occur mainly as a target-specific RNA replacement. Therefore, the mTERT-specific ribozyme will be of use for validation of TERT-targeting trans-splicing ribozymes as a cancer gene therapeutic agent in an immunocompetent mouse model with syngeneic tumors.

To use the mTERT-targeting ribozyme in a mouse model, we should take into consideration that significant differences exist in the dynamics and regulation of telomerase between the mouse and human. The telomeres of laboratory mice are much longer (40–60 kb) than those in the human (10 kb). Moreover, telomerase is widely expressed and activated in most of adult mouse tissues [5, 22]. Therefore, test of oligonucleotide-based inhibitors such as trans-cleavage ribozymes, antisense oligonucleotides, or siRNA for their repression of mouse telomerase expression in mice may be complicated with regard to their specificity and efficacy. In particular, there will be a lag phase between the time of mouse telomerase inhibition and the time to shorten the mouse telomere enough for cancer regression. In contrast, the trans-splicing approach will be more advantageous because it could overcome the lag phase by reducing the TERT level and simultaneously inducing cytotoxicity in the TERT-expressing cancer cells. Moreover, the tissue specificity for tumor cytotoxicity by the mTERT-targeting trans-splicing ribozyme can be improved through cancer- or condition-specific expression of the ribozyme [30, 32].
Fig. 3. Efficient and specific induction of suicide gene activity by the mTERT-specific trans-splicing ribozyme.
A. Vectors encoding the specific ribozymes with the herpes simplex virus thymidine kinase gene as 3’exon are represented. mTERT-expressing NIH3T3 cells (B), Hepa1-6 cells (C), hTERT+ Hep3B cells (D), or TERT-negative IMR90 cells (E) were cotransfected with the ribozyme-expressing vector (AS100, Rib67AS100-TK; AS300, Rib67AS300-TK) or positive control vector (CMV-TK) along with GFP-expressing vector. Viable cell numbers were counted after GCV treatment. The viability is represented as % relative to that of control cells transfected with CMV-Fluc. The average is presented with standard deviation of three independent determinants. Representative fluorescent microscopy results of each transfectant cell before and after GCV administration are shown.
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REFERENCES