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Specificity of intracellular *trans*-splicing reaction by *Tetrahymena* group I intron

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ABSTRACT

We have previously presented that *Tetrahymena* group I intron-based *trans*-splicing ribozyme could be utilized for the selective induction of new gene activities in target cells by recognizing the cell-specific RNA and catalyzing the in-frame fusion of desired RNA sequences. Especially, we have developed hTERT-targeting specific *trans*-splicing ribozyme to efficiently direct tumor-specific cytotoxicity by transcript targeting and RNA replacement. In this study, in order to observe therapeutic feasibility of this ribozyme, we analyzed *trans*-splicing specificity in cells. RT and RACE-PCR reaction and sequencing analysis of 19 *trans*-spliced products generated in the ribozyme-transfected hTERT-positive 293 cells revealed that all of the *trans*-splicing products resulted from reactions only with the targeted hTERT RNA. This study implies that the ribozyme exerted highly target RNA-specific intracellular *trans*-splicing activity. Therefore, RNA replacement by *trans*-splicing ribozyme could be a specific approach to cancer gene therapy.

Key words : cancer, gene therapy, group I intron, hTERT, RACE PCR, RNA replacement, *trans*-splicing ribozyme

Introduction

Specific cancer cell retardation is a major concern in applying cancer gene therapy into clinical settings. To overcome this limitation, most gene therapy protocols against tumors are based on transcriptional targeting using tissue- or cancer-specific promoters/enhancers (1). However, leaky promoter/enhancer activity in normal tissues could restrain their usefulness. Thus, more cancer-specific approach to gene expression or gene delivery will be required.

Tetrahymena group I intron ribozymes have been shown to target and revise mutant RNAs that cause human genetic or malignant diseases by *trans*-splicing with the target transcripts (2-5). Moreover, we have presented that *trans*-splicing ribozymes can specifically target and replace viral transcripts with a new RNA harboring anti-viral activity, and hence selectively stimulate a new gene activity in the viral RNA-expressing cells (6). Furthermore, we have recently developed specific *trans*-splicing ribozymes which can selectively induce cytotoxin gene activity in cancer cells expressing human telomerase reverse transcriptase catalytic subunit (hTERT) RNA with RNA replacement, and thereby specifically regress the survival of the cancer cells (7,8). Thus, the ribozymes will be novel tools for the expression of anti-cancer genes in cancer cells by selectively targeting and replacing cancer-specific transcripts.

In this present study, we analyzed specific activity of the *trans*-splicing reaction of the group I intron-based ribozymes which targets hTERT RNA in cells expressing the target RNA. To this end, we employed RT and RACE PCR of total *trans*-spliced products in hTERT-expressing cells which were transfected with the ribozyme-expressing vector. We detected *trans*-splicing products yielded with only the hTERT RNA, which suggests that the ribozyme specifically reacted

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with only targeted hTERT RNAs in cells.

Materials and methods

Ribozyme construct and transfection into cells

The ribozyme expressing construct, pRib39AS-Fluc, was constructed in the previous study (7). Briefly, the ribozyme was modified from pT7L-21 (9) by replacing internal guide sequence (IGS) 5′-GGAGGG-3′ with 5′-GGGGCT-3′, which recognizes +39 nt of hTERT RNA, and inserting synthesized complementary oligonucleotides containing an extended P1 plus a 6 nt P10 helix upstream to the modified IGS and 300 nt antisense sequence against the downstream region (+47 to +346 residue) of the targeted uridine of the hTERT RNA. In addition, firefly luciferase (Fluc) cDNA was inserted as 3′ exon of the ribozyme. The expression vector encodes the ribozyme under SV40 promoter.

For the analysis of *trans*-splicing products in cells, hTERT-expressing 293 cells were plated at 3.0×10^5 cells in 35 mm dishes 18-24 h prior to transfection. The cells were then transfected with 1 μg ribozyme expression vector, pRib39AS-Fluc, using 4 μl Lipofectamine (Invitrogen).

RACE PCR

Twenty four h after transfection of the specific ribozyme, total RNA was isolated from 293 cells with guanidine isothiocyanate supplemented with 20 mM EDTA. cDNA was constructed from 2 μg total RNA using random primer and AMV reverse transcriptase in the presence of 10 mM L-argininamide. Reaction products were purified by 2 M ammonium acetate ethanol precipitation and centrifugation at 1500 × g for 2 min with sephadex G-25 column. The cDNA products were then tailed at 37℃ for 15 min with 1 mM dATP, 25 mM $CoCl_2$, and 10 U terminal transferase (Roche) in 5X terminal transferase buffer. Terminal transferase was then inactivated at 80℃ for 3 min. PCR was performed with 2 μl of the reaction mixture which was cycled with 10 pmol adaptor 5′ primer and luciferase 3′ primer, 5′-GCGCAACTGCAACTCCGATAA (95℃, 30 sec; 55℃, 45 sec; 72℃, 1 min; × 30 cycles). Adaptor primers were designed from Takara with the known sequence of the manufacture. One fifth of the reaction product was analyzed with agarose gel electrophoresis. The amplified products were reacted with *Sca*I to remove any nonspliced ribozyme products, and moreover digested with *Eco*RI and *Hin*dIII at 37℃ for 3 h, then purified with phenol and ethanol. The final PCR products were subcloned in pUC19 and sequenced.

Results and discussion

Ribozyme structure

A specific group I ribozyme that recognizes uridine at position 39 (U39) of hTERT RNA was constructed because the

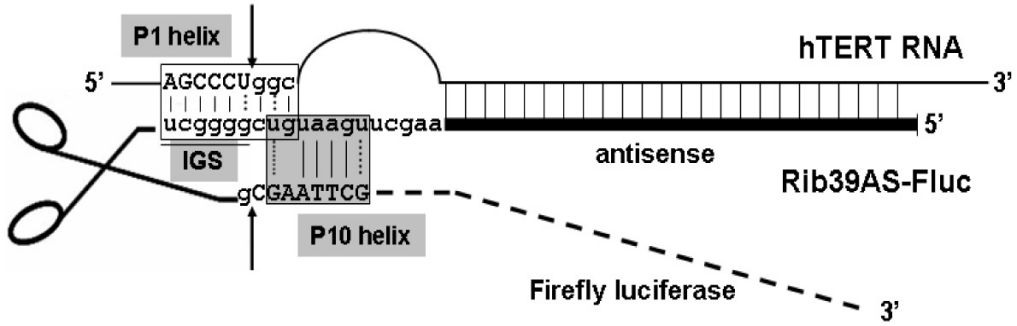

Fig 1. Schematic diagram of the *trans*-splicing ribozyme. The target hTERT RNA is presented. The *trans*- splicing ribozyme is shown with 3′ exon sequences, firefly luciferase RNA, capitalized. Potential base parings between the ribozyme and the target RNA are represented by vertical lines. Arrows depict 5′ and 3′ splicing sites.

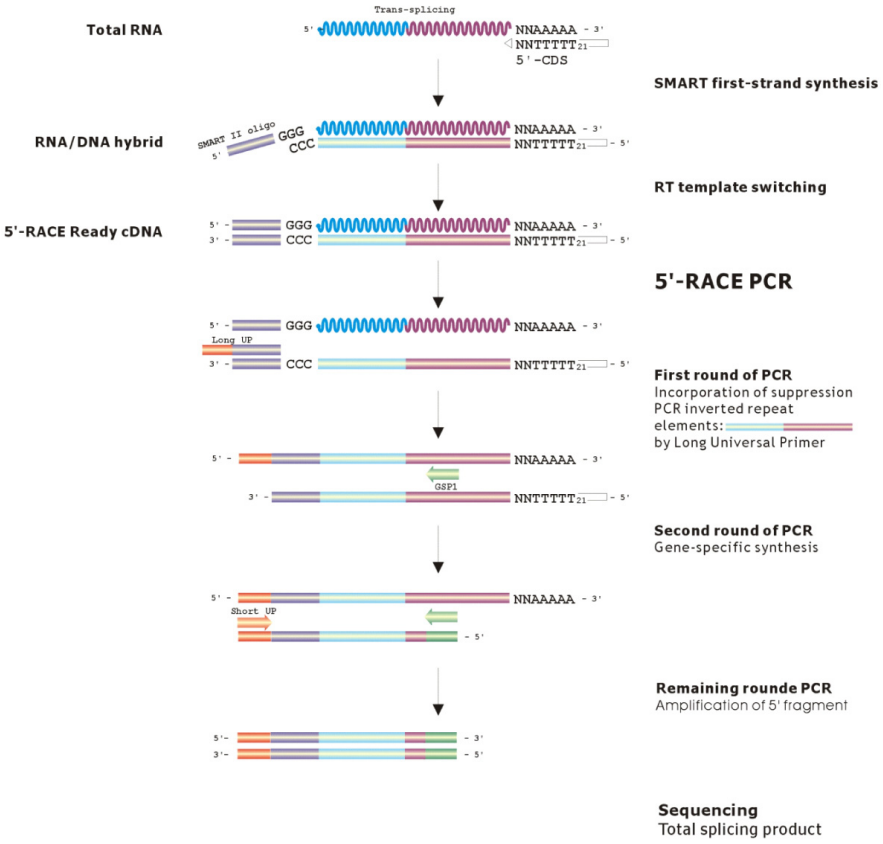

Fig 2. Schematic description of RACE PCR procedure

residues around U39 are predicted to be accessible to the ribozyme due to its presence in the loop region of the RNA from MULFOLD computer program (data not shown). IGS of the ribozyme was modified to contain an extension of P1 helix and an addition of 6 nt P10 helix combined with a 300 nt antisense sequence against the downstream region of the targeted uridine of the hTERT RNA for the functional expression in mammalian cells as described (7,10). In addition, firefly luciferase RNA was inserted as 3′ exon of the modified ribozyme, designated Rib39AS-Fluc (**Fig 1**). We constructed SV40 promoter-regulated expression vectors for expression of the ribozyme in cells.

This hTERT-targeting ribozyme was shown to accurately replace hTERT RNA with the intended sequence attached to the 3′ end of the ribozyme in cells as well as *in vitro* (7). In addition, the ribozyme can specifically inhibit telomerase function in the cells expressing the hTERT RNA by reducing the amount of the targeted RNA. Moreover, the ribozyme can selectively induce cytotoxin gene activity in cancer cells expressing the hTERT RNA and thereby specifically hampers the survival of these cells.

Specificity of intracellular *trans*-splicing reaction by specific ribozyme

RNA tagging approach was exploited to study the specificity of the ribozyme, Rib39AS-Fluc, in cells. In other words, by analyzing the total *trans*-spliced products in the cells, we determined whether the ribozyme was reacting with RNAs other than the targeted hTERT RNAs inside ribozyme-transfected cells. To address this question, an RT and 5′ end RACE (rapid amplification of cDNA ends) PCR was used to amplify the sequences of all tagged *trans*-splicing sites (**Fig 2**). Those amplified products were cloned, and the sequences of nineteen different clones were analyzed. The sequence analysis reveals that all of the *trans*-splicing products resulted from reactions only with the targeted hTERT RNA, suggesting that the ribozyme reacted with and tagged only

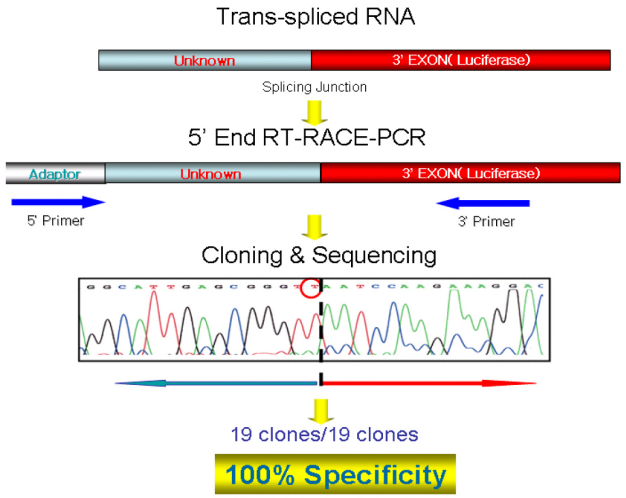

Fig 3. Specificity of intracellular *trans*-splicing reaction by specific *trans*-splicing ribozyme with 5′ end RACE PCR. The reaction products were analyzed by agarose gel electrophoresis. The amplified products from *trans*-splicing reaction between Rib39AS-Fluc and unknown RNA were isolated on the gel and cloned. The expected sequence around the splicing junction, indicated by the black dot line, was shown with the ribozyme recognition sequence in target RNA and the uridine at position 39 (circled).

targeted cellular transcripts, hTERT RNA, with its 3′ exon (**Fig 3**). Although assessment of more *trans*-spliced products might be needed, this result strongly implies that the group I intron-based ribozyme performed highly target RNA-specific *trans*-splicing activity in cellular milieu. Therefore, our ribozyme construct had minimized the degree to which transgene induction occurred via nonspecific targeting and RNA replacement.

Trans-splicing ribozymes will have the advantage over other anti-cancer modulators such that they can ruin the cancer-causing or cancer-associated specific transcripts via *trans*-cleavage of the RNAs and simultaneously induce cancer-destructive gene activity via specific RNA-targeting in the cancer cells. However, in order to successfully apply the strategy of ribozyme-mediated cytotoxin delivery to clinical cancer patient settings, specificity should be an important aspect. Most anti-cancer protocols are based on transcriptional targeting toward the expression of anti-cancer genes using tissue- and/or cancer-specific promoters/enhancers for specific tumor-targeted therapeutics (1). However, lack of specificity due to the possibility of leaky promoter activity and/or occlusion of the activity of the tissue-specific promoter or enhancer elements by enhancers of viral vector will limit their utility (11,12). In this study combined with the previous studies (7,8), group I-based *trans*-splicing ribozyme has been shown to stimulate transgene activity very specifically via cancer-specific RNA targeting. Together with the development of an efficient gene transfer system, a cancer specific RNA-targeted gene delivery strategy based on RNA replacement will be of effective and specific use for the treatment of human cancers.

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