JNBT
©2008INBDK

JNBT, Vol 5, No 1-2, pp. 13-17

Inhibition of telomerase expression *in vivo* by hTERT targeting *trans*-splicing ribozyme

Min-Sun Song[a], Jin-Sook Jeong[b] and Seong-Wook Lee[a,*]

[a]Department of Molecular Biology, Institute of Nanosensor and Biotechnology, Dankook University, Yongin 448-701, Korea
[b]Department of Pathology and Medical Research Center for Cancer Molecular Therapy, Dong-A University College of Medicine, Busan 602-103, Korea

ABSTRACT

A *trans*-splicing ribozyme which specifically targets and reprograms human telomerase reverse transcriptase (hTERT) RNA has been suggested as a potent anti-cancer gene therapeutic agent. In this study, we evaluated *in vivo* activity of the *trans*-splicing ribozyme in a nude mouse model of hepatocarcinoma-derived peritoneal carcinomatosis. To this effect, we constructed recombinant adenoviruses encoding for the hTERT-targeting *trans*-splicing ribozyme under the control of liver-selective phosphoenolpyruvate carboxykinase promoter or constitutively strong cytomegalovirus immediate early promoter. We observed that systemic delivery of both viruses efficiently inhibited telomerase expression in the established tumor nodules of the animal in a similar degree. Combined with target-dependent transgene induction activity of the *trans*-splicing ribozyme, the observed *in vivo* target gene deactivation activity indicates that RNA replacement using the *trans*-splicing ribozyme is an efficient anti-cancer approach.

Key words : *trans*-splicing ribozyme, cancer gene therapy, telomerase, hTERT

Introduction

The *Tetrahymena* group I intron-based ribozyme could target and reprogram a specific disease-associated RNA with therapeutic transgene transcript specifically in cells expressing the target RNA through *trans*-splicing reaction (1-3). We recently evidenced that the *trans*-splicing ribozyme could specifically and efficiently regress tumors in various different animal models through targeting and replacing of the cancer-specific transcript such as human telomerase reverse transcriptase (hTERT) RNA and then inducing therapeutic gene activity in the cancer cells expressing the RNA (4-7).

In addition to the targeted transgene activation, *trans*-splicing ribozyme will be more useful for anti-cancer therapeutics due to potential reduction activity of target gene through cleavage of the target RNA. In this study, we investigated whether telomerase activity could be significantly reduced in intraperitoneally hepatocarcinoma-established animals by delivery of the hTERT-targeting ribozyme. For delivery of the ribozyme *in vivo*, we constructed adenovirus that encoded the ribozyme under various promoters, systemically injected the virus in the tumor-embedded animal, and analyzed the ribozyme activity in the tumor nodules.

* Corresponding author :
Seong-Wook Lee
Tel : +82-31-8005-3195
Fax : +82-31-8005-4058
E-mail : SWL0208@dankook.ac.kr

Materials and Methods

Recombinant adenoviral vectors

Vectors which can express hTERT-specific *trans*-splicing ribozyme, Rib21AS targeting U21 on the hTERT, under the control of cytomegalovirus immediate early (CMV) or phos-

phoenolpyruvate carboxykinase (PEPCK) promoter were created as previously described (4,8). cDNA as a 3′ exon encoding for herpes simplex virus thymidine kinase (HSV*tk*) genes was incorporated into *Nru*I/*Xba*I downstream of the ribozyme expression construct. The resulting ribozyme cDNA flanked by the promoter and the HSV*tk* gene was inserted into the *Spe*I/*Bst*BI site of the pAdenoVator-CMV5-IRES-GFP shuttle vector (Qbiogene, Irvine, CA). Recombinant adenovirus vectors encoding for the ribozymes were then constructed through an *in vivo* homologous recombination procedure in bacteria (BJ5183) as follows. The shuttle plasmid first was linearized with *Pme*I, and then cotransformed into BJ5183 cells with an E1/E3 deleted adenoviral type5 backbone genome (pAdenoVator ΔE1/E3, Qbiogene). The recombinant vectors generated in the BJ5183 cells were isolated and linearized with *Pac*I. The linearized vectors were then transfected into 293 cells, and the generated recombinant adenoviruses were purified via three rounds of plaque purification and concentrated with Vivapure® AdenoPACK^{TM} 100 (Sartorius AG, Edgewood, NY). Titers of the recombinant adenovirus were analyzed using TCID50 method.

Animals

Four-to five-week-old male BALB/cAnNCrI nude mice (Orient Bio Inc., Seongnam, Korea) were used for this study. The animals were kept under specific pathogen-free conditions, acclimated to laboratory conditions for at least 1 week before use, and maintained in a Korean FDA animal facility in accordance with AAALAC International Animal Care policy (Accredited Unit-Korea Food and Drug Administration: Unit number-000996).

Peritoneal carcinomatosis model

For the peritoneal carcinomatosis model of Hep3B liver cancer cells, 2×10^7 Hep3B cells were injected intraperitoneally into male nude mice. The animals showed peritoneal carcinomatosis within 21 days, which was readily detectable by gross inspection using a binocular stereomicroscope. The mice after 2.5 weeks of intraperitoneal injection of Hep3B were randomized into the following groups: (1) Ad-PEPCK-LacZ, (2) Ad-PEPCK-Ribo-TK, and (3) Ad-CMV-Ribo-TK. We then intraperitoneally injected with 2.5×10^{10} v.p. of each adenovirus three times at two-day intervals. Two and half weeks after virus infection (total 5 weeks after Hep3B injection), the tumor nodules were collected and analyzed.

Immunohistochemistry for telomerase expression

For telomerase expression in the tumor nodules obtained from the above adenoviral-infected mice, immunohistochemistry was performed using the avidin-biotin-peroxidase complex technology with the DAKO EnVision Kit (Dako, Carpinteria, CA) on formalin-fixed and paraffin-embedded specimens that were dewaxed and rehydrated with graded alcohols. Endogenous peroxidase was prevented through the immersion of the sections in 3% aqueous hydrogen peroxide for 10 min. Antigens were retrieved via microwave treatment for 10 min in 10 mM of citrate buffer, pH 6.0. Primary antibodies against hTERT (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted into 1:100 and incubated for 1 h at room temperature. The sections were then treated with the secondary antibody and the avidin-biotin-peroxidase complex. The slides were counterstained with H&E.

Results and Discussion

To evaluate inhibitory effects of the hTERT-targeting *trans*-splicing ribozyme against telomerase expression in liver tumor tissue in animal, we constructed an adenoviral vector encoding for the specific ribozyme with HSV*tk* gene under the control of a PEPCK promoter (Ad-PEPCK-Ribo-TK) (**Fig 1**). In our previous *in vitro* study, the expression of the specific ribozyme with HSV*tk* under the PEPCK promoter was the most effective and specific with regard to the retardation of the growth of hTERT$^+$ liver cancer cells, when compared with other liver-specific promoters [8]. For a positive control and comparison of promoter activity, we generated an adenoviral vector encoding for the ribozyme under the control of the CMV promoter (Ad-CMV-Ribo-TK). In addition, we constructed adenovirus encoding for the bacterial β-galactosidase (*lac*Z) gene (Ad-PEPCK-LacZ,) as a negative control.

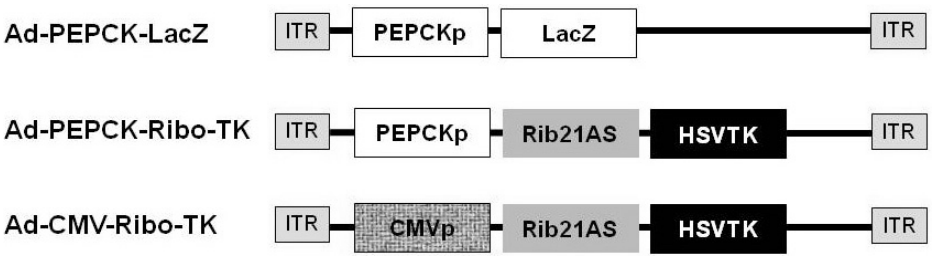

Fig 1. Schematic diagram of recombinant adenoviral vectors

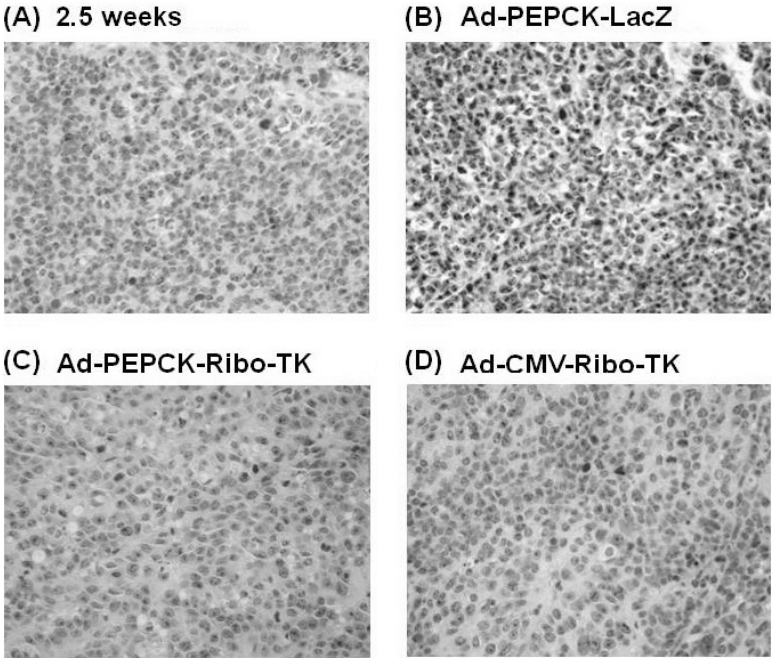

Fig 2. Representative immunohistochemical findings are shown for hTERT protein expression (Anti-hTERT, ×400) in the tumor masses of pretreatment group (A, 2.5 weeks) and groups which were injected with recombinant adenovirus (B, Ad-PEPCK-LacZ; C, Ad-PEPCK-Ribo-TK; D, Ad-CMV-Ribo-TK).

To evaluate the activity of the hTERT-targeting ribozyme *in vivo*, we established a human hepatocarcinoma-derived peritoneal carcinomatosis model through the intraperitoneal injection of Hep3B cells into nude mice. Multiple Hep3B tumor nodules were then generated in the mice abdomen 3 weeks after intraperitoneal tumor administration.

We infected recombinant adenoviral vectors intraperitoneally three times into the tumor-established mice, and then analyzed and compared telomerase expression in the liver tumor tissues using immunohistochemistry method after two and half week of first viral injection (**Fig 2**). In the mice treated with Ad-PEPCK-LacZ, telomerase expression in the tumor nodule was increased, as compared with pretreatment mice (**Fig 2A** and **2B**). Average tumor mass of mice with Ad-PEPCK-LacZ was sixteen times heavier than the mice of pretreatment group (data not shown). This suggests that tumors were grown very aggressively in the mice treated with the negative control adenoviral vector, resulting in increasing in telomerase expression. In sharp contrast, the Ad-PEPCK-Ribo-TK-treated mice, versus the pretreated or Ad-PEPCK-LacZ-treated mice, had markedly reduced telomerase expression in the tumor nodules (**Fig 2C**). Ad-CMV-Ribo-TK-treated mice also showed much decreased telomerase expression in the tumor nodules. No significant difference was observed in terms of inhibition

of telomerase expression between PEPCK- and CMV-derived expressions of hTERT-targeting *trans*-splicing ribozyme in the established liver tumors in animal. Consistent with the immunohistochemistry result, amount of hTERT mRNA was significantly reduced up to ~75% in the tumor nodules of Ad-PEPCK-Ribo-TK- and Ad-CMV-Ribo-TK-infected mice, when compared with mice infected with Ad-PEPCK-LacZ (data not shown).

These results indicate that the PEPCK promoter-driven *trans*-splicing ribozyme efficiently reduced target gene expression in liver tumor tissue *in vivo*, with an efficacy comparable to that associated with the strong CMV promoter-driven ribozyme.

A major advantage of RNA replacement approach using trans-splicing ribozyme for cancer therapy is that by targeting a tumor specific pre-mRNA, expression of the therapeutic gene product is stimulated specifically in cancer cells expressing the RNA [9]. In addition, significant decrease in the expression of the target gene product, as observed in this study, will effect in an additive and synergistic anticancer result with high efficacy as well as specificity.

Acknowledgements

This work was supported by the Korea Science & Engineering Foundation grant (M1053400000406N3400-00410) and grant from the National R&D Program for Cancer Control by Korean Ministry of Health & Welfare. M.S. Song is a recipient of Brain Korea 21 fellowship.

Reference

(1) Lewin AS, Hauswirth WW. (2001) Trends Mol Med **7**, 221
(2) Long MB, Jones JP III, Sullenger BA, Byun J. (2003) J Clin Invest **112**, 312
(3) Fiammengo R, Jaschke A. (2005) Curr Opin Biotechnol **16**, 614
(4) Kwon BS, Jung HS, Song MS, Cho KS, Kim SC, Kimm K, Jeong JS, Kim IH, Lee SW (2005) Mol Ther **12**, 824
(5) Hong SH, Jeong JS, Lee YJ, Jung HI, Cho KS, Kim CM, Kwon BS, Sullenger BA, Lee SA, Kim IH. (2008) Mol Ther **16**, 74
(6) Jeong JS, Lee SW, Hong SH, Lee YJ, Jung HI, Cho KS, Seo HH, Lee SJ, Park S, Song MS, Kim CM, Kim IH. (2008) Clin Cancer Res **14**, 281
(7) Song MS, Jeong JS, Bam G, Lee JH, Won YS, Cho KS, Kim IH, Lee SW. (2008) Cancer Gene Ther In press
(8) Song MS, Lee SW. (2006) FEBS Lett **580**, 5033
(9) Wood M, Yin H, McClorey. (2007) PLOS Genetics **3**, e109.

(Received Jul 6, 2008; Accepted Aug 15, 2008)