# Antitumor Effects of Systemically Delivered Adenovirus Harboring Trans-Splicing Ribozyme in Intrahepatic Colon Cancer Mouse Model

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## Abstract

**Purpose:** Our previous studies suggested that human telomerase reverse transcriptase (hTERT) RNA-targeting trans-splicing ribozyme could be a useful tool for cancer gene therapy. Here, we investigated whether adenoviruses harboring this ribozyme can be systemically delivered to mice, and whether they selectively mark tumors expressing hTERT and sensitize them to ganciclovir treatments.

**Experimental Design:** We constructed adenoviral vectors containing modified hTERT-targeting trans-splicing ribozyme with downstream reporter gene (Ad-Ribo-LacZ) or suicide gene (Ad-Ribo-HSVtk) driven by a cytomegalovirus promoter. The tumor-specific trans-splicing reaction and the tumor-killing effect of adenoviruses harboring ribozyme were investigated both in vitro and in vivo using mice with intrahepatic colon cancer metastasis via systemic administration. The safety of systemic administration of the viruses was also evaluated.

**Results:** We showed that Ad-Ribo-LacZ, when injected i.v., performs a highly specific trans-splicing reaction on hTERT mRNA and that it selectively marks tumors expressing hTERT in mice. More importantly, i.v. injection of Ad-Ribo-HSVtk plus ganciclovir significantly reduced tumor burden, with minimal liver toxicity, in mice with metastatic liver cancer, compared with the untreated group ($P = 0.0009$). Moreover, animals receiving Ad-Ribo-HSVtk showed improved survival compared with controls ($P < 0.0001$).

**Conclusions:** This study shows that systemically delivered adenovirus harboring trans-splicing ribozyme can recognize cancer-specific transcripts and reprogram them to combat the cancer cells. Use of trans-splicing ribozymes seems to be a potentially useful gene therapy for cancer.

**Colorectal cancer** is one of the most common cancers worldwide, both in terms of incidence and mortality (1). Despite progress in technologies for diagnosis and screening, liver metastasis occurs in 20% to 70% of patients with colorectal cancer and is the leading cause of death. Surgical resection or surgery coupled with systemic chemotherapy of liver metastasis is the treatment currently available for these patients (2). However, only 25% of liver metastases are amenable to surgery and ~60% of those patients relapse (3). Therefore, there remains a continued need for a universally effective treatment for cancer and for new therapeutic approaches.

Ribozymes are potential tools for gene therapy because of their ability to discriminate targets that differ by a single nucleotide, and splice and edit RNA transcripts (4, 5). The self-splicing group I intron from *Tetrahymena thermophila* was shown to catalyze its own excision without the aid of proteins and also perform a trans-splicing reaction that specifically cleaves target RNAs, leading to revision of gene information (6–8). In performing this trans-splicing reaction, group I ribozyme recognizes and binds a substrate RNA, cleaves the targeted RNA, and splices a 3′ exon sequence that is initially attached to the 3′ end of the ribozyme (9–11). The trans-splicing ribozyme has therefore been used to repair defective RNA messages such as mutant transcripts associated with genetic diseases (12–14) and cancer (15, 16).

A previous study suggested that group I trans-splicing ribozyme can be used to splice therapeutic RNA sequences...
onto a target transcript, resulting in the production of therapeutic gene activity specifically in the target RNA-associated cells (17). Extending this concept, we reported that human telomerase reverse transcriptase (hTERT) RNA-targeting trans-splicing ribozymes could be engineered to induce the hTERT-dependent expression of either a reporter gene (β-galactosidase; LacZ) or a prodrug-responsive gene (herpes simplex virus thymidine kinase; HSVtk) in tumor cells (18). Furthermore, we recently showed that adenoviral vector harboring the ribozyme can either selectively mark tumor cells or selectively make tumor cells sensitive to ganciclovir in hTERT-expressing cancer cell lines or tumor xenografts (19). These observations provide the basis for a novel approach to cancer gene therapy and suggest that trans-splicing ribozymes can be used as anticancer agents that recognize cancer-specific transcripts and reprogram them to combat the cancerous cells.

We have now further extended these observations and confirmed the feasibility of this approach by showing that systemically administered adenoviruses harboring hTERT-targeting trans-splicing trigger hTERT-dependent cytotoxicity in mouse model of hepatic colorectal oligometastases.

Materials and Methods

Generation of recombinant adenoviruses expressing trans-splicing ribozyme. Recombinant adenoviral vectors used in this study were produced as previously described (19), amplified in HEK293 cells, and purified by double cesium chloride gradient ultracentrifugation (20). We designated the adenovirus derivatives containing cytomegalovirus (CMV) promoter–driven ribozyme flanked by HSVtk as Ad-Ribo-HSVtk and LacZ as Ad-Ribo-LacZ, respectively. As a control, we used adenoviruses with HSVtk (Ad-HSVtk) or LacZ (Ad-LacZ) driven by the CMV promoter only. The Ad-Mock, which has the adenovirus backbone only, was used as negative control virus (Fig. 1A).

Cell studies. Cells used in this study were derived from human colon tumors (HT-29 and SNU-407), hepatoma (SNU-398), lung carcinoma (SBC-5), and telomerase-negative normal human lung fibroblasts (IMR-90). SNU-398 and SNU-407 were kindly provided by Dr. Jae-Gahb Park and Ja-Lok Ku (Korean Cell Line Bank, Seoul, Korea); HT-29 and IMR-90 cells were purchased from American Type Culture Collection; and SBC-5 cells were from Japanese Collection of Research Bioresources. The cells were cultured following the instructions provided by cell banks. Cell proliferation [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] assay was done using CellTiter 96 AQueous One solution reagent (Promega) according to the manufacturer’s instruction.

Quantitation of endogenous hTERT mRNA levels by real-time PCR. The endogenous hTERT mRNA levels were determined by real-time PCR using SYBR-Green Core Reagent Kit (Molecular Probes). Reverse transcription-PCR (RT-PCR) was done on total RNA (5 μg) obtained from HT-29 cells 2 days after infecting them with 200 multiplicities of infection of Ad-Mock or Ad-Ribo-HSVtk. The sequences of the primers used for the hTERT amplification were 5′-CGGAA-GAGTGTCTGGAGCAA-3′ and 5′-GGATGAAGCGGAGTCTGGA-3′. The conditions for the PCR were as follows: 95°C for 30 s, 55°C for 40 s, and 72°C for 1 min, for 40 cycles. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control for the standard curve in the reaction mix. To correct for minor variations in cDNA loading, we adjusted the threshold levels found in the GAPDH reaction.

Animal studies. Approval for the animal studies described here was obtained from the Animal Research Ethics Committee of Korean Food and Drug Administration in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International Animal Care policy (accredited unit-Korean Food and Drug Administration: unit number-000996). The animals were kept under specific

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**Fig. 1.** Therapeutic efficacy and specificity of Ad-Ribo-HSVtk in vitro. A, schematic diagrams of adenoviruses (Ad) used in this study. B, RNA analysis in the adenovirus-transfected cells. The hTERT-positive HT-29 and hTERT-negative IMR90 cells were transfected with Ad-Mock, Ad-LacZ, Ad-Ribo-HSVtk, or Ad-HSVtk at 150 multiplicities of infection (m.o.i.). The production of transgene RNA (TSM) is represented by amplification of HSVtk RNA (HSVtk) or spliced RNA (TSM), respectively. As an internal control, human GAPDH RNA was amplified (GAPDH). C, decrease of endogenous hTERT mRNA expression in the Ad-Ribo-HSVtk–infected HT-29 cells. Relative hTERT mRNA was determined with real-time PCR and quantitated as a percentage of the amount of cells transfected with control Ad-Mock. Columns, average of three independent determinants; bars, SD. **D,** efficacy of hTERT-specific cytotoxicity by Ad-Ribo-HSVtk in various cell lines. Either 5 × 10⁴ cells per well of cancer cell lines originated from colon (HT-29 and SNU-407), liver (SNU-398), and lung (SBC-5) were transfected with Ad-Ribo-HSVtk, Ad-HSVtk, or Ad-Mock at various multiplicities of infection. Points, mean for triplicate assays; bars, SD.
pathogen-free conditions and maintained in Korean Food and Drug Administration animal facility. Four- to five-week-old male BALB/cAnNCrj-nu/nu nude mice and female BALB/c mice (Charles River, Japan) were acclimated to laboratory conditions for at least 1 week before use.

Liver metastasis model. A mouse model of liver metastasis was established by intrasplenic inoculation of HT-29 cell as previously described, with minor modifications (21). Two days before the inoculation of cells, 50 μL of anti-asialo GM1 rabbit antibody (Wako Pure Chemicals) was administered i.p. to inhibit natural killer cell activity. A small longitudinal left upper flank incision was made to visualize the spleen, and 2 × 10⁶ HT-29 cells in 50 μL of PBS were injected under the spleen capsule with a 29-gauge needle. After removal of the needle, the injection site was pressed with an aseptic cotton sponge for several minutes to prevent further leakage. Then, the spleen was returned into abdominal cavity and the peritoneum and abdominal wall were sutured with silk. The animals showed multiple intrahepatic tumor nodules, easily detectable by gross inspection, within 8 to 10 days.

Adenovirus delivery and ganciclovir treatment. Adenoviral vectors were diluted to 100 μL volume with PBS before injection and injected into the mice through the tail vein with a 29-gauge needle. One day later, the animals were injected with ganciclovir (50 mg/kg) i.p., twice daily for 10 to 15 days.

Measurement of tumor growth. After euthanizing the animals, we measured the total mass of their liver lobes of each animal. Whole liver lobes were then fixed in 4% neutral buffered formalin, sliced to 5-mm thickness, and embedded in paraffin. The paraffin blocks were cut into 4-μm sections and stained with H&E. All sections of each lobe were examined, photographed, and the tumor mass was calculated. Three investigators independently did histologic determinations of the tumor sections. We calculated the mean values of tumor fraction in each group and estimated the actual tumor weights by the following formula: tumor weight = total liver mass (mg) × measured tumor fraction.

PCR analyses. The presence of adenoviruses was detected by PCR for inverted terminal repeat region of adenovirus and expression levels of primary transcripts of transfected viruses were determined by RT-PCR of LacZ or HSVtk RNAs as described in our previous report (19).

The production of trans-spliced molecules by adenovirus harboring ribozyme was investigated by nested RT-PCR. Total RNAs extracted from cells, livers, or tumor tissues transfected by adenoviruses were used for cDNA synthesis with primer for LacZ (5′-ACGGCAATTCCGCGCACATCTGAA-3′) or HSVtk (5′-CCGGGATCTCTACGACGTTGCTCCCATC-3′) in the presence of 10 mmol/L L-argininamide. The first PCR was done with a 5′ primer specific for the trans-splicing junction (5′-GGGGAATTCCGGCGGAGGCTCCGTCCCT-3′) and with a 3′ primer for the LacZ (5′-CCGCTCCAGCCGAGGCTCCGTCCCT-3′) or HSVtk (5′-GTATCGGCGGCGGCGCTGCTA-3′), respectively. Conditions for PCR reaction were as follows: preheating at 95°C for 3 min, followed by 30 cycles for PCR (95°C, 30 s; 57°C, 30 s; 72°C, 30 s), and final extension at 72°C for 5 min. Then, the second PCR was carried out with inward primers for LacZ (5′-CCTGCCTCTGAAACCGCCTGAC-3′) and 5′-GGGCTCGACGACGTTGCTGATAGCGGTCC-3′) or HSVtk (5′-GCTGGCGGCGGCTGCTA-3′) and 5′-CAGTACGGGCGGCTTTTTCC-3′), respectively. For this amplification, PCR mixtures were subjected to 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and polymerization for 1 min at 72°C. To evaluate specificity of trans-splicing reaction of ribozyme, the amplified product was eluted, cloned into pBluescript (SK+)/-, and sequenced.

Results

Ad-Ribo-HSVtk with ganciclovir treatment induces cytotoxicity in an hTERT-dependent manner. We investigated whether the cytotoxicity delivered by Ad-Ribo-HSVtk is due to a specific and high-fidelity trans-splicing reaction with hTERT RNA in hTERT-positive cells. The Ad-Ribo-HSVtk infection generated TSM in hTERT-positive HT-29 cells but not in hTERT-negative IMR-90 cells, although ribozymes (represented as HSVtk RNA) were expressed in the IMR-90 cells (Fig. 1B). Then, we assessed the efficacy of trans-splicing reaction by determining that Ad-Ribo-HSVtk could reduce intracellular levels of target RNA. As shown, we observed a 56.2% reduction in the steady-state level of endogenous hTERT mRNA in the Ad-Ribo-HSVtk–infected HT-29 cells, when compared with Ad-Mock–treated cells (Fig. 1C).

After showing that the trans-splicing ribozyme in adenovirus effectively replaced the target RNA by hTERT-specific transsplicing reaction, we examined for the ribozyme would trigger cytotoxicity in other hTERT-expressing cancer cell lines from various origins. We infected colon cancer (HT-29 and SNU-407), hepatoma (SNU-398), and lung cancer (SBC-5) cells with Ad-Ribo-HSVtk, Ad-HSVtk, or Ad-Mock at various multiplicities of infection and did MTS assay to determine cytotoxicity. We observed that Ad-Ribo-HSVtk reduced cell survivals in a viral dose-dependent manner with almost the same efficacy as Ad-HSVtk in all cell lines tested (Fig. 1D).

Systemically delivered Ad-Ribo-LacZ does not result in reporter gene expression in non–tumor-bearing mouse liver. We injected Ad-Ribo-LacZ [1 × 10¹¹ v.p. (viral particles)] into female BALB/c mice via tail veins (n = 9 per group); sacrificed three mice in each group 2, 7, and 14 days later; and examined their liver tissues. We chose only liver tissues for examination, because previous studies that analyzed expression of the CMV-driven LacZ gene in non–tumor-bearing mouse liver. We injected Ad-Ribo-LacZ [1 × 10¹¹ v.p. (viral particles)] into female BALB/c mice via tail veins (n = 9 per group); sacrificed three mice in each group 2, 7, and 14 days later; and examined their liver tissues. We chose only liver tissues for examination, because previous studies that analyzed expression of the CMV-driven LacZ gene in non–tumor-bearing mouse liver. We injected Ad-Ribo-LacZ [1 × 10¹¹ v.p. (viral particles)] into female BALB/c mice via tail veins (n = 9 per group); sacrificed three mice in each group 2, 7, and 14 days later; and examined their liver tissues. We chose only liver tissues for examination, because previous studies that analyzed expression of the CMV-driven LacZ gene in non–tumor-bearing mouse liver.
reporter gene in various mouse tissue by a variety of injection routes noted that >80% of virus injected in tail vein is sequestered in the liver (22). Using PCR analysis with vector-specific or LacZ RNA primers, we detected the presence of transfected adenoviruses and primary transcript of Ad-Ribo-LacZ and Ad-LacZ in the liver. PCR analysis detected inverted terminal repeat and LacZ RNA in liver tissues transfected by Ad-Ribo-LacZ and Ad-LacZ, suggesting that the viruses are efficiently dispersed in the mouse liver (Fig. 2A).

We further analyzed transgene expression by X-gal staining. Interestingly, mice injected with Ad-Ribo-LacZ displayed little or no reporter gene activity even in virus-laden tissues. Unlike in Ad-Ribo-LacZ–transfected mice, we observed diffuse and extensive expression of β-galactosidase in the liver of Ad-LacZ–transfected mice (Fig. 2B). We could not observe reporter gene expression on day 14, suggesting transient expression of transgene in adenovirus.

Ad-Ribo-LacZ specifically targets hTERT-expressing tumor. We tested whether hTERT-recognizing trans-splicing ribozyme targets hTERT-positive tumors developed in livers of BALB/c nude mice 12 days after intrasplenic injection of HT-29 cells (2 × 10⁶ per mouse). The tumor-bearing mice were then injected with Ad-Ribo-LacZ (1 × 10¹¹ v.p./mouse) via a tail vein (n = 6), and sacrificed 2 days later for examination of liver tissues. We examined reporter gene expression patterns in frozen sections of liver tissues after X-gal staining. As expected, we observed distinct β-galactosidase expression in metastasized tumors, whereas we detected almost no X-gal staining in normal mouse liver tissue areas, although some nonspecific blue-stained cells were observed (Fig. 3A, X-gal). To confirm that X-gal–positive regions are tumor nodules, we examined corresponding sections after H&E staining and found that developed tumors with loose reticulated inner side necrosis (Fig. 3A, H&E, marked by lines) were well matched with X-gal–positive blue-stained regions.

Next, we examined, using RT-PCR, whether Ad-Ribo-LacZ specifically targeted hTERT-positive tumor nodules. We found correctly targeted TSMs only in tumor nodules transfected by Ad-Ribo-LacZ. However, no TSM was detected in nontumorous mouse liver tissue, suggesting that the trans-splicing reaction specifically targeted human TERT only in vivo (Fig. 3C). We did not observe significant differences between individuals within groups. Sequence analysis of the amplified fragment confirmed that the Ad-Ribo-LacZ correctly targeted hTERT at the predicted site (Fig. 3D).

Determination of toxicity threshold of Ad-Ribo-HSVtk for systemic administration. We then determined maximal allowable dose of Ad-Ribo-HSVtk that can be administered to non–tumor-bearing mice without causing liver toxicity. For this purpose, we injected Ad-Ribo-HSVtk into male BALB/c nude mice via tail veins followed by ganciclovir treatment and monitored their survivals, compared with similarly treated Ad-HSVtk and Ad-Mock–injected groups. Mice were treated with 1 × 10¹¹ v.p. of viruses (n = 15/group) and, starting next day, injected with ganciclovir for 10 consecutive days. Animals injected with Ad-HSVtk died 6 days after the first injection of ganciclovir, whereas those injected with Ad-Ribo-HSVtk survived as long as 15 days, indicating lower toxicity of Ad-Ribo-HSVtk (Fig. 4A, a). To determine the maximal nontoxic dose of Ad-Ribo-HSVtk, we repeated the same experiments with decreasing viral doses (Fig. 4A, b and c). At a dose of 0.25 × 10¹¹ v.p., >90% of mice survived in the Ad-Ribo-HSVtk and Ad-Mock groups during and 30 days after treatment, whereas none survived for >9 days after ganciclovir treatment in Ad-HSVtk group (Fig. 4A, c). We conclude that 0.25 × 10¹¹ v.p./mouse is the maximal nontoxic dose for systemic administration of Ad-Ribo-HSVtk in mice.

Hepatotoxicity of Ad-Ribo-HSVtk in non–tumor-bearing athymic mice. We investigated whether the optimal dose of Ad-Ribo-HSVtk determined above causes any liver toxicity by evaluating liver morphology and liver enzyme levels [serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT)]. One day after injecting 0.25 × 10¹¹ v.p. of Ad-Ribo-HSVtk, Ad-HSVtk, and Ad-Mock (n = 15/group) into mice tail veins, we treated them with ganciclovir until euthanization. We sacrificed five mice from each group on days 2, 7, and 14 after the first ganciclovir treatment and collected liver and serum samples. In the Ad-HSVtk–treated group, only three mice survived to day 7 and none survived to day 14.

Microscopically, we observed no significant liver toxicity in Ad-Ribo-HSVtk–treated mice compared with controls during the observation period. In contrast, the livers of mice injected with Ad-HSVtk showed occasional mitoses at day 2 and massive necrosis with inflammation at day 7 (Fig. 4B). Also, there was no significant elevation of serum ALT/AST levels (IU/mL) in Ad-Ribo-HSVtk group compared with Ad-Mock group for.

Fig. 2. Reporter gene expression patterns of systemically delivered Ad-Ribo-LacZ and Ad-LacZ in the liver tissue of non–tumor-bearing mouse. A, PCR analysis of injected viruses in liver tissue of mice. Liver tissues were obtained for analysis at 2, 7, and 14 d after injection of Ad-Ribo-LacZ, Ad-LacZ, or PBS via tail vein. The presence of injected viruses and transgene expression were detected by PCR for the inverted terminal repeat region (ITR) and RT-PCR for LacZ RNA (LacZ), respectively. The amplified mouse GAPDH RNA (mGAPDH) served as an internal control. B, representative histochemical findings in liver tissues of A. After staining with X-gal, tissues were observed with the aid of a light microscope and photographed.
14 days. In contrast, statistically significant \( P = 0.0025 \) by Wilcoxon rank-sum test high levels in Ad-HSV\( \text{tk} \) (ALT/AST; 1,189 ± 158/978 ± 197) at day 7 compared with Ad-Mock (ALT/AST; 118 ± 134/183 ± 109) group were observed (Fig. 4C). Thus, treatment with Ad-Ribo-HSV\( \text{tk} \)/ganciclovir seems to be well tolerated with no liver toxicity, at the viral dose of \( 2.5 \times 10^{11} \) v.p., when delivered i.v.

**Regression in intrahepatic colorectal cancer by systemic delivery of Ad-Ribo-HSV\( \text{tk} \) plus ganciclovir.** We examined whether established metastases were regressed after systemic delivery of Ad-Ribo-HSV\( \text{tk} \) followed by ganciclovir treatment. Forty-nine mice were injected intrasplenic with HT-29 cells and randomly sorted into four experimental groups (\( n = 10/\text{group} \)) and a pretreatment group (\( n = 9 \)). Eleven days after inoculation with HT-29 cells, we injected the experimental groups with \( 0.25 \times 10^{11} \) v.p. of Ad-Ribo-HSV\( \text{tk} \), Ad-HSV\( \text{tk} \), Ad-Mock, and 100 \( \mu \)L of PBS, respectively. One day after virus injection (day 12), the three experimental groups were given ganciclovir or PBS for 10 days. The mice in the pretreatment group, in which mice received HT-29 and represented status of established tumors in liver at the start of treatment, were sacrificed on day 12 after cell injection and analyzed for tumor loads. All the mice injected with Ad-HSV\( \text{tk} \) died during the ganciclovir treatment within 10 days after start of ganciclovir treatment, apparently from liver failure due to nonspecific HSV\( \text{tk} \) expression. In contrast, 7 mice from the Ad-Ribo-HSV\( \text{tk} \) group, 10 from Ad-Mock, and 9 from PBS group survived until 29 days after cell injection (Fig. 5A).

These surviving were sacrificed and their liver tumors were weighed and assessed histopathologically. The average of tumor masses (mg) observed were as follows: 69.7 ± 21.9 for pretreatment group (median = 79.1, \( n = 9 \)), 4.5 ± 3.3 for Ad-Ribo-HSV\( \text{tk} \) group (median = 6.1, \( n = 7 \)), 96.2 ± 84.8 for Ad-Mock group (median = 76.2, \( n = 10 \)), and 139.6 ± 177.5 for PBS group (median = 78.0, \( n = 9 \)). We compared the Ad-Ribo-HSV\( \text{tk} \) and pretreatment groups for treatment effects and noted significant tumor load reduction in the Ad-Ribo-HSV\( \text{tk} \) group, compared with the pretreatment group (Wilcoxon rank-sum test, \( P = 0.0009 \)). Furthermore, there were significant differences in tumor weights among three treatment groups (Kruskal-Wallis test, \( P = 0.0015 \)). Pairwise comparisons using Wilcoxon rank-sum test also showed significant tumor reduction in Ad-Ribo-HSV\( \text{tk} \) compared with Ad-mock (\( P = 0.0006 \)) and PBS (\( P = 0.0036 \)), respectively (Fig. 5B).

We also observed that basophilic tumor regions, detected by H&E staining, matched with CK20-positive regions of the tumor, suggesting that established tumor nodules originated from HT-29 cells. The livers of mice treated with Ad-Ribo-HSV\( \text{tk} \) had either tiny or no metastatic tumors, whereas the livers from pretreatment had variably sized tumor nodules (Fig. 5C).

**Survival benefits from long-term Ad-Ribo-HSV\( \text{tk} \)/ganciclovir treatment.** We evaluated the ability of the Ad-Ribo-HSV\( \text{tk} \) to modulate survival of intrahepatic colon cancer–bearing animals. For these studies, the liver metastasis model was established by intrasplenic inoculation of HT-29 cell as previously described. Eleven days after HT-29 cells inoculation, we treated the animals with either the control or the HSV\( \text{tk} \)-encoding adenovirus and evaluated their survivals after ganciclovir treatment. We injected half of maximal allowable dose of adenovirus \( (0.125 \times 10^{11} \text{v.p.}) \) thrice at 30-day intervals. Experimental groups (\( n = 15 \) per group) included animals receiving tumor only (PBS), tumor followed by control adenoviruses (Ad-Mock and Ad-HSV\( \text{tk} \)), and Ad-Ribo-HSV\( \text{tk} \). We found significant differences in long-term survival after the
first virus injection among four treatment groups. Ad-HSVtk group had the worst survival and Ad-Ribo-HSVtk group had the most improved survival (Fig. 6A, log-rank test, \( P < 0.0001 \)). Further analysis using Cox proportional hazards model revealed that mice treated with Ad-HSVtk had 18 times higher risk of death compared with the ones that received tumor only (Ad-HSVtk versus PBS; hazard ratio, 18.3; 95% confidence interval, 5.5-61.1). In contrast, Ad-Ribo-HSVtk group had 74% lower risk of death compared with PBS group, whereas no significant difference in risks of death was observed in Ad-Mock group (Table 1).

We euthanized the mice surviving in Ad-Ribo-HSVtk therapy group (\( n = 10 \)) at day 100 after first virus injection and examined morphologically for evidence of tumor burden. We sacrificed two mice each from tumor only and control virus (Ad-Mock) groups, at day 88 after first virus injection for comparison. We observed multiple large tumor nodules on the surface of livers in control groups. In contrast, animals treated with the Ad-Ribo-HSVtk and ganciclovir had dramatically decreased tumor burdens with a reduction in the number and size of residual tumor nests (Fig. 6B).

**Discussion**

Because adenovirus has been used in several *in vitro* and *in vivo* preclinical analyses (23–26) and shown to transduce hepatocytes efficiently (27), we constructed adenoviral vectors containing modified hTERT-targeting *trans*-splicing ribozymes...
with downstream LacZ (Ad-Ribo-LacZ) or HSVtk (Ad-Ribo-HSVtk) driven by a CMV promoter. We showed that this viral system successfully delivered a ribozyme that did a highly specific trans-splicing reaction on hTERT RNA, resulting in hTERT-dependent expression of therapeutic or reporter gene in vitro and in vivo. This study thus showed that systemic delivery of adenovirus with hTERT-targeting ribozyme significantly reduces tumor burdens and provides survival benefits in mouse cancer model.

First, we determined that the specificity of the trans-splicing ribozyme for hTERT-expressing tumor cells is retained in the context of an adenoviral vector and these viruses are efficiently dispersed in the liver of mice. In the cellular studies, we confirmed that trans-splicing reaction is hTERT-specific by showing hTERT-dependent production of TSM and reduction of target RNA expression (endogenous hTERT mRNA) in hTERT-positive HT-29 cells. Real-time PCR study showed a 56.2% reduction of hTERT mRNA expression level, suggesting that >50% of target RNAs were involved in trans-splicing reaction. Moreover, we observed that Ad-Ribo-HSVtk reduced cell survivals with almost the same rate as Ad-HSVtk not only in colon cancer cell line HT-29 but also in another colon cancer (SNU-407), liver cancer (SNU-398), and lung cancer (SBC-5) cell lines, compatible with our previous studies (19). These results suggest that hTERT-targeting ribozyme could have potential use as universally applicable anticancer agent, regardless of the type of cancer. Furthermore, we evaluated whether systemically administered Ad-Ribo-LacZ provides tumor-specific reporter gene expression in mouse tumor model and confirmed that this viral system successfully delivered a

Fig. 5. Suppression of tumor growth by single injection of Ad-Ribo-HSVtk plus ganciclovir in metastatic colorectal cancer in the liver. A, survival of mice with metastatic cancer in the liver injected with 0.25 × 10⁸ v.p. of viruses and treated with ganciclovir (GCV). Ten mice for each group were injected with Ad-Ribo-HSVtk, Ad-HSVtk, Ad-Mock, or PBS via tail vein, on 10 d after intrasplenic inoculation of HT-29 cells. Arrow and arrowheads, virus injection and ganciclovir treatment, respectively. *: comparisons of intrahepatic tumor masses from mice of pretreatment and treatment groups. Tumor masses of the pretreatment (n = 9), the Ad-Ribo-HSVtk (n = 7), the Ad-Mock (n = 10), and PBS (n = 9) groups were determined and plotted as dots. Columns, average tumor mass; bars, SD. **, P = 0.0009 by Wilcoxon rank-sum test of Ad-Ribo-HSVtk compared with pretreatment group. ***, P = 0.0015 by Kruskal-Wallis test compared with corresponding control groups (PBS and Ad-Mock). C, representative histology of liver from pretreatment and Ad-Ribo-HSVtk-treated groups. Livers with the second highest tumor loads from each group were chosen for presentation. H&E findings and their corresponding anti-CK20 immunohistochemistry are shown in scanned photographs.
ribozyme that did a highly specific trans-splicing reaction on hTERT RNA, resulting in hTERT-dependent expression of reporter gene in vivo. In contrast, we did not detect β-galactosidase expression in nontumor tissues from normal or cancer model mice transfected with Ad-Ribo-LacZ. Furthermore, the absence of trans-spliced products in nontumor tissues provides strong evidence for the specificity of Ad-Ribo-LacZ in vivo.

We recognize that thorough toxicologic studies need to be conducted before Ad-Ribo-HSVtk can be considered for application in humans. The problems to be addressed include adenovirus vector-specific immune response leading to acute hepatotoxicity (28) and normal tissue damages due to overexpression or nonspecific expression of therapeutic gene resulted from imprecision of the targeting device. We could not assess the toxicity of the adenoviral vector itself, because we used the immunoincompetent nude mouse cancer model in our studies. We recognize that evaluation of toxicity by targeting mouse TERT in immunocompetent mice would have been ideal preclinical model for representing human situations. However, mouse TERT-targeting system could not be applied for cancer-targeting in mice, because mouse TERT mRNA is detectable in all tissues, especially prominent in liver and testis (29). Previous investigators, however, reported that i.v. inoculation of adenoviral vector at doses <1 × 10^{11} v.p./mouse in mice (30) or 5 × 10^{12} v.p./kg in nonhuman primates (31) usually leads to only limited hepatitis, suggesting that vector toxicity could be diminished by lowering the vector dose per inoculation. We therefore concluded that toxicity by virus itself can be ignored in our system, because we used one fourth to one eighth amount of viruses (0.125-0.25 × 10^{11} v.p./mouse or 0.625-1.25 × 10^{12} v.p./kg) in this study. However, the presence of preexisting adenovirus immunity in humans and development of immune response against vector backbone after the virus inoculation have impeded clinical use of adenovirus (28, 32). Recent advances in the strategies for immune evasion to improve the vector transfection efficiency and the duration of transgene expression would boost the utility of adenoviral vectors by establishing strategies for circumvention of vector immunity (33).

We did safety evaluation of Ad-Ribo-HSVtk/ganciclovir treatment in mice by analyzing liver toxicity, a major complication in using adenoviral vectors in gene therapy (27, 34). At the maximal allowable dose of Ad-Ribo-HSVtk (0.25 × 10^{11} v.p./mouse) followed by ganciclovir treatment, we did not observe any sign of liver toxicity in non-tumor-bearing nude mice. In contrast, we found a significant increase in liver enzymes and pathological changes in animals treated with Ad-HSVtk/ganciclovir. Furthermore, in our metastases model, >70% of mice survived with Ad-Ribo-HSVtk injection, whereas all the mice treated with Ad-HSVtk died within 10 days postinjection. These deaths seemed to be due to liver failure resulting from nonspecific HSVtk expression by Ad-HSVtk.

### Table 1. Hazard ratios for survival differences in three treatment groups, compared with PBS

<table>
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<tr>
<th>Treatment group</th>
<th>Hazard ratio (95% confidence interval)*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (reference group)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Ad-Mock</td>
<td>1.65 (0.75 – 3.63)</td>
<td>0.21</td>
</tr>
<tr>
<td>Ad-HSVtk</td>
<td>18.27 (5.46 – 61.07)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ad-Ribo-HSVtk</td>
<td>0.26 (0.09 – 0.74)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Hazard ratios (95% confidence interval) obtained from a multivariable Cox proportional hazards model.

P values from Wald test.
These results indicate that treatment with Ad-Ribo-HSVtk was well tolerated and showed no liver toxicity, below the viral dose of $0.25 \times 10^{11}$ v.p./mouse, when delivered i.v.

We also considered the adequacy of hTERT as tumor-specific target. Because human stem cells and germ cells in regenerating or normal human tissues have detectable telomerase activity (35, 36), systemic delivery of Ad-Ribo-HSVtk may produce toxic side effects. However, this toxicity could be minimized in stem cells because they proliferate intermittently and have negligible telomerase activity during quiescence (37). Moreover, liver tropism of adenovirus potentially limits infection of other stem cells (27). In addition, administration of Ad-Ribo-HSVtk by hepatic artery infusion (38) or direct intrahepatic injection (39) instead of systemic administration would provide added safety in humans.

Finally, we evaluated the efficacy of Ad-Ribo-HSVtk in the therapy of liver metastasis of colorectal cancer and were encouraged to find tumor reduction, both in terms of number and weight, after even a single viral injection. Moreover, the systemic administration of the Ad-Ribo-HSVtk and ganciclovir resulted in a survival advantage when compared with control animals. Two major drawbacks of targeted cancer gene therapy with adenovirus harboring a tissue/tumor–specific promoter are loss of specificity caused by cis–acting sequences in the adenoviral genome (40) and reduced expression levels compared with the current benchmark CMV promoter (41, 42).

The new therapeutic strategy we proposed here, based on targeting and replacing hTERT RNA by using a trans-splicing ribozyme, which is under the control of the CMV promoter, overcomes these drawbacks.

In the present study, we showed that the trans-splicing ribozyme both inactivates telomerase and sensitizes hTERT-expressing cancer cells selectively to prodrug treatment. This system thus provides a multifunctional gene therapy tool that both targets and treats the tumor. In our previous work, we observed the possibility that tumor specificity can be enhanced with reduced toxicity to normal tissue by replacing CMV promoter with tissue-specific promoter in vitro (43), although further explorations in in vivo system are required. We could increase targeting specificity of our system by using tissue/tumor–specific promoters combined with tumor/cell proliferation–specific ribozyme. In the future, one could design a trans-splicing ribozyme targeting a tumor-specific oncogene resulting in tumor growth inhibition by antisense effect of ribozyme. A target-specific therapeutic gene expression could have an additive, or perhaps a synergistic, anticancer effect. Furthermore, by selecting a drug-resistant gene as a target, one might expect to overcome drug resistance by reducing drug-resistant gene expression by trans-splicing reaction in addition to achieving the desired therapeutic effect by targeted expression of the therapeutic gene.

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References

Antitumor Effects of Systemically Delivered Adenovirus Harboring Trans-Splicing Ribozyme in Intrahepatic Colon Cancer Mouse Model


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