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Recombinant adenovirus infection suppresses hTERT expression through virus-associated RNA-mediated induction of type 1 interferon

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ABSTRACT

Adenovirus vector is one of the most widely used vectors in gene therapy applications for the treatment of diverse human diseases including cancer. In this study, we showed that infection with E1E3-deleted recombinant human adenovirus serotype 5 reduced human telomerase reverse transcriptase (hTERT) mRNA levels in hepatoma cell lines. We defined the mechanisms by which the recombinant adenovirus vector reduces hTERT mRNA levels as follows: Using the virus-associated RNA I/II (VAI/II) expression construct, we demonstrated that the expression of VAI and VAII RNAs led to an increase in IFN-α2 level, and IFN-α2 induction was responsible for the decrease in hTERT mRNA levels. We showed that the effects of VA RNAs were specific for the replication-incompetent E1E3-deleted adenovirus vector, because wild-type adenovirus affected neither IFN-α2 nor hTERT mRNA levels. Moreover, we demonstrated that adenovirus vector-mediated delivery of the hTERT-targeting anti-cancer reagent could additively reduce the levels of hTERT mRNA that were specifically overexpressed in most of the cancer cells. This study showed that E1E3-deleted adenovirus vector system reduced hTERT mRNA levels through VA RNA-mediated induction of type 1 interferon; hence the recombinant adenovirus itself could have anti-cancer activity. These results indicate that recombinant adenovirus vector could be an effective means to deliver anti-cancer reagents for combating cancerous cells more effectively.

1. Introduction

Adenovirus (Ad), which is a nonenveloped, double-stranded (ds) DNA virus of the *Adenoviridae* family, is the most extensively studied and developed as a delivery vehicle for gene therapy applications [1]. Among more than 50 different serotypes of adenoviruses, current vectors are primarily derived from group C viruses (serotypes 2 and 5), the most common serotypes to which most adults have been exposed [2]. E1E3-deleted replication-incompetent Ad vectors have been commonly used due to several advantages, including the ability to package large quantities of DNA and ease to produce and transfer genes into a wide spectrum of dividing and nondividing cells *in vitro* and *in vivo* [3]. However, infection with Ad vectors can activate innate immune responses induced by the viral particle or capsid independent of viral gene transcription [4,5]. Moreover, transcription-dependent activation of innate immunity can be mediated by Ad-associated RNA I (VAI) and VAII [6–8].

VAI and VAII, which are 157 and 158 nucleotide-long, respectively, are synthesized by polymerase III at high levels during Ad replication (VAI: ~10^8 molecules/cell, VAII: ~10^5 molecules/cell) and expected to form dsRNA-like secondary structures [9]. VA RNAs repress endogenous microRNA-mediated regulation by acting as a competitor for Exportin-5, Dicer, and RISC which are essential for the microRNA biogenesis pathway [10,11]. VA RNAs are processed in a manner similar to microRNAs and produce functional VA RNA derived microRNA [12–14]. Moreover, VAI was reported to specifically regulate gene expression by controlling the cytoplasmic stability and abundance of ribosome-bound mRNAs [15,16]. Initially, VA RNAs were known to competitively block the antiviral effect of interferon (IFN) through inhibition of dsRNA-induced protein kinase R that phosphorylates eukaryotic initiation factor-2α [17–19]. However, they can also induce the production of type I IFN (IFN-α and IFN-β) in a RIG-I dependent manner [6–8].

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IFNs constitute a family of cytokines that have antiproliferative, antiviral, and immunoregulatory activities [20,21]. IFN-α is currently used for treatment of hematologic malignancies as well as for solid tumors [22,23]. The antitumor activity of IFN-α is exerted indirectly through activation of immune cells such as T cells and natural killer cells, inhibition of vascularization, and induction of cytokines [24]. IFN-α also directly inhibits cancer cell growth through regulating cell cycle arrest, apoptosis, or differentiation [20,24,25]. Additionally, type I IFNs inhibit human telomerase reverse transcriptase (hTERT) activity by down-regulating hTERT expression in several cancer cell lines including malignant hematopoietic cell lines and ovarian cancer cells [26–29]. Taken together, the above studies suggest that Ad vectors themselves might play the role of an hTERT regulator through induction of VA RNA-mediated type 1 IFNs.

In the present study, we provided evidence that IFN-α induced by recombinant Ad-encoded VA RNAs could down-regulate hTERT mRNA levels in hepatoma cell lines. Down-regulation of the hTERT mRNA level by VA RNA-mediated type 1 IFNs was reversed by treatment with VA RNA-antagonizing anti-VAI RNA. Moreover, we confirmed that recombinant Ad vectors can additively inhibit hTERT mRNA expression when the Ad vector is incorporated with hTERT mRNA-targeting *trans*-splicing ribozyme as an antitumor therapy, which was previously developed by our group [30]. Our findings suggest a novel mechanism for the anti-telomerase action of recombinant Ad vectors, and they may provide a basis for future antitumor therapies.

2. Materials and methods

2.1. Constructs and viruses

VAI and VAII were amplified using PCR with primers (5′-ACGCGTCGACAGAAGCACCATGTCCTTG-3′ and 5′-CCCAAGCTTTTTTGAAAGTTAATCTCC-3′) with serotype 5 Ad vector as a template, and the amplified DNA was cloned into the *Hind*III and *Sal*I sites of the pUC19 vector. For the IFN-α2 construct, IFN-α2 cDNA was amplified using PCR with primers (5′-AAAAGTACTCGGCCTTGACCTTTGCCC-3′ and 5′-CCGCTCGAGTCATTCCTTACTCCTCAGGGACTC-3′), and the amplified DNA was cloned into the *Sca*I and *Xho*I sites of the pAVQ CMV5 tet-on vector. Recombinant adenovirus encoding GFP (Ad-GFP), herpes simplex thymidine kinase (Ad-HSVtk, CT), and hTERT-targeting *trans*-splicing ribozyme with HSVtk (Ad-Ribo-HSVtk, CRT) were generated as previously described [30]. Wild-type Ad5 was obtained from KCLB (Korean Cell Line Bank, Seoul, Korea) and VA-deleted Ad was kindly provided by Ramon Alemany (Bellvitge Biomedical Research Institute, Spain).

2.2. Cell culture

Huh-7 and Hep3B cells, which are human hepatoma cell lines, were cultured in Dulbecco's modified Eagle's medium and minimum essential medium, respectively, with high glucose and 10% fetal bovine serum (Thermo Scientific, Pittsburgh, PA).

2.3. Plasmid transfection and virus infection

Hep3B cells in a 6-well plate (4×10^5 cells/well) were cotransfected with VAI/II expression plasmid and anti-VAI or scrambled RNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To test the effect of IFN-α2 on hTERT, Hep3B cells were transfected with 3 μg of IFN-α2 expression plasmid using PEI transfection reagent (Polysciences Inc., Warrington, PA). At 48 h post transfection, total RNA was isolated for subsequent experiments. Hep3B or Huh-7 cells were infected with 10 MOI of adenoviruses and incubated for 48 h. In case of replication-competent Ad, Hep3B cells were infected with 0.5 MOI of wild-type Ad5 or VA-deleted Ad and maintained for 24 or 48 h.

2.4. RT-PCR analysis of hTERT mRNA

The level of hTERT mRNA was analyzed by reverse transcription (RT)-PCR and real-time PCR amplification. Total RNA was prepared from plasmid-transfected or Ad-infected cells using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Then, 1 μg of total RNA was reverse transcribed with random hexamers using MultiScribe™ Reverse Transcriptase (Life Technologies Inc., Carlsbad, CA). A 167-bp hTERT cDNA was amplified using the primer pair 5′-TATGGCTGCGTGGTGAACTTG-3′ and 5′-CATAGCTGGAGTAGTCGCTCT-3′.

2.5. Real-time PCR

Real-time PCR (qRT-PCR) was performed with 2 × real-time PCR premix (Solgent Co., Daejeon, Korea). Primers used for qRT-PCR were as follows: 18s; 5′-GTAACCCGTTGAACCCCATT-3′ and 5′-CCATCCAATCGGTAGTAGCG-3′, VAI RNA; 5′-GGGCACTCTTCCGTGGTCTG-3′ and 5′-AGGAGCGCTCCCCCGTTGTC-3′, VAII RNA; 5′-GGCTCGCTCCCTGTAGCCGG-3′ and 5′-AGGGGCTCGTCCCTGTTTCC-3′, IFN-α2; 5′-CTGCCTGGGATGAGACCC-3′ and 5′-CAGGCACATGGGCTGTATTT-3'. After an initial denaturation at 95 °C for 10 min, the cDNA samples were subjected to 40 cycles of PCR reaction (95°C for 30s, and 58°C for 30s, and 72°C for 30s). PCR reactions were performed in triplicate. Real-time PCR for hTERT was performed using the TaqMan gene expression assays kit (Cat. #4331182, Life Technologies Inc.) according to the manufacturer's instructions.

2.6. Statistics

The significance of differences between the mean values within groups was tested by a paired one-tailed t test (Student's t test). All data were expressed as the average ± standard deviation. Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Down-regulation of hTERT mRNA expression by adenovirus vector

In this study, we investigated whether intracellular infection with Ad would regulate hTERT expression in the cells and determined how Ad infection affects the hTERT level. To this end, we infected the human hepatoma cell line, Hep3B, with recombinant and replication-incompetent Ad-GFP and assessed the expression level of hTERT mRNA (Fig. 1). As a control, cells were infected with Ad-GFP inactivated by UV irradiation in a UV cross-linker [7]. Of note, Ad-GFP infection led to a decrease in the hTERT mRNA level of about ~58% compared with mock infection; in contrast, no effect on the hTERT mRNA level was observed when the virus was inactivated by UV irradiation (Fig. 1A and B). UV inactivation of Ad resulted in decrease in the VAI RNA level in the virus-infected cells compared with the original recombinant Ad (Fig. 1A and C), indicating that expression of Ad gene might be required for the regulation hTERT mRNA.

3.2. Effects of adenovirus virus-associated (VA) RNAs on the hTERT mRNA level

Since VA RNAs have been known to have multifunctional activities such as regulation of innate immunity [6–8,18], host RNAi

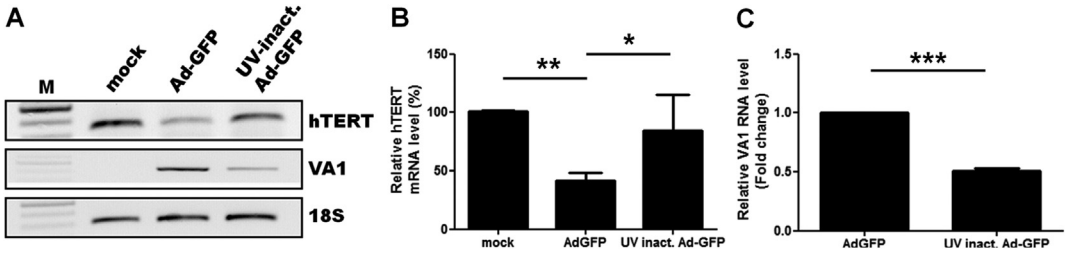

Fig. 1. Adenovirus vector down-regulates the hTERT mRNA level, dependent on its transcription. The effect of Ad-GFP and UV-inactivated Ad-GFP on the hTERT mRNA level in Hep3B cells was analyzed using RT-PCR (A) and qRT-PCR (B) at 48 h following infection with 10 MOI of the viruses. (C) qRT-PCR analysis of VAI RNA in Ad-GFP and UV-inactivated Ad-GFP infected Hep3B cells. Experiments were performed in triplicate with bars depicting standard error of means. Asterisks denote significance. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

pathway [10,11,14], and mRNA stability and translation [9,12,16], we determined whether VA RNAs are involved in the reduction of hTERT mRNA level (Fig. 2). To this end, we transfected the VAI/II expression construct together with VA RNAs antagonizing anti-VAI RNA or scrambled RNA into Hep3B cells. qRT-PCR analysis showed that transfection of anti-VAI RNA efficiently diminished both VAI and VAII RNA levels by about 2.5-fold (Fig. 2A). Of note, the expression of VAI/II efficiently reduced the hTERT mRNA level by up to 50% (Fig. 2B and C). In contrast, cotransfection of VAI/II expression plasmid with anti-VAI RNA resulted in an increase in the hTERT mRNA level by up to two-fold compared with cotransfection of the plasmid with scrambled RNA (Fig. 2B and C). These results suggest that VA RNAs are responsible for down-regulation of the hTERT mRNA level in the Ad-infected hepatoma cells.

3.3. Down-regulation of the hTERT mRNA level by VA RNA-induced type 1 interferon

We assessed how VAI/II regulates hTERT expression. IFN-α2 level was induced by about 2-fold when the VAI/II expression plasmid was transfected (Fig. 2D), similar to VA RNA-mediated type I IFN induction shown in other studies [6–8]. Cotransfection of VAI/II plasmid with anti-VAI RNA induced restoration of IFN-α2 back to normal levels, indicating VA RNA-specific IFN induction. The effect of IFN-α2 on the hTERT mRNA level was examined in Hep3B cells following transfection of IFN-α2 expression plasmid. As shown in Fig. 2E, expression of IFN-α2 efficiently reduced the hTERT mRNA level. qRT-PCR analysis showed that expression of IFN-α2 reduced the hTERT mRNA level by up to 60% (Fig. 2F).

3.4. Effects of adenovirus vector-delivered hTERT-targeting agent on the hTERT mRNA level

Based on the above results, we hypothesized that recombinant Ad vector-mediated delivery of antitumor reagents could enhance the antitumor effects due to antitelomerase activity of Ad vector. To this end, we infected hepatoma cell lines, Hep3B and Huh-7, with CT (Ad-HSVtk) or CRT (Ad-Ribo-HSVtk) which is an Ad vector encoding for hTERT-targeting *trans*-splicing ribozyme developed previously [30]. Like Ad-GFP, both CT and CRT diminished the hTERT mRNA level in Hep3B (Fig. 3A and B) and Huh-7 cells (Fig. 3E and F). These results suggest that down-regulation of hTERT mRNA level might generally occur after recombinant Ad infection of hepatoma cells. Of note, CRT infection more efficiently reduced the hTERT mRNA level than CT infection, by about 15–20% (Fig. 3B and F). Infection with these two Ad vectors induced similar expression

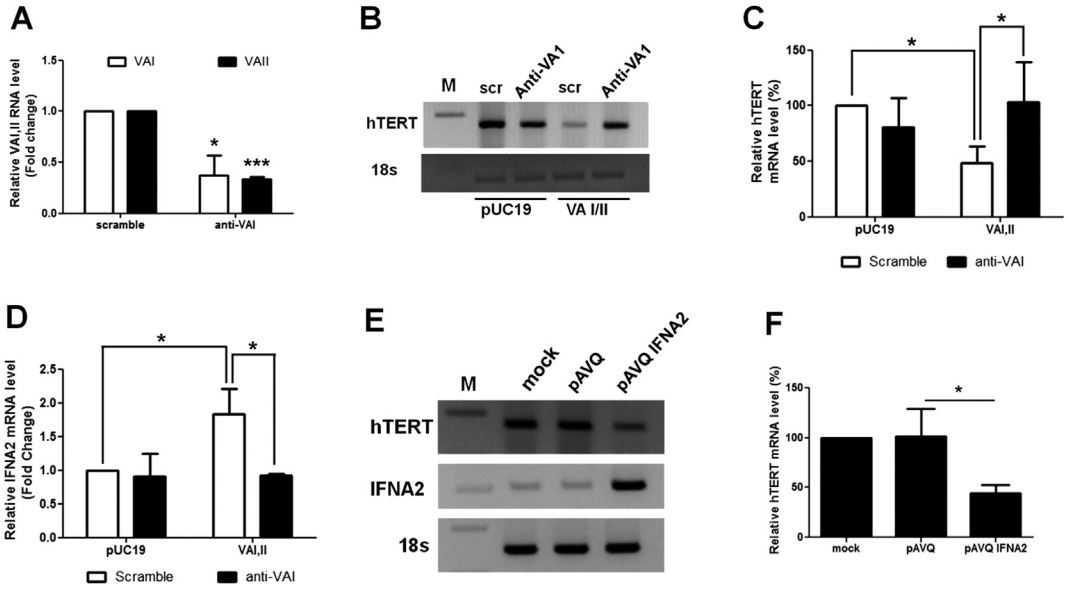

Fig. 2. VA RNA-mediated induction of IFN-α2 reduces the hTERT mRNA level. Hep3B cells were cotransfected with VAI/II expression plasmids and anti-VAI RNAs. At 48 h post-transfection, VA RNA (A), hTERT mRNA (B,C), and IFN-α2 mRNA levels (D) were analyzed using qRT-PCR (A,C,D) and expressed relative to the level in Hep3B cells cotransfected with pUC19 and scrambled RNAs or using RT-PCR (B). IFN-α2 expression plasmid was transfected into Hep3B cells. At 48 h post-transfection, IFN-α2 and hTERT mRNA levels were analyzed using RT-PCR (E). (F) hTERT mRNA levels were also analyzed using qRT-PCR and expressed relative to the level in mock-treated cells. Averages of measurements performed with 3 independent experiments are shown with standard deviation. Asterisks denote significance. $^{*}p < 0.05$.

of VA RNAs in both cell lines (Fig. 3C and G). IFN-α2 level was increased by infection with these two Ad vectors: CT infection induced a higher IFN-α2 level than CRT infection (Fig. 3D and H). Taken together, we speculated that combination of antitelomerase activity of recombinant Ad vector itself and *trans*-splicing activity of hTERT-targeting ribozyme by CRT additively affected the hTERT mRNA levels. These results suggest that Ad vector-mediated delivery tools could be more efficacious in cancer treatment.

3.5. Effects of wild-type adenovirus infection on the hTERT mRNA level

Next, we tested whether wild-type Ad induces down-regulation of the hTERT mRNA level like the recombinant Ad vector. To this end, we infected Hep3B cells with replication-competent wild-type or VA-deleted adenovirus and analyzed the VAI RNA, hTERT mRNA, and IFN-α2 mRNA levels (Fig. 4). In contrast to the recombinant Ad vectors, neither replication-competent wild-type nor VA-deleted adenoviruses affected the hTERT and IFN-α2 mRNA levels. These results indicate that modulation of the hTERT mRNA level could occur specifically in replication-incompetent recombinant Ad vector.

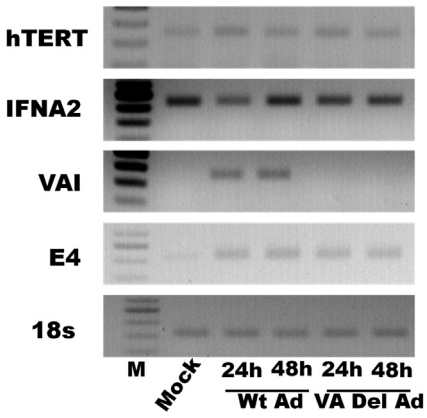

Fig. 4. Wild-type and VA RNA-deleted adenovirus do not modulate hTERT and IFN-α2 expression. Hep3B cells were infected with 0.5 MOI of wild-type or VA-deleted adenovirus. Expression levels of hTERT, IFN-α2, VAI RNA, and adenovirus E4 gene were analyzed using RT-PCR at 24 h and 48 h post-infection.

4. Discussion

Ad vectors are one of the most efficient gene transfer systems *in vivo* [1], representing delivery vehicles in 23.5% of human gene therapy trials which are presently being performed worldwide [31]. One of the limitations to be considered while using Ad vectors is the adaptive and innate immune responses evoked against the Ad capsid or intracellularly expressed Ad gene products [32,33]. To reduce the immune responses, "helper-dependent" or "gutted" Ad vectors and VA RNA-deleted Ad vectors have been developed [34,35]. However, with regard to cancer therapies, immune responses induced by intracellular infection of Ad might be helpful if they are specifically targetable to cancers because they can induce and/or enhance immunity against cancer cells in addition to direct regression of cancer cells through activities of transferred genes.

In this study, we demonstrated that infection with replication-incompetent E1E3-deleted Ad vectors could down-regulate the hTERT mRNA abundance in hepatoma cells through expression of viral genes (Fig. 1). VA RNAs were shown to be involved in the down-regulation of hTERT mRNA level through induction of type I IFN in the hepatoma cell lines (Fig. 2). However, the possibility that VA RNAs modulate hTERT mRNA directly or indirectly through microRNA pathways could not be completely excluded, since VA RNAs are known to inhibit microRNA biogenesis pathways as well as to act as a substrate for microRNA biogenesis pathways for generating viral microRNAs which can target cellular or viral genes [10–13]. Type 1 IFNs have been reported to suppress the activity of hTERT and down-regulate hTERT expression in several cell lines [26–29], but their roles in regulating hTERT mRNA levels in hepatoma cell lines were first described in this study. More studies are needed to determine whether type 1 IFNs can modulate hTERT expression in other cell lines. Noticeably, replication-competent wild-type Ad could not modulate either IFN-α2 level or hTERT expression unlike replication-incompetent Ad vectors (Fig. 4). This may be due to the expression of viral genes such as E1A and E1B in

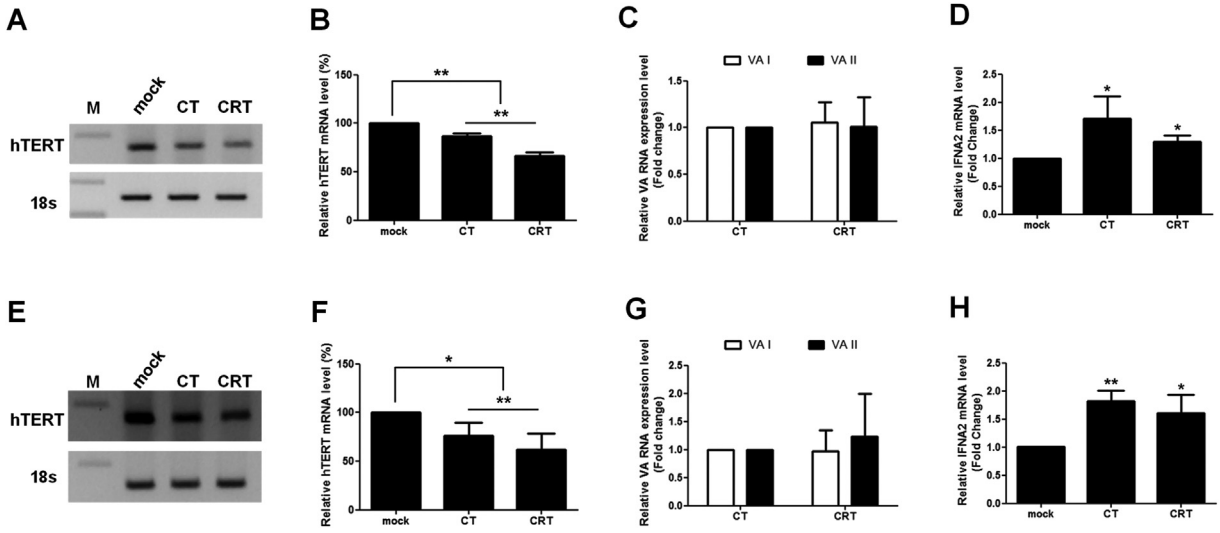

Fig. 3. Infection with adenovirus vector encoding for the hTERT-targeting *trans*-splicing ribozyme additively reduces the hTERT mRNA levels. Hep3B and Huh-7 cells were infected with CT or CRT. At 48 h post-infection, hTERT mRNA levels in Hep3B (A) and Huh-7 (E) cells were analyzed using RT-PCR. (B–D) show the qRT-PCR analysis of hTERT mRNA, VA RNA, and IFN-α2 mRNA levels in Hep3B cells. (F–H) depict qRT-PCR analysis of hTERT mRNA, VA RNA, and IFN-α2 mRNA levels in Huh-7 cells. Averages of measurements performed with 3 independent experiments are shown with standard deviation. Asterisks denote significance, $^{*}p < 0.05$, $^{**}p < 0.01$.

the wild-type Ad, which can inhibit numerous host signaling pathways including type 1 IFN pathways [4,36–38].

Human TERT is an attractive cancer target as it appears to be essentially required in most tumors for immortalization. Inhibition of hTERT expression in cancer cells leads to severe telomere shortening, senescence, and apoptosis [39]. Previously, we showed that Ad vector-mediated transfer of hTERT-specific *trans*-splicing ribozyme (Ad-CRT) efficiently regressed tumors *in vivo* [30,40]. Here, we showed that Ad-CRT infection additively reduced the hTERT mRNA level in cells (Fig. 3) most probably due to the anti-telomerase activity of Ad vector combined with the hTERT-targeting activity of the ribozyme. These results strongly suggest that Ad vectors will be more advantageous for delivering antitumor genes in cancer treatment. Moreover, since type 1 IFNs are mediators of innate and humoral immunity [41], recombinant Ad vector delivery may enhance immunity against cancers through induction of VA RNA-mediated type 1 IFNs, if specifically targetable to cancers.

In summary, we identified the ability of replication-incompetent recombinant Ad vectors to down-regulate the hTERT mRNA level at least in hepatoma cell lines. Reduction of the hTERT mRNA level would be derived from Ad vector-encoded VA RNAs through induction of type 1 IFNs, which might be dependent on RIG-I pathways [7,10]. This ability of the Ad vector could render it vector more useful as an anti-cancer gene delivery vehicle.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Transparency document

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