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Research paper

Liver-specific gene therapy of hepatocellular carcinoma by targeting human telomerase reverse transcriptase with pegylated immuno-lipopolyplexes

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ABSTRACT

The purpose of this study is to explore the possibility and feasibility of liver-specific gene therapy. A shRNA expression plasmid against human telomerase reverse transcriptase (hTERT) was constructed under the control of liver-specific promoter apolipoprotein A-I (ApoAI), designated as pApoAI-shTERT, and its liver-specific cytotoxicity and inhibition of telomerase activity were first evaluated in different cell lines, and its therapeutic effect was further studied in SMMC-7721 human liver tumor-bearing mice *in vivo*. The results showed that compared to pU6-shTERT, a shRNA expression plasmid against hTERT under the control of U6 promoter, pApoAI-shTERT only significantly diminished the cell viability in the telomerase positive hepatocarcinoma cells and showed no cytotoxicity in the telomerase negative cell lines as well as in the telomerase positive cell line of non-liver origin. Besides, pApoAI-shTERT only significantly reduced telomerase activity in the telomerase positive cell lines of liver origin. Intravenous administration of pegylated immuno-lipopolyplexes (PILP) formulated green fluorescent protein (GFP) expression plasmid under the control of ApoAI into liver tumor-bearing mice resulted in restricted GFP expression in liver and liver tumor. The treatment of pApoAI-shTERT formulated as PILP caused a 56% increase in the life span of SMMC-7721 tumor-bearing mice *in vivo* relative to the control, which was in agreement with the reduced tumor size and down-regulated hTERT mRNA level in the tumors. We conclude that the vector pApoAI-shTERT was able to cause liver-specific and hTERT target-specific cytotoxicity, and utilizing PILP to deliver pApoAI-shTERT is a promising strategy for liver-specific gene therapy.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and leads to nearly one million deaths annually worldwide because HCC responds poorly to conventional therapies [1,2] and frequently relapses shortly after surgical or nonsurgical treatments [3,4]. To overcome these clinical problems, newly established treatments, such as gene therapy, may be combined with the conventional treatments.

Telomerase activity could be detected in approximately 89.5% of hepatocarcinoma cells [5], whereas it is negligible in normal liver tissues. Therefore, telomerase is expected to be a good candidate for the therapy of hepatocarcinoma. Human telomerase reverse transcriptase (hTERT) is the rate-limiting factor of telomerase activity [6], suggesting that the control of telomerase activity is predominantly regulated at the expression of the hTERT gene. It was reported that high expression of hTERT could suppress apoptosis by multidifferentiation stimulation in some tumors [7]. More studies showed that inhibition of hTERT with the antisense or RNA interference (RNAi) technique could be a good antitumor strategy, which was successfully applied to reduce cancer cell growth [8,9]. Short hairpin RNA (shRNA) generated by a DNA vector, such as a plasmid, produces long-term, stable, and highly specific gene silencing. This is becoming the method of choice for RNAi-based gene therapy of malignancies [10]. Therefore, a shRNA-based anti-hTERT strategy would be a potential approach for hepatocarcinoma therapy.

However, the shortage of complete tumor selectivity due to telomerase expression in highly proliferating normal cells, such as hematopoietic stem cells, the lymphohematopoietic system, germ cells, and stem cell layers of the skin [11–13], can limit the therapeutic applicability of the shRNA-based anti-hTERT strategy. Ectopic expression of shRNA against hTERT under the influence of a widely expressed promoter such as CMV or U6 is expected in all kinds of tissues. Therefore, the development of modulated gene expression system such as tumor or organ-restricted gene

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expression through transcriptional targeting without affecting normal cells is required.

One of such approaches is to restrict expression of the transgene to the target organ. In liver, this has been achieved with the liver-specific promoter apolipoprotein A-I (ApoAI) [14] and a novel non-viral gene delivery system, pegylated immuno-lipopolyplexes (PILP) [15], which was developed by us recently. However, liver-specific gene therapy of hepatocellular carcinoma by targeting hTERT has not been reported before. Therefore, the purpose of the present studies was twofold: (1) construct an anti-hTERT shRNA expression plasmid under the control of ApoAI promoter, which was designated as pApoAI-shTERT and test its liver-specific cytotoxicity and inhibition of telomerase activity *in vitro*; (2) investigate the *in vivo* liver-specific expression of green fluorescent protein (GFP) expression plasmid under the control of ApoAI promoter and the therapeutic effect of pApoAI-shTERT delivered by PILP in an SMMC-7721 human liver tumor xenograft model in mice.

2. Materials and methods

2.1. Materials

POPC (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine), distearoylphosphatidylethanolamine (DSPE)-PEG2000, and DSPE-PEG2000-biotin were obtained from Avanti-Polar Lipids, Inc. (Alabaster, AL, USA). Protein G-Sepharose and Sephacryl S-300HR were purchased from GE Healthcare (Piscataway, NJ, USA). Bicinchoninic acid (BCA) protein assay reagents were obtained from BioTeke Corporation (Beijing, China). TRAPEZE® ELISA Telomerase Detection Kit was from Millipore (Bedford, MA, USA). ExGen 500 *in vitro* transfection reagent was from Fermentas, Inc. (Glen Burnie, MD, USA). Reverse Transcription System and the plasmid pCMV-GFP, a cytomegalovirus (CMV) promoter-driven GFP expression vector, were purchased from Promega (Madison, WI, USA). RNAiso Plus and SYBR® Premix Ex Taq™ were from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Polyethylenimine (MW 25 kDa, branched), streptavidin, and other molecular biology grade reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The rat anti-mouse transferrin receptor monoclonal antibody (TfRMAb) 8D3 and the murine anti-human insulin receptor monoclonal antibody (HIRMAb) 8314 were individually purified with protein G affinity chromatography from hydridoma-generated ascites. The plasmid pAI-W, carrying apolipoprotein A-I (ApoAI) promoter, was a kind gift from Dr. Kazuhiro Oka (Baylor college of Medicine, Houston, TX). Plasmids pU6-shTERT and pU6-shGFP, which are shRNA expression vectors against hTERT and green fluorescent protein (GFP), respectively, under the control of U6 promoter, were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The human cell lines SMMC-7721, HepG2, ACC-2, MCF7, IMR-90, and Lo2 were purchased from Institute of Biochemistry and Cell Biology, CAS (Shanghai, China).

2.2. Plasmid construction

The vector pApoAI-GFP, the GFP expression plasmid under the control of liver-specific promoter ApoAI, was constructed as described previously [14]. The pCMV-GFP vector was digested by *Nhe* I and *Pst* I restriction enzymes to remove GFP fragment to obtain the RNAi control plasmid pCMV-shRNA. ApoA-I promoter was PCR amplified from pAI-W vector and inserted into pCMV-shRNA to replace the CMV promoter, which was designated as pApoAI-shRNA. This is a liver-specific promoter-driven RNAi empty vector.

To construct the ApoAI promoter-driven shRNA expression vectors, pApoAI-shTERT and pApoAI-shGFP against hTERT and GFP, respectively, specific DNA oligonucleotides were chemically synthesized and annealed to form double-stranded DNA fragments, and the fragments were then inserted between the restriction sites EcoRI and XbaI in the RNAi empty vector. The following are the target sequences: 5′-ttc cgc cag gtg tcc tgc ctg aag gag ct-3′ (nucleotide 266–294) for hTERT and 5′-cac aag ctg gag tac aac tac-3′ (nucleotide 1030–1050) for GFP, which showed no homology to any other sequences by a blast search. All constructs were sequenced to confirm identity.

2.3. Preparation of pegylated immuno-lipopolyplexes

To prepare pegylated immuno-lipopolyplexes (PILP), pegylated lipopolyplexes (PLP) need to be prepared first as described previously [15]. The conjugate 8D3 MAb/streptavidin (8D3/SA) or 8314 MAb/streptavidin (8314/SA) was synthesized as described previously [16]. Once the pegylated lipopolyplexes are ready, equal amounts of 8D3/SA and 8314/SA were added at the molar ratio 1.5:1 of SA to biotin group in the PLP. The 8D3 MAb and 8314 MAb immediately conjugate to the PLP because of the high affinity between SA and biotin, which are called pegylated immuno-lipopolyplexes.

2.4. Cell cultures

HepG2, IMR-90, and Lo2 were cultured in DMEM (Hyclone, China) with 10% heat-inactivated FBS (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., China). MCF7, SMMC-7721, ACC-2, and TF-1α cells were grown in RPMI 1640 (Hyclone, China) supplemented with 10% FBS at 37 °C in an incubator with 5% CO_2.

2.5. Telomerase activity assay

Cells (SMMC-7721, HepG2, MCF7, ACC-2, and Lo2) were seeded in 6-well plates in suitable media as described previously. When the cells reached about 70–80% confluence, plasmids pApoAI-shTERT, pU6-shGFP, and pU6-shTERT were transiently transfected into different wells of cells using ExGen 500 at a dose of 3 μg of DNA per well. After a 72-h incubation at 37 °C, telomerase activity from 0. 5 mg of cell protein extracts was measured by telomeric repeat amplification protocol-enzyme-linked immunosorbent assay (TRAP-ELISA) method, using TRAPEZE® ELISA Telomerase Detection Kit as described previously [17]. Percent inhibition was calculated relative to the negative control pU6-shGFP where the negative control was defined as 0%. Each experiment was repeated three times.

2.6. MTT assay

Cells (IMR90, Lo2, SMMC-7721, and TF-1α) were seeded in 96-well plates one day before the transfection in suitable media as described previously. When the cells reached about 70–80% confluence, pApoAI-shTERT, pU6-shGFP, and pU6-shTERT were transiently transfected into different wells of cells using ExGen 500 at a dose of 0.6 μg of DNA per well. Forty-eight hours later, 20 μL of 3-(4,5-dimethylthiazolyl-2) 2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added into each well and incubated at 37 °C for 4 h. The media were carefully aspirated, and 200 μL of dimethyl sulfoxide (DMSO) was added to each well and pipetted up and down to dissolve crystals. Then, the cells continued to be incubated at 37 °C for 5 min before being measured for absorbance at 550 nm. The measured absorbance for test groups was expressed as percent of the control (cell viability %) where the control was defined as 100%. Each experiment was repeated three times.

2.7. In vivo liver-specific gene expression

Female BALB/c nude mice at age of 6–8 weeks were obtained from Shanghai Laboratory Animal Center, kept in filter-topped cages with standard rodent chow and water available ad libitum and a 12 h light/dark cycle. The experiments were performed according to the national regulations and approved by the local animal experiments ethical committee.

Subcutaneous hepatocellular carcinoma tumors were induced by the inoculation of 2×10^6 human SMMC-7721 cells in the right flank. When the tumor volume reached 400–500 mm^3, groups of mice ($n = 5$) were intravenously treated with either saline or 40 μg/mouse of pApoAI-GFP plasmid formulated as PILP. To prepare the PILP, both rat anti-mouse transferrin receptor monoclonal antibody 8D3 and mouse anti-human insulin receptor monoclonal antibody 8314 were used. Mice were sacrificed 48 h after the injection, and pieces of tumor, lung, liver, spleen, brain, heart, and kidney were removed and frozen in OCT (optimal cutting temperature) medium. Ten micron sections were prepared on a cryostat, and fluorescent microscopy was performed to visualize GFP expression using a Leica-SP2 fluorescent microscope with an argon laser for blue light excitation at 488 nm. Transfection efficiency was expressed as percent cells expressing GFP by counting the number of the cells that show GFP signal and also the number of the cells that do not have GFP signal in five areas (the upper left, the bottom left, the upper right, the bottom right, and the center) under the microscope.

2.8. In vivo therapeutic effect in a xenograft tumor model

The animal species and source are the same as described in the previous section. Subcutaneous hepatocellular carcinoma tumors were induced by the inoculation of 2×10^6 human SMMC-7721 cells in the right flank. Tumor size was measured in two dimensions with a caliper-like instrument. Individual tumor volumes (V) were calculated by the formula: $V = (\text{length} \times [\text{width}]^2)/2$. When the tumor volume reached 50–80 mm^3, groups of mice ($n = 12$) were intravenously treated with either saline or 40 μg/mouse of pApoAI-shGFP or pApoAI-shTERT plasmid formulated as PILP every five days. Growth curve was established by measuring tumor volume every five days. Mice were closely monitored for health conditions. When a mouse is in a moribund condition (e.g. hunched posture, rough hair coat, abnormal breathing, difficulty with ambulation, decreased food, or water intake), the mouse was euthanized and the tumor was removed for measurement of hTERT mRNA. One more day was counted toward to the total survival days of the mouse. Survival curve was established for each treatment.

2.9. Real-time RT-PCR analysis

Total RNA was extracted from the tumor tissue with RNAiso Plus following the protocol suggested by the manufacturer and quantified. cDNA was synthesized by RT with oligo dT priming and the Reverse Transcription System for TR-PCR. The real-time PCR was performed with SYBR® Premix Ex Taq™. PCR was run using a modified three-step amplification protocol followed by a melting curve to confirm the production of a single PCR product. The threshold cycle number (Ct) was calculated for hTERT and β-actin using the Mx3000P™ Stratagene software. β-Actin gene (a common housekeeping gene) was used as an inner control. PCR primers were designed using the Beacon Designer software and obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The hTERT PCR primers (forward, 5′-cgg aag agt gtc tgg agc aa-3′ and reverse, 5′-gga tga agc gga gtc tgg a-3′) amplified a region of 144 nucleotides (nt); β-actin PCR primers (forward, 5′-gcg gga aat cgt gcg tga cat t-3′ and reverse, 5′-gat gga gtt gaa ggt agt ttc gtg-3′) produced a DNA fragment of 232 nt. The expression of hTERT mRNA was analyzed by the 2 (-Delta Delta C (T)) relative-quantitative method as described previously [18].

2.10. Statistical analysis

Data are presented as the mean ± SE of each group. Statistical differences between groups were evaluated by an analysis of variance (ANOVA) followed by Dunnett post-test. A P value of less than 0.05 was considered significant. The survival rate was compared between different groups with Fisher exact test.

3. Results

3.1. Liver-specific cytotoxicity of pApoAI-shTERT

One tumor cell line derived from human liver carcinoma (SMMC-7721), two cell lines of non-liver origin (IMR90 and TF-1α), and one nontumorigenic liver cell line (Lo2) were transiently transfected with pU6-shGFP (negative control), pU6-shTERT, or pApoAI-shTERT, respectively. Vectors pU6-shTERT and pU6-shGFP are the shRNA expression plasmids against hTERT and GFP, respectively, under the control of U6 promoter; pApoAI-shTERT is the shRNA expression plasmid against hTERT under the control of the liver-specific promoter ApoAI. Seventy-two hours later, cell viability was determined by MTT assay. The result showed that pU6-shTERT significantly reduced cell viability in the two telomerase positive cell lines (SMMC-7721 and TF-1α) to 42% and 76%, respectively, and did not decrease the cell viability in the two telomerase negative cell lines (IMR90 and Lo2). In contrast, pApoAI-shTERT only significantly diminished the cell viability in the telomerase positive liver carcinoma SMMC-7721 cells to 50% and showed no cytotoxicity in the telomerase negative cell lines (IMR90 and Lo2) as well as in the telomerase positive cell line of non-liver origin (TF-1α) (Fig. 1). These results suggest that pApoAI-shTERT is able to cause liver and telomerase-specific cytotoxicity.

3.2. Liver-specific telomerase activity inhibition of pApoAI-shTERT

Four telomerase positive cancer cell lines (SMMC-7721, HepG2, MCF7 and ACC-2) and one telomerase negative cell line (Lo2) were

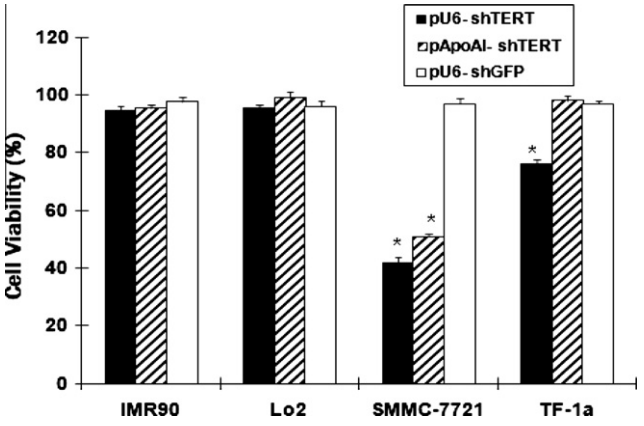

Fig. 1. Liver-specific cytotoxicity induced by pApoAI-shTERT. The cells were seeded in 96-well plates and incubated in DMEM or 1640 medium supplemented with 10% serum at 37 °C. pApoAI-shTERT, pU6-shTERT, or pU6-shGFP (negative control) plasmid were transfected into different wells of cells with ExGen 500 at a dose of 0.6 μg of DNA per well. Seventy-two hours later, MTT assay was performed to evaluate the cell viability (%). Data are presented as mean ± SE ($n = 3$). *$P < 0.05$, compared with the negative control pU6-shGFP, which was 100%.

used in this experiment. SMMC-7721, HepG2 and Lo2 are of liver origin, and MCF7 and ACC-2 are of non-liver origin. The result showed that pU6-shTERT caused near 80% inhibition of telomerase activity relative to the negative control pU6-shGFP in all the telomerase positive cancer cell lines, but caused almost no inhibition in the telomerase negative cell line (Lo2). In contrast, pApoAI-shTERT only significantly reduced telomerase activity in the telomerase positive cell lines of liver origin (SMMC-7721 and HepG2) by 60% (Fig. 2). These results suggest that pApoAI-shTERT can cause liver-specific telomerase activity inhibition.

3.3. In vivo liver-specific gene expression

The GFP expression vector pApoAI-GFP was constructed under the control of the liver-specific promoter ApoAI, formulated as PILP, and intravenously injected into SMMC-7721 human liver tumor-bearing mice. At 48 h, the mice were sacrificed, various organs were removed, and fluorescence microscopy was performed to visualize GFP expression. The result showed that there was only GFP expression in liver and liver tumor with a transfection efficiency of 45 ± 7% and 55 ± 8%, respectively, whereas there was no detectable GFP expression in any organs of non-liver origin, like lung, spleen, brain, heart, and kidney (Fig. 3A). High-resolution images clearly show the GFP signal in the cells of liver and tumor from pApoAI-EGFP-treated mice (Fig. 3B). In contrast, there was no GFP expression in all the organs in the saline-treated control group (Fig. 3A and B).

3.4. In vivo therapeutic effect of pApoAI-shTERT in SMMC-7721 human liver tumor-bearing mice

SMMC-7721 tumor-bearing mice were intravenously treated with either saline, pApoAI-shGFP (anti-unrelated plasmid DNA), or pApoAI-shTERT, respectively. After five treatments, the time at which half of the mice were dead (ED_{50}) was 34 days in the control group (saline) and the anti-unrelated plasmid pApoAI-shGFP did not significantly change the life span of the mice with an ED_{50} of 35 days. However, the ED50 for the pApoAI-shTERT treatment group is 53 days (Fig. 3A). Therefore, pApoAI-shTERT treatment caused a 56% increase in life span of the tumor-bearing mice. The growth curves show that pApoAI-shTERT treatment significantly inhibited tumor growth with a growth inhibition rate of 39% at 30 days post-implantation relative to saline, whereas the control vector pApoAI-shGFP did not cause any growth inhibition (Fig. 4B). Furthermore, pApoAI-shTERT treatment markedly down-regulated hTERT mRNA level by about 58% relative to the control, whereas pApoAI-shGFP did not significantly change hTERT mRNA expression level in the tumors (Fig. 3C). These results suggest that the therapeutic effects were caused specifically by the downregulation of hTERT by pApoAI-shTERT.

4. Discussion

In order to succeed in cancer gene therapy, the efficient delivery of therapeutic genes to a target site is a major challenge. We have developed a novel and efficient non-viral gene delivery system—pegylated immuno-lipopolyplexes [15], which is a ternary complex formed with anionic liposomes, cationic polymer, and DNA. This is the second generation of non-viral gene delivery vectors that can improve gene transfer compared to the first generation of non-viral gene delivery vectors represented by lipoplexes and polyplexes. The surface of the lipopolyplexes is decorated with strands of polyethylene glycol (PEG) to promote stabilization in the bloodstream, and the tips of the PEG strands are conjugated with a targeting monoclonal antibody (MAb). This peptidomimetic MAb triggers receptor-mediated endocytosis into tumor cells. The pattern of gene expression *in vivo* is determined by the receptor specificity of the targeting MAb [15]. Our previous work has also shown that by utilizing liver-specific promoter and the PILP technology, expression of an exogenous gene can be restricted in liver [14].

In this study, a shRNA expression plasmid against hTERT was constructed under the control of the liver-specific promoter ApoAI and its liver-specific cytotoxicity and inhibition of telomerase activity were first evaluated in different cell lines. pU6-shTERT, a shRNA expression plasmid under the control of U6 promoter which is active in all kinds of tissues, significantly reduced cell viability in the two telomerase positive cell lines (SMMC-7721 and TF-1α) and showed no effect on the cell viability in the two telomerase negative cell lines (IMR90 and Lo2). Furthermore, pU6-shTERT caused nearly 80% inhibition of telomerase activity in all the telomerase positive cancer cell lines, but caused almost no inhibition in the telomerase negative cell line (Lo2). In contrast, pApoAI-shTERT only significantly diminished the cell viability in the telomerase positive liver carcinoma SMMC-7721 cells and showed no cytotoxicity in the telomerase negative cell lines (IMR90 and Lo2) as well as in the telomerase positive cell line of non-liver origin (TF-1α). Besides, pApoAI-shTERT only significantly reduced telomerase activity in the telomerase positive cell lines of liver origin (SMMC-7721 and HepG2). It is well known that holoenzyme of telomerase is composed of telomerase RNA, telomerase-associated protein, and telomerase reverse transcriptase (TERT). Among them, TERT is the key factor of telomerase activity, which is more closely correlated with cancer than telomerase [19]. Thus, knockout or downregulation of TERT gene at the transcriptional level could downregulate telomerase activity, accelerate neoplasm cell

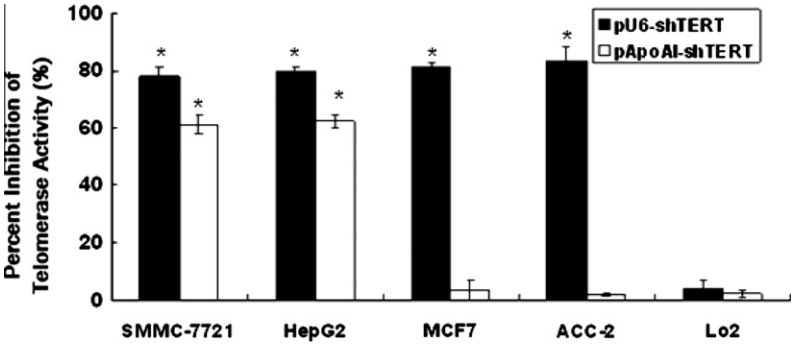

Fig. 2. Liver-specific telomerase activity inhibition by pApoAI-shTERT. The cells were seeded in 6-well plates and incubated in DMEM or 1640 medium supplemented with 10% serum at 37 °C. pApoAI-shTERT, pU6-shTERT, or pU6-shGFP (negative control) plasmid were transfected into different wells of cells with ExGen 500 at a dose of 3 μg of DNA per well. Seventy-two hours later, telomerase activity was detected and percent inhibition was calculated relative to the negative control pU6-shGFP. Data are presented as mean ± SE ($n = 3$). $^*P < 0.05$, compared with the negative control pU6-shGFP, which was 0%.

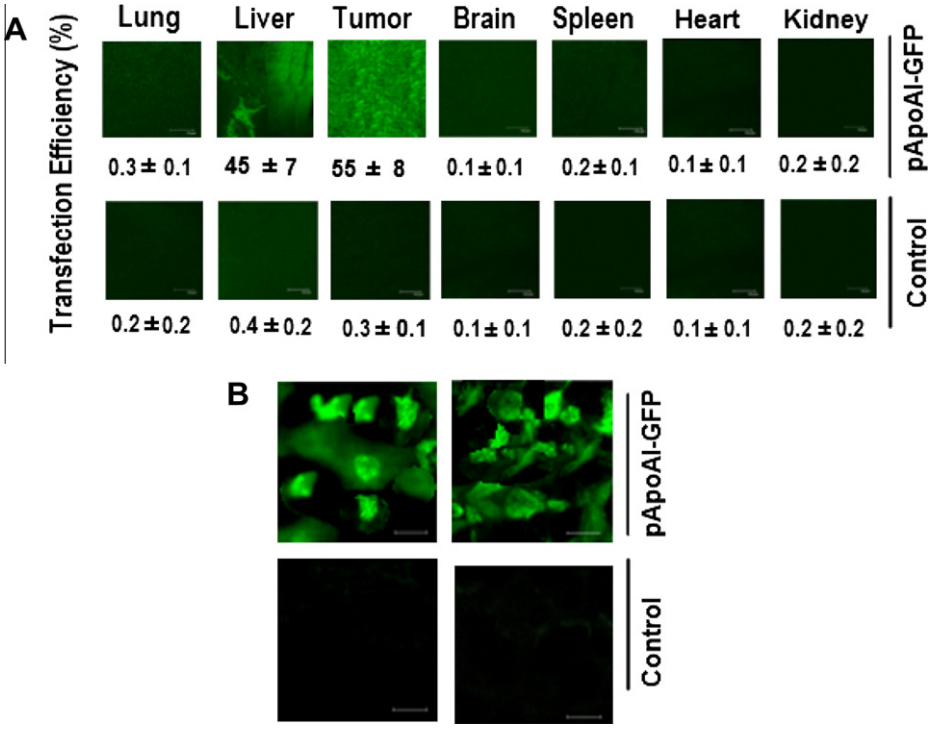

Fig. 3. Liver-specific GFP expression. (A) GFP expression in liver, tumor, lung, brain, spleen, heart, and kidney was determined by fluorescent microscopy at 48 h after intravenous injection of either saline (control) or 40 μg/mouse of pApoAI-GFP plasmid formulated as PILP to female BALB/c mice ($n = 5$). Scale bars in all panels are 150 μm. Transfection efficiency was expressed as percent cells expressing GFP. (B) High-resolution images of GFP expression in liver and tumor determined by fluorescent microscopy at 48 h after the administration. Scale bars in all panels are 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

apoptosis, and inhibit the growth of tumors [20]. Our results suggested that pApoAI-shTERT vector was able to cause liver-specific and hTERT target-specific cytotoxicity.

Both vectors pApoAI-GFP and pApoAI-shTERT are under the control of the liver-specific promoter ApoAI, and their *in vivo* liver-specific gene expression and therapeutic effect were evaluated in SMMC-7721 human liver tumor xenograft model in mice by utilizing the PILP gene delivery technology. In these studies, two different monoclonal antibodies (MAb) 8D3 and 8314 were conjugated with the gene delivery system. 8D3, the rat anti-mouse transferrin receptor MAb, was used to carry the therapeutic gene in the particles across the murine blood vessels that perfuse the tumor, and 8314, the murine anti-human insulin receptor MAb, was used to target the therapeutic gene to human cancer xenograft in mice. The result showed that intravenous administration of the PILP formulated GFP expression plasmid resulted in restricted GFP expression in liver and liver tumor, suggesting that the ApoAI promoter enables liver-specific gene expression, which is consistent with our previous data [14]. Furthermore, when the ApoAI promoter was constructed in the hTERT shRNA expression plasmid, pApoAI-shTERT treatment caused a 56% increase in the life span of the tumor-bearing mice relative to the controls, which is in agreement with the reduced tumor size and down-regulated hTERT mRNA level in the according tumors. This suggests efficient liver-specific gene therapy of hepatocellular carcinoma with the liver-specific promoter ApoAI.

It was reported that lipopolyplexes could initiate a potent cytokine response and consequently inhibit tumor growth, and the antitumor activity was not observed in immunodeficient mice [21]. Besides, it is well established that unmodified siRNA can activate innate immune response, and therefore, there is real potential for siRNA to elicit non-specific therapeutics effects in a wide range of disease models including tumor models [22]. Either siRNA or shRNA could cause specific or non-specific off-target effects [23]. In order to explore the possible non-specific therapeutic effect, in the *in vivo* experiment, we have set up a group of animals treated with pApoAI-shGFP, which is a shRNA vector unrelated to the target TERT. Compared to saline, we have found that the treatment pApoAI-shGFP has not caused any effects on life span, tumor volume, and hTERT mRNA downregulation. It should be noted that our experiment was performed in immunodeficient mice, suggesting that the antitumor activity should not be related to cytokine response based on Whitemore's report [21]. Actually, our previous report already showed that our PILP system did not cause cytokine production *in vivo* [15]. Taken together, it is suggested that the therapeutic effects we have observed for pApoAI-shTERT are not non-specific effects related to cytokine production, or any other off-target effects.

There are two possible ways to achieve liver-targeted gene therapy: one is liver-specific ligand-mediated gene therapy, which has been extensively studied. Since hepatocytes uniquely express asialoglycoprotein receptors (ASGP-R), several ligands like galactose, asialo-feutin, asialo-transferrin, asialo-ceruloplasmin, asialo-lactoferrin, asialo-orosomucoid, lac-BSA, and hepatoglobulin have been explored for hepatocyte-selective transgene expression with non-viral gene delivery systems [24]. ASGP-R is expressed strongly in normal liver cells, whereas the expression is decreased in various chronic liver diseases and hepatoma [25]; especially, when the tumor reaches a higher grade (grade III or IV), the ASGP-R expression becomes less [26]. This may cause a problem of inefficient gene delivery for practical gene therapy of human liver cancer. The other is liver-specific promoter-mediated gene therapy like the study reported here, which is much less extensively studied. An efficient gene delivery system is the requisite. Our data here further support that our pegylated immuno-lipopolyplexes is an efficient non-viral gene delivery system for gene therapy of liver cancer.

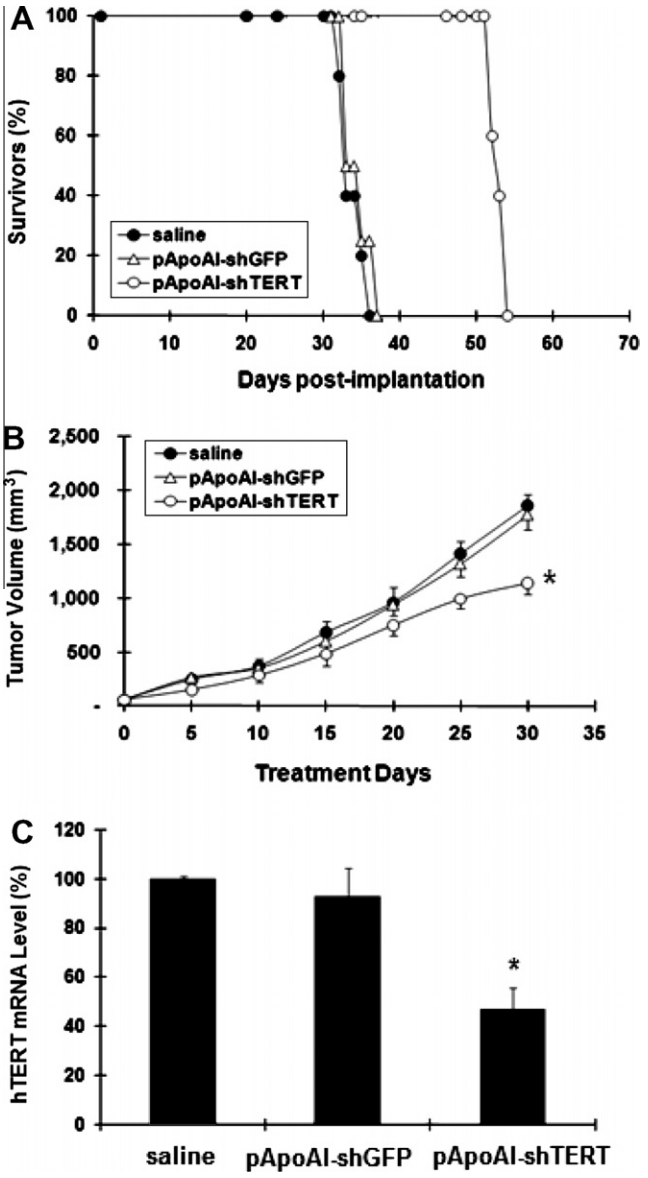

Fig. 4. Therapeutic effects of pApoAI-shTERT on SMMC-7721 human liver tumors in mice. Subcutaneous hepatocellular carcinoma tumors were induced by the inoculation of 2×10^6 human SMMC-7721 cells in the right flank of female BALB/c nude mice. When the tumor volume reached 50–80 mm^3, groups of the mice ($n = 12$) were intravenously treated with either saline (control) or 40 μg/mouse of pApoAI-shGFP (anti-unrelated plasmid) or pApoAI-shTERT plasmid formulated as PILP every five days. (A) Effect of pApoAI-shTERT on the life span of the liver tumor-bearing mice; (B) effect of pApoAI-shTERT on tumor growth; (C) effect of pApoAI-shTERT on the hTERT mRNA level in the tumors. $^*P < 0.05$, compared with the control saline group.

In summary, liver-specific gene therapy of hepatocelluar carcinoma is made possible by utilizing the PILP gene delivery system to deliver shRNA expression plasmid against hTERT under the control of the liver-specific promoter ApoAI.

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