Combination of HSV1-TK/shTERT by retrovirus vector inhibits hepatocellular carcinoma cell growth in vitro and in vivo

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Abstract. Hepatocellular carcinoma (HCC) is a common malignant carcinoma worldwide and the third leading cause of cancer mortality. However, current treatment strategies are not potent enough to combat this disease. Therefore, identification of novel and more effective treatments is crucial. Of the current methods, gene therapy, which targets cancer-specific expression and limits toxicity, is a new strategy for treating cancers. In this study, we developed a retroviral vector containing herpes simplex virus type-1-thymidine kinase (HSV1-TK) and a short hairpin RNA for the human telomerase reverse transcriptase (hTERT) gene and investigated the antitumor effects in an in vitro and in vivo mouse model of liver cancer, monitored by PET image. In vitro experiments on HCC cells in the TK-shTERT treatment group showed significant accumulation of 18F-FHBG, which preferentially inhibits HCC cell growth with extremely limited toxicity in normal cells. In vivo studies showed a significant reduction of growth in the TK-shTERT treatment group. In conclusion, these findings showed that combination HSV1-TK/hTERT gene therapy effectively and safely inhibits HCC cell growth in vitro and in vivo and is worthy of development in clinical trials for the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant carcinoma worldwide and the third leading cause of cancer mortality (1,2). Although its mortality decreased along with great advances in surgical resection and chemotherapy, the long-term prognosis remains unsatisfactory (3,4). Thus, effective and innovative therapeutic procedures are required to combat this disease. Evidence has shown that in addition to current chemotherapy, gene therapy using small-interfering RNA (RNAi) is a potent therapeutic approach in HCC therapy (5).

Telomerase is a ribonucleoprotein complex that is involved in tumor growth and progression in part through maintaining the ends of chromosomes (6). Human telomerase reverse transcriptase (hTERT), an essential subunit of the telomerase complex, maintains the length of telomeres by reverse transcription and the addition of TTAGGG repeats onto the telomeric ends of the chromosomes (7). Moreover, in various human cancer cells, such as liver and ovarian cancer cells, a close correlation between hTERT expression and telomerase activity has been identified (8-10). Previous findings showed that the hTERT appeared to be the major determinant of telomerase activity (11). These observations indicated that hTERT expression and telomerase activity serve as useful diagnostic and/or prognostic markers in many types of human malignancies. Accordingly, the potential of telomerase inhibition by targeting hTERT as an effective therapy for cancer treatment has been demonstrated (12,13). hTERT was previously found to be expressed in most cancer cells but not in normal immortal cells (14). However, previous results showed that hTERT can be detected in both malignant and normal tissues (15-17). Thus, to use this target by silencing hTERT expression in the treatment of cancer requires identification of a method with tumor cell-specificity and low toxicity, which can effectively inhibit hTERT gene expression in tumor cells but cannot affect hTERT activity in normal immortal cells.

Different approaches are currently under investigation to develop gene therapy of cancer. However, experience from clinical trials of cancer gene therapy indicates that no single therapeutic strategy can effectively eradicate cancer, whereas combined poly-gene therapy is a more reliable approach to combating cancer. A well-established radionuclide-based reporter gene system is the herpes simplex virus type-1-thymidine kinase (HSV1-TK) enzyme, which can specifically infect a variety of cancer cells but not normal mammalian cells (18,19). This reporter gene can itself be the therapeutic gene, and anticancer gene therapy using HSV1-TK can be coupled with imaging of the accumulation of radio-labeled probes such as 9-[(4-fluoro-3-(hydroxymethyl)butyl]guanine (FHBG) or 5-iodo-20-fluoro-20deoxy-1-b-D-arabinofuranosyluracil (FIAU) (20,21), which can be imaged using PET. Thus, co-expression of a reporter gene and a therapeutic gene...
may allow for delivery of therapeutic gene to cancer cells efficiently and safely and treat cancer effectively. Consequently, we constructed triple-expressing vector in which the HSV1-TK gene and shTERT were driven by the pLXSN and the U6 promoters and detected the antitumor effects on cells in vitro as well as tumor inhibition in vivo. In this study, the combination of hTERT shRNA/HSV1-TK preferably targeted HCC cells and significantly suppressed the tumor growth in liver tumor xenograft. Moreover, hTERT shRNA/HSV1-TK showed virtually no toxicity in normal cells, suggesting that the hTERT shRNA/HSV1-TK vector may be exploited as a potential new therapeutic strategy for HCC.

Materials and methods

Cell lines and culture. Human HCC cell lines (BEL-7402, HCC36, HepG2, HA22T and Hep3B), normal mammary epithelial (L-02, QSG-7701), and normal lung fibroblasts (WI-38) were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO2/95% air.

Construction of recombinant retroviral vectors and vector titration. The siRNA sequence targeting hTERT corresponded to the coding region from 385 to 393: 5'-aaGCaCTTCCTCT-3' and reverse: 5'-CaaGCTTCTCGa-3'. to the coding region from 385 to 393: 5'-aaGCaCTTCCTCT-3'. to the coding region from 385 to 393: 5'-aaGCaCTTCCTCT-3'. Titration.

Construction of recombinant retroviral vectors and vector titration. The siRNA sequence targeting hTERT corresponded to the coding region from 385 to 393: 5'-AAAGCAGAATCCTCTC TTCACGCT-3'. The oligonucleotides with a sequence predicted to induce efficient small-interfering RNA (RNAi) of hTERT (containing sense and antisense sequences linked by the hairpin loop: CGaA) were synthesized as follows: Forward: 5'-CGTGAATCACTTCTC GTAGGCTGC-3' and reverse: 5'-CAATCTGGCATCG TACGTAGGATTCCTC-3'. These oligonucleotides were annealed in STE buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA) at 94°C for 5 min and cooled gradually. The double-stranded product was cloned downstream to the human U6 promoter of the pGEM/U6 vector (Genscripts). The recombinant retroviral vector pLXSN (Clontech) was employed to develop the pLXSN-TK-U6-shTERT vector. Full-length HSV1-TK cDNA (kindly provided by Dr Qiu Xinfang, Fudan University, Shanghai, China) sequence coupled to U6 promoter was removed from pRNAT-shTERT vector, and a Nhel-SalI restriction site was introduced into the pRNAT-shTERT vector by PCR. HSV1-TK cDNA was amplified by PCR using the sense primer, which contains a Nhel restriction site, and the anti-sense primer, which contains a SalI restriction site. Following restriction digestion with Nhel and SalI of pRNAT-hTERT shRNA, the HSV1-TK sequence was ligated into the pRNAT-shTERT vector. The resulting vector containing shTERT sequence and HSV1-TK cDNA coupled to the U6, was designated as pLXSN-TK-U6-shTERT vector, and confirmed by DNA sequencing. A retroviral vector containing the shTERT sequence (pLXSN-U6-hTERT shRNA) was also developed. The U6 promoter was inserted into the vector as described above.

Plasmid vectors were transfected into the amphotropic packaging cell line 293 by using the calcium phosphate transfection system (Invitrogen Life Technologies, Carlsbad, CA, USA) reagents, as previously described (22). Transfected cells were selected in a medium containing G418 (Invitrogen) and single cell-derived clones were isolated and expanded to cell lines. Viral titer, determined by infection of NIH3T3 with virus-containing supernatants from single cell-derived clones of 293 producer cells as described previously (22), ranged from 10⁴ to 10⁶ pfu/ml. The supernatant from the producer cell clones with higher viral titer was used to transduce target cells.

MicroPET imaging of ¹⁸F-FHBG in vivo. When flank tumors reached 200 mm³ in size, 0.2 ml pLXSN-U6-shTERT (5x10⁶ pfu/ml) or 0.2 ml pLXSN-TK-U6-shTERT (5x10⁶ pfu/ml) were injected directly into the center of the tumor. Mice were anesthetized via inhalation of isoflurane (1-1.5%) with an oxygen flow rate of 2 l/min. Depth of anesthesia was monitored by respiratory rate and eye and footpad reflex. A total of 150 MBq (0.2 ml) ¹⁸F-FHBG was injected into the tail vein to acquire dynamic volumetric data for 210 min. Volumetric images were reconstructed with filtered back projection after the data were corrected for uniformity, scatter, attenuation, decay, and injected activity using the software AspIm 4.1 provided by the manufacturer. Time-activity curves were generated from the selected regions of interest including pLXSN-U6-shTERT (on the left flank), pLXSN-TK-U6-shTERT tumor (on the right flank).

Western blot analysis. The cells were lysed in mammalian protein extraction reagent (Pierce Biotec, Rockford, IL, USA). Cell lysates were collected and protein concentration of the cell lysates was measured. Proteins (10-20 µg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were then incubated with primary antibodies in 3% bovine serum albumin/Tris-buffered saline/Tween-20 at 4°C overnight, followed by incubation with secondary antibodies at room temperature for 1 h. The protein signals were detected by ECL method. Western blotting reagents were obtained from Pierce Biotec. The antibodies to hTERT and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell viability assay. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were plated at a density of 5x10⁵ cells/well in 96-well tissue culture plates and subjected to different treatments. Following a 72-h incubation at 37°C in a humidified atmosphere containing 5% CO2/95% air, the cells were incubated for another 4 h with MTT reagent. The formazan product was dissolved in dimethyl sulfoxide and read at 570 nm on a Victor 3 Multi Label plate reader (PerkinElmer, Boston, MA, USA).

In vivo combination therapy. When tumor masses reached average volume of ~200 mm³, pLXSN-U6-shTERT and pLXSN-TK-U6-shTERT were initiated. Tumor xenograft mice were randomly divided into the control, pLXSN-U6-shTERT and pLXSN-TK-U6-shTERT groups. The mice in each group (n=5) received intratumor injections of either 0.2 ml pLXSN-U6-shTERT (5x10⁶ pfu/ml) or 0.2 ml pLXSN-TK-U6-shTERT (5x10⁶ pfu/ml) on continuous injection for five days. The mice
in the control group received saline injections at the same time. Individual tumor size was measured every 2 days by use of a caliper and tumor volumes were determined by measuring the length (L) and width (W) of the tumors and calculating using the following formula: \( V = \frac{LW^2}{2} \).

Animal maintenance and experimental procedures were approved by the Nanfang Hospital Animal Ethics Committee.

**In vivo apoptosis assays.** At the end of the experiment (5 weeks after intratumor injection), the mice were humanely euthanized and tumors were surgically dissected. The tumor specimens were fixed in 4% paraformaldehyde for TUNEL staining (Roche Applied Sciences, Indianapolis, IN, USA) according to the manufacturer’s instructions. Briefly, the slides were incubated with 50 ml of TUNEL reaction mixture in a humidified atmosphere for 1 h at 37°C in the dark. The percentage of TUNEL apoptotic cells was analyzed by randomly selecting five independent fields for each sample.

**Statistical analysis.** The results are given as mean ± SD. Student’s t-test was used to analyze the significance of differences. The significance level was set at \( P<0.05 \).

**Results**

**Construction and identification of pLXSX-TK-U6-shTERT recombinant plasmid.** Recombinant plasmids pGEM-T-shTERT, pGEM-T-U6, pUC19-EGFP and pUC19-TK were constructed. BamHI/SalI and SalI/HindIII double enzyme digestion methods were selected to obtain pUC19-EGFP-U6 and pUC19-EGFP-U6-shTERT respectively, EcoRI/XhoI double enzyme digested pUC19-EGFP-U6-shTERT and pLXSX plasmids to obtain pLXSX-EGFP-U6-shTERT, BamHI/SalI double enzyme digested pGEM-T-U6 and pUC19-TK plasmids to obtain pUC19-TK-U6, and SalI/EcoRI double enzyme-digested recombinant plasmids pLXSX-EGFP-U6-shTERT and pUC19-TK-U6 to obtain pLXSX-TK-U6-shTERT.
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Figure 1a. Restriction enzyme identification of recombinant plasmids are shown in Fig. 1B.

pLXSN-TK-U6-shTERT recombinant plasmid preferentially inhibits HCC cell growth in vitro with extremely limited toxicity in normal cells. To determine whether the pLXSN-TK-U6-shTERT plasmid can specifically target HCC cells, the expression of hTERT was detected by western blotting 48 h after transient transfection in BEL-7402 and HepG2 HCC cells (Fig. 2A). The results showed that pLXSN-TK-U6-shTERT inhibited cell growth in vitro in a dose-dependent manner (Fig. 2B). Moreover, we examined the killing effects of pLXSN-TK-U6-shTERT in a panel of HCC and normal cell lines. The results showed that the pLXSN-TK-U6-shTERT inhibited cell growth more effectively than pLXSN-U6-shTERT in vitro (Fig. 2B). However, in normal cells, the cell killing activity of pLXSN-U6-shTERT was more potent than that of pLXSN-TK-U6-shTERT (Fig. 2C). Thus, the cytotoxic effect of pLXSN-TK-U6-shTERT is potent in cancer cells but limited in normal cells, indicating that pLXSN-TK-U6-shTERT is tumor-specific in vitro and is a potential therapeutic agent that can be used for the treatment of HCC.

Detection of anatomic sites of 18F-FHBG sequestration with microPET. When tumor masses reached an average volume of ~200 mm³, the mice received intratumoral injections of 0.2 ml pLXSN-U6-shTERT (5x10⁵ pfu/ml, left flank) or 0.2 ml pLXSN-TK-U6-shTERT (5x10⁵ pfu/ml, right flank). Compared with shTERT tumors (left flank), TK-shTERT tumors of mice showed significant accumulation (right flank) over time as a result of intracellular entrapment of HSV-TK-phosphorylated 18F-FHBG, 150 min after 18F-FHBG injection, when the uptake value reached its greatest level (Fig. 3A and B). The liver and kidney concentration of 18F-FHBG was significantly greater, and no radioactive distribution was observed in the brain (Fig. 3B).

pLXSN-TK-U6-shTERT recombinant plasmid exerts a significant antitumor effect in mouse xenograft model of HCC. To investigate the therapeutic effects of pLXSN-TK-U6-shTERT in vivo, we established a mouse xenograft model with the BEL-7402 HCC cell line. When tumor masses reached an average volume of ~400 mm³, the mice in each group received intratumoral injections of pLXSN-U6-shTERT plasmid or pLXSN-TK-U6-shTERT plasmid of continuous injection for
Five weeks after the first treatment, the pLXSN-TK-U6-shTERT group significantly inhibited tumor growth compared with the pLXSN-U6-shTERT group (Fig. 4A and B). These findings were further supported by the increase in apoptosis and decrease in proliferation in the tumors of the pLXSN-TK-U6-shTERT-treated groups. Notably, although the two treatment groups showed a statistically significant trend of increase in TUNEL-positive cells and decrease in Ki67-positive cells compared with the control, pLXSN-TK-U6-shTERT showed significantly higher activities than pLXSN-U6-hTERT shRNA, suggesting that pLXSN-TK-U6-shTERT has higher targeting power over pLXSN-U6-shTERT (Fig. 4C and D). Collectively, these results showed that pLXSN-TK-U6-shTERT consistently exerts strong antitumor effects on liver tumor in vivo and induces apoptosis with high-tumor specificity.

Discussion

Cancer cells frequently overexpress hTERT, a determinant of telomerase activity, resulting in enhanced proliferation and tumor progression (23,24). hTERT associates with human telomeres and may enhance genomic stability and DNA repair in human cancer cells (25). Thus, hTERT is an attractive therapeutic target in malignant tumor treatment. A variety of gene-targeting approaches were found to interfere with hTERT function (26-28). Among them, RNAi-mediated hTERT gene silencing provides an efficient method to inhibit telomerase activity for human cancer therapy (29-31). RNAi is known to be very effective and selective in vitro, however, issues, including incomplete suppression of target genes, requirement of cytotoxic transfection reagents and/or enhancers, positively impairing normal cellular functions and lack of effective delivery system, hamper the development of this novel therapy for cancer treatment. Thus, the development of low-toxic and potent shRNA delivery systems is a crucial step for the success of RNAi-based cancer therapy.

The most studied suicide gene is HSV1-TK. HSV-TK-based suicide gene therapy has been used to target cancers and its role has been assessed in several clinical trials. The reporter gene can itself be the therapeutic gene or can be coupled with the therapeutic gene (32). Clinical trials using this approach have been conducted in patients with gliomas and no serious adverse events were reported (33). The successful employment of several candidate gene
combinations for HSV1-TK suicide gene therapy have been reported including IL-2 (34), STAT3 (35), cytosine deaminase (36), nitroreductase (37), and carboxylesterase (38). Non-invasive imaging should be accompanied with gene therapy approaches for treatment response monitoring as well as for assessment of distribution, extent and duration of transgene expression. Rapid washout of activity from the blood significantly decreased sensitivity and specificity of tracer accumulation in HSV1-TK-expressing tumors. ¹⁸F-FHBG has emerged as the most reliable agent for treatment response monitoring. In this study, we demonstrated the applicability of non-invasive imaging using ¹⁸F-FHBG for monitoring cancer gene therapy in an experimental animal model of HSV1-TK-expressing tumor xenografts, which is also in concordance with a previous report (39). In the present study, we demonstrated the in vitro and in vivo therapeutic efficacy of retroviral vector-mediated combined HSV1-TK suicide and hTERT gene therapy for HCC. The use of an LXSN-TK-based retroviral vector allows high transfection efficiency of hTERT expression and selective targeting of cancer cells, while sparing normal cells in vitro.

Although retrovirus vectors increasingly receiving attention in the field of liver cancer gene therapy due to their hepatic tropism and high titers, they have one major drawback of in vivo delivery and transduction (40,41), which may be relatively to obtain in liver cancer in which percutaneous locoregional treatment is easy to perform. In the current study, we also carried out in vivo transduction by repeated intratumoral injection of the vector. When tumors were transduced in vivo with TK-shTERT retroviral vector, we observed a strong antitumor effect, with complete regression of tumors composed of transduced cells compared with the shTERT group, showing wide apoptotic areas and markedly decreased Ki67-positive cells in the residual tumor (Fig. 4), thus confirming the efficacy of the vector and the possibility to transduce a sufficient amount of tumor cells by locoregional treatment, as also confirmed by the in vivo experiment.

Taken together, our study has demonstrated enhanced antitumor effects by the use of combination gene therapy using TK-shTERT for tumor xenografts in mice as compared with shTERT single therapeutic approach. Furthermore, therapeutic response monitoring was possible by serial non-invasive in vivo imaging using a reporter gene system. These results suggest that shRNA targeting hTERT, coupled with the HSV-TK suicide gene, may be therapeutically useful for HCC and potentially for other malignancies.
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References


