# Anticancer activity of oncolytic adenoviruses carrying p53 is augmented by 11R in gallbladder cancer cell lines *in vitro* and *in vivo*

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Abstract. Gallbladder cancer (GBC) is a rare disease associated with an extremely poor patient prognosis, and occasionally, aberrant expression of p53 is present. Considering that p53 is one of the most widely studied tumor-suppressor genes, we used a cell-penetrating peptide, 11R, to enhance the transferring efficiency of the oncolytic adenovirus carrying the p53 gene by constructing SG7605-11R-p53, a gene-viral therapy system which has higher specificity, enhanced safety, and efficacy. After infection with SG7605-11R-p53 at a multiplicity of infection (MOI) of 1 PFU/cell in vitro, the survival rate of EH-GB1 cells was lower than 50%, and that of EH-GB2 cells was lower than 40%, while the survival rate was higher than 90% for BJ human fibroblast cells, demonstrating that SG7605-11R-p53 has potent specific cytotoxicity against GBC cells. The tumor growth was greatly inhibited in nude mice bearing EH-GB2 xenografts when the total dose of SG7605-11R-p53 was 1x109 PFU, and terminal dUTP nick end-labeling (TUNEL) revealed that the apoptotic rate of cancer cells was

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Abbreviations: MOI, multiplicity of infection; TUNEL, terminal dUTP nick end-labeling; GBC, gallbladder cancer; CPP, cell penetrating protein

*Key words:* gallbladder cancer, p53, gene therapy, cell-penetrating peptide, 11R

 $66.75\pm6.702\%$ . Compared with existing gene therapy with longstanding shortcomings, our new system offers an additional option for patients with advanced GBC and other cancers who may not be suitable for chemotherapy, radiotherapy or who are not indicated for surgical treatment.

### Introduction

Gallbladder cancer (GBC) is a rare but highly lethal disease, known to humans since 1777 (1,2). It is now recognized as one of the most common biliary tract malignancies (3). Up to 75% of such patients suffer from this unresectable disease at the time of surgical evaluation (4).

Currently available treatments such as chemotherapy and radiotherapy have little effect on advanced GBC, and the 5-year survival rate is only ~5% in such cases (5). Therefore, development of new treatment modalities, such as gene therapy, merits high priority. Yet, few studies are available concerning GBCs.

Gene therapy has been widely accepted as an important strategy for treating malignancies (6). Yet, the clinical application of tumor gene therapy still has some limitations, such as low gene transferring efficiency, poor transgene expression, and limited target specificity to tumors (7). Among the possible candidate genes is the gene that codes for the p53 protein, whose product is mainly enriched around the nucleoli of normal cells and can specifically bind with DNA. The protein activity is regulated by phosphorylation and is readily degraded (8,9). In contrast, the p53 protein loses its function quite readily due to its gene mutation, especially on its 393 amino acid residue (10).

The wt-p53 gene has been a popular candidate for gene replacement therapy since it suppresses tumor growth in various types of solid tumors (11-15). Mutation in the p53 gene may cause various types of cancers. Researchers have also proven that the growth of tumors with p53 gene mutation may be inhibited even in the late stages, and the function of p53 may still be recovered by clinical therapy. As a result, p53 has already been considered as a target for gene therapy for various types of cancers such as gastric (16), colon, ovarian (17) and

liver cancer (18). Studies have long confirmed that mutation in the p53 gene occurrs in GBC as well (19-21).

As a delivery method for gene therapy, viral vector system-mediated gene therapy for targeting of tumor-suppressor genes such as p53 has been popular for over 15 years. Currently, the viral vector has been accepted as a useful media to transduce exogenous genes into various types of human cells in vitro and in vivo. One small element used in gene therapy systems is known as the cell-penetrating protein (CPP) which can penetrate the cell membrane to the nucleus through the cytoplasm even without any additional substance such as Lipofectamine 2000 (LF2000) (22), and does not destroy the cell membrane structure. Hence, CPPs could effectively carry macromolecules such as Taxol (23), CyA (24) and methotrexate (25). p53 has already been connected with CPPs to treat oral carcinoma (26), bladder carcinoma (27) and glioma (28), but reports are rare on gene strategy related to CPP proliferation in cells.

A protein transduction method using 11 polyarginine peptides (11R) has proven to be useful in the delivery of protein to cells. Studies have also shown that p53 protein-fused 11R (11R-p53) is efficiently delivered to cancer cell lines *in vitro*. These existing studies have implied that a combination of the 11R-mediated protein transduction method and viral vectormediated gene transduction therapy may be a practical solution for gene therapy of unresectable cancer.

In the present study, we constructed a penetrating peptide 11R-mediated oncolytic adenoviral vector carrying tumor-suppressor gene p53, and demonstrated its replicative activity and antitumor specificity *in vitro* ant its oncolytic efficacy *in vivo*. Our goal was to attempt to establish a new and improved wide spectrum, specific, safe and highly effective antitumor gene therapy strategy.

## Materials and methods

*Cell lines and culture*. Gallbladder carcinoma cell line GBC-SD was obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. EH-GB1 and EH-GB2 cells were reserved at the Eastern Hepatobiliary Surgery Hospital (29,30). Normal fibroblast BJ cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured with Dulbecco's modified Eagle's medium (DMEM; Gibco, UK) containing 100 ml/l fetal bovine serum (FBS), 100 kU/l penicillin, and 100 mg/l streptomycin at 37°C and in an atmosphere containing 5% CO<sub>2</sub>.

*Construction of plasmids and recombinant viruses*. Using PPE3-enhanced green fluorescent protein (EGFP) as the template, AT236 as primers for PCR (primer nucleotide sequence, CGACGGCGACGGCGAAGAAGGGTGAGCAA GGGCGAGG) and AT237 (CGCGGATCCTTATTATCTAG ATCCGGTGGATCTGAGTCCGGAC), we obtained a 753-bp product EGFP fragment. Then, using the 753-bp product as the template, GT358 as primers for PCR (primer nucleotide sequence, CCGGAATTCACCATGCGCCGCAGGAGAGAGA CGGCGACGGCGA) and AT237 (CGCGGATCCTTATTATCTAT CTAGATCCGGTGGATCTGAGTCTGAGTCCGGACGCCGCAGGAGACGA CGGCGACGGCGA) and AT237 (CGCGGATCCTTATTAT CTAGATCCGGTGGATCTGAGTCCGGAC), we obtained 11R-EGFP. 11R-EGFP and PENTER17 were digested by EcoRI and BamHI, and then the 2 digested products were combined by ligase in the following ratio as 11R-EGFP/ *Eco*RI + *Bam*HI to PENTER17 = 4:1 to get PENTER17-11R-EGFP. The combined plasmid PENTER17-11R-EGFP was identified using EcoRI/BamHI to get two 792+3011 bp segments and XbaI/XhoI to get three 206+3967+2630 bp segments. After the identification of the plasmid PENTER17-11R-EGFP, it was recombined with PPE3-RC, and PPE3-11R-EGFP was obtained through Gateway recombination. Using large scale preparation of the plasmid, PPE3-11R-EGFP was co-transfected with T4-TP-122/142 into 293 cells by the Lipofectamine® 2000 kit. Ten days later, virus plaques appeared and were collected, from which adenoviral DNA was extracted using QIAamp DNA Blood Mini kit following the manufacturer's instructions and confirmed by PCR analysis using the forward and reverse primers of 11R-EGFP. The confirmed recombinant adenoviruses were named SG7605-EGFP, SG7605-p53, SG7605-11R-EGFP and SG7605-11R-p53.

In vitro viral replication assay. Monolayer cells, including logarithmically growing cancer cells EH-GB1, EH-GB2, GBC-SD (10<sup>5</sup> cells/well) and contact-inhibition normal cells BJ (10<sup>6</sup> cells/well) were cultured in a 6-well plate overnight and infected with SG7605-p53 or SG7605-11R-p53 at a multiplicity of infection (MOI) of 5.0 PFU/cell. Virus inocula were removed after 2 h. The cells were then washed twice with phosphate-buffered saline (PBS) and incubated with 2% serum medium at 37°C for 0, 12, 24, 48 or 96 h. Lysates of cells were prepared with 3 freeze-thaw cycles. Serial dilutions of the lysates were titered in human embryonic kidney 293 cells with tissue culture infectious dose 50 PFU method, and the results were normalized with that at the beginning of infection, and reported as multiples. The adenovirus SG7605 and SG7605-p53 were also used as controls.

Western blot analysis of 11R-p53. Cells were counted and seeded on a 24-well plate at a density of 5x10<sup>4</sup>/well for 24 h. SG7605, SG7605-p53 and SG7605-11R-p53 were separately added to the system at a MOI of 5 PFU/cell, and SG7605 at MOIs of 5 and 20 PFU/cell. Cells were then gently shaken for 2 h; the supernatant was then discarded and 2% serum medium was added for a 48-h culture. Cells were collected and lysed with PER Mammalian Protein Extraction reagent (Pierce, Rockford, IL, USA); the proteins were harvested and maintained at -80°C after quantification by a BioPhotometer (Eppendorf AG, Hamburg, Germany).

SDS-PAGE gel electrophoresis solution (10%) was used to separate the proteins at 70 V. The proteins were transferred to a Protran nitrocellulose transfer membrane (Schleicher and Schuell Inc., Keene, NH, USA) and was then blocked with blocking buffer (0.5% FBS, 50 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 0.1% Tween-20, 0.02% NaN<sub>3</sub>) for 1 h, washed with 1X Tris-buffered saline-Tween-20 (TBST, 50 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 0.1% Tween-20) for 3 times, each time for 5 min. Then the membrane was incubated with the monoclonal antibodies against adenovirus p53 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at  $4^{\circ}$ C overnight, then washed with 1X TBST 3 times, each time for 5 min. The membrane was then allowed to react with the

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horseradish peroxidase (HRP) goat anti-mouse IgG antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) at room temperature for 1 h, then washed with 1X TBST 3 times, each time for 5 min. Finally, color development solution LumiGLO<sup>®</sup> chemiluminescent reagent and peroxide (Cell Signaling Technology, Inc.) were added for visualization on X-ray film.

Quantitative real-time PCR (qRT-PCR) for detection of the expression of the p53 gene. For p53 gene expression, total cellular RNA was extracted from 5x10<sup>5</sup> GBC or BJ cells using the RNeasy Mini kit from Qiagen (Valencia, CA, USA), followed by treatment with DNase to remove any possible contaminating DNA from the RNA samples. qRT-PCR was used to detect p53 expression. The fluorescent TaqMan probe (6FAM-TGACGACCCCATAGAGGAACATAAAAAGCAT) and the primer pair (forward, TGGAAGGCTGGGAGCCA and reverse primer, GAAAGCGCAACCGGACG) were used for qRT-PCR in analysis of the p53 mRNA and were designed using Primer Express 1.0 and synthesized by Applied Biosystems (Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. A negative control with no template was included for each reaction series. qRT-PCR reaction was carried out using a LightCycler<sup>TM</sup> system (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions, under the following amplification conditions: 2 min at 50°C, 30 min at 60°C, 5 min at 95°C, then 40 cycles of 20 sec at 94°C, and 1 min at 62°C. The amplified fragment was 450 bp. GAPDH was taken as the internal control, with the primers being AT094, 5'-ACCACAGTCCATCAC-3' and AT095, 5'-TCCACCACCCTGTTGCTTGTA-3'. Data were analyzed with LightCycler software.

Viability test in vitro. The cytotoxic effect of the virus on different cell lines was examined by Cell Proliferation Kit I (MTT) (Roche Diagnostics GmbH, Mannheim, Germany). To determine the optimal cell concentration, cells in the exponential phase were collected, counted, and were made into a single-cell suspension of a series of concentration gradients (from  $2x10^4$ /ml to  $2x10^5$ /ml). The suspensions were seeded into a 96-well plate (each concentration in 8 wells and 100  $\mu$ l suspension in each well) and cultured for 24 h. After another 7 days of culture, 100 µl PBS (0.1 mol/l) and 10 µl MTT labeling reagent were added to each well to achieve a final concentration of 0.5 mg/ml, and the cells were cultured for another 4 h; then 100  $\mu$ l/solubilization solution (10% SDS in 0.01 mol/l HCl) was added to each well, and the cells were placed in an incubator overnight. Model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA) was used to determine the absorbance at 570 nm, with the reference wavelength at 655 nm. A cell growth curve was plotted to confirm the optimal concentration of cells.

Next, an MTT assay under different MOI values was performed. Cells in exponential phase were collected, counted, and cultured with 10% serum medium. Single-cell suspensions were prepared according to the above determined optimal cell concentration. The suspensions were seeded into a 96-well plate for a 24-h culture, with 100  $\mu$ l suspension in each well. The viruses were diluted with serum-free medium and 100  $\mu$ l of the virus (with MOIs ranging from 0.01 to 100 PFU/ cell) was added to the plate, with each MOI value assigned to 8 wells. After another 7 days of culturing, MTT assay was carried out as described above.

Animal experiments. The protocol for the in vivo mouse xenograft models was approved by the Second Military Medical University's Medical Experimental Animal Care. Surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. A total of  $1 \times 10^7$ logarithmically growing EH-GB2 cells (a human GBC cell line established and characterized by our laboratory) was injected s.c. into the right flank of each BALB/c nude mouse (Institute of Animal Center, Chinese Academy of Sciences, Shanghai, China) aged 6-8 weeks. From 10 to 14 days after inoculation, when the subcutaneous nodules grew and reached 7-9 mm in diameter, the mice were randomly assigned to 3 treatment groups (SG7605, SG7605-p53 and SG7605-11R-p53) and one control group (treated with virus preservation buffer). There were 11 mice in each group. Pre-established tumors were then injected with 100  $\mu$ l control buffer or 2x10<sup>8</sup> PFU viruses in the same medium, respectively. The injections were repeated every other day for 5 times with a total dosage of 1x10<sup>9</sup> PFU. Tumor growth was monitored by periodic measurements with calipers, and the tumor volume was calculated using the following formula: Volume = (maximal length) x (perpendicular width) $^{2}/2$ . All of the animals were sacrificed 28 days after treatment, and the tumors were resected and weighed. The tumor reduction rate was calculated using the following formula:

 $\begin{array}{l} Tumor \ reduction \ rate = [1 - (V_{treatment \ group \ endpoint} - V_{treatment \ group \ initial} \\ \ point)/(V_{control \ group \ endpoint} - V_{control \ group \ initial \ point})] \ x \ 100\%. \end{array}$ 

Another set of male 50 BALB/C nude mice aged 4-6 weeks, was used to establish the EH-GB2 xenografts for observing the survival time, and 32 mice with 5-7 mm xenograft tumors were treated in the same way as described above. The survival status of the mice due to heavy tumor burden was observed until the experiments were terminated. All the procedures in the study were performed in accordance with institution guidelines and were approved by the Committee on the Use and Care of Animals of the Second Military Medical University.

*Histology and immunohistochemistry*. All of the excised tumors were fixed in 10% neutral formaldehyde for 6 h, and embedded with paraffin. Tumor sections were subjected to H&E staining and immunohistochemistry. The expression of p53 was evaluated through immunohistochemistry using the mouse anti-human p53 antibody (Biodesign International). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was carried out using the *In situ* Cell Death Detection kit (Roche Diagnostics GmbH) according to the manufacturer's instructions.

Statistical analysis. ANOVA was used to test the differences in any of the parameters. Survival was analyzed by the Kaplan-Meier method, and results were compared for statistical significance using the generalized Wilcoxon test. Chi-square analysis was used to test the difference in survival rates in each group. All statistical analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). The difference was considered statistically significant at a P-value <0.05.



Figure 1. Schematic diagram of the recombinant adenoviruses. Compared with the original type of adenoviruses, hTERTp was used to replace Ela-pro; Elb-pro and Elb were replaced as micRNA. The expression cassette of p53 or enhanced green fluorescent protein (EGFP) was inserted into the adenoviral genome E3 region and generated the recombinant adenoviruses SG7605-p53 or SG7605-EGFP. The cell penetrating peptide, 11R, was used to ferry inserted protein into cells. ITR, inverted terminal repeats; Ψ, adenovirus 5 packaging signal; Ela-pro, the wild-type of Ela promoter; Elb-pro, the wild-type of Elb promoter, including Elb-55 kDa and Elb-19 kDa; CMV, cytomegalovirus promoter.

#### Results

*Construction of adenovirus SG7605-11R-p53*. A novel conditionally replicative adenovirus SG7605-11R-p53 was constructed, in which the E1A gene was placed under the control of the hTERT promoter plus 3 extra E-boxes, and the mouse endostatin-expressing cassette was inserted between the adenoviral package signal and the hTERT promoter (Fig. 1). Adenoviral DNA was extracted, and recombination was confirmed by PCR amplification without contamination by wild-type adenoviruses. SG7605-p53, SG7605-EGFP and SG7605-11R-EGFP were also constructed for use in subsequent studies as described below.

Specific gene expression mediated by SG7605-11R-p53. EGFP was strongly expressed in cancer cells infected with the 11R-penenetrating peptide-mediated tumor-specific adenovirus harboring EGFP (SG7605-11R-EGFP), but was only weakly expressed in normal cells after infection (P<0.0001) (Fig. 2A). To prove that the specificity of expression was due to the difference between cancer cells and normal cells, SG7605-EGFP was used to infect BJ, GBC-SD, EH-GB1 and EH-GB2 cells at MOIs of 5 and 20 PFU/cell. SG7605-EGFP-mediated expression of EGFP was enhanced with an increase in MOIs in BJ, GBC-SD, EH-GB1 and EH-GB2 cells. Compared with SG7605-EGFP, SG7605-11R-EGFP mediated a higher level of EGFP expression in the GBC-SD, EH-GB1 and EH-GB2 cells (P<0.0003), but there was no significant difference in BJ cells when infected at the same MOI of 5 PFU/cell (P=0.5601) (Fig. 2B). 11R and p53 were positively expressed in cancer cells infected with the 11R-penetrating peptide-mediated tumor-targeting adenovirus SG7605-11R-p53, but were not or only weakly expressed in normal cells. SG7605-p53 did not express 11R at the indicated MOI, and expression of p53 was greatly lower than SG7605-11R-p53 (Fig. 2C and D), indicating the 11R-penetrating peptide-mediated tumor-targeting adenovirus specifically proliferated in the cancer cells.

Oncolytic effect of SG7605-11R-p53 on cell viability. For assessing the selective killing capability of SG7605-11R-p53 in comparison with SG7605-p53, a panel of tumor cells was planted in 96-well plates at a density of  $1x10^4$  cells and infected with SG7605-11R-p53 and, 24 h later at a final MOI from 0.001 to 100 PFU/cell, SG7605-p53. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was then employed to quantify cell viability and compare the MOI which was associated with 50% cell viability (IC<sub>50</sub>). We found that when the MOI was 1 PFU/cell, the survival rate of EH-GB1 cells infected with SG7605-11R-p53 was <50% and the survival rate of EH-GB1 cells infected with SG7605-p53



Figure 2. Specific proliferation of the 11R-mediated oncolytic adenovirus. (A) When the multiplicity of infection (MOI) was 5 PFU/cell, SG7605-11R-EGFP proliferated in cancer cells and resulted in highly enhanced green fluorescent protein (EGFP) expression; while EGFP expression was low in the BJ cells. In contrast, SG7605-11R-EGFP mediated a higher level of EGFP expression in cancer cells (EH-GB1, EH-GB2) and gallbladder cancer (GBC)-SD cells and a similar level of EGFP expression in normal BJ cells when infected at the same multiplicity of infection (MOI) of 20 PFU/cell; original magnification, x200. (B) The EGFP-positive cell percentage of every tested cell line was counted for 5 medium-power fields (original magnification, x200) under a microscope. Data are shown in histograms; \*\*P $\leq$ 0.01. (C) Relative expression level of p53 in EH-GB1, EH-GB2 and GBC-SD cell lines was confirmed when infected with SG7605-p53 or SG7605-11R-p53 using qRT-PCR, and SG7605-11R-p53 resulted in a significantly higher level of p53 or SG7605 or SG7605-p53.

was >90%, suggesting that SG7605-11R-p53 had a stronger cytotoxic effect against EH-GB1 cells. A similar result was also observed in the EH-GB2 cells. IC<sub>50</sub> value of SG7605-11R-p53 was markedly lower than that of SG7605-p53 (P<0.0001 for IC<sub>50</sub>) (Fig. 3). The SG7605-p53/SG7605-11R-p53 ratios of IC<sub>50</sub> values were 13422.03-, 79.61- and 6576.72-fold in the EH-GB1, EH-GB2 and GBC-SD cells, respectively.

Antitumor effects on cancer xenografts in the nude mouse models. Gallbladder carcinoma cell line EH-GB2 was implanted subcutaneously in BALB/c nude mice, and the mice were divided into different groups on the 14th day when the diameter of the tumors reached ~5 mm. The mice were treated once every other day for a total of 5 times, each time with a dose of  $2x10^8$  PFU. Ten days after the initial treatment, SG7605-11R-p53 achieved a noticeable therapeutic effect compared with SG7605-p53; 17 days after initial treatment, SG7605-p53 also achieved a noticeable therapeutic effect. By the end of the experiment (31 days later), SG7605-11R-p53 achieved a significantly greater therapeutic effect than that of SG7605-p53 (P<0.01) (Fig. 4A). The mice were sacrificed 35 days later by cervical dislocation. Tumors were removed and examined pathologically (Fig. 4B). A significantly improved survival rate was observed in mice treated with SG7605-11R-p53 (Fig. 4C). The mice first began to die in the control group on day 14, then in the SG7605 and SG7605-p53 groups on days 15 and 24, respectively, and finally in the SG7605-11R-p53 groups on day 41. At the end of the experiment, all of the mice were dead in the control group, whereas in the SG7605-11R-p53 group 3 out of 8 mice were alive (Fig. 4B). The survival rate of the SG7605-11R-p53 group was 37.5% (P<0.05), compared with that of the other virustreated groups (0%). The causes of mouse death indicated the heavy tumor burden and consequent organ failure or cancer cachexia. As determined from the autopsy of the mice, tumor metastasis to other organs was not noted.

Based on the H&E staining result, there were many necrotic foci in the tumor tissues of the SG7605-11R-p53-treated group. Around the necrotic areas, most cancer cells were positive for p53 expression, but there were also rare cancer cells that were positive for p53 in the SG7605 and control groups (Fig. 5A). In the virus-treated groups, including the SG7605,



Figure 3. Cytotoxic effect of tumor-specific SG7605-11R-p53 against cancer cells. Cells were seeded into a 96-well plate at  $10^4$  cells/well and were infected with SG7605-11R-p53 and SG7605-p53 with a multiplicity of infection (MOI) of 0.01 to 100 PFU/cell, and SG7605 was used as the control. The cytotoxic effects of SG7605-11R-p53 and SG7605-p53 were compared in the cancer cells vs. the normal cells. \*P<0.05; \*\*P<0.01.



Figure 4. Antitumor effect of SG7605-11R-p53 in nude mice. (A) SG7605-11R-p53 and SG7605-p53 both showed apparent therapeutic effects in mice bearing gallbladder cancer EH-GB2 xenografts, and SG7605-11R-p53 exhibited an even better antitumor efficacy than SG7605-p53. \*P<0.05; \*\*P<0.01. (B) Compared with the control group, tumors treated with SG7605-11R-p53 and SG7605-p53 were reduced in size. (C) Mice treated with SG7605-11R-p53 survived noticeably longer than the other groups, and mice treated with SG7605-11R-p53 presented with a longer median survival time.



Figure 5. p53 expression and apoptosis-inducing role of SG7605-11R-p53 in nude mice. (A) H&E staining, immunostaining, and terminal dUTP nick end-labeling (TUNEL) indicating pathomorphological changes, p53 expression and apoptosis in the cancer cells. (B) The percentage of cells positive for p53 and the percentage of apoptotic cells were counted in 5 high-power fields. \*P<0.05; \*\*P<0.01.

SG7605-p53, SG7605-11R-p53 groups, different numbers of TUNEL-positive cancer cells were noted, but this effect was most profound in the SG7605-11R-p53 groups (Fig. 5B). The positive indices for TUNEL staining, which was defined as the percentages of positive cells counted in 5 randomly selected high-power fields, were  $19.50\pm5.00$  (for the SG7605 group), 28.75±2.754 (for the SG7605-p53 group), and  $66.75\pm6.702$  (for the SG7605-11R-p53 group). In the control group, there were a few TUNEL-positive cancer cells, with a positive index of  $18.25\pm2.986$  (Fig. 5B).

# Discussion

Gene therapy for cancer treatment has been widely used for a long time, but major advances were not made until 2009, with several cutting-edge discoveries (31-35). These improvements have brought new inspiration for gene therapy research. On the other hand, rather than being a single-gene controlled disease, cancer has proven time and again to be a multigene-related disease (36,37). As a result, the progress made thus far in gene therapy research for cancer has been mostly concerned with effective tumor-suppressor gene screening, gene vector efficiency and vector system safety.

Among the candidates for gene therapy, p53 is one of the most significant genes due to its tumor-suppressor function. It effectively downregulates the expression of genes involved in angiogenesis through the angiogenesis-inhibiting properties of the wild-type (wt) p53 protein (38). Another mechanism of p53 tumor suppression is the increase in the sensitivity of tumor cells to radiotherapy and chemotherapy (38). However, p53 mutates easily, which occurs in approximately half of all human tumors. Prevention of the p53 mutation to restore wt-p53 activity, thus has become an attractive anticancer therapeutic strategy. Mechanisms of p53 therapy for tumors include the reversal of the resistance or augmenting the sensitivity of cancer cells to chemotherapeutic agents and radiation therapy (12,39,40).

The most effective payload needs an equally effective delivery method. Targeted gene therapy using an oncolytic adenovirus system has been a heavily sought-after treatment strategy. By improving and transforming the adenovirus gene, the oncolytic adenovirus system shows good proliferation specificity and achieves its goal of cancer cell necrosis. Because of its sensitization to tumor cells, the adenovirus has the added benefit of enhancing the sensitivity of tumor cells to radiation and chemotherapy.

Unfortunately, this method is not without issues. Safety issues such as inflammatory responses, toxicity, and random integration of viral vector DNA into the host chromosomes have hindering the application of viral-mediated gene therapy. Other alternative methods, such as liposomes, do exist, yet it is well known that although liposomes are able to transfer exogenous genes with minimal toxicity *in vivo*, their transduction efficiency is worse than that of the virus-mediated gene transfer method.

Currently, one of the most prominent roadblocks to this type of gene therapy for cancer is efficacy of gene expression and local concentration of antitumor protein. Even though the adenovirus can increase the copy number of the antitumor gene, the vector often fails to penetrate tissue or cells and hence exhibits a worse in vivo antitumor effects than expected. This is why application of penetrating peptides brings new hope to developing effective cancer treatments using viral vectors. Of particular interest to us is the HIV type 1 Tat protein, which can enter cells in cell culturing medium. The PTD3 of the Tat protein contains a high proportion of arginine and lysine residues, and is thought to have the ability to penetrate the plasma membrane. It is proven to be able to carry and direct the uptake of heterologous proteins into cells by generating genetic in-frame PTD fusion proteins. Similarly, studies have shown that polyarginines (a group of molecules to which our 11R molecule belongs) also have the same transduction activity as the PTD of the Tat protein.

We hope to apply our gene delivery strategy to GBC, one of the most aggressive cancers whose therapy strategy options are limited apart from surgical resection. Given the clinical consideration, an improved safe and effective gene therapy system for GBC is urgently needed.

Based on the aforementioned points, we aimed at combining the advantages of the oncolytic adenovirus, p53 gene treatment and cell penetrating peptide 11R. We successfully constracted the oncolytic adenovirus expressing 11R and p53 fusion protein; it can release even more cell-penetrating p53 product while it replicates. In this way, SG7605-11R-p53 produces more oncolytic adenovirus with which to attack tumor cells nearby after lysing the host tumor cells. In addition, the 11R-p53 fusion protein has better cell penetrating ability due to the fact that 11R confers to the construct a stronger potency to kill tumor cells. On the other hand, inserting the CPP gene into the adenovirus gene broadened the taxis of the adenovirus vector without affecting the original route of the virus-infecting tumor cells, and provided us with a novel idea of transforming the adenovirus aside from the CAR infection route. Examining the expression of 11R with EGFP or the target gene p53 in the oncolytic adenovirus system validated that the oncolytic adenovirus with 11R had high proliferative activity, high transferring efficiency and high p53 expression in p53-positive GBC cells.

Our new gene therapy vector system bearing the p53 gene shows improved efficiency compared with SG7605-p53, and exhibited better tumor regression ability in a nude mouse model. Hence, the oncolytic adenovirus with 11R-penetrating peptide-p53 fusion protein had increased killing ability of tumor cells and higher expression of the target gene than the traditional non-proliferative adenovirus vector, yet posed no significant toxicity to normal cells. In the nude mouse tumor model, SG7605-11R-p53 exhibited favorable *in vivo* capability to kill tumor cells and express the target gene p53. It also remedied issues that have traditionally hampered research in adenovirus therapy, such as poor transgene expression and targeting of specific tumors. This new oncolytic adenovirus system has better therapeutic effect than the traditional Ad-p53 therapy regimen.

In conclusion, SG7605-11R-p53 brings new hope in the clinical application of gene therapy for GBC and perhaps even for other types of cancers.

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