A novel triple-regulated oncolytic adenovirus carrying p53 gene exerts potent antitumor efficacy on common human solid cancers

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Abstract
Conditionally replicating adenoviruses (CRAd) can replicate specifically in cancer cells and lyse them. The CRAds were widely used in the preclinical and clinical studies of cancer therapy. We hypothesize that more precisely regulated replication of CRAds may further improve the vector safety profile and enhance its antitumor efficacy. Here, a triple-regulated CRAd carrying p53 gene expression cassette, SG600-p53, was engineered. In SG600-p53, the E1a gene with a deletion of 24 nucleotides within CR2 region is controlled under the human telomerase reverse transcriptase (hTERT) promoter, the E1b gene expression is directed by the hypoxia response element (HRE), whereas the p53 gene is controlled by the cytomegalovirus promoter. The precise triple-regulation endows SG600-p53 with enhanced antitumor potential and improved safety profile. The tumor-selective replication of this virus and its antitumor efficacy were characterized in several tumor cell lines in vitro and in xenograft models of human non-small cell lung cancer in nude mice. With the selective replication and oncolysis, it was found by ELISA assay that SG600-p53 expressed p53 efficiently in cancer cells. In NCI-H1299 tumor xenograft models, SG600-p53 displayed a tumor-selective killing capacity. At a dose of 2 × 10⁸ plaque-forming units, SG600-p53 could completely inhibit the tumor growth and more effective than replication-defective Ad-p53. Histopathologic examination revealed that SG600-p53 administration resulted in cancer cell apoptosis. We concluded that the triple-regulated SG600-p53, as a more potent and safer antitumor therapeutic, could provide a new strategy for cancer biotherapy. [Mol Cancer Ther 2008;7(6):1598–603]

Introduction
Conditionally replicating adenoviruses (CRAd) can replicate specifically in cancer cells and lyse them. When the CRAd vectors are armed with the antitumor transgene, the transgene copies and expression may be increased markedly along with the preferable replication of CRAds in tumor cells but not in normal cells, so that the antitumor efficacy was enhanced (1). Thereby, the CRAds overcome the disadvantages of traditional gene therapy, including the low transfection, no specificity, and poor efficacy. The previous study from van Beuschem et al. (2) showed the enhanced antitumor effect of CRAd with wild-type p53 gene in the treatment of glioma. However, the replication of CRAds is not always precise under the control of single cis-acting element; sometimes, it replicates to some extent in normal cells and results in side toxicity. It is necessary to improve the CRAd safety with multiple regulating mechanisms.

Based on two common characteristics of telomerase activation and low oxygen tension environment in most solid cancers (3, 4), a human telomerase reverse transcriptase (hTERT) promoter-regulated CRAd (5, 6), CNHK300 with the E1a gene controlled by the hTERT promoter, and a dual-regulated CRAd, CNHK500, with the E1a and E1b genes controlled by the hTERT promoter and the hypoxia response element (HRE; ref. 7), respectively, were reported previously. They showed that the simultaneous regulation of E1a and E1b transcription by two promoters can greatly increase the tumor specificity of CRAds and reduce adverse side effects in normal cells. Otherwise, the loading of antitumor transgene in CRAds is also an effective means to enhance the antitumor efficacy. Among many candidates, the p53 gene plays an important role in cancer gene therapy, which has received preclinical validation by developing anticancer agents that specifically reactivate p53 function (8). To explore the efficacy of wild-type p53 reactivation as a tumor therapy, Ventura et al. (9) showed that restoring endogenous p53 expression in mouse
led to regression of autotchthonous lymphomas through cellular apoptosis pathway and sarcomas through cellular senescence in mice but without affecting normal tissues. By RNA interference to conditionally regulate endogenous p53 expression in a mosaic mouse model of liver carcinoma, it was found that even brief reactivation of endogenous p53 in p53-deficient tumors could produce complete tumor regressions through the induction of a cellular senescence that was associated with differentiation and up-regulation of inflammatory cytokines (10). These studies indicated that the p53-triggered tumor regression is not only due to the cellular apoptosis but the cellular senescence program, which is dependent on the tumor types.

In this study, we constructed a triple-regulated replicative adenovirus, SG600, in which the CR2 region of E1a gene was partly deleted, and the E1a and E1b genes were controlled by the hTERT promoter and the HRE, respectively. These modifications were expected to increase the capacity of viral replication and oncolysis specifically targeting the cancer cells and to decrease the viral cytotoxicity to normal cells. When SG600 was used to be a vector and armed with the wild-type p53 gene, we believe that not only the antitumor effect of the generated SG600-p53 is improved but also the expression of p53 gene is increased markedly.

Materials and Methods

Cell Culture and Virus Preparation

Human cancer cell lines (A549, NCI-H1299, H446, HepGII, Hep3B, PANC-1, HeLa, and L02), human fibroblast lines (MRC-5, HEL-1, HPF-1, and BJ), and human embryo kidney cell line (HEK293) were purchased from the American Type Culture Collection. The human cancer cell lines (SGC-7901 and SMMC-7721) were obtained from the Institute of Cell Biology, Chinese Academy of Sciences. All cell lines were cultured according to the instructions of the providers.

Overlap PCR was done to delete the E1a promoter, the E1b promoter, and the 24 nucleotides of adenovirus E1a CR2 region, by which the nucleotides 464 to 550 of pXC1 (Microbix Biosystems) were deleted and replaced by multiple cloning site 1 and the nucleotides 1,634 to 1,712 were deleted and replaced by multiple cloning site 2. The synthesized hTERT promoter (-212 to +46 bp) and HRE (five copies of TCCACAGTGCATACGTGGGCTCCAA-CAGGTCTCT, with 60-bp additive nucleotides) were cloned into multiple cloning sites 1 and 2, respectively, and generated pSG600. The p53 expression cassette containing the cytomegalovirus promoter, p53 cDNA, and SV40 polyA, released from pCA13-p53, which we constructed previously (data not shown), was subcloned into the multiple cloning site 1 of pSG600 between the expression cassettes of E1a and E1b genes and then generated the adenoviral plasmid pSG600-p53. The plasmids pSG600, pSG600-p53, and pCA13-p53 were respectively transfected into HEK293 cells using the Effectene Transfection Reagent (Qiagen) together with the adenovirus packaging plasmid pPE3. After homologously recombining in HEK293 cells, we obtained two CRAds named SG600 and SG600-p53 and one replication-deficient adenovirus named Ad-p53.

Adenovirus Replicative Assay In vitro

To investigate the replication of SG600-p53, the cancer cell and normal cell lines were seeded in six-well plates at a density of 1 x 10^5 per well and infected with SG600-p53 at a multiplicity of infection (MOI) of 5 plaque-forming units (pfu)/cell. The supernatants and cells were titered at 0, 48, and 96 h after infection by TCID50 method as described previously (5).

Western Blot Analysis for E1A and E1B Expression

HepGII, Hep3B, MRC-5, and BJ cell lines were seeded in six-well plates at a density of 5 x 10^5 per well, cultured for 24 h, and infected with viruses at a MOI of 1 pfu/cell. Two days after infection, cells were harvested and measured the expression of E1A and E1B-55-kDa by Western blot using mouse anti-adenovirus E1A monoclonal antibody M73 (Santa Cruz Biotechnology) or rat anti-adenovirus E1B-55-kDa monoclonal antibody (Oncogene Research Products) as described in earlier report (5).

ELISA for p53 Expression

In the in vitro experiments, NCI-H1299 cells were seeded in six-well plates at a density of 5 x 10^5 per well. To investigate the relation of viral dose and p53 expression, the cells were infected with SG600-p53 and Ad-p53 at MOI of 5, 1, 0.5, 0.1, and 0.01 pfu/cell. At 72 h after infection, the cells were collected and lysed, and the supernatants of cell lysates were used to detect p53 expression by ELISA as described previously (6). To investigate the relation of time and p53 expression, the cells were infected with SG600-p53 and Ad-p53 at a MOI of 1 pfu/cell, and the supernatants of cell lysates were collected at 0, 24, 48, 72, and 96 h after infection for detecting p53 expression.

Adenovirus Oncolytic Assay In vitro

To evaluate the cytotoxicity of adenovirus vectors, a panel of tumor cells was planted at a density of 1 x 10^5 in 96-well plates and infected with SG600-p53 and Ad-p53 at a gradient of MOI from 0.001 to 100 pfu/cell. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay according to the instructions of Cell Proliferation Kit I (Roche Molecular Biochemicals).

Animal Experiments

BALB/c nude mice (nu/nu) were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences. NCI-H1299 cancer cells in log phase were s.c. injected into the right flanks of mice (1 x 10^7 per mouse). Four weeks later, the tumor xenografts were established, when the mice were allotted randomly into seven groups: SG600-p53 I, SG600-p53 II, SG600-p53 III, SG600, Ad-p53, ONYX-015, and control (10 mice per group). Mice were given total 1 x 10^8 pfu viruses by five times of intratumoral injections, one time every other day, in the SG600-p53 II, SG600, Ad-p53, and ONYX-015 groups and total 5 x 10^8 and 2 x 10^8 pfu viruses by five times in the SG600-p53 I and SG600-p53 III groups, respectively. In the control group, 100 μL viral preservation solution [10 mmol/L Tris-HCl (pH 8.0), 2 mmol/L MgCl2, 4% sucrose] per mouse per time.
was injected intratumorally. Tumor volume was estimated with the formula: 
\[
\text{Volume} = \frac{\text{maximal diameter}}{2} \times \frac{\text{perpendicular diameter}}{2}.
\]

**Histology and Immunohistology**

Mice were sacrificed 35 days later according to the institutional guidelines. The tumors were removed, 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 located using mouse anti-p53 antibody (Biodesign International) by immunohistochemistry. The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay was done using the *In situ* Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer’s instructions.

**Statistical Analysis**

Data from *in vitro* experiments were assessed by Student’s *t* test and data from *in vivo* experiments were assessed by ANOVA. Findings of *P* < 0.05 were considered significant.

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### Results

#### Conditional Replication of the p53 Armed CRAd

A series of tumor and normal cell lines were planted in six-well plates (10⁶ per well) and infected with indicated viruses at a MOI of 5 pfu/cell. The cell lysates and supernatants at 48 and 96 h after infection were titered by TCID₅₀ method in HEK293 cells and normalized to virus production per cell. SG600-p53 replicated preferably in all cancer cell lines but markedly decreased in normal cell lines. The replicative capability of SG600-p53 ranged from 632.91- to 4,062.50-fold increase in cancer cells at 48 h after infection and from 793.97- to 4,062.50-fold increase at 96 h, whereas from 1.60- to 13.50-fold increase in normal cells at 48 h after infection and from 1.60- to 50.50-fold increase at 96 h (Table 1).

In SG600 and SG600-p53, E1A and E1B-55-kDa expressions were controlled by the hTERT promoter and the HRE, respectively (Fig. 1A). By Western blot, the expressions of E1A and E1B-55-kDa were positive in SG600-p53-infected cancer cell lines and no or weak expression in SG600-p53-infected normal cells (Fig. 1B).

#### SG600-p53-Mediated p53 Expression and Cancer Cell-Killing Effect

The p53-deficient lung cancer cell line NCI-H1299 was cultured in six-well plates at a density of 5 × 10⁵ per well and infected with SG600-p53 and Ad-p53 at different MOI. The cells were collected at 24, 48, 72, and 96 h after infection and detected p53 expression. The results showed that SG600-p53 expressed p53 with high efficiency in cancer cells compared with Ad-p53. At 72 h after infection with SG600-p53 and Ad-p53, the expression level of p53 was increased gradually along with the increase of MOI (Fig. 2A). At a MOI of 1 pfu/cell, the expression level of p53 was increased gradually along with time prolonging (Fig. 2B). For assessing the selective killing effect of SG600-p53 in comparison with Ad-p53, a panel of tumor cells was planted in 96-well plates at a density of 1 × 10⁴ cells and infected with SG600-p53 and Ad-p53 24 h later at the final

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**Table 1. Selective replication of SG600-p53 in cancer cell lines compared with that in normal cell lines**

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<th>96 h</th>
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<td>HPF-1</td>
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<td>50.50</td>
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<tr>
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</tbody>
</table>

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**Figure 1.** Expressions of E1A and E1B-55-kDa under the control of hTERT promoter and HRE. **A,** in SG600, the hTERT promoter and HRE were used to regulate the E1a and E1b genes, respectively. A p53 gene expression cassette containing cytomegalovirus promoter, p53 cDNA, and SV40 polyA was inserted into the genome of SG600 and then generated SG600-p53. ITR, inverted terminal repeats; ψ, adenovirus type 5 packaging signal. **B,** cells were seeded in six-well plates at a density of 5 × 10⁵ per well and infected with viruses at a MOI of 1 pfu/cell, and the cell lysates at 48 h were examined for E1A and E1B-55-kDa expression by Western blot. The expression of E1A or E1B-55-kDa mediated by SG600-p53 was positive in cancer cell lines and no expression in normal cells but always positive in the wild-type adenovirus-infected BJ cells.
MOI from 0.001 to 100 pfu/cell. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was then measured to quantify cell viability and compared their MOI associated with 50% cell viability (IC50) and 90% cell viability (IC90). IC50 and IC90 values of SG600-p53 were lower markedly than those of Ad-p53 ($P = 0.0256$ for IC50 and $P = 0.0049$ for IC90; Fig. 3). The Ad-p53/SG600-p53 ratios of IC50 values were 1,176.67-, 156.75-, 128.83-, 1,167.64-, 9,500.00-, 110.43-, and 173.07-fold in A549, NCI-H1299, NCI-H446, SMMC-7721, Hep3B, SGC-7901, and PANC-1, with IC90 values being 501.76-, 24.71-, 185.43-, 108.21-, 8,738.33-, 111.01-, and 117.42-fold in these cell lines, respectively.

**Antitumor Efficacy of SG600-p53 on Tumor Xenografts**

We evaluated the antitumor efficacy of SG600-p53 on NCI-H1299 tumor xenografts established in nude mice. The tumor-bearing mice were given five intratumoral injections of different viral doses, one time every other day. At the same dose of total $1 \times 10^8$ pfu viruses, SG600-p53 achieved the best among the virus-treated groups, and the antitumor effect of Ad-p53 was poorer than that of other viruses (Fig. 4A). Thirty-five days later after treatment, the groups injected with viruses achieved excellent antitumor efficacy when compared with the control group ($P = 0.0001$ for SG600-p53 I, SG600-p53 II, SG600-p53 III, and SG600 groups; $P = 0.0002$ for ONYX-015 group; $P = 0.0121$ for Ad-p53 group). To investigate the antitumor efficacy of SG600-p53 in different doses, $2 \times 10^8$, $1 \times 10^8$, and $2 \times 10^7$ pfu doses of SG600-p53 were administrated. The result showed that $2 \times 10^7$ pfu of SG600-p53 could completely inhibit the tumor growth (Fig. 4B).

**Histopathologic Confirmation for Cancer Cell Apoptosis Mediated by the p53 Armed CRAd**

Mice were killed 35 days later by cervical dislocation. Tumors were removed and examined pathologically. There were many necrotic foci in tumor tissues of SG600-p53-treated groups. Around the necrotic areas, most cancer cells were positive for p53 expression (Fig. 5A), but there were no cancer cells positive for p53 in SG600 and control groups (Fig. 5B). In the virus-treated groups, including the SG600-p53 I, SG600-p53 II, SG600-p53 III, SG600, Ad-p53, and ONYX-015 groups, there were different amounts of cancer cells positive for the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling but more obviously in SG600-p53 groups (Fig. 5C). The positive indices for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining, which was counted the percentages of positive cells from five randomly selected high-power fields, were 89.3 ± 11.4, 83.0 ± 13.1, 67.3 ± 10.8, 44.7 ± 12.3, and 25.8 ± 9.2% for SG600-p53 I, II, III, IV, and V, respectively.

**Figure 2.** Transgene expression by SG600-p53. NCI-H1299 cells were seeded in six-well plates at a density of $5 \times 10^4$ per well and infected with the adenovirus SG600-p53 and Ad-p53 at MOI of 5, 1, 0.5, 0.1, and 0.01 pfu/cell. At 0, 24, 48, 72, and 96 h after infection, the cells were collected and the supernatants of cell lysates were used to detect p53 expression by ELISA. **A**, SG600-p53 expressed p53 with high efficiency in cancer cells compared with Ad-p53 at 72 h after infection and increased gradually along with the increase of MOI. **B**, at a MOI of 1 pfu/cell, the expression level of p53 was increased gradually along with the time prolonging.

**Figure 3.** IC50 and IC90 values by cell viability at various MOI of viral infection. A density of $10^4$ cells per well cultured in 96-well plates were infected with indicated viruses at a wide range of MOI, ranging from 0.001 to 100 pfu/cell, and 7 days later, the cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. IC50 and IC90 values of SG600-p53 were lower markedly than those of Ad-p53.
F9.5, 35.7 ± 10.5, and 32.3 ± 8.5 in SG600-p53 I, SG600-p53 II, SG600-p53 III, SG600, Ad-p53, and ONYX-015 groups, respectively. In control group, there were a few cancer cells positive for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling with the positive index of 5.3 ± 2.1 (Fig. 5D).

Discussion

The approach to treat cancers with viruses, named virotherapy, was attended recently with the development of CRAds (11, 12). CRAds are the viruses that do not replicate in normal cells but selectively replicate in tumor cells, kill them, and subsequently infect adjacent tumor cells. They hold the promise of being effective agents for the treatment of solid tumors (7, 13). However, the treatment for cancers with CRAds solely is suboptimal. The virotherapy with CRAds is based on their cancer selectivity by confining viral replication in cancer cells. In the further study, CRAds need to be optimized to improve their safety for the patients with cancers and to maximize their antitumor potential as critical anticancer agents.

Both E1a and E1b genes are necessary for efficient viral replication. For the purpose of improving the safety of CRAds, we modified SG600 by controlling the E1a gene with the hTERT promoter and the E1b gene with the HRE.
Two common characteristics were found in most human solid tumors: the existence of hypoxic areas in the tumor tissues and the expression of telomerase in the cancer cells (14–17). A few investigations showed that the existence of hypoxic areas in tumor tissues and the expression of telomerase in cancer cells can be employed to achieve cancer targeting treatment (18–20). Accordingly, our modifications to regulate SG600 with two promoters could restrict adenoviral replication to tumors and thus achieve better tumor-selective oncolysis. SG600 was synchronously deleted 24 nucleotides in E1a-CR2 region that is responsible for binding/inactivating pRb family members, which may partly preserve pRb functions and exert the inhibition of tumor growth.

For the purpose of fully realizing the antitumor potential of CRAds, we modified SG600 by the payload of p53 gene. The p53 is one of the most important tumor suppressor gene, and the transfer of wild-type p53 gene is an important method to cure the p53-deficient cancers (21, 22). Considering that the tumor-specific CRAds can selectively replicate in tumor cells, the construction of a gene-viral vector, SG600-p53, by inserting the anticancer gene p53 into the genome of the tumor-specific CRAds may increase the expression of p53 and be able to combine the advantages of gene therapy and virotherapy. Hence, this strategy of gene-viral therapy overcomes the obstacles of traditional gene therapy and enhances the anticancer efficacy (1).

The in vitro and in vivo experiments showed that SG600-p53 replicated preferably in cancer cells and expressed E1A and E1B-55-kDa, whereas hardly replicated and no E1A and E1B-55-kDa expressed in normal cell lines, suggesting that SG600-p53 has a high selectivity to cancer cells and a low toxicity to normal cells. SG600-p53 expressed p53 with high efficiency in cancer cells compared with Ad-p53, showing that the expression of p53 was increased along with the viral replication in cancer cells. Both the high selectivity of viral replication and the high efficiency of p53 expression ensured the efficient oncolytic effect and inhibition effect of SG600-p53 specifically on cancer cells. In NCI-H1299 tumor xenografts, the SG600-p53-treated groups achieved the significant antitumor efficacy. The antitumor effect of SG600-p53 was the best among the groups and was related to the doses of viruses. SG600-p53 (2 × 10⁸ pfu) exerted higher antitumor efficacy and could completely inhibit the tumor growth compared with 5 × 10⁷ or 1 × 10⁹ pfu of viruses. By pathologic examination, we found that the virus administration resulted in cancer cell apoptosis, especially in the groups of SG600-p53, whereas the wide area of necrosis in the tumor tissues appeared after the treatment of SG600-p53.

In conclusion, we successfully constructed an efficient tumor-selective oncolytic adenovirus SG600-p53, which was regulated under both hTERT promoter and HRE and synchronously carried the p53 transgene and E1a-CR2 partially deletion. SG600-p53 holds an increase of anticancer efficacy and an improvement of the safety as a critical anticancer agent. It is a new strategy for cancer biotherapy by the use of SG600-p53.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank L.F. Li, L.H. Jiang, and Y.Z. Qian for assistance with cell culture and J.Z. Gu for help with animal studies.

References
Molecular Cancer Therapeutics

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