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The therapeutic effects of recombinant adenovirus RA538 on human gastric carcinoma cells *in vitro* and *in vivo*

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Abstract

AIM To evaluate the potential of RA-538 gene therapy for gastric carcinoma.

METHODS Human gastric carcinoma cell line SGC7901 treated with Ad-RA538 or Ad-LacZ were analysed by X-gal stain, MTT, DNA ladder, Tunel, flow cytometric analysis, PCR, and Western Blot *in vitro*. The tumorigenicity and experimental therapy in nude mice model were assessed *in vivo*.

RESULTS Ad-LacZ could efficiently transfer the LacZ gene into SGC7901 cells. X-gal-positive cells at MOI 25, 50, 100, and 200 were 90%, 100%, 100%, and 100% respectively. Ad RA538 could strongly inhibit cell growth and induced apoptosis in SGC7901 cells. The proliferation of the Ad-RA538-in fected SGC7901 cells was reduced by 76.3%. The mechanism of killing of gastric carcinoma cells by Ad-RA538 was found to be apoptosis by DNA ladder,Tunel and flow cytometric analysis. The tumorigenicity in nude mice using Ad-RA538 showed that all three mice failed to form tumor from 7 to 30 days compared with Ad-LacZ and parent SGC7901 cells. Experimental therapy on the nude mice model bearing subcutaneous tumor of SGC7901 cells showed that intratumor instill ation of Ad-RA538 inhibited the growth of the tumors. Ad-RA538-treated tumors were inhibited by 60.66%, compared with that of the tumor injected with Ad-LacZ and mock.

CONCLUSION The expression of Ad-RA538 can inhibit growth and induce apoptosis of gastric cancer cell *in vitro* and *in vivo*. Ad-RA538 can be used potentially in gene therapy for gastric carcinoma.

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INTRODUCTION

Gastric carcinoma is one of the most common malignant tumors in the world. It is treatable by surgical resection in the early stages, but advanced gastric carcinoma does not usually respond to conventional therapy[1-6]. Therefore gene therapy represents an attractive alternative for the treatment of gastric carcinoma. Present studies, suggest that overexpression of oncogenies, with or without functional loss of tumor suppressor genes, is responsible for the progression of human malignancies through multistep processes[7-13]. On the basis of this multiple hit model of carcinogenesis, cancer gene therapy has rapidly developed as alternative to conventional cancer therapy. It is widely accepted that c-myc gene plays a pivotal role in regulat ing cell proliferation and differentiation. Some studies indicated that c-myc gene has a close relationship with carcinogenesis[14-16]. Using subtr active hybridization strategy, RA538 was isolated, a cDNA colon from a human esophageal cancer cell line before and after RA-treatment[17]. It is proved that RA538 can induce differentiation and apoptosis of tumor cell and down-regulate c-myc gene expression[18-21]. In the present study, we treated a human gastric carcinoma cell line with adenovirus-recombinants carrying RA538 or LacZ gene.

MATERIALS AND METHODS

Cell lines and culture

Human gastric carcinoma cell line (SGC 7901) was obtained from the Academy of Military Medical Sciences, China. Human embryonic kidney cell line (293) was kindly provided by Professor Zhan Qi-Ming (Academy of Sciences, China). SGC 7901 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Two hundred and ninety-three cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Recombinant adenovirus production

The recombinant, replication-deficient type 5 adenoviruses that have been developed for gene therapy contained deletions of the E1 and E3 regions. The vector was constructed by homologous recombination in 293 cells of the PJM17 plasmid containing the right end of Ad5 (Microbix Biosystems Inc., Canada) and a plasmid containing 0-16 m.u. of left end of pAd CMV (a gift from Zhang Wei-Wei). A recombination adenovirus vector, called Ad-RA538 containing the human RA-5 38 cDNA fragment, the total 3.8kb of RA538 suppressor gene was constructed. Ad-RA538 and Ad-LacZ were constructed by National Laboratory of Molecular Oncology, Department of Cell Biology, Chinese Academy of Medical Sciences. The resulting vector was plaque-purified twice on 293 cells and propagated. Virus stocks were titered by plaque-forming assay on 293 cells. Ad-RA538 and Ad-LacZ were identified by polymerase chain reaction (PCR).

Recombinant adenovirus infections of the cell lines were carried out by dilu tion of viral stock to certain concentrations, addition of viral solution to cell monolayers (0.5mL per 6cm dish), and incubation at room temperature for 30min with agitation every 10min. This was followed by addition of culture medium and the return of the infected cells to the 37℃ incubator.

Adenovirus transduction efficiency

Gastric carcinoma cells were seeded in 6cm culture plates at a density of 1×10^6 cells /dish and cultured 12 hours. Cells were infected with Ad-LacZ at a multiplicity of infection with 25, 50, 100 and 200 (MOI). After 48 hours, the cells were washed with phosphate-buffered saline (PBS), treated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). X-gal-positive cells were revealed by a blue precipitate in the cell.

Assay of cell growth

Cells were seeded at 8×10^3 cells/ well in 96-well plates and were infected with either Ad-RA538, Ad-LacZ and PBS (Mock) at 25, 50, 100 and 200 MOI within 12-24 hours. At different time after adenoviral infection, 3-2, 5-diphenyltet razolium bromide (MTT) assay was performed. Cell viability is proportional to the absorbance at the test wave length (525nm).

DNA extraction and gel electrophoresis[22]

Cells were lysed in gunaidine-isothiocyanate and DNA was extracted by benzyle chloride using Herrmann M'methord[5]. RNA was digested with 1μg/mL RNase A 50μg of each DNA sample was loaded on to a 18g/L agarose gel.

TUNEL assay

Air dried cell samples were fixed with paraformaldehyde solution for 30min . Slides were rinsed with PBS and incubated with blocking solution (0. 3% H_2O_2 in methanol) for 30min. Slides were rinsed with PBS and incubated in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2min in ice. TUNEL reaction mixture were added with 50μL in sample. Slides were incubated in a humidified chamber for 60min at 37℃, and a nalysed under a fluorescence microscope. Coverter-POD were added with 50μL in sample. Slides were incubate in a humidified chamber for 30min at 37℃. Slides were rinsd with PBS for three times. DAB substate solution were added with 50μL-100μL for 15min.Slides were rinsd with PBS for three times and were stained with hemotolin. Slides were mountd under glass coverslip and analysed under light microscope.

Flow cytometry

The cells were fixed in 70% ethanol, treated with 0.1g/L Rnase A, and with 100mg/L propidium iodide. Cell cycle phase distribution were analysed by flow cytometry (Coulter Epice, ELITE, ESP, USA).

Polymerase chain reaction

The polymerase chain reaction (PCR) was designed to amplify a 800bp fragment of the adenovirus gene using primer (5'-primer TCGTTTCTCAGCAGCTGTTG; 3'- primer CATCT-GAACTCAAA GCGTGG) and a 300bp fragment of the RA538 gene using primer (5' primerATGGGT-GAACA ACAGAAGAG-3'primer TTAGA-GATTCAGATTTGGCT). Hot-start PCR am plification was performed. In a Pekrin-Elmer 2400 thermocydcer using the following program: 1×95℃ for 5min; 30×95℃ for 1min; 59℃ for 50sec; 72℃ for 50sec; and 1×72℃ for 7min. The final concentration for all PCR components in a 25μL volume was as follows: 100μM of each of extracted genomic DNA and 1 unit of Taq polymerase in 1×Taq polymerase buffer. PCR products were run on 1% agarose gels.

Western blot analysis

Total cell lysated were prepared by lysing the cell

with sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer after rinsing the cells with PBS. For the SDS-PAGE analysis, each lane was loaded with cell lysates equivalent to 6×10^4 cells (30μL). The protein in the gel were transferred to nitrocellulose filter (NC filter, Bio-Rad Compan y, California, USA). The membranes were blocked with 0.5% dry milk in PBS. The primary antibodies used were mouse anti-human c-myc or β-actin monoclonal antibody (Santa Cruz, California, USA), and the secondary antibody was horseradish peroxidase-conjugated rabbit anti-mouse IgG (Amersham, Uppsala, Sweden). The membranes were hybridized according to the Amersham's ECL protocol. The membrane was exposed to X-ray film.

Tumorigenicity assay of SGC7901 cell growth after treatment with Ad-RA538

The gastric cancer cells were infected with Ad-RA538 and Ad-LacZ at a dose of 100 MOI. An equal number of cells was treated with PBS and mock infection. Twenty-four hours after infection, the treated cells were harvested and rinsed with PBS. For each treatment, 10.6 cells in 0.1mL were injected to BALB /c male nude mice. Tumor formation was evaluated after 4 weeks.

Adenovirus treatment in vivo

Mice (BALB/c nude mice aged 5 weeks) were inoculated with 10^6 SGC 7901 cells into the flank. Tumors were allowed to grow to 5mm in diameter. The animals were divided into three groups: Ad-RA538 injection; Ad-LacZ inject ion and PBS injection. There were five nude mice in each group. Ad-RA538 or Ad-LacZ (1×10^9 pfu/each/100μL) or PBS/100μL were directly injected into the tumor centre of each nude mouse at d1, d3 and d5. Tumor sizes were observed 4 weeks after the injection, and estimated with calipers.

Statistics

Data are presented as means ± standard errors of the means. Comparisons among different groups of samples were made by two-tailed *t* test and χ^2 test.

RESULTS

Recombinant adenovirus prediction

Ad-RA538 and Ad-LacZ were propagated on 293 cells. Their titers were 3.0×10^9 pfu/mL and 3.0×10^{11}pfu/mL respectively. Ad-RA538 and Ad-LacZ were identified by PCR. Ad-RA538 showed expression of RA538 gene. Ad-LacZ show ed expression of adenovirus gene.

Adenovirus transfection efficiency in SGC7901 cell line

The time course of β-galactosidase (β-Gal) expression was first determined by counting the percentage of X-Gal-positive cells at 48h after infection with Ad-LacZ. Ad-LacZ could efficiently transfer the LacZ gene into SGC7901 cells. And X-gal-positive cells at MOI 25, 50, 100 and 200 were 90%, 100% 100% and 100% respectively.

Inhibition of SGC 7901 cells growth

The degree of growth inhibition was measured by MTT assay.The growth rates of Ad-RA538 infected SGC 7901 cells were inhibited by 76.3 % (MOI 200, 8 day) as compared to Ad-LacZ and Mock (P>0.01) (Table 1). Ad-RA538 induced apoptosis in SGC 7901 cells.

DNA fragmentation

SGC7901 cells were treated with MOI 100 Ad-RA538 for 2, 4 and 6 days. Figure 1 shows that the electrophoresis pattern after treatment with Ad-RA538. DNA fragmentation became apparent at d2, d4 and d6. The peak of ladder pattern was detected at d6. Ladder pattern was not detected in Ad-LacZ treated cells.

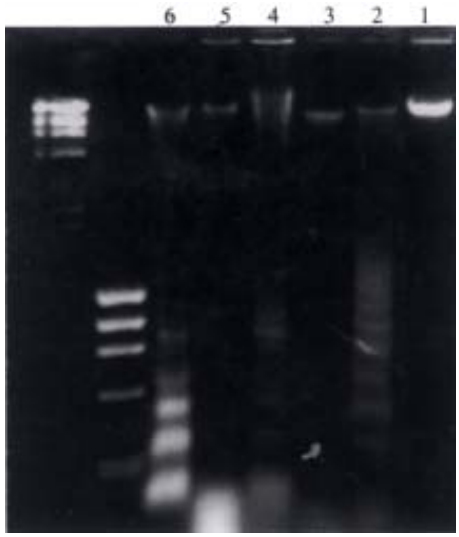

Figure 1 DNA ladder of Ad-RA538 on SGC7901 cells.
lane 1: Ad-lac Z (2d); lane 2: Ad-RA538 (2d);
lane 3: Ad-lacZ (4d); lane 4: Ad RA538 (4d);
lane 5: Ad-lacZ (6d); lane 6: Ad-RA538 (6d).

TUNEL assay

SGC 7901 cells treated with Ad-RA538 were assayed for apoptosis by TUNEL. SGC 7901 cells could induce apoptosis. The abnormal chromatin clumps, nuclear membrane wrinkling, nuclear collapse, cytoplasm bubble and cytomembrane wrinkling had appeared after treatment with Ad-RA538. Apoptotic cells were not shown by TUNEL assay in Ad-LacZ treated cells. In this kit terminal deoxynucleotidyl transferase, which catalyzes polymerization of nucleotides to free 3'-OH DNA ends in a template-independent manner, was used to

label DNA strand breaks. After substrate reaction, stained cells can be analyzed under light microscope (Figure 2).

Flow cytometric analysis

A flow cytometric analysis was performed on SGC 7901 cells infected with Ad-RA 538 and Ad-LacZ. Apoptotic cells of Ad-RA538 infected cells played a peak in t he flow cytometry histogram. Apoptotic peak was 34.2% at d2. The cell cycle G-2M arrest were shown at d2, d4 and d6. The apoptosis peak and cell cycle arrest were not found in Ad-LacZ treated cells (Table 2) .

c-myc expression in Ad-RA538 infected cells

The expression of c-myc protein in SGC 7901 cells was detected by western blot analysis after Ad-RA538 infection for 1, 3, 5 or 7 days. Ad-RA538 may down- regulate expression of c-myc gene (Figure 3).

Inhibition of tumor growth in vivo

The tumorigenicity in nude mice of using Ad-RA538 showed that three of these mice failed to form tumor from d7 to d30 compared with Ad-LacZ and parent SGC 7901 cells (P>0.01) (Figure 4). The tumorigenicity in nude mice using Ad-LacZ and parent SGC 7901 cells was 100%.

Experimental therapy on the nude mice model bearing subcutaneous tumor of SGC 7901 cell showed that intratumor instillation of Ad-RA538-treated tumor were inhibited by 60.66%, compared with that of the tumor injected with Ad-lacZ and mock (Figures 5,6).

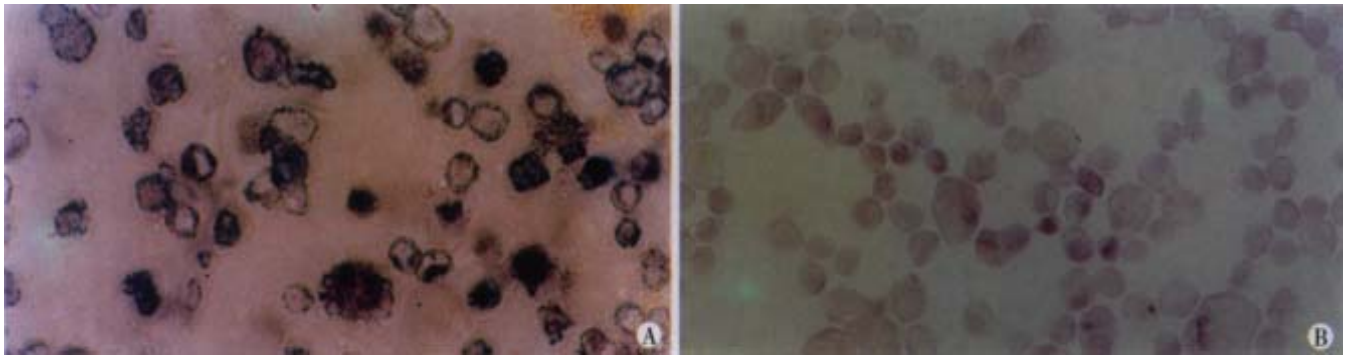

Figure 2 TUNEL assay of Ad-RA538 and Ad-LacZ on SGC 7901 cells.

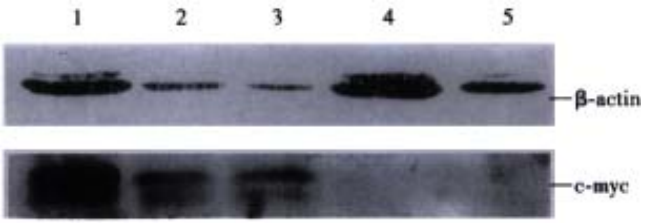

Figure 3 Western blot analysis c-myc and β -actin expression of SGC7901 cell infected with Ad-RA538.
Lane 1: control, Lane 2-5: 1d, 3d, 5d, 7d

Figure 4 Tumorigenicity assay in nude mice follow AdRA538.

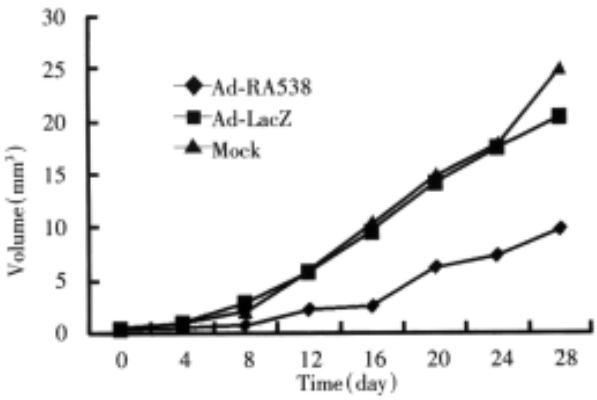

Figure 5 *In vivo* therapy of Ad-RA538 on SGC7901 tumors in nude mice.

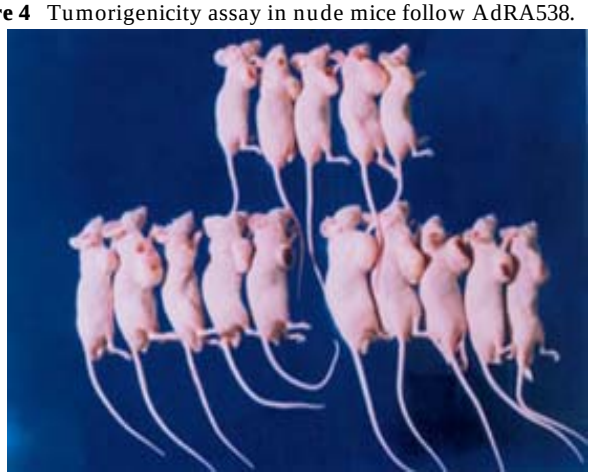

Figure 6 Experimental therapy on the nude mice model of Ad-RA538.

Table 1 Anti-proliferative effect of Ad-RA538 on SGC7901 cells (8d, *x*±*s*)

MOI	Ad-RA538		Ad-LacZ		Mock	
	A	Survival(%)	A	Survival(%)	A	Survival(%)
25	1.062±0.05	95.0±4.5	1.120±0.077	100±6.9	1.31±0.065	100±6.3
50	0.941±0.067	84.0±6.0	0.984±0.024	87.5±2.1	1.29±0.034	100±5.2
100	0.534±0.091	47.4±8.1[a]	1.195±0.095	100±8.5	1.28±0.041	99.8±7.0
200	0.265±0.090	23.7±8.0[b]	1.241±0.091	100±8.0	1.23±0.057	98.0±4.5

[a]*P*>0.05 *vs* Ad-LacZ or Mock; [b]*P*>0.01 *vs* Ad-LacZ or Mock.

Table 2 Flow cytometry analysis of cell cycle effects of Ad-RA538 on SGC7901 (%)

Type of adenovirus	12h			1d			2d			4d			6d		
	G1	G2M	S	G1	G2M	S	G1	G2M	S	G1	G2M	S	G1	G2M	S
Ad-RA538	74.6	13.0	12.4	55.7	28.2	17.2	69.9	14.0[a]	16.1	39.7	26.6[b]	33.8	44.4	20.9[c]	34.7
Ad-LacZ	66.5	15.3	18.2	59.2	18.1	22.7	59.5	22.8	17.3	71.0	12.4	16.6	73.3	8.4	18.3

[a]*P*>0.01 *vs* Ad-LacZ; [b]*P*>0.01 *vs* Ad-LacZ; [c]*P*>0.01 *vs* Ad-LacZ.

DISCUSSION

The ability to infect numerous different cell types and the absence of requi rement for dividing cells make adenovirus an attractive candidate for *in vivo* gene therapy[23]. They can be grown to a very high titer, and can be easily concentrated to reach titers of 10^{13}-10^{14} particles per milli liter. Adenoviral vectors are very effective agents for gene transfer with extremely high transduction efficiency for a wide variety of cell types. In this study, adenovirus vector has high transduction efficiency for SGC 7901 cells, and can mediate a high level of expression of interested gene in transducted cells. The percentage of X-gal staining positive SGC 7901 cells 48h after infection with Ad-LacZ was 90% at MOI 25. Ad-RA538 could strongly inhibit growth and induce apoptosis of SGC 7901 cells. It may be related to high trasduction efficiency of adenovirus for SGC 7901 cells.

It is known that activation of proto-oncogene and inactivation of tumor suppre ssive gene are the most common genetic alternation in tumor[24-33]. Using subtractive hybridization strategy, RA538 was isolated, a cDNA colon from a human esophageal cancer cell line before and after retinoic acid (RA) treatment. RA can induce differentiation and apoptosis of tumor cell[34-37] . It is proved that RA538 can induce differentiation and apoptosis of tumor cell and down-regulate c-myc gene expression[17,18,38-41]. Reducing the expression of c-myc may effectively suppress the proliferation and malignant phenotype of cancer cells[42-48] and to counter c-myc gene amplification in gastric carcinoma cells[49]. The effects of Ad-RA538 on human gastric carcinoma cell line were examined both *in vitr o* and *in vivo*. Ad-RA538, containing the human RA538 cDNA fragment, was constructed. Firstly, Ad-LacZ could transfer the LacZ gene into more than 90% of gastric carcinoma cells. Ad-RA538 could successfully inhibit expressi on of c-myc protein. These data showed the capability of adenovirus to transfer exogenous genes efficiently into gastric carcinoma cell line. Secondly, the growth inhibitory effect of Ad-RA538 was then examined. In c-myc gene amplificati on gastric carcinoma cell line SGC7901, the growth inhibition by infection with Ad-RA538 correlated with both transduction efficiency and adenovirus dose. The growth rates of Ad-RA538 infected SGC 7901 cells were inhibited by 76.3%. DNA fragmentation, TUNEL and flow cytometric analysis suggested that Ad-RA538 could strongly induce apoptosis of gastric carcinoma cells. Some reported that tumor suppressive gene BCL-XS could inhibit the proliferation and malignant phenotype of cancer cells[50], Bouillellet[51] reported that stra 6, a retinoic acid-responsive gene could induce apoptosis of tumor cells. These are the same with our studies about Ad-RA538. Our studies with the tumorigenicity in nude mice an d experimental therapy on the nude mice model using Ad-RA538 also supported these findings and showed that Ad-RA538 may be useful to inhibit the growth of gastric tumors. Our studies suggested that adenoviral-mediated RA538 overexpression may result in the elimination of tumor cells by apoptosis, and inhibition of proliferation, thus reducing the tumor burden. In conclusion, these finding sindicated that the Ad-RA538 might promote efficient tumor cell death and inhibit tumor cell proliferation. The use of these vectors may be a potential tool for reducing tumor growth *in vivo* and treatment of gastric carcinoma.

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