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• *BASIC RESEARCH* •

Reversal of the phenotype by *K-ras*^{val12} silencing mediated by adenovirus-delivered siRNA in human pancreatic cancer cell line Panc-1

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Abstract

AIM: To investigate the *in vitro* antitumor effect of adenovirus-mediated small interfering RNAs (siRNAs) on pancreatic cancer and the associated mechanism.

METHODS: A 63-nucleotide (nt) oligonucleotide encoding *K-ras*^{val12} and specific siRNA were introduced into pSilencer 3.1-H1, then the H1-RNA promoter and siRNA coding insert were subcloned into pAdTrack to get plasmid pAdTrackH1-*K-ras*^{val12}. After homologous recombination in bacteria and transfections of such plasmids into a mammalian packaging cell line 293, siRNA expressing adenovirus AdH1-*K-ras*^{val12} was obtained. Stable suppression of *K-ras*^{val12} was detected by Northern blot and Western blot. Apoptosis in Panc-1 cells was detected by flow cytometry.

RESULTS: We obtained adenovirus AdH1-*K-ras*^{val12} carrying the pSilencer 3.1-H1 cassette, which could mediate gene silencing. Through siRNA targeted *K-ras*^{val12}, the oncogenic phenotype of cancer cells was reversed. Flow cytometry showed that apoptotic index of Panc-1 cells was significantly higher in the AdH1-*K-ras*^{val12}-treatment group (18.70% at 72 h post-infection, 49.55% at 96 h post-infection) compared to the control groups (3.47%, 3.98% at 72 and 96 h post-infection of AdH1-empty, respectively; 4.21%, 3.78% at 72 and 96 h post-infection of AdH1-p53, respectively) (P<0.05).

CONCLUSION: These results demonstrate that adenoviral vectors can be used to mediate RNA interference (RNAi) to induce persistent loss of functional phenotypes. In gene therapy, the selective down-regulation of only the mutant version of a gene allows for highly specific effects on tumor cells, while leaving the normal cells untouched. In addition, the apoptosis of pancreatic cancer cell line Panc-1 can be induced after AdH1-*K-ras*^{val12} infection. This kind of adenovirus based on RNAi might be a promising vector for cancer therapy.

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Key words: Pancreatic cancer; siRNA; Adenovirus; Phenotype

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INTRODUCTION

Pancreatic carcinoma is a very aggressive carcinoma and has the worst prognosis among common gastrointestinal cancers. Its carcinogenesis is a multistep process, often requiring 7 to 10 discrete (epi) genetic events that endow the cells with an ever-increasing proliferative advantage[1]. These multiple genetic alterations include dominant mutant oncogenes. It is often not clear which of these oncogenes is continuously required, and which may inhibit tumorigenesis when inactivated. In order to develop effective anti-cancer therapies, it is essential to know which of these events is still required late in the process of tumor progression to maintain an oncogenic phenotype. In 85% cases of pancreatic carcinoma, *Ras* genes were mutated[2,3]. The proteins encoded by the *Ras* genes (*K-ras*, *H-ras*, and *N-ras*) are guanine nucleotide binding proteins that associate with the inner plasma membrane and transduce external signals to the interior of cells. They regulate a broad spectrum of cellular activities, including cell proliferation, differentiation, and survival[4]. Mutant *Ras* oncogenes often contain point mutations that alter only a single amino acid, which locks the oncogenic Ras proteins in a persistently activated GTP-bound state[5,6]. Single amino acid substitutions at codons 12, 13 or 61 of Ras p21 protein result in aberrant Ras proteins that contribute to the formation of malignancy due to the disruption of normal GTPase activity of proteins[7].

The codon 12 mutation has been identified as the most predominant mutation among the hot spots[8]. However, the variability of residue substitutions at this position seems limited. The substitution of the normal codon 12 Gly residue by Val residue has been documented[8]. In mouse models of cancer, somatic activation of oncogenic *K-ras* is necessary for early onset of tumors, and its continuous production for maintenance of tumor viability[9-12]. A difficulty in using *Ras* oncogenes as targets in anti-cancer therapy is that at present, it is not possible to specifically inhibit only the oncogenic *Ras* alleles[3,7,9]. This may be essential, since the wild-type *K-ras* gene appears to be required for viability, as evidenced by the embryonic lethal phenotype of mice nullizygous for *K-ras*[13]. Therefore, tools are required to effectively inhibit the activity of oncogenic *K-ras*, but not that of the wild-type *K-ras* protein in normal tissues. We report here that oncogenic alleles of *K-ras* could be specifically and stably inactivated in human pancreatic cancer cells using a viral RNA interference (RNAi) vector, leading to loss of tumorigenicity.

RNAi is a process during which double-stranded RNA induces the homology-dependent degradation of cognate Mrna[14]. In several organisms, introduction of double-stranded RNA has been proven to be a powerful tool to suppress gene expression through a process known as RNAi[15]. However, in most mammalian cells this provokes a strong cytotoxic response[16]. This non-specific effect could be circumvented by use of synthetic siRNAs [21- to 22-nucleotides], which could mediate strong and specific suppression of gene expression[17]. Ever since synthetic 21-23 nucleotide siRNA has been shown to induce efficient RNAi in mammalian cells[17,18], siRNA is routinely used in gene silencing by transfection of chemically synthesized siRNA[19]. However, this reduction in gene expression is transient, which would severely restrict its applications. To circumvent the high cost of synthetic siRNA and establish stable gene knock-down cell lines by siRNA, several plasmid vector systems have been designed to produce siRNA inside cells driven by RNA polymerase III-dependent promoters, such as U6- and H1-RNA promoters[20-24]. With these plasmid vectors, the phenotypes of gene silencing could be observed by stable transfection of cells[20]. Nevertheless, transient siRNA expression, low and variable transfection effciency remain the problems for such plasmid vector derived siRNA. Recently, several virus vectors have been developed for efficient delivery of siRNA into mammalian cells[25-27]. Retroviral vectors have been designed to produce siRNA driven by either U6- or H1-RNA promoter for efficient, uniform delivery and immediate selection of stable knock-down cells[25,26]. But retroviruses can integrate into genome and have a narrower spectrum of cell types than adenoviruses[28-30].

Decades of studies of adenovirus biology have resulted in a detailed picture of the viral life cycle and the functions of the majority of viral proteins. The genome of the most commonly used human adenovirus (serotype 5) consists of a linear, 36-kb, double-stranded DNA molecule. Both strands are transcribed and nearly all transcripts are heavily spliced. Additionally, high titers of viruses and high levels of transgene expression generally can be obtained. Recombinant adenoviruses currently are used for a variety of purposes, including cancer gene therapy[28-30]. Two approaches are traditionally used to generate recombinant adenoviruses. The first involves direct ligation of DNA fragments of the adenoviral genome to restriction endonuclease fragments containing a transgene[31,32]. The low efficiency of large fragment ligations and the scarcity of unique restriction sites have made this approach technically challenging. The second and more widely used method involves homologous recombination in mammalian cells capable of complementing defective adenoviruses ("packaging lines")[33,34]. The desired recombinants are identified by screening individual plaques generated in a lawn of packaging cells[35]. Though this approach has been proven extremely useful, the low efficiency of homologous recombination, the need for repeated rounds of plaque purification, and the long time required for completion of the viral production process have hampered more widespread use of adenoviral vector technology. The problems noted above have stimulated novel methods for generating adenoviral vectors. Six years ago, a simplified system (AdEasy system) for generating recombinant adenoviruses was developed[36]. This system results in highly efficient viral production procedures that often obviate the need for plaque purification and significantly decrease the time required to generate usable viruses.

In this study, we developed a simple adenovirus system utilizing the well-defined polymerase III H1-RNA promoter to drive efficient expression of siRNA in human cancer cells. Our results demonstrate efficient and specific knock-down of *K-ras*^{val12} in Panc-1 pancreatic carcinoma cell lines and indicate a promising application of this adenovirus system in cancer gene therapy.

MATERIALS AND METHODS

Plasmids, cell lines, medium, and reagents

Plasmids pSilencer3.1-H1 and pSP72 were purchased from Ambion Inc. (TX) and Promega Corp. (WI), respectively. Plasmid pAdTrack was kindly provided by Dr. Bert Vogelstein (Johns Hopkins Oncology Center, USA). Plasmid pSUPER-p53 was kindly provided by Dr. Reuven Agami (Center for Biomedical Genetics, Netherlands). All cell lines (Panc-1, HeLa) were purchased from the American Type Culture Collection. These lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, CA) supplemented with 100 mL/L fetal bovine serum (FBS, HyClone, UT), 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37 ℃ in 50 mL/L CO_2. Lipofectin was purchased from Invitrogen Corp. (CA). The antibodies used in this study were human K-ras (F234, mouse monoclonal IgG), p53 (DO-1, mouse monoclonal IgG) and actin (I-19, goat polyclonal IgG) from Santa Cruz Biotechnology Inc. (CA). ^{32}P-DNA probe was from Promega Corp. (WI).

Plasmid constructs

The 63 nt oligonucleotides encoding human *K-ras*^{val12} specific siRNA were 5'gatccgttggagctgttggcgtagttcaagagactacgcca acagctccaacttttggaaa3' and 5'agcttttccaaaaaagttggagctgttggc

gtagtctcttgaactacgccaacagctccaacg3'. These oligonucleotides were annealed and ligated to the *Bam*HI and *Eco*RI sites of pSilencer3.1-H1 to get plasmid pSilencer3.1-H1-*K-ras*^{val12}, subcloned into pSP72 (with *Hin*dIII and *Eco*RI) and then cloned into pAdTrack (with *Hin*dIII and *Bgl*II) to obtain plasmid pAdTrackH1-*K-ras*^{val12}. pAdTrackH1-p53 (the pSUPER-p53 positive control contains the oligonucleotides encoding p53-specific siRNA) and pAdTrackH1-empty were constructed according to the procedures as described above. The inserted sequences were confirmed by dideoxy sequencing.

Production of recombinant adenovirus and cell infection

We employed an efficient homologous recombination system as described[36]. The shuttle vectors pAdTrackH1-empty, pAdTrackH1-p53 and pAdTrackH1-*K-ras*^{val12} were linearized with *Pme*I and transformed into BJ5183 cells (harbor pAdEasy-1) by electroporation. Positive clones were selected and confirmed by DNA miniprep and *Pac*I digestion. Plasmids from correct clones were amplified by transforming into DH10B cells. Miniprep plasmid DNA was prepared by a standard alkaline lysis procedure. The resulting adenoviral DNA (AdEasyH1-*K-ras*^{val12}, AdEasyH1-empty and AdEasyH1-p53) was linearized with *Pac*I and purified by ethanol precipitation. The packaging cell line Ad-293 was grown in DMEM supplemented with 100 mL/L FBS, 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere containing 50 mL/L CO_2 in air at 37 ¡æ. Twenty-four hours before transfection, 0.3×10^6 cells were plated in a 6-well plate to reach a 50-70% confluency. Cells were transfected according to the manufacturer's instructions (Invitrogen, CA). After 4-6 h, the medium containing the transfection mix was replaced with the growth medium. Transfected cells were incubated for an additional period of 7-10 d and medium was changed every 2-3 d. Viruses were harvested, amplified and titered according to the manufacturer's instructions (Stratagene, CA). The efficiency of packaging and amplification could be observed under the fluorescence microscope at 470 nm everyday.

Pancreatic carcinoma Panc-1 cells and cervical carcinoma HeLa cells were grown in DMEM supplemented with 100 mL/L FBS, 100 U/mL penicillin and 100 mg/mL streptomycin at 37 ℃ in 50 mL/L CO_2. On the day before virus infection, 0.3×10^6 Panc-1 or HeLa cells were plated in each well of 6-well plates. On the following day, the cells were incubated with recombinant viruses (AdH1-*K-ras*^{val12}, AdH1-p53 or AdH1-empty) at a multiplicity of infection (MOI) of 10-20 at 37 ℃. After adsorption for 1-2 h, 2 mL of fresh growth medium was added and cells were placed in the incubator for an additional period of 3-5 d.

Western blot analysis

Panc-1 and HeLa cells were infected by adenoviruses and harvested after 60 h infection, washed once with cold phosphate buffered saline (PBS, pH 7.0) and lysed in lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 2 mmol/L EDTA, 1% NP-40) containing protease inhibitors (Boehringer Mannheim, Germany). Total protein (30 μg per lane) was resolved on SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane and incubated with anti-*K-ras*^{val12}, anti-p53 and anti-actin antibodies, followed by incubation with corresponding secondary antibodies. The bands were visualized by using the enhanced chemiluminescence system (Pierce, Rockford, IL). As a control, Panc-1 and HeLa cells without adenovirus infection were used for detecting *K-ras*^{val12}.

Northern blot analysis

Panc-1 cells were infected as described above, and total RNA (30 μg) was extracted 60 h later. RNA was loaded on 11% denaturing polyacrylamide gels, and separated and blotted as described[37]. Anti-sense strand of *K-ras*^{val12} was labeled by ^{32}P. The control 5S-rRNA band was detected with ethidium bromide (EB) staining.

Flow cytometry analysis

At 72 and 96 h post-infection with viruses, Panc-1 cells were trypsinized, washed with PBS. The harvested cells were fixed in 750 mL/L ethanol overnight at 4 ℃, washed with PBS, digested by DNase-free RNase A (to a final concentration of 100 μg/mL) for 30 min at 37 ℃, stained with 50 mg/mL propidium iodide (PI) in PBS (1 h on ice in the dark), and then measured by flow cytometer (Beckman, IL) for relative PI fluorescence (FL-2). The apoptotic index was a mean of four independent experiments.

Measurement of cell proliferation

In DMEM containing 100 mL/L serum, 2×10^4 Panc-1 and HeLa cells were infected with either AdH1-*K-ras*^{val12}, control AdH1-p53 or AdH1-empty viruses. Then the cells were resuspended in 2 mL of 4 g/L low melting point agarose and seeded in duplicate into six-well plates coated with 10 g/L low melting point agarose in DMEM containing 100 mL/L FBS and adenoviruses. The number of foci was scored after 3 wk.

Statistical analysis

Results were expressed as mean±SD and the mean values were compared by using the ANOVA (SNK, Student-Newman-Keuls test) in the SAS 8.1 software. $P<0.05$ was considered statistically significant.

RESULTS

Generation of siRNA-expressing adenoviruses: AdH1-K-ras^{val12}, AdH1-empty and AdH1-p53

We utilized an AdEasy-1 system by which adenoviruses were generated and a pSilencer™ Kit pSilencer 3.1-H1 containing the well-defined polymerase III H1-RNA promoter to deliver siRNA expressing cassettes into cells and to silence a specific gene in human cancer cells. After several rounds of cloning, the H1-RNA promoter and the 63 nt oligonucleotide were cloned into the shuttle vector pAdTrack to get pAdTrack-H1, which could drive the expression of siRNAs targeting different genes in recombinant adenoviruses (Figure 1). Here, the 63 nt oligonucleotide directed against *K-ras*^{val12} was introduced into pAdTrack, then homologously recombined with pAdEasy-1 in *E. coli*

BJ5183 and packaged in 293 cells, after which adenovirus AdH1-*K-ras*^{val12} was obtained (Figures 1, 2). In the same way, AdH1-empty and AdH1-p53 were obtained.

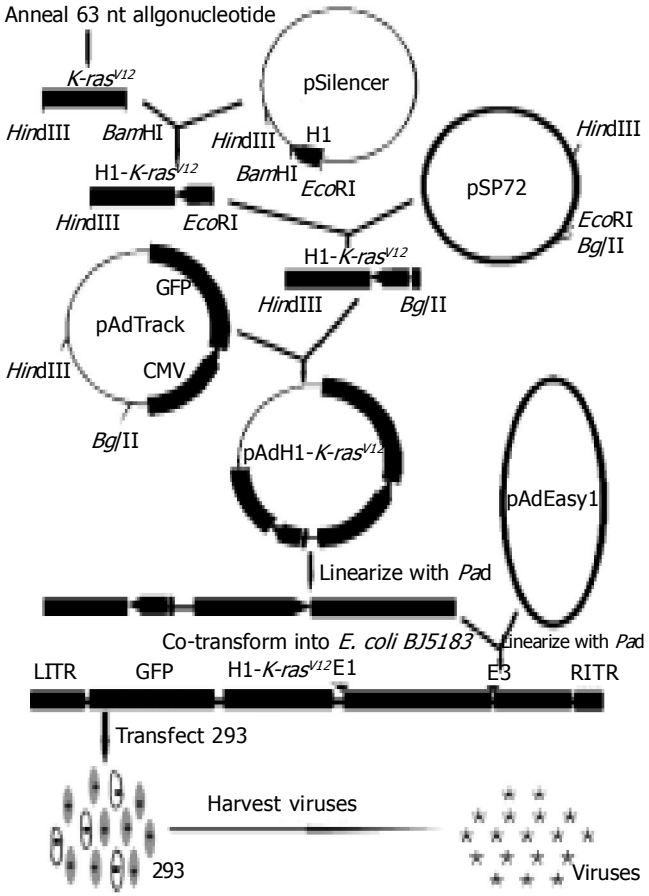

Figure 1 Schematic outline of siRNA expressing adenovirus AdH1-*K-ras*^{val12}, AdH1-p53 and AdH1-empty.

Effect of siRNA-expressing adenoviruses at the molecular level

In this study, we tested our system by targeting *K-ras*^{val12} gene as a model. To study the inhibitory effects of oncogenic *Ras* expression on the tumorigenic phenotypes of human cancer cells, we targeted the expression of endogenous mutant *K-ras*^{val12} alleles with siRNA vector in the human pancreatic cell line Panc-1 (Figure 3). To target specifically the mutant *K-ras*^{val12} alleles, the H1-RNA promoter was used in our strategy. We cloned a 19 nt targeting sequence spanning the region encoding valine 12 of mutant *K-ras* into the pSilencer 3.1-H1 vector, and the yielded pSilencer 3.1H1-*K-ras*^{val12} (Figure 1) showed that human pancreatic carcinoma Panc-1 cells transiently transfected with pSilencer3.1H1-*K-ras*^{val12} had significant suppression of endogenous *K-ras*^{val12} expression, whereas control p53 protein levels were unaffected (data not shown). We then cloned the pSilencer3.1H1-*K-ras*^{val12} cassettes into the pAdTrack to generate the adenovirus AdH1-*K-ras*^{val12}. Viral stocks (AdH1-empty and AdH1-p53 viral stocks were used for control infections.) were used to infect Panc-1 cells that harbored *K-ras*^{val12}. In AdH1-*K-ras*^{val12} neither AdH1-empty nor AdH1-p53 infected Panc-1 cells, *K-ras*^{val12} gene was efficiently silenced 60 h post-infection. After viral infection, Western blot analysis with anti-K-ras-specific antibodies revealed that the *K-ras*^{val12} expression in AdH1-*K-ras*^{val12}-infected Panc-1 cells was markedly suppressed compared to the control infections (Figure 4).

Consistent with this, Northern blot analysis with the anti-sense strand of *K-ras*^{val12} as a probe detected *K-ras*^{val12} mRNA generated in the control groups (AdH1-empty and AdH1-p53) but not in AdH1-*K-ras*^{val12} group (Figure 5).

Effect of siRNA-expressing adenoviruses at the cellular level

Flow cytometry analysis was performed to observe the apoptosis. As shown in Figure 6, knocking down *K-ras*^{val12} Panc-1 displayed increased apoptotic cells compared to the

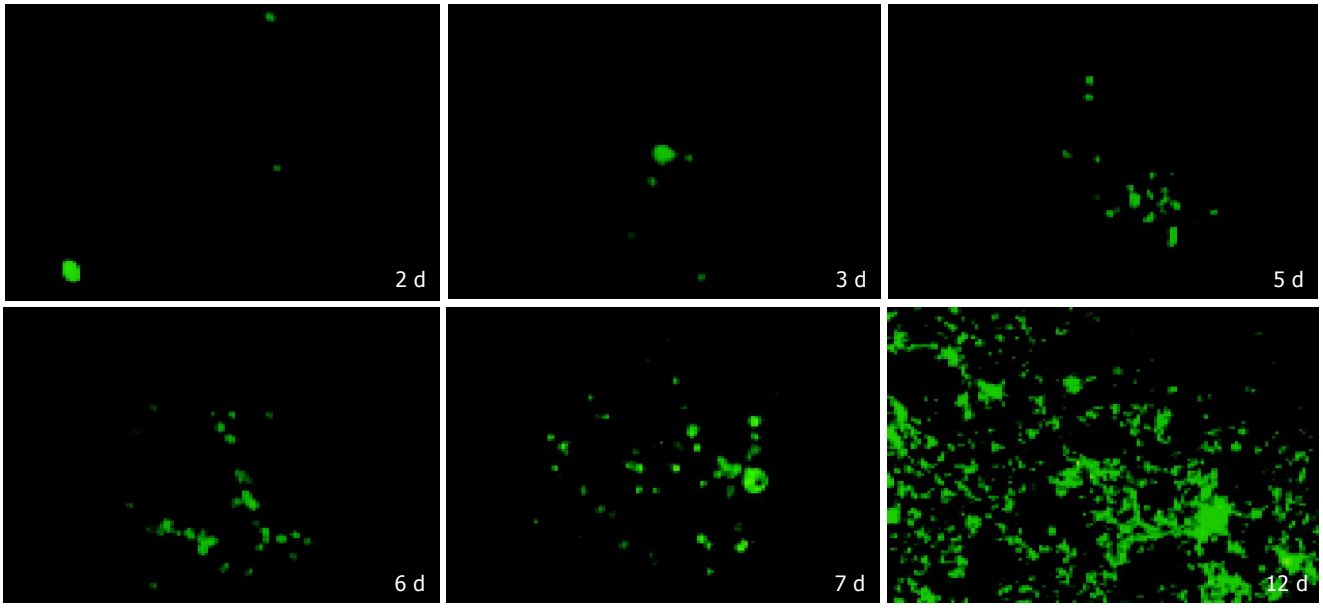

Figure 2 Foci produced by adenoviruses on d 2-7 and 12 after transfection of 293 cells.

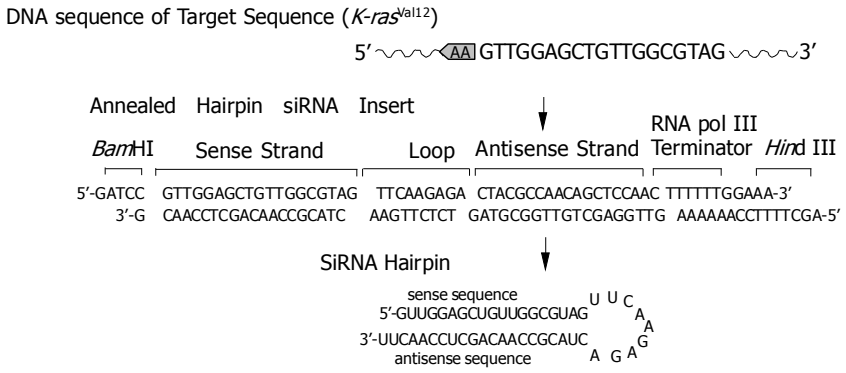

Figure 3 Sequence (for detail, refer to[46]) of Val^{12} mutant alleles of human *K-ras* and the predicted mutant-specific short hairpin transcripts encoded by AdH1-*K-ras*^{val12}.

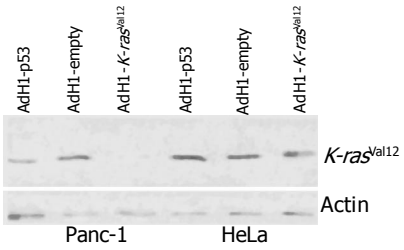

Figure 4 Representative Western blot analysis for *K-ras*^{val12} expression in Panc-1 and HeLa cells 60 h after infection with viral stocks (AdH1-*K-ras*^{val12}, AdH1-empty, AdH1-p53) of three separate experiments.

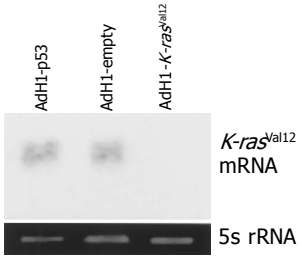

Figure 5 Northern blot analysis with total RNA from same infected Panc-1 cells of single experiment.

control groups treated with AdH1-p53 or AdH1-empty. The percentage of apoptotic cells in the treatment group (18. 70% 72 h post-infection, 49.55% 96 h post-infection) was significantly higher than that in the control groups (3. 47%, 3.98% 72 and 96 h post-infection of AdH1-empty, respectively; 4.21%, 3.78% 72 and 96 h post-infection of AdH1-p53, respectively) (P<0.05).

The presence of oncogenic *K-ras* alleles was frequent in human tumors, but almost invariably associated with other genetic events[3]. To address the question of whether the oncogenic phenotype of late stage human tumors still depended on the expression of oncogenic mutant *K-ras*, we again used Panc-1 cells. One phenotype associated with tumorigenicity had the ability to grow independent of anchorage when plated in a semisolid media (soft agar assay). We infected Panc-1 and HeLa cells with either AdH1-*K-ras*^{val12}, control AdH1-p53, or AdH1-empty viruses. A total of 2×10^4 cells were plated in soft agar and allowed to grow for three weeks. As expected from transformed human tumor cell lines, both Panc-1 and HeLa cell lines were able to grow and form colonies when infected with AdH1-empty and AdH1-p53 control viruses (Table 1). In contrast, infection of AdH1-*K-ras*^{val12} abolished almost completely the colony growth of Panc-1 cells in this assay. Importantly, the effect of AdH1-*K-ras*^{val12} was specific, as soft agar growth of HeLa cells (which contained *H-ras* oncogene) was unaffected (Table 1). Our results demonstrated a significant down-regulation of *K-ras*^{val12} expression in Panc-1 cells.

Table 1 Cell proliferation assay in soft agar (mean±SD)

Cell line	AdH1-*K-ras*^{Val12}	AdH1-empty	AdH1-p53
Panc-1	4±1.53	202±7.77	201±6.56
HeLa	300±2	299±12.06	301±5.57

The number of soft agar colonies from three independent experiments is presented (P<0.05).

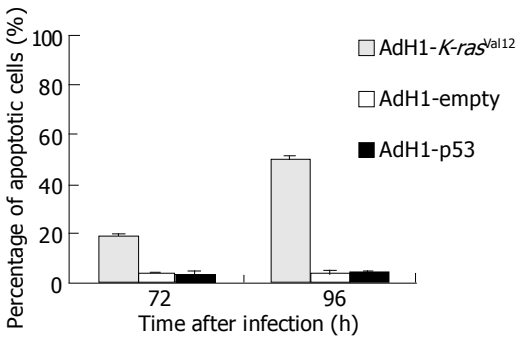

Figure 6 Apoptotic indices shown as the mean of four separate experiments for each group (P<0.05).

Test of the specificity of siRNA-expressing adenovirus AdH1-K-ras^{val12}

We tested the specificity of our targeting construct by examining the expression of wild type *K-ras*. We used HeLa cells, which could endogenously express wild type *H-ras* and

K-ras alleles. Western blot analysis revealed that comparable levels of wild type *H-ras* and *K-ras* proteins were expressed in HeLa cells, irrespective of whether they were infected with the same AdH1-*K-ras*^{val12}, AdH1-p53, or AdH1-empty adenoviral stocks used for the Panc-1 cells (data not shown). In contrast, p53 expression was suppressed equally by AdH1-p53 in both HeLa and Panc-1 cell types, ruling out the possibilities that the HeLa cells were not infected or lacked components necessary for RNAi (Figure 7). In addition, *K-ras*^{val12} was also measured at the protein level. It showed that *K-ras*^{val12} expressed in Panc-1 but not in HeLa (Figure 8). Thus, the RNAi response provoked by AdH1-*K-ras*^{val12} adenoviruses was powerful and sufficiently selective to distinguish the wild type *K-ras* alleles from the *K-ras*^{val12} alleles, which differed in 1 bp only.

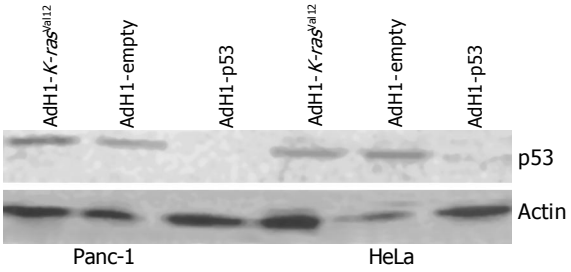

Figure 7 Stable polyclonal pools of Panc-1 and HeLa cells harboring wild-type *H-ras* infected with the indicated viral stocks and immunoblotted for the detection of p53 and actin proteins in single experiment.

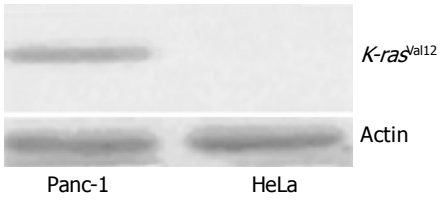

Figure 8 Detection of *K-ras*^{val12} at protein level in Panc-1 and HeLa cells without virus infection in single experiment.

DISCUSSION

In cancer gene therapy, successful strategy depends on the cancer specificity and the efficient delivery into mammalian cells. In this study, RNAi technique and the human adenovirus serotype 5 were used to reach the goal. The availability of a high virus titer, infection of a broad spectrum of cell types and independence on active cell division make adenovirus the vector of choice for siRNA delivery. In the AdEasy-1 system, the backbone vector containing most of the adenoviral genomes, is used in supercoiled form, obviating the need to enzymatically manipulate it. The recombination is performed in *E. coli* rather than in mammalian cells. There are no ligation steps involved in generating the adenoviral recombinants, as the process takes advantage of the highly efficient homologous recombination machinery present in bacteria. The vector contains a green fluorescent protein (*GFP*) gene incorporated into the adenoviral backbone, allowing direct observation of the efficiency of transfection and infection. In the system, the process of viral production can be directly and conveniently followed in the packaging cells by visualization of the GFP reporter. The packaging cell line 293 is highly transfectable by lipid-DNA complexes and constitutively expresses the *E1* gene products required for propagation of all recombinant adenoviruses. The system described herein has several advantages in terms of easy manipulation and speed. The ability to recover reasonable quantities of homogeneous viruses, without plaque purification, represents a major practical advantage. The GFP tracer makes it possible to follow all stages of the viral production process in a convenient fashion. In the case of cells that are inefficiently infected with adenoviruses, the GFP tracer additionally makes it possible to isolate expressing cells through fluorescence-activated cell sorting, thus facilitating several kinds of experiments. To generate viruses, we must attach importance to the following considerations. First, high efficient *E. coli* BJ5183 competent cells containing supercoiled circular pAdEasy-1 and constructs can completely digest with *Pme*I, guaranteeing the successful homologous recombination. Second, to produce viruses, recombinant plasmids can be digested with *Pac*I to liberate linear adenoviral genomes, then transfected into 293 cells. It is critical to linearize the vectors at the *Pac*I sites, as transfection of circular plasmids yields no viruses (data not shown). Third, homologously recombinant plasmids could be produced directly from *E. coli* BJ5183 cells with a relatively low yield. Therefore, miniprep DNA from *E. coli* BJ5183 cells should be used to transform DH10B cells, a *recA* strain in which high-quality and high yields of plasmid DNA could be obtained more easily. The *E. coli* strain BJ5183 is not *recA,* but it is deficient in other enzymes that mediate recombination in bacteria. It could be chosen from several strains mutated in *recA*, *recBCD*, *recJ*, or *recF*[38,39], because of its higher efficiency of transformation and stable propagation of plasmid DNA in experiments. Once recombination is achieved and verified, the adenoviral recombinant DNA can be simply transferred to *recA* and *endA* strains (such as DH10B) for greater yields of DNA if desired. (Because of its *recA* status, DH10B can not be used to generate adenoviral recombinants by homologous recombination.). In contradiction to the previous literature[36], we found that it was not important to place the insert in head-to-tail orientation when the siRNA expressing cassettes were cloned into pAdTrack. But we could not explain the phenomenon. If other kinds of expressing cassettes could be constructed like this, the system would be more convenient for the production of adenoviruses. Consistent with the observation of several groups[25-27], these viral vectors could infect cells uniformly and rapidly. Given the simplicity of this system, which employs readily available reagents, most laboratories have the ability to silence their genes by recombinant adenoviruses.

RNAi has been widely used in identification and characterization of genes[40]. In functional genomics, a large number of genes controlling cell division and metabolism have been identified by screening with RNAi. Another

potential application is in the area of gene therapy[41]. It has been shown recently that RNA viruses are sensitive to RNAi[42-44]. Nevertheless, our results indicate that adenoviral vectors could also be used to mediate efficient integration of pSilencer3.1-H1 cassettes in human cells and direct the synthesis of siRNAs to suppress gene expression. It is suggested that the expression of siRNAs may depend on RNA polymerase III promoter but not on any kind of vectors.

To detect the effect of AdH1-*K-ras*^{val12} at the cellular level, flow cytometry analysis and measurement of cell proliferation are employed. Determination of the relative DNA content of apoptotic nuclei (which are hypodiploid due to loss of fragments) by PI staining and cytofluorimetric analysis is a more sensitive way to demonstrate apoptotic cell death[45]. As shown in Figure 6, after AdH1-*K-ras*^{val12} infection, the apoptosis of Panc-1 cells is detected by flow cytometry. But, in the study of Brummelkamp *et al*[46], flow cytometry analyses showed that knocking down *K-ras*^{val12} had no significant effect on the ability of Capan-1 cells to proliferate adherently under standard tissue culture conditions. We think that it may be due to different viral vectors or different cell lines. Further researches are needed to explain the contradiction. Consistent with Brummelkamp *et al*[46], soft agar assay demonstrates that viral vectors could be used to mediate RNAi to induce persistent loss of functional phenotypes, suggesting that siRNAs-expressing adenoviruses can be used for cancer therapy at the late stage of human tumors. Nevertheless, adenoviruses must be employed repeatedly.

In mammalian cells, dsRNA larger than 30 bp was reported to induce generally non-specific suppression of gene expression by activating the antiviral interferon response[14]. This obstacle has been overcome by the discovery that the effector in RNAi is an siRNA which is 21-23 nt long with 2 nt 3'-overhang and 5'-phosphate. The successful gene silencing with chemically synthesized 21-22 nt siRNAs can rapidly trigger its wide application in mammalian cells[14,17,18]. It has been demonstrated that a vector derived from polymerase III-dependent H1-RNA gene promoter can produce siRNA and cause efficient and specific down-regulation of gene expression, resulting in functional inactivation of the targeted genes[20]. Almost all the elements of H1-RNA promoter are located upstream of the transcribed region, and it is ideally suitable for the expression of about 19 nt siRNAs. It has been reported that stem-loop precursor transcripts are generated and processed to functional siRNA by cellular enzymes[20]. This small size of siRNA could prevent activation of the dsRNA inducible interferon system present in mammalian cells and avoid the non-specific phenotypes normally produced by dsRNA larger than 30 bp in somatic cells[20,46]. Similarly, our results showed the specificity. Adenovirus-delivered siRNAs may be an ideal method for tumor-specific gene therapy.

In conclusion, a simple siRNA delivery strategy can be developed by combination of well-defined H1-RNA promoters and pAdEasy-1 adenovirus system. Vectors like these have at least two potential applications. In gene therapy, the selective down-regulation of only the mutant version of a gene allows for highly specific effects on tumor cells, while leaving the normal cells untouched. This feature greatly reduces the need to design viral vectors with tumor-specific infection and/or expression. By designing target sequences that span chromosomal translocation breakpoints found in cancer, these vectors may also be used to specifically inhibit the chimeric transcripts of these translocated chromosomes. The recent demonstration that siRNAs can inhibit gene expression *in vivo* provides further support for the notion that oncogene-specific RNAi may be a viable approach to the treatment of cancer[47,48]. This technology has a foreseeable wide application in experimental and clinical work for cancer therapy. In addition, these vectors can be used to efficiently identify the genetic events that are required for cancer cells to manifest a tumorigenic phenotype. Through the use of this technology, out of the many genetic alterations present in most human cancer cells, the most effective targets for drug development can be rapidly identified[46]. Furthermore, an apparent apoptosis in Panc-1 cells has been mediated by replication-incompetent adenoviruses. The mechanism needs to be further investigated. Since this kind of viruses transiently expresses exogenous genes, in order to get a persistent gene expression, we are developing a conditionally replicative virus that can express siRNA for cancer therapy.

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