ORIGINAL RESEARCH

Effects of survivin interference RNA on non-small cell lung carcinoma

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Manuscript submitted 4th May, 2009

Manuscript accepted 13th September, 2009

Clin Invest Med 2009; 32 (6): E225-E231.

Abstract

Objectives: The primary purpose of this study was to investigate the *in vitro* and *in vivo* effect of survivin interference RNA (siRNA) on non-small cell lung cancer.

Methods: Lentivirus was used as a vector to transfer siRNA into human lung cancer A549 cells. The proliferation of the cancer cells was assessed by MTT

[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The lentivirus-mediated siRNA was also injected into the transplanted A549 tumor tissues in mice. Tumour growth was assessed after 11 injections over a period of 21 days.

Results: Compared with the placebo and the blank lentiviral vector groups, the siRNA treatment group had reduced cell growth rate following 4 days of the treatment ($P < 0.01$). The average size of the transplanted A549 tumours in the siRNA treatment group ($0.75 \pm 0.16 \text{ cm}^3$, $n=8$) was smaller than in the placebo ($2.09 \pm 0.22 \text{ cm}^3$, $n=6$) or the blank lentiviral vector groups ($1.89 \pm 0.18 \text{ cm}^3$, $n=6$) ($P < 0.01$). The tumour growth inhibition rate in the siRNA groups was 46.1%.

Conclusion: Lentivirus-mediated siRNA therapy inhibits the growth of human lung cancer cells *in vitro*. The siRNA therapy also suppresses the growth of the transplanted lung cancer in mice.

Survivin gene plays an important role in the pathogenesis of several cancers such as lung and prostate cancer.^{1,2} Therefore, removal of this gene's action may be an effective strategy in the management of these cancers. RNA interference is a process of post-transcriptional gene silencing in which dsRNA inhibits gene expression in a sequence-dependent manner via degradation of the corresponding mRNA.³ RNA interference against survivin gene may block the expression of this gene, which may inhibit the growth of tumour cells.

Lentivirus is a potential vector for RNA interference or gene therapy. This virus enters susceptible target cells through the combination of capsid glycoprotein and specific receptors on the cell membrane.⁴ The viral RNA is reversed into double-stranded linear DNA, which is then transferred into nucleus of the host cells. The viral DNA integrates into the host's chromosome permanently, becoming provirus which

is copied to the offspring cells identical to the host genome through cell proliferation in the target cells.⁴

Researchers have used antisense oligonucleotides and ribozyme to inhibit the expression of survivin gene successfully⁵ but these methods are time consuming and expensive. The primary purpose of this study was to construct a survivin interference RNA (siRNA) and its lentiviral vector. The *in vitro* and *in vivo* effects of siRNA on the proliferation of lung cancer cells were investigated.

Materials and Methods

This study was approved by the institution review board of Liaocheng People's Hospital. siRNA, lentiviral vector, and 293T cell lines were purchased from Shanghai Genechem Co. (China). Dulbecco's Modified Eagle Medium (DMEM-F12) was provided by GIBCO (USA). Trizol, fetal calf serum and 0.25% trypsin were purchased from Invitrogen Co. (USA). Restriction enzymes, T4 DNA ligase, and DNA markers were obtained from Takara (Japan). Large-scale plasmid DNA purification kits and gel extraction kits were provided by Qiagen (Germany). BALB/C nude mice (male, weight 17-18g), and human lung adenocarcinoma cell lines A549 were obtained from the Cancer Institute of the Chinese Academy of Medical Sciences (Beijing, China).

siRNA preparation

Synthesis of DNA fragments against survivin was performed according to the manufacturer's instruction (Genechem, Shanghai, China). Double-stranded oligo DNA was prepared as below:

Positive chain –

5'-GAGGCTGGCTTCATCCACTGC-3',

Antisense chain –

5'-GCAGTGGATGAAGCCAGCCTC-3'

DNA samples were incubated at 90°C for 4 min, and 70°C for 10 min. After slowly cooling to room

temperature, 12% non-denaturing PAGE gel was used to test the efficiency of the double-stranded formation.

Lentiviral vectors

Lentiviral vector system was composed of pGCL-green fluorescent protein (GFP) vector, pHelper 1.0 (gag/pol element) carrier, and pHelper 2.0 (VSVG element) three-vector plasmid. pGCL- GFP vector contained U6 promoter and was able to continually express small interfering RNA in the host cells. The plasmid could express CMV promoter-driven green fluorescent protein, which can be used to evaluate the efficiency of viral transfection while packaging, as well as the efficiency of host cells infection.

pHelper 1.0 plasmid contained HIV gag gene encoding the main structure of the virus protein, pol gene encoding the virus-specific enzyme, and rev gene encoding gag and pol regulation of gene expression regulator. pHelper 2.0 plasmid had the VSVg gene of herpes simplex virus gene, which provides the virus packaging capsid protein.

pGCL-GFP, pHelper 1.0 and pHelper 2.0 were packaged into 293T cells. The 293T cells supernatant was collected 72 h after transfection. Cell debris was removed by centrifuging. The supernatant was then filtered by a 0.45µm filter at 25000 rpm centrifuging for 90 min. The virus precipitation was resuspended, and dissolved at 4°C overnight. Partied viruses were preserved at -70°C in a low-temperature refrigerator. Lentiviruses were packaged in 293T cells and being tested for the multiplicity of infection (MAOI) value.

The lung cancer A549 cells were subsequently infected with lentivirus-mediated siRNA (RNAi group). A non-purpose gene expression vector (blank vector) was used as normal control (NC group).

Determination of viral titer

A549 cells were placed on 96-well culture dish, where 4×10^4 cells were plated in 100µl DMEM medium. Viral suspension was added to the wells in the dish for incubation for 4 h. Fresh medium (100µl) was then

added. The expression of fluorescent cells was observed after 4 days. The number of fluorescent cells in the wells was counted and divided by the dilution multiples to generate the viral titer.

PCR identification of the positive clones

The siRNA oligonucleotide against survivin gene sequence was annealed to form double-stranded DNA. The DNA was ligated into pGCL-GFP vector, and transformed into *E. coli* DH5 α . The positive clones were selected and identified by PCR.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay of cell proliferation

The yellow tetrazolium MTT was reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan was solubilized and quantified by spectrophotometric method.

Lentivirus particles were added to 2×10^5 cultured cells. The culture medium was replaced 12 h later

with fresh medium. The culture was continued for another 3 days. Cells were then suspended at 6×10^3 cells per well and were infected with the lentivirus for 5 days. MTT reagent was added to each well. The cell culture plate was incubated at 37°C for 3 h. DMSO (200 μ l) was used to dissolve the crystal. Cell growth curves were constructed.

In vivo experiment

BALB/C nude mice were vaccinated with A549 cell suspension (8×10^6 cells per mouse) by subcutaneous injection to the right armpit. When the tumour grew to 10×5 mm in size, the animals were randomly divided into placebo (normal saline), blank vector group (normal control or NC group), and lentiviral vector-mediated siRNA treatment group (RNAi). Placebo, blank vector or 50 μ l of siRNA lentiviral vector were injected directly into the tumour every second day for 11 times, with a total duration of therapy of 22 days.

Each group had 6-8 mice. The size of the tumour was measured with a vernier caliper every three days. Relative tumour volume (RTV) was calculated by the following formula: tumour volume V (mm^3) = $\frac{1}{2} \times ab^2$ (a for longer track, b for short track); $\text{RTV} = V_t/V_0$ (V_0 was the tumour size measured for the first time, V_t was the tumour size at the end of therapy).

The inhibitory rate (IR) was calculated as following: $\text{IR} = (1 - T_{\text{RTV}}/C_{\text{RTV}}) \times 100\%$ (T_{RTV} : RTV of the treatment group, C_{RTV} : RTV of the control group).

After the treatment, the tumour from each animal was harvested and weighed.

Statistics

SPSS 10.0 software was used to analyze the data, which were expressed as means $\pm$ SD. Comparisons of numerical data within or between groups were performed by ANOVA. Categorical data were compared by Chi-square test. $P < 0.05$ was considered statistically significant.

FIGURE 1. PCR identification of positive clones. 1. Marker: 5,3,2,1.5,1 Kb, 750,500,250,100 bp; 2. negative control (no load); 3. Positive control (insert); 4. Psc 131.

Results

PCR identification of the positive clones

The PCR product from recombinant bacterial cloning was 410bp (insert fragment was 115bp). The pGCL-GFP empty vector was used as a control for the PCR product 295bp (Fig 1), confirming the insertion of siRNA.

FIGURE 2. (A) Viral titer test results in the blank vector group ($\times 200$; viral titer: $10e^{0-6}$). (B) Viral titer test results in the survivin interference RNA group ($\times 200$; viral titer: $10e^{0-6}$).

Titer of the lentivirus

The titer of the lentivirus in the blank vector and RNAi groups is shown in Fig 2A and 2B. After transfection with the plasmid, the A549 cells showed green luminescence, which suggests the expression of lentivirus-mediated siRNA in these cells. A549 cells were co-transfected with a plasmid expressing lentivirus-mediated siRNA or a non-purpose gene expression vector. A plasmid expressing GFP was also co-transfected to monitor transfection efficiency. Fluorescent microscopy at 72 h post-transduction revealed that transduction with a non-purpose gene expression vector had no effect on GFP expression levels (Figure 2A), whereas transduction with lentivirus-mediated siRNA resulted in almost complete inhibition of GFP expression (Figure 2B).

Tumour cell proliferation

All infected tumour cells had a green fluorescent appearance. Under the fluorescence microscope, after 24 h of lentivirus treatment, GFP expression in all groups of tumour cells was 100%. This level of expression persisted in the next three days.

Seven days after the viral infection, the cell proliferation in the blank vector (NC group) was not affected,

FIGURE 3. Cell proliferation in the RNAi and blank vector (NC) group ($\times 200$). Fluorescent microscopy at 72 h post-transduction revealed that transduction with a non-purpose gene expression vector had no effect on GFP expression levels, whereas transduction with lentivirus-survivin-siRNA after 72 h resulted in a dramatic reduction in the levels of GFP compared to the NC group.

FIGURE 4. Cell growth curve in the four groups. PC: placebo; NC: blank vector, RNAi: survivin interference RNA group.

but it was significantly inhibited in the RNAi group (Fig 3). The cell growth in the RNAi group (MOI 50 and MOI 150) was lower than in the placebo and the NC group (Fig 4, $P < 0.01$).

Effect on tumour growth in vivo

There were no mortalities or discernable adverse effects during the 3-week treatment. The weight and volume of the tumour in the RNAi group were significantly lower than in the placebo and the NC group (Table 1 and 2, respectively, $P < 0.01$).

The tumour inhibition rate of the RNAi group was 46.1%. There was no tumour growth inhibition in the placebo group.

Discussion

The major findings of this study are: 1) Lentivirus can be used as a reliable vector for survivin RNA interference and therefore gene therapy; 2) Lentivirus-mediated survivin RNA interference inhibits the growth of human lung cancer cells *in vitro*; 3) Survivin interference RNA, when injected directly into tumour tissues, suppresses the growth of transplanted lung cancer cells in mice.

TABLE 1. The weight of the tumour after treatment.

Group	Number	Animal weight (g)		Tumour weight (g)
		Before	After	
Placebo	6	17.6±1.7	19.5±1.4	2.07±0.65
NC	6	18.7±1.0	19.3±0.6	1.94±0.29
RNAi	8	18.8±1.8	19.8±1.9	1.12±0.42*

* $P < 0.01$ vs placebo and blank vector (NC) groups.

TABLE 2. The volume of the tumor after treatment.

Group	Number	Volume of tumour (cm ³)			
		Day 0	Day 7	Day 14	Day 21
Placebo	6	0.23 ± 0.17	1.10 ± 0.32	1.53 ± 0.11	2.09 ± 0.22
NC	6	0.18 ± 0.14	0.86 ± 0.19	1.31 ± 0.14	1.89 ± 0.18
RNAi	8	0.19 ± 0.08	0.27 ± 0.12*	0.51 ± 0.18*	0.75 ± 0.16 *

* $P < 0.01$ vs placebo and blank vector (NC) groups.

Survivin is a direct inhibitor of caspase-3 and caspase-7, acting as a blocker of apoptosis.^{6,7} CDE and CHR in the survivin gene group are suppression elements of the G1 phase of the cell cycle, regulating the expression of G2/M phase regulator's half-life.^{8,9} Survivin also interacts with the cell cycle regulator Cdk4, leading to Cdk2/Cyclin E activation and Rb phosphorylation. As a result of Survivin/Cdk4 complex formation, p21 is released from its complex and interacts with mitochondrial procaspase 3 to suppress Fas-mediated cell death.^{10,11}

Lentiviral vectors have large gene carrying capacity, low immunogenicity and are able to infect non-division cells.¹² These viruses are also able to integrate into the genome of host cells with ease.¹² As a result, lentiviral vectors have become a popular option in gene transfer research and cancer therapies. Although the recent discovery of survivin interference RNA has brought new hopes to cancer gene therapy, there are still many barriers to overcome. These barriers include how to get the survivin interference RNA into the targeted cells more efficiently, and to avoid degradation of the gene during the process. The other barriers are how to determine the appropriate concentration of survivin interference RNA to achieve the

optimal therapeutic effect and to lower adverse reactions; as well as how to ensure the specificity of interference RNA and to avoid the effects of foreign target (off-target effect).

There have been several recent studies on gene-based therapies for non-small cell lung cancer.¹³⁻¹⁵ At present, most of the reported gene carriers or vectors lack target cell selectivity. These vectors integrate into the tumour cells as well as normal host cells.¹⁶ As a result, the therapeutic effects and the safety profile from *in vitro* tests are not always mirrored by the *in vivo* studies. In the present study, we adopted a lentiviral vector to investigate the effect of survivin interference RNA on cancer cell lines as well as on transplanted human lung adenocarcinoma in mice. Three weeks of treatment resulted in a smaller tumour and 46.1% tumour inhibition rate, with no mortality observed in the treated animals. These results provide an important basis for further *in vivo* trials on the long-term efficacy and safety of this lentivirus-mediated gene therapy.

The exact signaling pathways by which survivin interference RNA suppresses tumour cell growth in the present study are unclear. The *in vivo* metabolic mechanism of lentivirus RNA is also uncertain. There is some evidence that lentivirus RNA may undergo a degradation process *in vivo*, compromising its therapeutic efficacy.¹⁷ After silencing the survivin gene expression, lentiviral RNA's ability of inhibiting cell proliferation and colony-forming will decline, largely due to the increased apoptosis and the loss of survivin-driven cells.¹¹ Therefore, the long-term *in vivo* anti-cancer effect of the lentivirus-mediated RNAi therapy requires further studies.

One limitation of this study is that the effect of siRNA on the level of survivin protein in the *in vitro* lung cancer A549 cells, and in the transplanted cancer cells in the mice, was not investigated. Further studies are required to ascertain if the *in vitro* and *in vivo* cancer cell inhibition following this siRNA treatment is due to the low expression of the surviving protein in these targeted cancer cells. In addition, the long-term

effect of this siRNA therapy on the tumour and the normal tissues or cells is unknown and requires further investigation.

In conclusion, over-expression of survivin gene is associated with the pathogenesis of a variety of human cancers. Lentivirus can be used as a vector of the survivin interference RNA to treat some of these cancers. This survivin interference RNA treatment has been shown in the present study to suppress the proliferation of tumour cells *in vitro*. In addition, short course of survivin interference RNA treatment in mice inhibited the growth of the transplanted human lung cancer. Further studies are required to assess the long-term safety and efficacy of this novel therapy.

Acknowledgements

This study was supported by a research grant from Bureau of Science and Technology, Liaocheng City, China (No: 2007024802).

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