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Induction function of siRNA-mediated survivin gene silencing on nasopharyngeal carcinoma cell apoptosis

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ABSTRACT. We examined the function of survivin gene expression in patients with nasopharyngeal carcinoma (NPC), as well as small interfering RNA (siRNA) on controlling CNE-2 NPC proliferation and apoptosis. Immunohistological methods, *in situ* hybridization, and reverse transcription-polymerase chain reaction technique were used to detect survivin protein and mRNA expression. We designed an siRNA sequence to inhibit survivin gene expression. The MTT method was used to examine the function of siRNA on controlling cell growth and proliferation. Induction of cell apoptosis by siRNA was examined by flow cytometry; electron microscopy was used to observe ultrastructure changes in CNE-2 cells. Western blotting was used to detect survivin gene expression. The survivin protein was expressed in 71.9% of cells, while its mRNA was expressed in 65.6% of cells. Relative mRNA expression was 4.16×10^{-2}; these data for the control groups were 23.3, 33.3, and 4.42×10^{-4}, respectively. Following transfection with 3 different siRNA sequences, survivin mRNA expression in CNE-2 cells was decreased. Inhibition of cell proliferation and rate of apoptosis increased with

increasing siRNA concentration. Western blotting revealed decreased survivin expression and electron microscopy revealed ultrastructural changes in cancer cells. Survivin gene expression in NPC generally increased. *In vitro* transcription of siRNA decreased CNE-2 survivin gene expression, and different sequences of siRNA decrease gene expression in CNE-2 cells to varying degrees. Transfected siRNA3 can effectively inhibit CNE-2 cell proliferation and induce apoptosis; gene silencing using siRNA may represent a new treatment for NPC.

Key words: Gene silencing; Nasopharyngeal carcinoma; siRNA; Survivin

INTRODUCTION

Nasopharyngeal carcinoma (NPC), a common head and neck cancer, is typically observed in Asian populations in southern China. This cancer is most frequently observed in Guangdong, Guangxi, Hainan, Hunan, Fujian, and other provinces, as well as autonomous regions and other Southeast Asian countries. Because of the anatomical location of NPC and its sensitivity to radiation, the use of high-energy ionizing radiation is the most prevalent treatment method (Pastorino et al., 2002). However, survival in these patients remains low and the treatment is very harmful to the human body; additionally, repeated treatments can increase the tolerance of NPC cells to radiation. Hence, development of a new method with fewer side effects is necessary. The progression and metastasis of NPC are controlled by genes. Research examining molecular target therapy has identified cell receptors, key genes, and regulatory molecules involved in NPC (Cheng and Ming, 2012). Various oncogene proteins are involved in tumor progression, and the functional effects of the genes should be examined to understand uncontrolled cell proliferation and inhibition of apoptosis.

A recent study revealed that survivin is a member of inhibitor of the apoptosis protein family (Li 2005), participates in apoptosis, and is often expressed in tumor tissues. RNA interference (RNAi) can be used to block gene transcription. To investigate the usefulness of survivin small interfering RNA (siRNA) for NPC gene therapy, we conducted immunohistochemistry (IHC), *in situ* hybridization (ISH), and reverse transcription-polymerase chain reaction (RT-PCR) to detect survivin protein and mRNA expression. Additionally, we synthetized siRNA that was transferred to CNE-2 cells using the liposome method. RT-PCR was then utilized to determine the function of siRNA in controlling survivin gene expression. The ability of siRNA to control the growth and proliferation of cells and the function of siRNA on the induction of cell apoptosis were examined. Ultrastructural changes in CNE-2 cells and survivin gene expression were also detected.

MATERIAL AND METHODS

Patients

Sixty-four patients with NPC and 30 control subjects from the Province People's Hospital of Hainan were recruited for the study between May 2005 and September 2006. Among the 64 patients, 43 were males and 21 were females, with an average age of 50 years (22-77

years). Except for 1 patient with vesicular nucleus cell carcinoma, the remaining patients showed poorly differentiated squamous cell carcinoma. Among the 64 NPC patients, detailed clinical data were obtained for 22. According to a National NPC Meeting held in Fuzhou in 1992 (Min et al., 1994), we categorized the cases as stages I and II in 4 cases and III and IV in 18 cases.

Methods

IHC staining with streptavidin-peroxidase (SP) method

The expression of survivin in paraffin-embedded sample from NPC patients was determined by IHC staining with the SP method according to kit instructions (Fuzhou Maixin Biotech. Co., Ltd., China). The sliced 4-μm sections of paraffin-embedded samples that were pre-fixed in 10% formalin were mounted on silicon-coated slides for 24 h. Afterwards, samples were treated successively with steps of dewaxing (treated with xylene I for 20 min and xylene II for 20 min), hydration (treated with 100% ethanol for 10 min, 95% ethanol for 5 min, 80% ethanol for 5 min and 70% ethanol for 5 min). Endogenous peroxidase was inactivated by incubating with fresh 3% H_2O_2 for 10 min, and then rinsed 3 times with PBS for 3 min. Afterwards, antigen retrieval was conducted by immersing sample in 10 mM citrate buffer, pH = 6, and then heating at 100°C for 15 min in microwave oven. Sample was rinsed with tap water for 10 min and then soaked in PBS for 5 min after sample had cooled down in room temperature. Sample was blocked with non-immune animal serum work solution at 37°C for 10 min, and then the solution was discarded. Solution containing primary rabbit polyclonal antibodies against human survivin was added to sample and incubated for 1 h at room temperature, and then rinsed 3 times with PBS. Afterwards, solution containing biotin-labeled anti-rabbit polyclonal antibodies was added and incubated for 10 min, and then also rinsed 3 times with PBS. SP work solution was added to sample and incubated at 37°C for 10 min. One or two drops of 3',3'-diaminobenzidine (DAB) were added as chromogenic reagent to each slide, and the consistency of the staining was evaluated under light microscopy 2 or 3 min later. Once proper staining was present, it was immediately terminated by rinsing with tap water for 5 min. Afterwards, sample was successively treated with hematoxylin re-staining, tap water rinsing, enhancing differentiation with lithium carbonate and reversing blue by water rinsing for 10 min. Lastly, sample was successively treated by dehydrating with graded alcohol, hyalinizing with xylene, drying and fixing with neutral balsam.

ISH to detect survivin mRNA

Survivin mRNA detection was conducted according to kit instructions of BOSTER Immunoleader (Pleasanton, CA, USA). Paraffin-embedded samples from NPC patients who were pre-treated with dewaxing, hydration and deactivating endogenous peroxidase were digested with pepsin (100 μL concentrated pepsin dissolved in 1 mL 3% sodium citrate) at 37°C for 10 min, and then rinsed 3 times with distilled water. Sample was then fixed with 1% polyoxymethylene (dissolved in DEPC-PBS) for 10 min and rinsed 3 times with distilled water. Sample was treated in a closed container at 39°C for 2 h with pre-hybridization solution and then discarded. Afterwards, to each slide it was added a 20-μL hybridization solution that contains digoxin-labeled oligonucleotide probe for detecting survivin mRNA and then incubated in a wet closed container at 39°C overnight. Then, free probes were removed by

soaking each slide at 37°C with the following procedures: soaking in 2X SSC for 5 min 2 times, soaking in 0.5X SSC for 15 min and soaking in 0.2X SSC for 15 min. Afterwards, the following steps were performed: incubation at 37°C for 30 min with the addition of a blocking solution, discarding of the blocking solution, incubation at 37°C for 1 h with the addition of biotinylated mouse anti-digoxin antibodies, rinsed 4 times with DEPC-PBS, incubation at 37°C for 20 min with the addition of SABC, rinsed 5 times with DEPC-PBS. The coloring with DAB and subsequent procedures was the same as that of IHC staining with SP method.

CNE-2 cell culture

CNE-2 cells were cultured in a constant temperature incubator with RPMI 1640 supplemented with 10% FBS, 50 U/mL penicillin and 50 µg/mL streptomycin. CNE-2 cells are subcultured when they are 85-90% confluent in flask. Trypsin-EDTA (0.05%) was added to release cells, and digestion was immediately neutralized by adding 10 mL culture medium when cells are in a single-cell suspension under microscope.

RNAi experiment

Using the tool from the Ambion website, we designed 3 siRNA sequences (sense strand and antisense strand) for the human survivin gene mRNA and one negative control with the following sequences: siRNA1: 5'-GGC AGU GGC CUA AAU CCU Utt-3'; 5'-AAG GAU UUA GGC CAC UGC CTT-3'. siRNA2: 5'-GGC AGU GUC CCU UUU GCU Att-3'; 5'-UAG CAA AAG GGA CAC UGC CTT-3'. siRNA3: 5'-GGU UCC UUA UCU GUC ACA Ctt-3'; 5'-GUG UGA CAG AUA AGG AAC CTT-3'. Negative control: siRNA: 5'-UUC UCC GAA CGU GUC ACG UTT-3'; 5'-ACG UGA CAC GUU CGG AGA ATT-3'. Liposomes were transfected into cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) with Opti-MEM medium and diluted siRNA for transfection; siRNA concentration was 40 nM. The same concentration of siRNA that did not interfere with any genes was added to the negative controls; additionally, an equal volume of PBS was added to a control group. RNA was extracted for detection after 24 h.

MTT method for detecting CNE-2 cell proliferation rate

According to the three siRNA interference effects detected by fluorescence quantitative RT-PCR method, we used siRNA3 to conduct the following interference experiment.

Flow cytometry detection of cell apoptosis

Cells in the logarithmic growth phase were inoculated at 10^6 cells per bottle and the cells were cultured in DMEM without antibiotics for 24 h. The 4 experimental groups were transfected with siRNA2 at concentrations of 25, 50, 100, and 200 nM; the negative control group contained no siRNA. After 24 h, the culture medium was removed and PBS was used to wash the cells twice; ice-cold 70% ethanol was added and the cells were incubated overnight at 4°C. Cells were digested using trypsin-EDTA in medium containing fetal calf serum. The cells were pelleted by low-speed centrifugation, the supernatant was removed, and PBS was used to wash the cells twice. The cells were centrifuged for 5 min at 250 *g* to collect approximately

5 x 10^5 cells. Binding buffer was added to the cells, and 5 μL Annexin V-fluorescein isothiocyanate (FITC) and 5 μL propidium iodide were added; the excitation wavelength was 488 nm and the emission wavelength was 530 nm. The green fluorescence of Annexin V-FITC used the FITC channel (FL1), while propidium iodide red fluorescence used the propidium iodide channel (FL3).

CNE-2 cells and NPC tissue semi-quantitative RT-PCR

Total RNA was extracted and real-time RT-PCR was conducted; the SYBR ExScript™ RT-PCR kit (Sigma, St. Louis, MO, USA) was used according to manufacturer instructions.

Electron microscopy of ultrastructural changes

Cancer cell ultrastructure changes were observed under a transmission electron microscope (JEM - 1010, JEOL, Akishima-Shi, Tokyo, Japan).

Western blot

After interference using siRNA3 for 24 h, a kit was used to extract nuclear and cell plasma proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate proteins and a standard wet film gauge (Bio-Rad, Hercules, CA, USA) was used to transfer proteins to a nitrocellulose membrane. Coomassie brilliant blue stain was used to dye the transferred proteins; after blocking with non-fat dry milk, survivin IgG primary antibody was incubated and horseradish peroxidase/DAB was added as the secondary antibody for visualization.

RESULTS

RNAi

After transfecting siRNA into CNE-2 cells for 24 h, the 3 siRNA sequences affected CNE-2 cells; siRNA2 and siRNA3 showed a significant difference with the negative control group. The interference effect of siRNA3 was the highest, as shown in the Table 1.

Table 1. Fluorescence quantitative RT-PCR for detecting mRNA expression in cells.

	Survivin gene mRNA expression			Average
	Repeat 1	Repeat 2	Repeat 3	
siRNA1	1.40×10^{-4}	4.08×10^{-6}	8.72×10^{-7}	7.93×10^{-6}
siRNA2	1.97×10^{-10}	4.24×10^{-12}	1.62×10^{-12}	1.11×10^{-11}
siRNA3	2.20×10^{-12}	7.82×10^{-12}	2.33×10^{-11}	3.42×10^{-12}
Negative control	6.04×10^{-5}	5.57×10^{-5}	3.35×10^{-5}	4.83×10^{-5}
Blank control	2.01×10^{-3}	2.67×10^{-3}	5.48×10^{-3}	3.09×10^{-3}

CNE-2 cell proliferation rate by MTT

After transfection of CNE-2 cells with different concentrations of siRNA3 for 24 h,

cell proliferation was increasingly inhibited with increasing siRNA3 concentrations (Table 2).

Table 2. CNE-2 cells transfected with different concentrations of siRNA3 for 24 h.

	siRNA3 concentration (nM)	Inhibition rate (%)
Experimental group 1	200	41.48
Experimental group 2	100	23.50
Experimental group 3	50	16.22
Experimental group 4	25	8.11
Experimental group 5	12.5	5.29
Control group	0	

Cell apoptosis by flow cytometry

When transfected with 25, 50, 100, and 200 nM siRNA3 and for negative control cells, apoptosis rates were 11.8, 13.8, 15.6, 17.0, and 8.51%, respectively; the apoptosis rate increased siRNA3 concentration. The apoptosis rate was the highest following transfection with 200 nM siRNA3.

Semi-quantitative RT-PCR results

After amplification of survivin gene cDNA fragments, fluorescence measurements revealed that the growth curve showed a characteristic "S" shape. Software was used to automatically calculate the relative content of initial cDNA (mRNA) as well as the ratio of housekeeping genes glyceraldehyde 3-phosphate dehydrogenase. The relative content of the survivin gene in CNE-2 cells was 2.48×10^{-3}. Survivin gene in 38 cases of nasopharyngeal carcinoma relatively expressed the highest level at 10^{-5}-10^{-3} in the NPC group, accounting for 70.37% of cells (38/54); while in the chronic nasopharyngeal group, 14 cases relatively expressed the highest level at 10^{-5}-10^{-4}, accounting for 50% (14/28). Average relative survivin mRNA gene expression in NPC tissue was 4.16×10^{-2}, while the average value in the chronic nasopharyngeal group was 4.42×10^{-4}. The SAS software (SAS Institute, Cary, NC, USA) was used to determine that the data showed a non-normal distribution; the rank sum test and Z-statistics were -2.43 and the approximate χ^2 test was 5.93 ($P < 0.05$), indicating that the relative expression of the survivin gene in NPC tissue was significantly higher than that in chronic nasopharyngeal tissue.

Ultrastructure changes of cells

As shown on the left in Figure 1, CNE-2 cells in growth phase had an unequal size distribution, the nucleoplasm was large, and the nuclear chromatin and nucleosome were increased. One or 2 large nucleoli were clear and contained several organelles. The right of this figure shows the cell after RNA interference. The cells were dispersed and volume was clearly decreased, electron density was increased, the nucleoplasm concentrated, and the nuclear envelope had broken down.

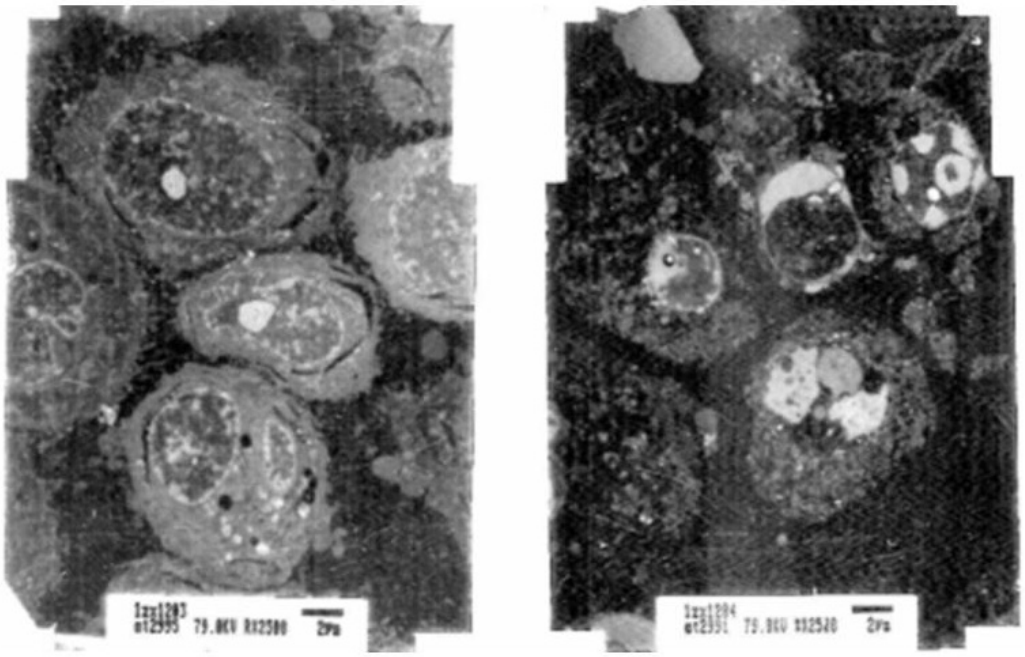

Figure 1. Electron microscopy images.

Western blot detection

After the siRNA interference in CNE-2 cells, the survivin protein was transferred to a nitrocellulose membrane, but the bands were unclear, indicating that survivin gene expression was inhibited by siRNA. This result may be useful for future RNA interference therapy of NPC using the survivin gene as a target.

DISCUSSION

The 15-kb survivin gene is located on chromosome 17q25. The survivin protein has 142 amino acids and a double function in inhibiting cell apoptosis and regulating cell division. Survivin gene expression is highly specific to tumor cells, and the protein is not expressed in most normal tissues (Xiang et al., 2006; Nikitakis et al., 2009), making survivin a target for cancer therapy. Currently, there are 2 treatment methods targeting survivin: antisense oligodeoxy nucleotide and RNAi. RNAi silences genes after transcription into double-stranded RNA, and this very specific and effective method has become a powerful tool for examining gene function and disease treatments such as tumor and viral therapies (Devi, 2006; Shabalina and Koonin, 2008; Umbach and Cullen, 2009). Survivin expression can enhance the radiation sensitivity of tumor cells and improve the effectiveness of cancer treatments (Zhang et al., 2007; Li et al., 2009). Because of its high degree of malignancy and early transfer, the clinical treatment of NPC shows poor long-term efficacy (Wee, 2008). Therefore, effective methods for treating NPC must be developed.

RNAi uses the specificity of double-stranded RNA to degrade complementary homologous mRNA sequences, strongly inhibiting the expression of a target protein. Because siRNA molecules under RNAi do not affect the nonspecific interferon pathway, which could inhibit the expression of target genes, using siRNA for gene therapy is a widely applicable and promising treatment (Holcik et al., 2000). RNAi functions in post-transcriptional gene silencing, which combines with double-stranded RNA to deteriorate mRNA and result in gene silencing (Filipowicz et al., 2005). In our study, after survivin gene silencing in CNE-2 cells, the growth rate of cells decreased significantly. This inhibition resulted from changes in the cell cycle, among which cell increased during the G1 phase and decreased during the S phase. In this study, after survivin gene silencing, the numbers of CNE-2 cell clones reduced, suggesting that CNE-2 cell proliferation decreased. This result is consistent with those of Koike et al. (2011) in prostate cancer. Through fluorescent quantitation and RT-PCR detection, siRNA2 was found to disperse and shrink the volume of cells; additionally, electron density increased, the nucleoplasm became concentrated, and the nuclear envelope had broken down. This study further confirmed the influence of the survivin gene on the growth and proliferation of NPC cells associated with cell cycle changes. Our results reveal a relationship between the survivin gene and NPC, laying a foundation for targeted gene therapy.

The RNAi technique uses double-stranded RNA to degrade homologous mRNA and block corresponding gene expression, and thus has become a popular method for studying gene function and gene therapy in recent years. In this study, siRNA3 showed the largest effect and was selected for further study. In subsequent studies, we will develop a short-hairpin RNA plasmid expression vector to produce effective survivin siRNA, which can continue to express short-hairpin RNA in mammalian cells. Processing of short-hairpin RNA to form siRNA molecules will enable inhibition of gene expression over a longer period of time and lead to gene silencing. Moreover, because the plasmid can be amplified, compared with chemical synthesis, this method may significantly reduce costs related to siRNA preparation, which is more suitable for developing therapeutic drugs.

The methyl thiazolyl tetrazolium (MTT) and flow cytometry results showed that transient transfection survivin siRNA3 effectively inhibited CNE-2 cell proliferation and moderately increased the tumor cell apoptosis rate. Therefore, from a clinical perspective, using combination therapy (radiotherapy and chemotherapy and/or joint survivin siRNA) may have unexpected effects. Overall, survivin gene expression in patients with NPC increases; *in vitro* synthesis of specific siRNA can effectively decrease CNE-2 survivin gene expression. Different siRNA sequences have varying abilities to decrease CNE-2 gene expression. Transfection with siRNA3 can effectively restrain CNE-2 cell proliferation and induce apoptosis. Thus, siRNA can induce gene silencing and lead to gene loss, offering a new method of anti-NPC gene therapy.

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