The Effect of Survivin siRNA on Apoptosis, Proliferation and Invasion by a Colon Carcinoma Cell Line

Jingdong He[1]
Kangqun Yang[1]
Zhenshu Zhang[2]
Yiyang Zhang[1]
Xiaofei Chen[1]
Rong Wu[1]

[1] Department of Oncology, Huai'an No. 1 Hospital Affiliated to Nanjing Medical University, Huai-an, Jiangsu 223300, China.
[2] Department of Digestive Medicine, Nanfang Hospital, Guangzhou 500060, China.

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Correspondence to: Zhenshu Zhang
E-mail: zzs6689@163.com

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OBJECTIVE To study the efficiency of silencing survivin gene expression by an anti-survivin small RNA expression plasmid and to examine its impact on apoptosis, proliferation and invasive ability of the SW480 colorectal cancer cell line.

METHODS An expression plasmid of survivin siRNA (pRNAT/sur-siRNA) was constructed and transduced into SW480 cells. Western blot analysis and a semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) were used to measure the change in expression level of the survivin protein and mRNA after transduction of the expression plasmid into the SW480. Apoptosis, proliferation, and invasive ability of the SW480 cells was evaluated by flow cytometry, MTT assay and Boyden Chamber Assay, respectively.

RESULTS The expression plasmid (pRNAT/sur-siRNA) against survivin down-regulated survivin protein and mRNA expression dramatically, with a down-regulation of 85% and 80%, respectively. After transfection of the pRNAT/sur-siRNA to the SW480 cells, the apoptotic rate of the pRNAT/sur-siRNA/SW480 cells was 16.9%, and the proliferation rate was 37.4%, significantly different compared to the controls ($P<0.01$). Cell invasive studies (Boyden Chamber Assay) showed that number of cells penetrating the membrane for the pRNAT/sur-siRNA/SW480, pRNAT/SW480 and the SW480 cells was 153±66, 505±65 and 578±98, respectively ($P<0.01$).

CONCLUSION siRNA against survivin can significantly inhibit the expression of survivin in SW480 cells, and thus promote apoptosis, inhibit proliferation and invasive ability.

KEYWORDS: survivin, RNA interference, colon carcinoma, gene therapy.

Survivin is one of the apoptotic profilins. Although it is not expressed in mature tissues, it may be expressed in tumors such as colorectal carcinoma.[1] Gene therapy against the survivin gene provides a new mode for the treatment of colorectal carcinoma.[2] The purpose of this study was to analyze the impact of silencing survivin gene expression by an anti-survivin small RNA expression plasmid, and to determine the effect on apoptosis, proliferation and invasive ability of the colorectal cancer SW480 cell line.

MATERIALS AND METHODS

Materials and reagents

The pRNAT-U6.1/Neo carrying agents were obtained from the GeneScript Co., USA. The restriction enzymes Hind III and BamH 1 were from Promega, USA. The nucleotides for the survivin shRNA series were synthesized by the Shanghai Boya Biology Co. Ltd., the mono-

clonal antibodies for survivin were obtained from Santa Cruz Biotechnology Inc., the Lipofectin 2000, a cellular transfection reagent, from the Invitrogen Life Co. and Trizol LS Reagent, a total RNA extraction reagent from the Gibco BRL Co. Kits for the extraction of cell protein and the estimation of apoptosis were purchased from the Bi-yuntian Biology Co., Jiangsu, the PVDF membrane from the Pall Co., USA, and the MTT from the Shanghai Bioengineering Co. The colon carcinoma SW480 cell line was maintained by our laboratory. The Transwell used in the Boyden Chamber was purchased from the Millipore Co. , and Matrigel, the man-made basal lamina from the BD Co., UK. Other common biochemical reagents were purchased in China.

Design and construction of the pRNAT/sur-siRNA expression plasmid

The GeneScript Co. supplied the software, e.g. GeneScript's siRNA Target Finder and siRNA Construct Builder, for the shRNA array. The on-line design was conducted based on the survivin gene (NM_001168). The shRNA array was CCG CAT CTC TAC ATT CAA GAA, with 100 as the initiation position. The siRNA insertion element was 76 bp with the array constructed as follows (Fig.1).

The two nucleotide chains were annealed according to the following reaction system, i.e., 95℃×10 min, 25 ℃×1 h and the product was diluted to 40 ng/μl, and based on the 20 μl reaction volume, with 1 μl 5'-3' nucleotide, 1 μl 3'-5' nucleotide, 1 μl 20×SSC and 17 μlH_2O.

The pRNAT -U6.1/Neo vector was digested with the restriction enzymes Hind III and BamH 1 and a 1% agar gel electrophoresis (AGE) was used to purify the digested vector.

The siRNA insertion element was cloned into the pRNAT-U6.1/Neo vector, and positive clones were screened using antibiotics followed by validation of the constructed pRNAT/sur-siRNA expression plasmid.

Cell transfection

The expression plasmid of pRNAT/sur-siRNA and vacant plasmid of pRNAT were respectively dissolved in serum-free medium, and the Lipofectamine2000 and nucleotide were mixed in a polystyrene test tube. Transfection was completed after 30 min standing at room temperature. The positive clone cell line (pRNAT/sur-siRNA/SW480 cell) was screened by G418 400 μg/L.

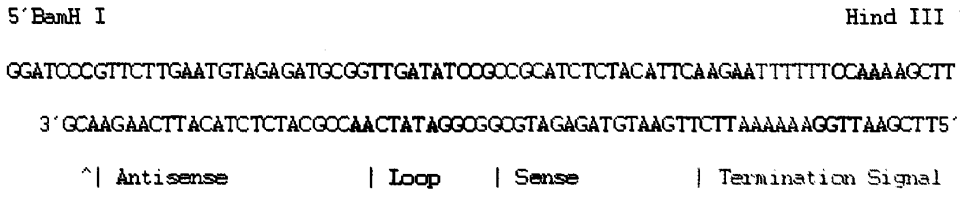
RT-PCR determination for expression of survivin mRNA

Trizol was used to extract total cytoplamic RNA resulting in an A260/A280 ratio for each total sample of 1.90~1.96. After electrophoretic separation of the total cytoplamic RNA, distinct 28 S and an 18S RNA bands were seen. Moreover, density of the 28 S RNA was 1.5~2.0 times that of the 18 S. The RT-PCR was conducted in two steps. The RT-PCR reaction system was 20 μl and the toal cytoplamic RNA 1 μg. Synthesis of the first cDNA chain was carried out for 10 min at 30 ℃ and 30 min at 42 ℃, and inactivation of AMV reverse transcriptase for 5 min at 99 ℃. The PCR reaction system was 50 μl. The upstream and downstream primers of the survivin were 5'-TTC ATC CAC TGC-CCC ACT-3' and 5'-CCT TTC CTA AGA CAT TGC-TAA-3', respectively. The PCR reaction conditions included denaturization for 30 s at 94 ℃, annealing for 30 s at 50 ℃ and extension for 90 s at 72 ℃, with a total of 32 cycles.

Western blot determination for expression of the survivin protein

Following disruption of the cells, the protein was quantified by the Bradford method and 20 μg of the extracted proteins was taken. After 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the gel was electro-transferred using 100 V of constant voltage for 1 h, to a polyvinylidene difluoride (PVDF) blotting membrane. After blockage with BSA for 1 h, the rabbit anti-human survivin polyclonal antibody (1: 200 dilution) was added as the first antibody, and was

Fig.1



rocked at room temperature for 2 h. After TBST membrane-washing, the sheep anti-rabbit IgG (1:1000 dilution) was added as the second antibody. TBST (0.05% Tween20 was added to the TBS) membrane-washing was then conducted and the intensity of chemoluminescence determined.

Flow cytometer determination of SW480 cell apoptosis

After transfection of the SW480 cells for 48 h, the cells were collected by centrifugation followed by washing (2 times) with phosphate buffer, after which 70% absolute alcohol was added to suspend the cells. The cells were maintained for 6 h at 4 ℃, washed twice with PBS, 200 μl RNase A added, followed by filtering through a 400-mesh nylon screen mesh, and kept at 37℃ for 30 min. Propidium iodide (PI) was added at a final concentration of 10 μmol/L. The degree of apoptosis was assayed after 30 min of incubation at normal temperature with protection from light. The SW480 cells transfected with the vacant vector were set as the controls.

MTT assay for cell proliferation

The pRNAT/sur-siRNA/SW480 cells, pRNAT/SW480 cells and SW480 cells were treated with 0.25% trypsin digestion at the logarithmic growth phase and were used to prepare unicellular suspensions (5×10^5 cells/ml) using 10% calf serum. Then the cells were transferred to a 96-well plate (100 μl/well). After 24 h of culture, the supernatant was changed to serum-free medium for an additional 48 h. There were 6 wells for each group. MTT (5 mg/ml, 10 μl/well) was added 6 h before termination of the culture. At the end of the culture, 100 μl/well of 20% SDS was added. On the next day, the absorbance (A) at 570 nm was determined using an enzyme-linked immune mark analyzer. The following formula was used to calculate inhibition of cell growth:

The inhibition ratio of the cell growth R = [1-(Experiment group A/Blank group A)]×100%.

Cellular in vitro invasion assay

The Boyden Chamber Assay method was employed. The Transwell chamber was put in the cell culture wells of a 24-well plate. At the bottom of the orifice, there was a filter membrane with a 6.5 mm diameter containing many micropores with a 12.0 μm pore size. The serum-free 1640 medium containing Matrigel was added on the filter membrane of the upper layer chamber, and was held at 37℃ for 2 h to allow the Matrigel to form a gel film. After dissociation and washing of the cells, they were resuspended in 1640 medium with 0.1% bovine serum albumin, and the cell concentration adjusted to 2×10^6/ml. A 100 μl cell suspension was added to the upper-layer chamber and a 300 μl NIH-3T3 serum-free medium supernatant was added to the underlayer. After the cells were cultured for 24 h, under 5% CO_2 by volume at 37℃, they were removed from the upper layer chamber and cotton swabs were used to wipe the cells inside the chamber and the Matrigel. H&E staining was conducted after 95% alcohol fixation of the cells which were examined under the microscopes on the lateral surface of the micropore. The cell number at the under surface of the filter membrane invading the basal membrane was randomly counted in 5 visual fields under the microscope (× 400). The process was repeated 3 times for each sample and the total assay conducted in triplicate. The relative number of invading tumor cells indicated their invasive capacity.

Statistical methods

The *t* test was used and the data expressed by $\bar{x}\pm s$. The SPSS 11.5 software was employed for statistical treatment.

RESULTS

Construction of the pRNAT/sur-siRNA expression plasmid

The siRNA expression plasmid against survivin was successfully constructed (pRNAT/sur-siRNA). The insertion element of the siRNA was 76 bp. Both ends were digestion sites for BamH I and Hind III, which was in accord with the sequencing and designing series (Fig.2). After transfection of the SW480 colon cancer cells by the pRNAT/sur-siRNA expression plasmid, a green fluorescence occurred in the cell cytoplasm, indicating the correct expression of the pRNAT/sur-siRNA (Fig.3).

Inhibition of survivin expression by the pRNAT/sur-siRNA plasmid

Analysis by RT-PCR and Western blot examination showed that the pRNAT/sur-siRNA inhibited the expression of survivin mRNA and survivin protein of the SW480 cells, with an inhibition ratio of 80% and 85%, respectively (Figs.4,5).

Enhancement of apoptosis by the pRNAT/sur-siRNA expression plasmid in SW480 cells

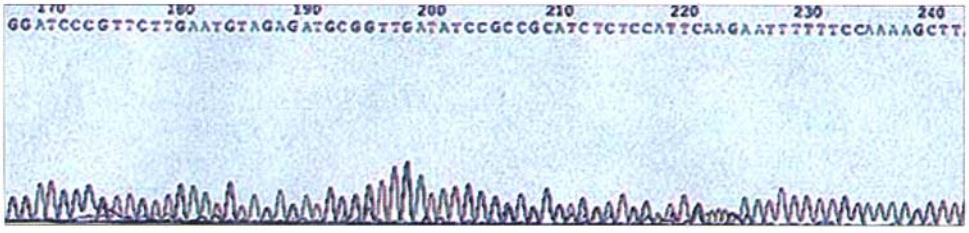


Fig.2. Sur-shRNA /pRNAT recombinant plasmid sequence map.

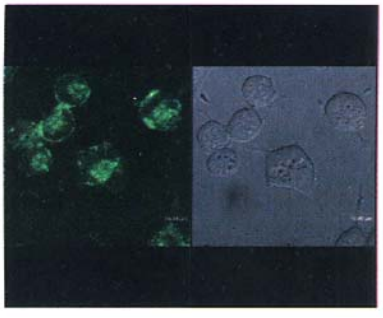


Fig.3. Laser confocal microscope examination showed that pRNAT/ sur-siRNA-transfected the SW480 cytoplasm. For details, see the expression of the green fluorescent protein (GFP).

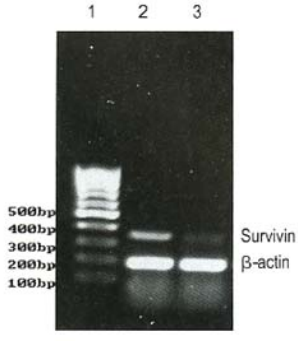


Fig.4. RT-PCR analysis: Sur-siRNA inhibits survivin mRNA expression.
1 100 bp DNA molecular weight standard
2 Expression of SW480 cell survivin mRNA
3 Survivin mRNA expression of Sur-siRNA/SW480 cells

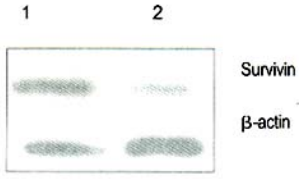
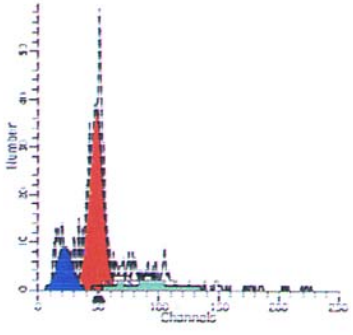
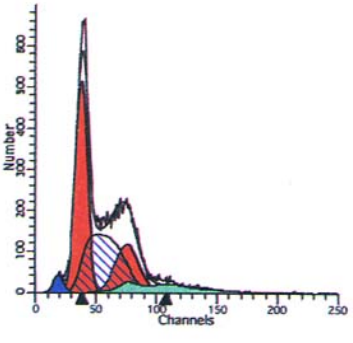


Fig.5. Western blot analysis: Sur-siRNA inhibits survivin protein expression.
1 Survivin protein expression of SW480 cells
2 Survivin protein expression of sur-siRNA/ SW480 cells

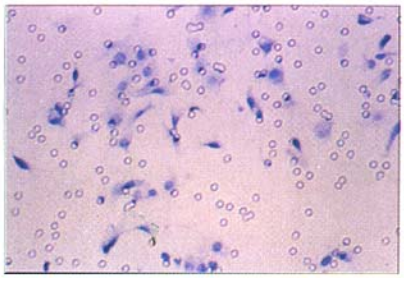


siRNA/SW480

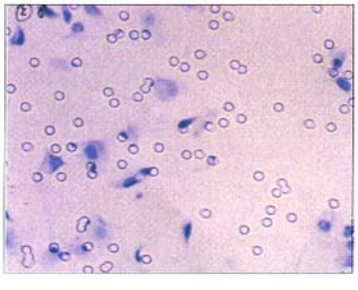


pRNAT/SW480

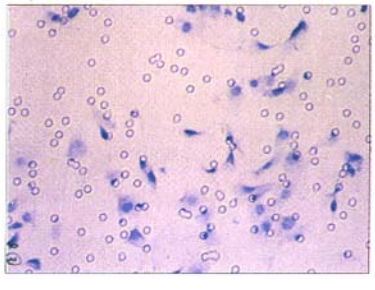
Fig.6. Flow cytometry for determination of apoptosis. The apoptotic rate of the Sur-siRNA/SW480 cells was 16.9%, while the apoptotic rate of the pRNAT/SW480 cell line was 2%.



SW480 cells



pRNAT/SW480 cells



siRNA/SW480 cells

Fig.7. The cell penetration number in the visual field shown by cell invasion. The penetration number of the siRNA/SW480 cells was less (× 400).

The apoptotic rate of the sur-siRNA/SW480 cells determined by flow cytometry was 16.9% and that of the RNAT/SW480 cells was 2.0% . Apoptosis of the pRNAT/sur-siRNA/SW480 cells was significantly higher compared to the controls (Fig.6).

pRNAT/sur-siRNA inhibition of SW480 cell multiplication

See Table 1 for the cell-proliferation inhibition ratio of the RNAT/Sur-siRNA/SW480 cells, control group (pRNAT/SW480) and SW480 cells. The results showed that the proliferation of pRNAT/Sur-siRNA/SW480 cells was significantly inhibited (P<0.01).

Table 1. The effect of pRNAT/sur-siRNA on the SW480 cell proliferation (n=6)

Experimental groups	OD value ($\bar{x}\pm s$)	Inhibition Ratio(%)
Sur-siRNA/ SW480	0.523 ± 0.0373*	37.4
pRNAT/ SW480	0.795 ± 0.0267*	4.4
SW480	0.835 ± 0.0362	0.0

*P<0.01

The in vitro invasion capacity of the cells

The total chamber membrane was microscopically scanned, and the penetration numbers for 5 randomized visual fields for pRNAT/sur-siRNA/SW480 cells, pRNAT/SW480 cells and SW480 cells were 153±66, 505±65 and 578±98, respectively. This indicated that penetration number of the pRNAT/sur-siRNA/SW480 cells was decreased (P<0.01, Fig.7).

DISCUSSION

The RNA interference phenomenon widely exists in nature from prokaryotes to humans. The specificity and efficiency of RNA interference to inhibit target genes was 10 times greater compared to antisense nucleotides, supporting the application of RNA interference to be used in suppressing gene function for tumor therapy.[3] Survivin is one of the profiling members for cell apoptosis, which has a powerful anti-apoptotic effect, and plays an important role in maintenance of mitosis and the regulation of angiopoiesis.[4] It also plays a key role in the pathogenesis of colorectal cancer.[5,6] Our study employed RNA interference technology to silence changes in survivin gene expression of SW480 cells.

In this study, the specific RNA interference fragment against the survivin mRNA was designed and synthetized based on the survivin gene sequence, and was cloned into the pRNAT -U6.1/Neo vector to construct a survivin shRNA eukaryotic expression plasmid (pRNAT/Sur-shRNA). RT-PCR and Western blot analysis showed that Sur-shRNA inhibited expression of survivin. Results of the studies indicated that after the SW480 cell survivin gene was silenced, SW480 cell apoptosis was significantly increased, the cells were blocked at the G1 phase and cell proliferation of the tumor was significantly reduced. The mechanism might be that expression of the survivin gene mainly had its effect at the G2/M transition. Survivin may competitively combine with Cdk4/p16INK4a to form a survivin/Cdk4 dimer, which resulted in the dissociation of the p16INK4a protein from the Cdk4/p16INK4a, thus activating the Cdk2/CyclineE and phosphorylation of Rb protein, resulting in mitosis and cell multiplication. After down-regulation of the survivin proteins, the cells in the G1 phase increased and these in the S phase were reduced, causing a decrease in cell proliferation.[7] Affter survivin of the SW480 cells was silenced, their invasion capability markedly decreased, thus suggesting there was a correlation between expression of survivin and invasion by the colon cancer cells. It is possible that after the survivin gene was silenced, the cells were blocked at the G2/M transition because of an influence on the assemblage of the microtubules and microfilaments. Under the changing conditions of the micro-tubular structure, the SW480 cells might undergo a decrease in the cell viscoelasticity coefficient, resulting in a decrease in cell invasion capability.[8] In addition, inhibition of survivin expression may reduce expression of the tumor angiogenesis factor, resulting in suppression of invasion by the SW480 cells.[9,10] The results of this study suggest that antagonism of the survivin gene by siRNA might be an effective method to treat colon cancer.

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