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Inhibitory effect of *survivin*-targeting small interfering RNA on gastric cancer cells

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ABSTRACT. A pair of inverted repeated sequences of the gene *survivin* was designed for stable double-stranded RNA establishment. After stable transfection, the biological behaviors of gastric cancer cells were observed. The interference rates of *survivin*-targeting siRNA (siRNA-*survivin*) in BGC823, MKN45, SGC7901, and cisplatin-resistant SGC7901 groups were 55.363 ± 3.974, 71.433 ± 3.774, 69.433 ± 7.336, and 76.767 ± 3.541%, respectively, compared with those in the control group. After siRNA-*survivin* interference, survivin protein expression noticeably decreased, apoptotic rates markedly increased, and cell proliferation was inhibited to varying degrees. Mitochondrial cytochrome C protein expression decreased and the levels of cytoplasmic cytochrome C and caspase-3 increased, which showed significant differences compared with values before transfection. pRNA-shSU eukaryotic expression vectors were constructed. After plasmid transfection, green fluorescent protein expression increased and survivin protein expression noticeably increased in BGC823 and SGC7901. siRNA-*survivin* promotes GC cell apoptosis and inhibits cell proliferation by downregulating *survivin* mRNA and protein expression. The underlying mechanisms

are correlated with a decrease in mitochondrial cytochrome C and cytoplasmic cytochrome C and caspase-3.

Key words: Survivin; Gastric cancer; Small interfering RNA; Apoptosis

INTRODUCTION

Gastric cancer (GC), one of the most common malignancies in China, is characterized by a pathological process involving multiple factors, genes, and links. GC has the fourth highest incidence rate and the second highest fatality rate among malignancies worldwide (Brummelkamp et al., 2002). In China, it has the highest incidence rate among various tumors, and approximately 170,000 patients die of this condition annually, accounting for nearly 1/4 of malignancy-related deaths. Therefore, it is a serious life-threatening disease. GC occurs at any age, but more commonly between 40 and 60 years. Males are more affected than females with a 2:1 gender ratio. Current therapies for GC, such as therapeutic endoscopy, surgery, radiotherapy, chemotherapy, interventional therapy, biotherapy, and immunotherapy fail to achieve satisfactory results. The five-year survival rate of GC patients is below 20% (Li et al., 1998).

The gene *survivin* was first isolated through screening by hybridization of the human genomic library using effector cell protease receptor-1 (EPR-1) cDNA by Ambrosini et al. (1997). Since this gene enables to prolong cell growth, it was so named. *survivin* expression has apparent characteristics of cell cycle and tissue distribution. Its overexpression may lead to tumorigenesis with uncontrolled cell cycle progression. Conversely, cells lacking *survivin* have abnormal cell division (Van Antwerp et al., 1998; Vaira et al., 2007).

The *survivin*-encoded product survivin is a new member of the inhibitor of apoptosis (IAP) family and has multiple functions. It inhibits cell apoptosis, promotes cell transformation, participates in cell division, vascularization, and the generation of tumor cell drug resistance, and so on. It enters cells under the stimulation of the Fas gene and cell proliferation, wherein it binds the cyclin CDK4, leading to CDK2/cyclin E activation and Rb phosphorylation. These processes promote DNA replication and shorten the G1/S course (Sommer et al., 2003). Furthermore, the formation of the survivin/CDK4 complex allows p21waf1/cip1 to be released from the p21waf1/cip1-CDK4 complex. The released p21waf1/cip1 then interacts with procaspase-3 to prime the inactivation of caspase-3, thereby inhibiting Fas-mediated apoptosis. Survivin serves as the direct repressor of caspase-3 and caspase-7 and blocks the process of cell apoptosis (You et al., 2004).

Survivin is extensively expressed in embryonic tissues, such as the kidneys, brain, and lungs. In healthy adults, it is only weakly expressed in the thymus and genital glands. However, it has extensive expression in most malignant tumor tissues. These characteristics of survivin suggest that *survivin* may stay in an out-of-control status during tumorigenesis. Nakamura found that survivin is much more highly expressed in GC tissue than atrophic gastritis tissue, but not expressed in normal gastric tissue (Nakamura et al., 2004). Its expression level negatively correlates with patients' survival. A lower differentiation level of GC tissue indicates higher survivin expression and poorer prognosis (Fortugno et al., 2002). Our previous study proved that survivin expression is noticeably increased in GC tissue, and that survivin overexpression is correlated with the histological grade and pathological stage of GC (Altieri, 2003a).

In this study, we observed the biological behaviors of tumor cells after transfecting

survivin-targeting (siRNA-*survivin*) into these cells. We also designed and synthesized a pair of inverted repeated sequences of *survivin* with the expectation to provide a new method for GC gene therapy.

MATERIAL AND METHODS

Cell strain screening and culture

GC BGC823, MKN45, SGC7901, and cisplatin-resistant SGC7901 strains were selected as the subjects. They were supplied by Dr. Min Chen at the Nanjing Drug Tower Hospital affiliated to Nanjing University Medical School. The strains were cultured in RPMI-1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biology Technology Co., Ltd., China), 100 U/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2 at 37°C using the adherence method. When the cells nearly reached confluence, they were digested with 0.25% pancreatin for subculture 2-3 times per week. Cells in logarithmic phase were harvested.

Mitochondrial protein extraction

The cells were washed and counted. About 5 x 10^7 cells were added to 1.5 mL of pre-chilled Lysis Buffer-1 for cell resuspension. They were ground at 0-4°C and then centrifuged at 800 rpm for 5 min at 4°C. About 0.5 mL homogenized supernatant was carefully added to another pre-chilled centrifuge tube containing 0.5 mL Medium Buffer. Centrifugation at 15,000 rpm at 4°C was performed for 10 min. After centrifugation, cytoplasmic components were contained in the supernatant and mitochondria precipitated. The supernatant was transferred to another centrifuge tube. The mitochondrial pellets were resuspended with 0.2 mL Wash Buffer and then centrifuged at 15,000 rpm at 4°C for 10 min. The supernatant was discarded.

Total protein extraction and quantitation were performed. About 10 μL phosphatase inhibitor, 1 μL proteinase inhibitor, and 5 μL 100 mM PMSF were applied per mL cold Lysis Buffer and then shaken. The mixture was allowed to stand for a few minutes on ice. About 20 μL pelleted mitochondria were added to 200 μL prepared Lysis Buffer and then softly shaken at 4°C for 15 min. The mixture was centrifuged at 14,000 rpm at 4°C for 15 min. The supernatant was total extracted protein. Protein quantitation was performed using the Bradford (bicinchoninic acid) method. The product was stored at -70 °C.

Western blot analysis

Total protein extraction and quantitation were performed using the previously described methods. The extraction products were subjected to SDS-PAGE electrophoresis for one to two hours. The separation gels of the proteins with corresponding molecular weights were taken and blocked in 5% skimmed milk for 1 h and then incubated with primary antibodies (1:1000 dilution; Abcam, USA) overnight. β-actin (1:3000 dilution; Multi-Sciences, China) was used as the internal reference. After TBST washing, the samples were incubated with secondary antibodies (KPL, USA) for 2 h. Photographs were taken and scanned using a gel or photograph transient display system. Optical density (OD) values of the target bands were analyzed using the Quantity One image analysis software. The ratios between the OD of the

target bands and those of their corresponding β-actin were calculated.

Immunofluorescence

The cells were inoculated on gelatin-pretreated cover slips in a 6-well culture plate. After handing, they were washed twice with PBS and fixed in cold acetone at 4°C for 30 min. After washing, they were blocked in goat serum (Boster, China) for 1 h and then incubated with primary antibodies (1:200; Abcam, USA) at 4°C overnight. After PBS washing, anti-rabbit fluorescence-labeled secondary antibody (Molecular Probe, USA) was applied for 2 h of incubation. DAPI solution was applied for 5 min of nuclear staining. Outcomes were observed under a fluorescence microscope within 1 h. Photographs were taken.

Real-time polymerase chain reaction (PCR)

The upstream and downstream primers of *survivin* were 5'-GGC ATG GGT GCC CCG ACG TT-3' and 5'-AGA GGC CTC AAT CCATGG CA-3' and those of GAPDH were 5'-CAT CTT CCA GGA GCG AGA-3' and 5'-TGT TGT CAT ACT TCT CA-3'. RNA extraction, reverse transcription, and PCR detection were performed according to kit instructions (TaKaRa, Japan). Real-time PCR was conducted on an ABI7500 real-time quantitative PCR instrument. The reaction system with a volume of 20 μL contained 2 μL first-strand cDNA, 10 μL SYBR Premix Ex TaqTM (2X), 0.4 μL ROX Reference Dye II (50X), 0.4 μL *survivin* upstream primer, 0.4 μL *survivin* downstream primer, and 6.8 μL deionized water. The amplification conditions consisted of 95°C for 30 s, followed by 40 cycles of 95°C for 3 s, 60°C for 30 s, 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The $2^{-\Delta\Delta Ct}$ method was used for outcome analysis. The amplification system of GAPDH was consistent with that of *survivin*.

Cell apoptosis detection

The digested cell suspension was cultured on a 6-well plate with 1 mL (containing about 2 x 10^5 cells) in each well. They were further cultured with PPI or chemotherapeutic drugs according to requirements. The suspended cells were centrifuged at 2000 rpm for 5 min and collected, whereas the adherent cells were subjected to EDTA-free trypsinization (the digestion time should not be too long in case of false positivity) and then collected. They were washed twice with 0.01 M PBS and then centrifuged at 2000 rpm for 5 min. About 1-5 x 10^5 cells were collected. Binding Buffer (500 μL) and Annexin V-FITC (5 μL; KeyGENE Biotech, China) were added and mixed. About 5 μL PI was added and shaken. The mixture was allowed to react away from light at room temperature for 5-15 min. Flow cytometry was performed within 1 h.

Cell proliferation ratio detection

A 96-well plate was used for cell proliferation experiments with about 8000 cells per 100 μL of the culture solution in each well (the exact cell number was determined by their size and proliferation ratio). The cells were cultured in accordance with the experimental requirements. Meanwhile, they were stimulated with 0-10 μL specific drugs.

About 10 µL CCK-8 solution (KeyGENE, China) were added to each well with a total culture volume of 100 µL. Wells containing corresponding volumes of cell culture solution and CCK-8 solution rather than cells were taken as blank controls. For drug interference detection, those containing corresponding volumes of cell culture, drug, and CCK-8 solutions were taken as blank controls. The cells were continuously grown for 0.5-4 h (1 h was enough in most situations). Culture time spans were determined according to cell type and density. Microplate assays were performed at 0.5, 1, 2, and 4 h, respectively, and then the most appropriate detection time point was determined. Absorbance (*A*) was read at 450 nm.

siRNA-*survivin*

According to the gene sequence of *survivin*, two specific siRNA and two nonsense siRNA were designed and synthesized *in vitro* (aided by the Thermo-Fisher Company) as follows: *survivin* siRNA (+): 5'-GGA CCA CCG CAU CUC UAC AdTdT-3' and 3'-dTdTC CUG GUG GCG UAG AGA UGU-5'; control *survivin* siRNA (-): 5'-CGU ACG CGG AAU ACU UCG AdTdT-3' and 3'-dTdTG CAU GCG CCU UAU GAA GCU-5'.

The cells in logarithmic phase were digested and counted 1 day before transfection. They were inoculated with BGC823, MKN45, SGC7901, and cisplastin-resistant SGC7901 strains according to 8 x 10^3 cells/well on a 96-well plate and 2 x 10^5 cells/well on a 24-well plate overnight. Cell strain transfection was performed using Lipofectamine 2000 liposomes (Invitrogen, USA) bound with 100 nM siRNA. The cells were collected for RNA interference effects using immunoblotting, immunofluorescence, and real-time PCR.

Vector construction

Based on the coding region of *survivin* 5'-GGA CCA CCG CAT CTC TAC-3', two DNA sequences were synthesized by Sangon Biotech (China) as follows:

1) 5'-GAT CCG GGA CCA CCG CAT CTC TAC ATC AAG AGT GTA GAG ATG CGG TGG TCC TTT TTT GAA TTC A-3'

2) 5'-AGC TTG AAT TCA AAA AAG GAC CAC CGC ATC TCT ACA CTC TTG ATG TAG AGA TGC GGT GGT CCC G-3'

About 10 pmol of each of the above mentioned primers were added to annealing buffer solution (containing 10 mM Tris, pH 8.0, 1 mM EDTA, and 10 mM NaCl) and the mixture then placed in a water bath at 95°C for 5 min. A centrifuge tube was placed in 1 L of water at 95°C, and the water was cooled gradually to room temperature. DNA vectors at 1 µL (1 µg) were digested in digestion buffer (3 µL 10X K buffer, 24 µL ddH_2O, 1 µL *Bam*HI, and 1 µL *Hin*dIII) for 2 h and then purified with agarose gels. The digested pRNAT-U6.3 vectors at 5 µL, the annealed fragments at 5 µL, 10X ligation buffer at 2 µL, and ddH_2O at 7 µL were ligated with 1 µL T_4 DNA ligase at 16°C overnight. The ligated products at 10 µL were applied to 100 µL DH5α competent cells. They were subjected to 0°C for 30 min, 42°C for 2 min, and 0°C for 3 min. Next, 1 mL 37°C-preheated LB medium was added for 30-min homoisothermal culture at 37°C. The obtained product was spread on an Amp^r plate. Monoclonal bacteria were cultured using small-volume (2 mL) culturing. Plasmids were extracted using a plasmid mini kit. They were respectively digested with *Bam*HI, *Eco*RI, and *Hin*dIII and then subjected to electrophoresis.

Statistical analysis

All measurement data are reported as means ± standard error. Statistical analysis was carried out by the SPSS 13.0 software. One-factor ANOVA was used to compare between groups. Differences of $P < 0.05$ were considered to be statistically significant.

RESULTS

survivin mRNA expression

The interference effect of siRNA-*survivin* interference on *survivin* mRNA expression was detected using real-time PCR. As shown in Figure 1, siRNA-*survivin* successfully interfered with *survivin* mRNA expression in all four cell strains. The interference efficiency of negative siRNA in BGC823 was set at 1. The interference efficiency of siRNA-*survivin* in BGC823, MKN45, SGC7901, and cisplatin-resistant SGC7901 was 55.363 ± 3.974, 71.433 ± 3.774, 69.433 ± 7.336, and 76.767 ± 3.541%, respectively. These findings indicated that siRNA-*survivin* had an inhibitory effect on *survivin* mRNA expression in all four GD cell strains.

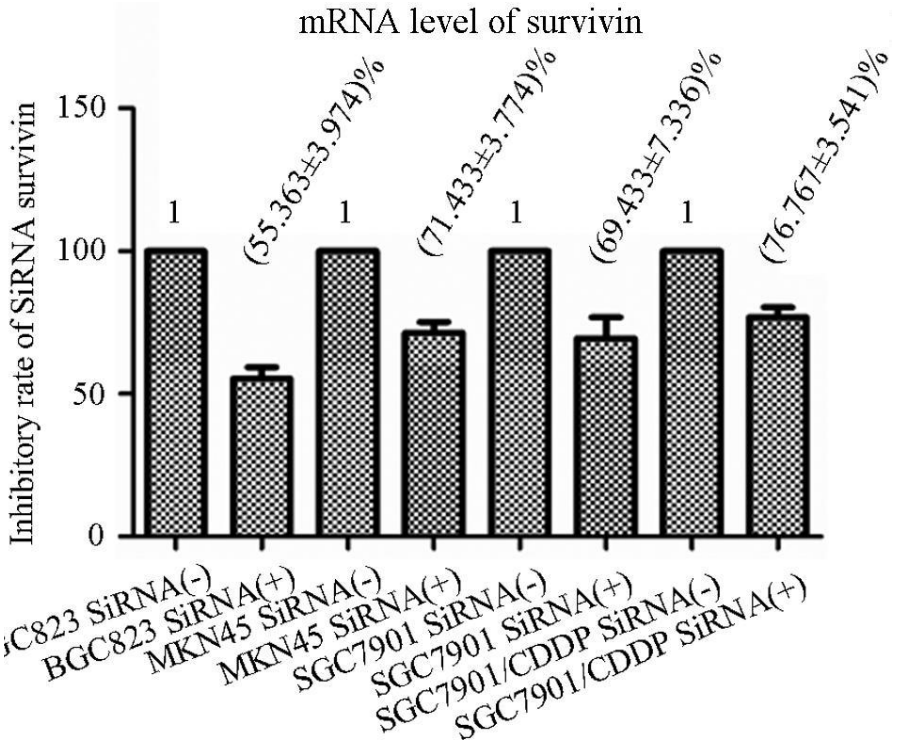

Figure 1. Effects of siRNA-*survivin* interference on *survivin* mRNA expression and cell proliferation (*survivin* mRNA level).

Survivin protein expression

Survivin protein expression was detected after 48-h interference with siRNA. The results showed that siRNA-*survivin* had noticeably inhibitory effects on survivin protein expression in the four cell strains. For MKN45, the relative optical density (OD) value of the survivin protein expression after positive interference was 0.453 ± 0.019, whereas that after negative interference was 0.541 ± 0.019. For BGC823, the relative OD value of the survivin protein expression after positive interference was 0.293 ± 0.017, whereas that after negative interference was 0.453 ± 0.019. For SGC7901, the relative OD value after positive interference was 1.114 ± 0.025, whereas that after negative interference was 1.678 ± 0.035. For cisplatin-resistant SGC7901, the relative OD value of the survivin protein expression after positive interference was 1.678 ± 0.034, whereas that after negative interference was 1.831 ± 0.093. All cell strains showed significant differences in OD values after positive and negative interference ($P < 0.05$). The results are shown in Figure 2.

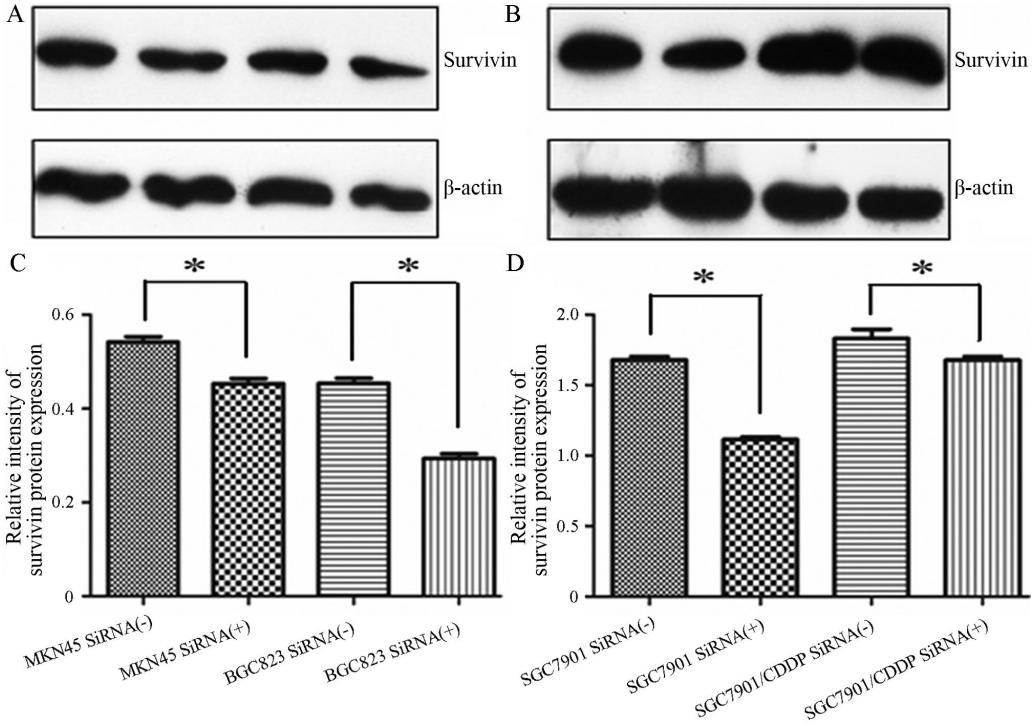

Figure 2. Effects of siRNA-survivin interference on *survivin* protein expression in the four GC cell strains (*$P < 0.05$).

Intracellular survivin protein localization

As shown in Figure 3, intracellular survivin protein location changed in all the GC cells strains after 48 h of siRNA-*survivin* interference. Before interference, survivin was primarily localized in nucleus. After interference, intranuclear survivin markedly decreased. This finding indicated the effective interference of siRNA-*survivin*.

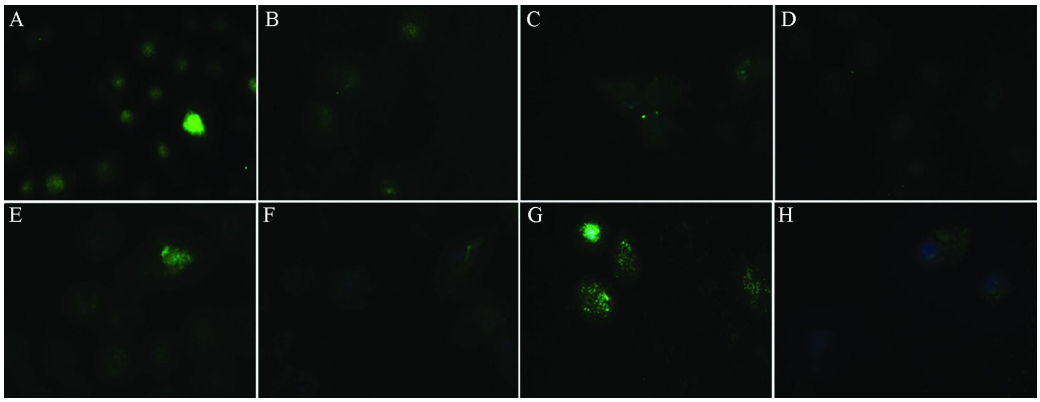

Figure 3. Effects of siRNA-*survivin* interference on the intercellular survivin protein localization in BGC823, MKN45, SGC7901, and SGC7901/CDDP. **A.** BGC823 siRNA (-). **B.** BGC823 siRNA (+). **C.** MKN45 siRNA (-). **D.** MKN45 siRNA (+). **E.** SGC7901 siRNA (-). **F.** SGC7901 siRNA (+). **G.** SGC7901/CDDP siRNA (-). **H.** SGC7901/CDDP siRNA (+).

Cell apoptosis

Cell apoptosis after siRNA interference in survivin expression was observed using flow cytometry. The results showed that after siRNA-*survivin* interference, the apoptosis of the four strains noticeably increased, particularly early apoptosis. In the MKN45 group, the early apoptotic rate after positive interference was 10.3%, whereas that after negative interference was 7.2%. In the BGC823 group, the early apoptotic rate after positive interference was 22.5%, whereas that after negative interference was 3.4%. In the SGC7901 group, the early apoptotic rate after positive interference was 12.3%, whereas that after negative interference was 6.9%. In the cisplatin-resistant SGC7901 group, the early apoptotic rate after positive interference was 35.4%, whereas that after negative interference was 26.1%. The results are shown in Figure 4.

Cell proliferation

Cell proliferation after siRNA-*survivin* interference was detected using CCK-8 kits. The results showed that cell proliferation of the four GC cell strains was significantly inhibited (Figure 5). In the BGC823 group, the OD value after positive interference was 0.511 ± 0.043, whereas that after negative interference was 0.802 ± 0.178 ($P < 0.05$). In the MKN45 group, the OD value after positive interference was 0.701 ± 0.099, whereas that after negative interference was 0.775 ± 0.133 ($P < 0.05$). In the MKN45 group, the OD value after positive interference was 0.701 ± 0.099 and that after negative interference was 0.775 ± 0.133 ($P > 0.05$). In the SGC7901 group, the OD value after positive interference was 0.384 ± 0.079 and that after negative interference was 0.400 ± 0.041 ($P > 0.05$). In the cisplatin-resistant SGC7901 group, the OD value after positive interference was 0.301 ± 0.014 and that after negative interference was 0.372 ± 0.027 ($P > 0.05$).

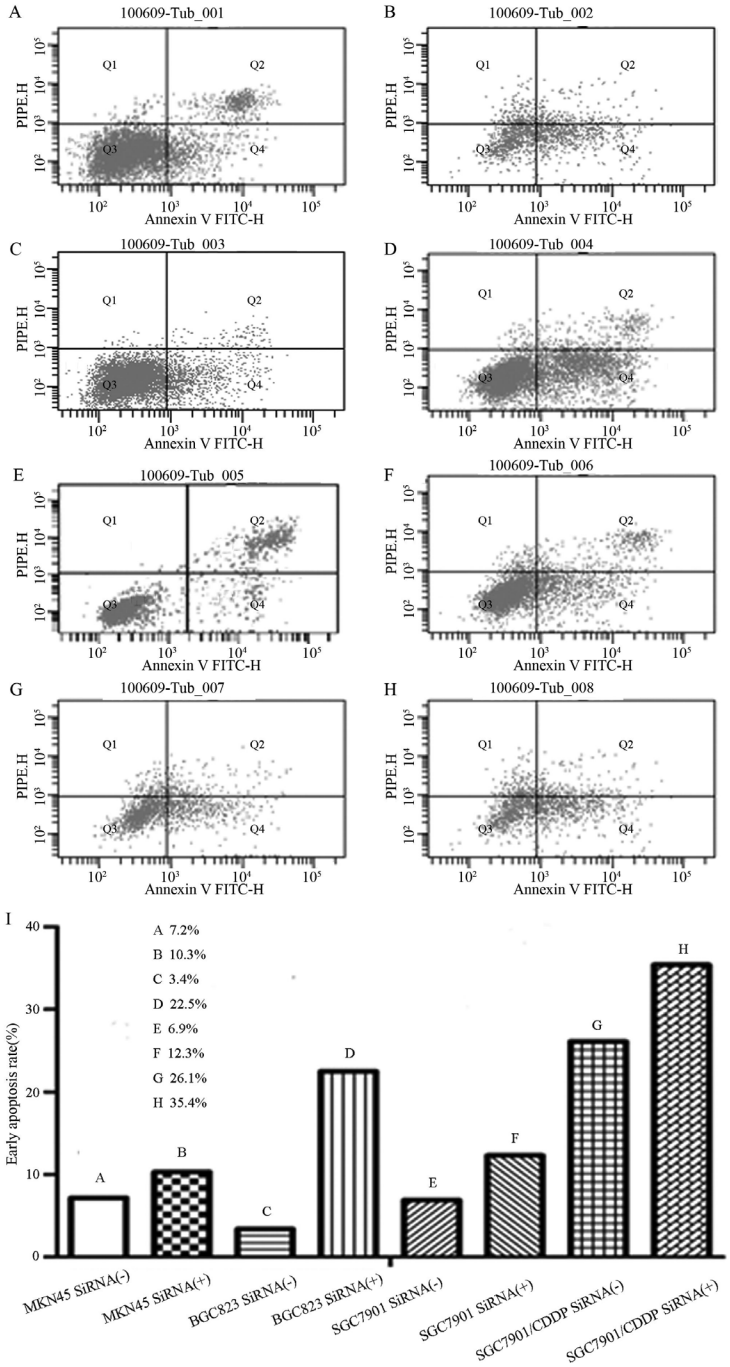

Figure 4. Effects of siRNA-*survivin* interference on early cell apoptosis of BGC823, MKN45, SGC7901, and SGC7901/CDDP. **A.** MKN45 siRNA (-). **B.** MKN45 siRNA (+). **C.** BGC823 siRNA (-). **D.** BGC823 siRNA (+). **E.** SGC7901 siRNA (-). **F.** SGC7901 siRNA (+). **G.** SGC7901/CDDP siRNA (-). **H.** SGC7901/CDDP siRNA (+).

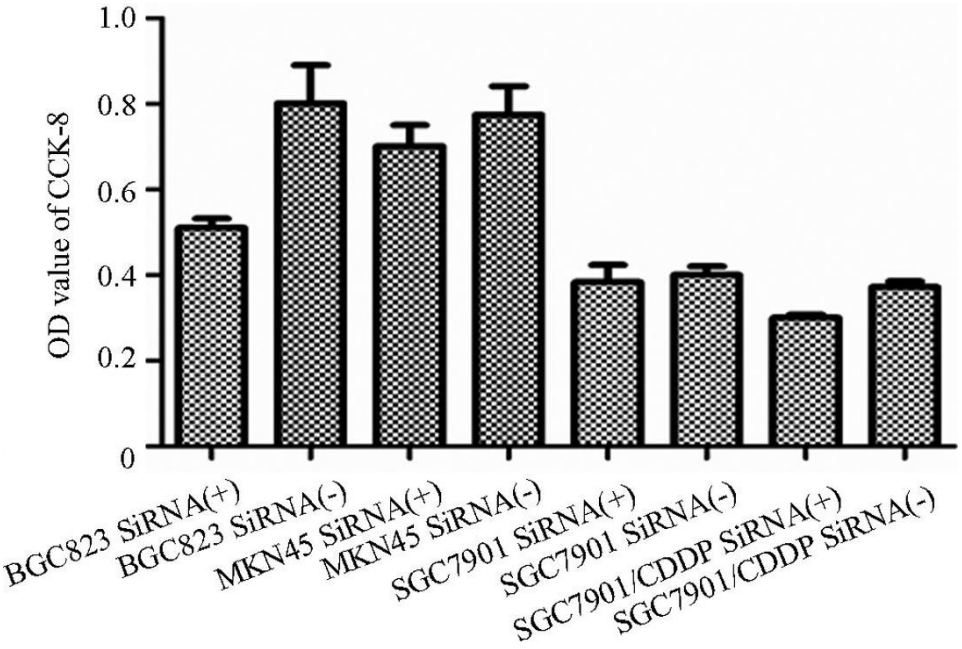

Figure 5. Effects of siRNA-*survivin* interference on *survivin* mRNA expression and cell proliferation (changes in cell proliferation).

siRNA (+)/siRNA (-) ratios

With the siRNA (+)/siRNA (-) ratio as the ordinate, the ratios between the *survivin* mRNA levels, protein levels, and OD values after positive and negative interference were localized for comparison. The results showed that siRNA-*survivin* had the best interference effect on BGC823, which was followed by SGC7901 (Figure 6A). With the siRNA (+)/siRNA (-) ratio as the ordinate, the ratio between the early apoptotic rates after positive and negative interference were localized. The results showed that siRNA-*survivin* had a better early apoptosis-promoting effect on BGC823 than any other cell strain. The results are shown in Figure 6B.

Caspase-3 protein

As shown in Figure 7, after 48-h siRNA interference, caspase-3 protein expression significantly increased in all four siRNA-*survivin* interfered cell groups compared with those in their corresponding negative control groups. This finding demonstrated that interference in survivin expression upregulated caspase-3 expression to some degree.

Mitochondrial and cytoplasmic cytochrome C

The relative OD values of the mitochondrial cytochrome C protein expression in the positively interfered BGC823, MKN45, SGC7901, and SGC7901/CDDP were 0.353

± 0.017, 0.821 ± 0.106, 0.501 ± 0.097, and 0.369 ± 0.060, whereas those in the negative control cell strains were 1.383 ± 0.087, 1.093 ± 0.042, 0.709 ± 0.109, and 1.024 ± 0.059, respectively, which showed significant differences (^P < 0.01 and * P < 0.05; Figure 8A, B). These results indicated that siRNA-*survivin* significantly decreased mitochondrial cytochrome C protein expression. The relative OD values of the cytoplasmic cytochrome C protein expression in the positively interfered BGC823, MKN45, SGC7901, and SGC7901/CDDP were 0.993 ± 0.083, 1.156 ± 0.202, 1.057 ± 0.047, and 1.078 ± 0.066, whereas those in the negative control cell strains were 0.255 ± 0.061, 0.850 ± 0.062, 0.328 ± 0.061, and 0.778 ± 0.035, respectively, which showed significant differences (P < 0.01 and P < 0.05; Figure 8C, D). These results indicated that siRNA-*survivin* significantly decreased cytoplasmic cytochrome C protein expression.

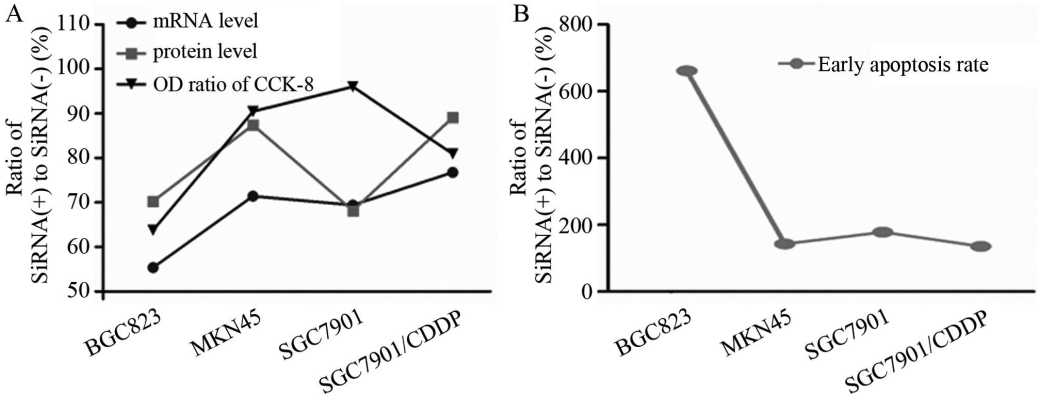

Figure 6. Comparisons of the mRNA levels, protein levels, relative OD values, and early apoptotic rates after positive [siRNA (+)] and negative [siRNA (-)] interference in the four GC cell strain groups. **A.** mRNA levels, protein levels, relative OD values, and early apoptotic rates after siRNA (+) and siRNA (-) interference. **B.** early apoptotic rates after siRNA (+) and siRNA (-) interference.

pRNA-shSUR identification

The interfering sequence was synthesized using the loop-stem structure tcaagag and identified by adding an *Eco*RI restriction enzyme cutting site (Figure 9A). pRNA-shSUR was digested by *Bam*HI, *Eco*RI, and *Hin*dIII and then subjected to 0.8% agarose gel electrophoresis. The result showed only one specific band (Figure 9B). The macrorestriction map was identified correctly. After plasmid transfection, the GFP fluorescent expression increased in BGC823 and SGC7901 (Figure 9C-F).

pRNA-shSUR and liposomes were mixed and then transfected into the GC cells. At 48 h, survivin protein expression in the cells was analyzed using Western blotting. The relative OD values of the survivin protein expression in BGC823 and SGC7901 after shRNA transfection were 0.116 ± 0.021 and 0.132 ± 0.011, whereas those after negative control plasmid transfection were 0.468 ± 0.094 and 0.156 ± 0.037, which showed significant differences (P < 0.01 and P < 0.05: Figure 10).

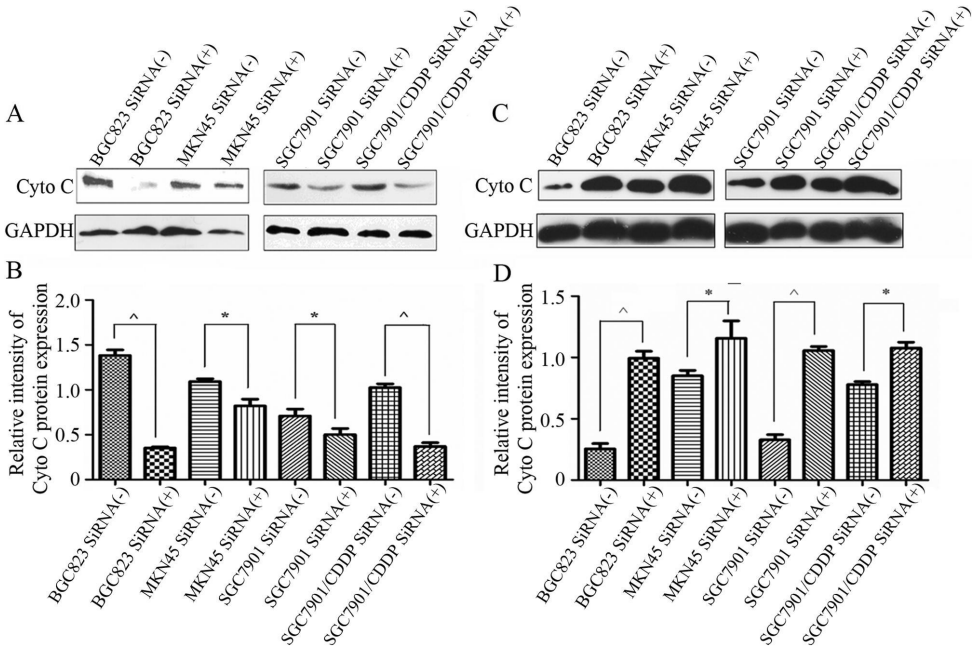

Figure 8. Effects of siRNA-*survivin* interference in *survivin* expression on mitochondrial and cytoplasmic cytochrome C protein expression (^P < 0.01 and *P < 0.05). **A.** and **B.** Mitochondrial cytochrome C protein expression, **C.** and **D.** cytoplasmic cytochrome C protein expression.

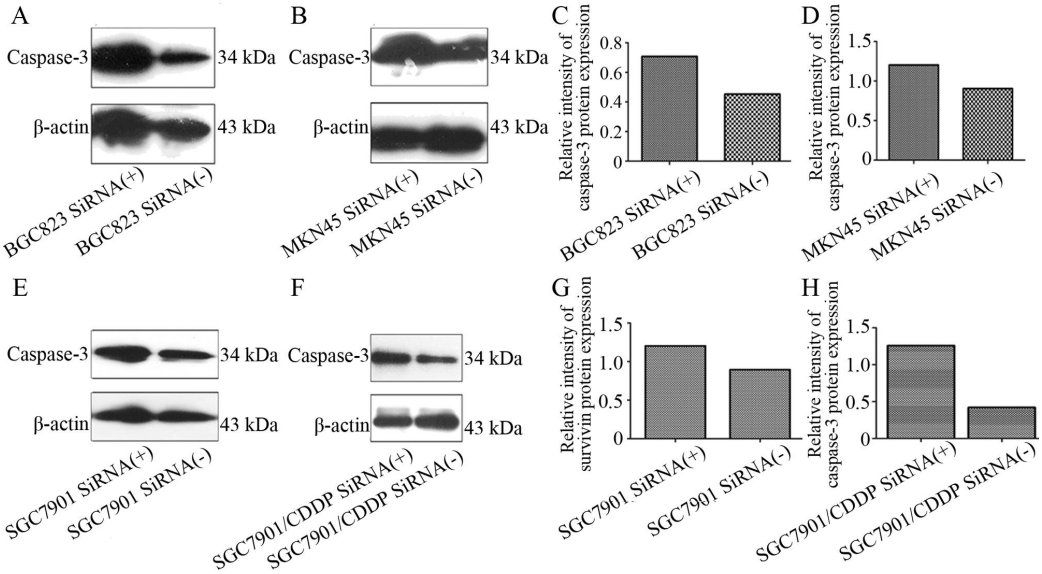

Figure 7. Effects of siRNA-*survivin* interference on caspase-3 protein in BGC823, MKN45, SGC7901, and SGC7901/CDDP.

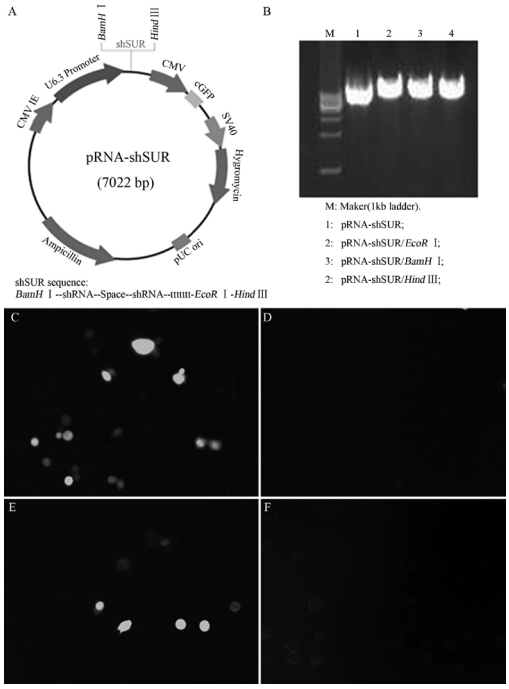

Figure 9. Structural schematic diagram (**A**), digestion identification (**B**), and post-transfection GFP identification (**C-F**) of the recombinant plasmid pRNA-shSUR. **A.** the structural schematic diagram of pRNA-shSUR. **B.** digestion identification. **C.** BGC823 after pRNA-shSUR transfection. **D.** BGC823 after pRNA-shControl transfection. **E.** SGC7901 after pRNA-shSUR transfection. **F.** SGC7901 after pRNA-shControl transfection.

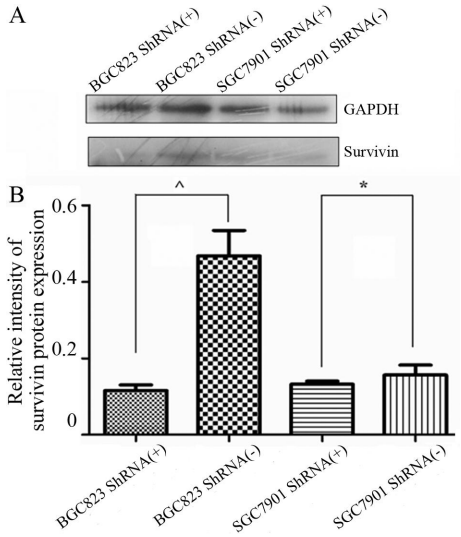

Figure 10. Effect of pRNA-shSUR transfection on survivin protein expression in the GC cell strains (^$P < 0.01$ and *$P < 0.05$).

DISCUSSION

Tumorigenesis is a multiple-link process underlying which oncogene activation functions as a critical pathological mechanism. Therefore, specific anti-oncogene activation has become an important research direction of oncotherapy. Considering that RNAi technique has the characteristics of high efficiency and strict sequence specificity, we designed specific oncogene-directed siRNA molecules in this study. These molecules were expected to act on abnormal genes rather than normal genes, thereby realizing the aim of tumor treatment. Brummmelkamp et al. (2002) once constructed the siRNA expression system of the mutant K-RASV12, namely, pSUPER-K-RASV12. They transfected it into the human pancreatic cancer CAPAN-1 cell strain and observed its inhibitory effect on the expression of endogenous K-RASV12. Their results showed that pSUPER-K-RASV12 noticeably decreased K-RASV12 mRNA expression but had no influence on the expression of cyclin D1 (the control). K-RASV12 carrying siRNA noticeably inhibited the cloning and growth of CAPAN-1 cells and prevented tumorigenesis in nude mice, whereas it promoted cell growth and cloning in the control group and tumorigenesis occurred in nude mice within 5 weeks. Their findings indicated that siRNA had remarkable specificity and inhibitory effect on tumorigenesis, and can thus provide a new technical scheme for tumor research.

Survivin has the functions of regulating cell cycle and inhibiting cell apoptosis. *survivin* is not expressed in G1 phase; its expression increases six times in S phase and 40 times in G2/M phase (Li et al., 1998). This finding suggested that it is primarily expressed in G2/M phase and serves as a regulatory gene in this phase. This characteristic of *survivin* is attributed to three cell cycle-dependent elements (CDEs) and a cell cycle homology region (CHR) among its primers. CDEs and CHR belong to suppression elements in G1 phase, and they regulate the half-life of gene expression in G2/M phase, which enable survivin to be specifically expressed in G2/M phase. Survivin regulation is correlated with cell cycle-related transcription; meanwhile, it is upregulated by NF-κB, insulin-growth factor 1/mTOR signals, the ras oncogene family, signal transducer and activator of transcription 3 (STAT 3), and wnt-2 (Van Antwerp et al., 1998; Sommer et al., 2003; You et al., 2004; Vaira et al., 2007); furthermore, its regulation correlates with its intracellular localization and the generation of different spliceosomes during translation. Survivin has two types of subcellular locations, namely, intranuclear and/or intracytoplasmic (Fortugno et al., 2002). This distributional difference is caused by different post-transcriptional modifications of survivin. The selective splicing of survivin pre-mRNA can generate different splicing isoforms, and total length of survivin, survivin-2B, and survivin-Δ Ex-3 are the three known isoforms. However, the functions of survivin isoforms in different tumors remain to be explored.

Numerous studies of survivin expression in tumors have been reported (Altieri, 2003a). Survivin increases the resistance of tumor cells to apoptosis signals through caspase-dependent or non-dependent channels, thereby blocking apoptosis; conversely, tumor cell antagonizing survivin leads to apoptosis (Ambrosini et al., 1997; Li et al., 1998; Altieri, 2003a,b).

Survivin decomposition in HeLa cells causes abnormal mitoses and polyploidy (Li et al., 1999), whereas *survivin* knock-out mice suffer from early abortion due to microtubule constitution disorders and polyploid formation (Uren et al., 2000). Studies have shown that survivin overexpression correlates with the increase in proliferation index (Sui et al., 2002; Takai et al., 2002; Fields et al., 2004; Morinaga et al., 2004), decrease in apoptosis rate (Kawasaki et al., 1998; Tanaka et al., 2000), chemotherapeutic drug administration (Tran et al., 2002;

Zaffaroni et al., 2002), and tumor recurrence (Swana et al., 1999).

A number of studies have shown that survivin is noticeably overexpressed in GC tissue (Tsuburaya et al., 2002; Li et al., 2004; Lu and Liu, 2004). Furthermore, the GC apoptotic index in the survivin-positive group is significantly lower than that in the negative group, and survivin expression correlates with the differentiation and pathological staging of GC tissue, which suggest that an increase in survivin may serve as a clue for poor prognosis of GC (Li et al., 2004). However, some authors disagree with these findings (Okada et al., 2001). These inconsistencies may be ascribed to differences in survivin protein detection methods and intracellular localization or different spliceosomes involved. Survivin expression in surgical specimens is associated with the size, invasion depth, lymphatic metastasis, staging and prognosis of tumors; furthermore, survivin plays an important role in tumorigenesis by stimulating tumor angiogenesis (Lee et al., 2006). Survivin is overexpressed in tumor cells while almost not expressed in normal tissues, which determines the *survivin* gene as a suitable target for tumor treatment.

survivin-targeting double-stranded RNA noticeably reduces mRNA transcription and translation of *survivin* and decreases survivin protein expression in the pancreatic cancer cell line PANC-1, thereby resulting in cell apoptosis, whereas it has no noticeable influence on the cell cycle (Tsuji et al., 2005).

Based on the aforementioned findings, we constructed a double-stranded siRNA and then transfected it into GC cell strains. The results of real-time PCR showed that siRNA interfered with the mRNA levels in all the cell lines investigated. For BGC823, the mRNA level after siRNA-*survivin* positive interference was 55.363 ± 3.974% of that after negative interference; for MKN45, the mRNA level after positive interference was 71.433 ± 3.774% of that after negative interference; and for SGC7901 and cisplatin-resistant SGC7901, the mRNA levels after positive interference were 69.433 ± 7.336 and 76.767 ± 3.541% of those after negative interference, respectively. These findings suggest that the synthesized siRNA markedly decomposed *survivin* mRNA in this study, and prove that the designed and synthesized double-stranded RNA was valid.

Immunoblotting was then performed. For BGC823, the relative OD value of survivin protein expression after positive interference was 0.293 ± 0.017, whereas that after negative interference was 0.453 ± 0.019; for MKN45, SGC7901, and cisplatin-resistant SGC7901, the OD values after positive interference were 0.453 ± 0.019, 1.114 ± 0.025, and 1.678 ± 0.034, whereas those after negative interference were 0.541 ± 0.019, 1.678 ± 0.035, and 1.831 ± 0.093, respectively. Significant differences were observed in all the groups after positive and negative interference ($P < 0.05$). This finding was also evident in the immunofluorescence results: survivin protein was expressed in nuclei; however, after siRNA interference, intranuclear survivin protein expression significantly decreased.

The primary function of survivin expression in tumor cells is to antagonize cell apoptosis. In this study, flow cytometry was performed using Annexin V-FITC-PI double staining kits. The results showed that the biological behaviors of GC changed after interference with survivin expression: for BGC823, the early apoptotic rate after positive interference was 22.5%, whereas that after negative interference was 3.4%; for MKN45, SGC7901, and cisplatin-resistant SGC7901, the early apoptotic rates after positive interference were 10.3, 12.3, and 35.4%, whereas those after negative interference were 7.2, 6.9 and 26.1%, respectively. These results suggest that the RNAi technique promoted noticeable GC cell apoptosis by interfering with survivin expression.

Miao et al. (2007) successfully interfered with survivin protein expression in the GC cell strain SGC7901 using siRNA; they also found that the inhibition of GC cell growth was correlated with the GC cell apoptosis-inducing effect of siRNA *in vitro*. Ma et al. (2007) found that *survivin*-targeting antisense oligonucleotides did not only induce the apoptosis of SGC7901 but also increased its sensitivity to paclitaxel.

The anti-cell apoptosis mechanism of survivin is realized by inhibiting the activation of caspase-3 and caspase-7. Caspases are a group of cysteine-containing proteases that promote cell apoptosis. Their enzymatic substrates include themselves and other factors. They transduce apoptosis signals or directly serve as apoptosis effector molecules by hydrolyzing their substrates to promote cytoskeleton degradation and DNA fragmentation. Normally, most capspases exist in the form of inactive precursors, namely, caspase proenzymes. The activation of these proenzymes is usually caused by the upper stage of caspase splicing, forming a cascade effect; therefore, they are autocatalyzed (Altieri, 2001; Wakana et al., 2002). The usual channel whereby caspases cause cell apoptosis is the receipt of dead signals by dead receptors, which causes the activation of the upstream procaspase-8 and leads to the activation of procaspase-9. These processes ultimately result in cytoarchitectonic damage. Survivin inhibits the caspase activation induced by cytochrome C or caspase-8, and also prevents caspase-3 and caspase-7 (Kania et al., 2003). Therefore, survivin overexpression in GC inhibits cell apoptosis or promotes mitosis, which benefits the survival and growth of tumor cells.

Cytochrome C was previously assumed to primarily participate in controlling cellular energy metabolism. Recently, it has been thought as an important factor participating in apoptosis. The loss of mitochondrial respiratory activity is only part of its participation in apoptosis. What is more important is its release from mitochondria. Normally, cytochrome C is a water-soluble protein located in the mitochondrial membrane space with a relative molecular mass of 1.45×10^4. It binds the mitochondrial inner membrane in an electrically stable manner and cannot penetrate through the outer membrane. During apoptosis, cytochrome C is released through the mitochondrial outer membrane, and afterwards binds to apoptotic protease activating factor-1 (Apaf-1) through ATP/dATP to form oligomers. Apaf-1 interacts with the anterior region of procaspase-9 via the amino terminal of the oligomer to activate caspase-3 and downstream caspases. In this study, after *survivin* in GC cells was interfered, the cytochrome C level in mitochondria decreased, whereas that in cytoplasm increased. Presumably, *survivin*-targeting interference causes the release of cytochrome C from mitochondria into cytoplasm, as well as an increase in caspase-3 protein expression, which indicates that *survivin*-targeting siRNA promotes GC cell apoptosis through the classic mitochondrial pathway.

Abnormal cell proliferation occupies an equally important position with abnormal apoptosis in the genesis of tumors, and malignant tumor cells display reproductive activity as vigorous as embryonic tissues. Survivin has a cell proliferation-promoting function. Sui et al. (2002) analyzed 103 cases of ovarian neoplasms to explore the influence of survivin expression on cell proliferation and curative effect of tumors. They found that the survivin positive rates in malignant and borderline tumors were significantly higher than that in benign tumors (the detection rates were 21.2, 47.8 and 51.1%, respectively); survivin expression positively correlated with the activity of tumor cell proliferation; its overexpression plays critical roles in both the development and prognosis prediction of ovarian neoplasm. Survivin is positively expressed in 88% (46/52) of patients with pancreatic cancer; it is closely correlated with tumor cell proliferation and growth; the cell proliferation index in the survivin-positive group is sig-

nificantly higher than that in the negative group (Sarela et al., 2002). *Survivin*-targeting mixed double-stranded RNA inhibits the tumor progression in animals transplanted with rhabdomyosarcoma, suggesting a possible gene therapeutic effect (Caldas et al., 2006).

In addition, the results of the CCK-8 experimental in this study proved that interference in survivin expression can further inhibit the reproductive activity of GC cells. The results of cytometry showed *survivin*-targeting siRNA vectors induced the early apoptosis of the four cell strains investigated in this study. Thus, this study successfully proved the validity and early cell apoptosis-promoting effect of *survivin*-targeting siRNA vectors from multiple perspectives.

Tumorigenesis is a process involving numerous factors. Survivin overexpression in tumor cells noticeably inhibits cell apoptosis and allows tumor cells to avoid identification and removal by the immune system. Survivin possesses high conservation and a cell apoptosis inhibiting role. Therefore, it can only provide a new research direction for tumor treatment in expression specificity in tumor cells. *survivin* is a newly discovered anti-apoptosis gene. It promotes tumor cell proliferation, playing an important role in the development of tumors. As *survivin* is selectively expressed in tumor tissues, it may serve as a specific target to open up a new path to tumor gene therapy.

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