GASTRIC CANCER

Downregulation of survivin by RNAi inhibits growth of human gastric carcinoma cells

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Abstract

AIM: To investigate the inhibitory effect of a specific small survivin interfering RNA (siRNA) on cell proliferation and the expression of survivin in human gastric carcinoma cell line SGC-7901.

METHODS: To knockdown survivin expression, a small interfering RNA targeting against survivin was synthesized and transfected into SGC-7901 cells with lipofectamineTM2000. The downregulation of survivin expression at both mRNA and protein levels were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. Cell proliferation inhibition rates were determined by methyl thiazolyl tetrazolium (MTT) assay. The effect of survivin siRNA on cell cycle distribution and cell apoptosis was determined by flow cytometry (FCM).

RESULTS: RNA interference could efficiently suppress the survivin expression in SGC-7901 cells. At 48 h after transfection, the expression inhibition rate was 44.52% at mRNA level detected by RT-PCR and 40.17% at protein level by Western blot analysis. Downregulation of survivin resulted in significant inhibition of tumor cell growth *in vitro*. The cell proliferation inhibition rates at 24, 48 and 72 h after survivin siRNA and non-silencing siRNA transfection, were 34.06%, 47.61% and 40.36%, respectively. The apoptosis rate was 3.56% and the number of cells was increased in G₀/G₁ phase from 38.2% to 88.6%, and decreased in S and G₂/M phase at 48 h after transfection.

CONCLUSION: Downregulation of survivin results in significant inhibition of tumor growth *in vitro*. The inhibition of survivin expression can induce apoptosis of SGC-7901 cells. The use of survivin siRNA deserves further investigation as a novel approach to cancer therapy.

INTRODUCTION

Survivin was initially identified as a member of the inhibitor of apoptosis protein (IAP) family. Up to the present, overexpression of survivin has not been reported in differentiated normal tissues with exception of thymus, basal colonic epithelium endothelial cells and neural stem cells during angiogenesis^[1]. However, the highly specific expression of survivin was found in many human cancers, including gastric cancer. By inhibiting apoptosis and promoting mitosis, survivin facilitates cancer cell survival and growth^[2-6]. Several studies have revealed that survivin as an indispensable factor can regulate and assist completion of cytokinesis^[7-10]. Recently, it has been also proven that overexpression of survivin can protect cells from apoptosis by inhibiting pro-apoptotic caspases-3 and caspases-7; when acting as a microtubule stabilizer during mitosis, it promotes cell cycle progression as well^[11-14]. In addition, Asanuma K *et al*^[15] reported that survivin enhanced the expression of the Fas ligand in cancer cells through up-regulation of specific protein-1-mediated gene transcription, and enabled cancer cells to counter-attack immune cells by inducing FasL-triggered apoptosis of cells in the immune surveillance system.

RNAi is a genetic interference phenomenon that is effective for suppressing gene expression. It involves post-transcriptional gene silencing *via* a process in which double-stranded RNA (dsRNA) inhibits gene expression in a sequence-dependent manner through degradation of the corresponding mRNA. Its blocking action on gene expression has been successfully observed in rat and human cells cultured *in vitro*, and the knockdown of genes in cells has been achieved^[16,17].

In the present study, siRNA targeting to the survivin gene was introduced into gastric carcinoma cell line SGC-7901, which overexpresses survivin. Its effect on cancer cell growth was investigated.

MATERIALS AND METHODS

Cell lines and culture

Human gastric carcinoma cell line SGC-7901 was a present from Dr. Wei Liu. The cells were cultured in RPMI 1640 medium supplemented with 100 mL/L fetal bovine serum (FBS), 8×10^5 U/L penicillin and 0.1 g/L streptomycin in humidified incubator containing 50 mL/L CO₂ at 37°C.

siRNA preparation and transfection of short interfering RNA

siRNA oligonucleotides with two thymidine residues (tt) at the 3' end of the sequence were designed for survivin (sense, 5'-GCAUUCGUCCGGUUGCGCUtt-3'; antisense, 5'-AGCGCAACCGGACGAAUGCtt-3'). Cells were treated in parallel with a non-silencing-siRNA (sense, 5'-UUCUCCGAACGUGUCACGUt-3'; antisense, 5'-ACGUGACACGUUCGGAGAAtt-3') as control oligonucleotides were synthesized by Shanghai Genechem Co. These cells were cultured in medium without antibiotics, and 24 h before transfection resulting in a confluence of the cell monolayer by 50%-70%. Specific survivin siRNA or non-silencing siRNA (70 nmol) were mixed with lipofectamineTM 2000 (Invitrogen) according to manufacturer's recommendation and added to the cells. After 6 h at 37°C, the medium was changed, and the cells were cultivated in RPMI 1640 supplemented with 10% heat-inactivated FBS.

3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay

SGC-7901 cells (5×10^5) were placed onto 96-well plates in RPMI-1640 containing 10% FBS in a final volume of 0.1 mL. The next day, the cells were treated with siRNA. MTT was added (20 μ L/well of 5 g/L solution in PBS) after culture for 24 h, 48 h and 72 h. When incubated at 37°C for 4 h, the reaction was stopped by addition of 100 μ L DMSO. The reaction product was quantified by measuring the absorbance at 490 nm using an ELISA reader (WALLAC 1420 VICTOR 2, Victor Co, Finland) and Software HT-Soft (Perkin-Elmer). All samples were assayed repeatedly in six wells.

Reverse transcription polymerase chain reaction

SGC-7901 cells (5×10^5) were seeded onto 6-well plates. Total RNA was extracted 48 h after transfection using trizol reagent. Reverse transcription was performed using one step RT-PCR kit. The primers of human survivin were 5'-GGACCGCCTAAGAGGGCGTGC-3' (forward primer) and 5'-AATGTAGAGATGCGGTGGTCCTT-3' (reverse primer). The primers of human β -actin were 5'-GTGGGCATGGGTCTAGAAG-3' (forward primer) 5'-GAGGCGTACAGGGATAGCAC-3' (reverse primer). Thermal cycle conditions were as follows: 42°C for 30 min, 94°C for 2 min, followed by 28 cycles of 94°C 15 s, 55°C 30 s, 72°C 1 min, with a final extension at 72°C for 10 min. RT-PCR products were visualized by ethidium bromide-stained agarose gels.

Western immunoblot analysis

SGC-7901 cells (5×10^5) were seeded onto 6-well plates.

Forty-eight hours after transfection, cells were collected and washed twice by cold PBS, and each well was treated with 50 μ L lysis buffer (2 mmol/L Tris-HCl pH 7.4, 50 mmol/L NaCl, 25 mmol/L EDTA, 50 mmol/L NaF, 1.5 mmol/L Na₃VO₄, 1% Triton X-100, 0.1% SDS, supplemented with protease inhibitors 1 mmol/L phenylmethylsulfonylfluoride, 10 mg/L pepstatin, 10 mg/L aprotinin, and 5 mg/L leupeptin) (all from Sigma). Protein concentrations were determined using the bicinchoninic acid protein assay. Equal amounts of protein (40 μ g) were separated on a 15% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond C, Amersham, Freiburg, Germany). Membranes were blocked in 5% nonfat dry milk in TBS for 1 h at room temperature and probed with rabbit antisurvivin antibodies (dilution, 1:500 Santa Cruz Biotechnology, USA) overnight at 4°C. After 3 times washing with TBS containing 0.1% Tween 20, membranes were incubated with anti-rabbit IgG-horseradish-peroxidase (1:5000, Santa Cruz Biotechnology, USA), and developed by luminol mediated chemiluminescence (Appylgen Technologies Inc, China). To confirm equal protein loading, membranes were reprobed with a 1:1000 dilution of an anti-actin antibody (Santa Cruz Biotechnology, USA). Densitometric analyses were performed using Scion Image software.

Flow cytometry

SGC-7901 (5×10^5) cell lines were seeded in triplicate onto 6 well-plates, and cultured in RPMI-1640 supplemented with 100 mL/L FBS. When transfected for 48 h, the cells were collected and washed with ice-cold PBS, and fixed in 70% ethanol overnight at 4°C. The fixed cells were pelleted, washed in PBS, resuspended in PBS containing 0.1 mg/mL of propidium iodide, and analysed by flow cytometry.

Statistical analysis

Data were expressed as mean $\pm$ SD. All statistical analyses were performed using the SPSS 10.0 software package for Windows (SPSS Inc., Chicago, IL). One-way ANOVA followed by Bonferroni correction was used to compare the data among three or more groups. A value of $P < 0.01$ was considered statistically significant.

RESULTS

Expression of survivin gene in SGC-7901 cells

To examine the specific effect of survivin siRNA treatment on survivin expression in SGC-7901 line, the survivin mRNA and protein expression levels were determined quantitatively with RT-PCR and Western blot analyses, respectively. Results are displayed in Figure 1 and Figure 2. Survivin mRNA and protein were strongly expressed in gastric cancer SGC-7901 cells as reflected by RT-PCR and Western blot. The inhibition rate of survivin mRNA after transfection with specific survivin siRNA was 44.52%. The inhibition rate of survivin protein after transfection with specific survivin siRNA was 40.17%. Survivin expression was decreased significantly at 48 h after transfection with specific survivin siRNA.

Figure 1 Expression of survivin mRNA in different groups of SGC-7901 cells. **A:** Survivin mRNA expression in RT-PCR assay; **B:** Survivin mRNA expression in SGC-7901. 1: treated with survivin siRNA; 2: treated with non-silencing siRNA; 3: untreated. Data are expressed as mean $\pm$ SD of three experiments, survivin siRNA group vs non-silencing siRNA group, ^b $P < 0.01$; survivin siRNA group vs untreated group, ^b $P < 0.01$.

Figure 3 Effects on cell viability after non-silencing siRNA and survivin siRNA treatment. 1: untreated; 2: non-silencing siRNA; 3: survivin siRNA. Data are expressed as mean $\pm$ SD, survivin siRNA group vs non-silencing siRNA group, ^b $P < 0.01$; survivin siRNA group vs untreated group, ^b $P < 0.01$.

Effect of survivin siRNA on cell proliferation of gastric cancer cells

To determine whether inhibition of survivin affects cell proliferation in SGC-7901, metabolic activity at 24, 48, and 72 h was determined by the MTT assay. The cell viability was reduced significantly after treatment with specific survivin siRNA ($P < 0.01$) at 24, 48, and 72 h as compared with non-silencing siRNA treatment or untreated controls (Figure 3). The cell proliferation inhibition rates were

Figure 2 Western blot assay of survivin protein expression in SGC-7901 cells. **A:** Survivin protein expression in Western blot assay; **B:** Survivin protein expression in SGC-7901. 1: untreated; 2: non-silencing siRNA; 3: survivin siRNA. Data are expressed as mean $\pm$ SD of three experiments, survivin siRNA group vs non-silencing siRNA group, ^b $P < 0.01$; survivin siRNA group vs untreated group, ^b $P < 0.01$.

Table 1 Effects of treatment with survivin-siRNA on cell cycle distribution and apoptosis 48 h after transfection

Groups	Cell cycle			Apoptosis rate
	G ₀ /G ₁	S	G ₂ /M	
Untreated	38.21 $\pm$ 3.39	47.71 $\pm$ 2.73	14.00 $\pm$ 0.92	0.83 $\pm$ 0.34
Non-silencing	42.53 $\pm$ 2.78	43.28 $\pm$ 4.12	14.20 $\pm$ 2.90	1.08 $\pm$ 0.33
Sur-siRNA	88.60 $\pm$ 4.04 ^b	12.00 $\pm$ 3.42 ^b	0.10 $\pm$ 0.07 ^b	3.56 $\pm$ 0.91 ^b

Data are expressed as mean $\pm$ SD of the three experiments, sur-siRNA group vs non-silencing group, ^b $P < 0.01$; sur-siRNA group vs untreated group, ^b $P < 0.01$.

34.06%, 47.61% and 40.36%, respectively.

Effect of survivin siRNA on cell cycle distribution and apoptosis

Survivin has been reported to express in the G₂/M phase of the cell cycle and to contribute to the aberrant progression of cancer cells through mitosis. In our study, in comparison of non-silencing siRNA group and untreated controls, specific survivin siRNA caused accumulation in the G₀/G₁ phase, and decreased the number of cells of G₂/M phase and increased hypodiploid DNA content ($P < 0.01$) at 48 h after transfection. Apoptosis rate was 3.56% in the group of specific survivin siRNA transfection (Table 1).

DISCUSSION

The balance between apoptosis and anti-apoptosis signal pathways plays a role in the pathogenesis of a variety of

cancers. It has been demonstrated that the inhibition of apoptosis promotes the mitotic progression in cancer cells^[18]. Based on its specific overexpression in a vast majority of solid cancers and its anti-apoptotic function, survivin represents a suitable target for antitumor approaches. The inhibition of survivin would block the anti-apoptosis and mitotic progression in tumor cells^[19], as a result, tumor development is suppressed. So, survivin has attracted considerable attention as a novel member of IAP family.

RNAi is an evolutionarily conserved phenomenon in which gene expression is suppressed by the introduction of homologous double-stranded RNAs (dsRNAs). Synthetic siRNA can trigger an RNA interference response in mammalian cells and induce strong inhibition of specific gene expression^[20]. Therefore, it can be used as a powerful approach to silence mammalian gene expression for gene function studies^[21-24].

Since survivin is important for the survival of various human tumors, survivin siRNA could become an effective therapeutic agent for tumors with overexpression of survivin. Survivin has been successfully down-regulated by RNAi in some previous studies^[25-29]. However, the down-regulation of survivin expression by RNAi in gastric cancer SGC-7901 cells has not been performed until now.

In addition, it is reported that the effect of RNAi may be greatly different, targeting to different sequence of the gene^[30-32]. It is therefore, very important to find the proper sequence. The sequence designed by Williams *et al*^[26] has been confirmed effective in their research. So in our study, we synthesized this siRNA duplex with a thymidine overhang on 3' terminus *in vitro* to diminish the survivin expression of gastric carcinoma SGC-7901 cell line and to impair its growth potential. We observed that SGC-7901 cells transfected with survivin siRNA grew slowly as compared with the control groups. Survivin siRNA showed anti-proliferation function. RT-PCR and Western blot findings demonstrated that survivin mRNA and protein expression were reduced by over 40%, and survivin gene was blocked in SGC-7901 cells transfected with surviving siRNA at the level of transcription and protein expression. Flow cytometry revealed that the cell growth inhibition by survivin siRNA was a result of cell cycle arrest at G₀/G₁-phase and induction of apoptosis indicated by increased hypodiploid DNA content. The possible mechanism is that survivin is characterized by cell cycle dependent expression, i.e., it is expressed in G₂/M. Ito and co-workers transfected survivin into four human hepatocellular carcinoma cell lines and found that the number of cells in G₀/G₁ was remarkably reduced, and the number of cells in S or G₂/M increased. In our study, after SGC-7901 cells were transfected with survivin siRNA, the number of cells in G₀/G₁ was remarkably increased, and the number of cells in S or G₂/M decreased. The effect showed the same mechanism. However, in the study by Kappler and co-workers, five human sarcoma cell lines transfected with specific survivin siRNA showed G₂/M arrest^[26]. Ning *et al*^[33] also observed a specific G₂/M arrest when using survivin-directed siRNA as a gene therapeutic approach to human bladder cancer cells. The reason remains unclear, and it may be due to different cell lines.

The above-mentioned findings confirm that chemically synthesized siRNAs can specifically block survivin gene expression, inducing cell apoptosis and inhibiting the growth of carcinoma cells.

In conclusion, our data suggest that survivin gene can be regarded as a very good target gene in genetic therapy for gastric carcinomas and the use of survivin siRNA deserves further investigations as a novel approach to cancer therapy.

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