Synergism between a siRNA targeted to survivin and 5-FU in inhibiting MCF-7 cell proliferation *in vitro*

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Abstract: **Objective** To investigate the role of small interfering RNA (siRNA) targeted to survivin in combination with 5-fluorouracil (5-FU) in inhibiting the proliferation of MCF-7 cells. **Methods** A siRNA targeted to survivin was synthesized and transfected into MCF-7 cells via lipofectin. Changes of the cell growth activity in response to combined treatment with survivin siRNA and 5-FU or 5-FU treatment alone was evaluated by MTT assay. The *Q* method of Jin Zhenjun was used to evaluated synergism between the synthesized siRNA and 5-FU. **Results** Treatment with 5 nmol/L siRNA reduced the IC_{50} of 5-FU from 4.42 to 1.18 μg/ml, and the inhibitory effect of combined treatment on MCF-7 cells was higher than that of 5-FU alone ($F=26.74$, $P<0.01$). Synergism effect ($Q \geqslant 1.15$) was observed between 5-FU at lower concentrations and survivin siRNA. **Conclusion** siRNA may enhance the effectiveness of 5-FU in inhibiting the proliferation of MCF-7 cells.

Key words: RNA interference; chemotherapy; combined-modality therapy; MCF-7 cells; breast cancer

The response of malignant tumors to chemotherapy varies even if the tumors arise from the same organ or show similar histopathologic origins and clinical stage, often resulting in unsatisfactory clinical outcomes of patients receiving conventional chemotherapy. One possible solution of this problem lies in the strategy of improving the chemosensitivity and decreasing drug resistance of the cancer cells. Apoptosis is the predominant cell death pathway of the cancer cells in response to chemotherapy or radiotherapy, and in fact, increasing the susceptibility of tumor cells to apoptosis, hence the overall chemosensitivity of cancers, has been one of the major targets in cancer therapy research. Survivin, an apoptosis inhibitor known to protect from cell apoptosis, is frequently found overexpressed in human tumor cells. Survivin phosphorylation on Thr^{34} may regulate apoptosis at cell division via an interaction with caspase-9, and survivin overexpression may help cells overcome the G_2/M apoptotic checkpoint, and promote resistance to cancer therapy[1-3].

RNA interference (RNAi) is an evolutionary conserved mechanism that is operative in insects, plants, and mammalian cells. In this process, sequence-specific posttranscriptional silencing is initiated by the introduction into cells of double-stranded annealed sense and antisense RNAs that are homologous to the sequence of the silenced gene[4-6]. Importantly, RNAi can be achieved in mammalian cells following transfection with synthetic 21- and 22-nucleotide (nt) small interfering RNAs (siRNA), indicating that RNAi may serve as a powerful technology to specifically block the expression of target genes[7, 8].

In this study, we synthesized siRNA targeting survivin and tested its feasibility to induce apoptosis and increase chemosensitivity of breast cancer cell line MCF-7.

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MATERIALS AND METHODS

Drugs and chemicals

5-fluorouracil (5-FU) was the product of Tianjin Renmin Pharmaceutical Factory. 3-(4,5-Dimethylthiazole-2-yle)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co and Lipofectamine™ 2000 from Invitrogen Co. Powdered RPMI 1640 medium was obtained from Gibco. Newborn calf serum was supplied by Beijing Medical Center of Veterinary Sciences. All other chemicals used were of reagent grade.

Cell culture

Human breast cancer cell line MCF-7 was cultured in RPMI 1640 medium supplemented with 10% newborn calf serum (decomplemented by processing for 1 h at 56 ℃), 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were grown at 37 ℃ in 5% CO_2/95% air, and passaged with 0.25% trypsin plus 0.02% EDTA.

Synthesis of survivin siRNA

Selection of the siRNAs was carried out according to the characterization of siRNA by Elbashir *et al* [9]. Using simple T_7 RNA polymerase-directed *in vitro* transcription, a survivin siRNA sequence was screened, whose sequence was compared against an appropriate genome database to eliminate the possibility of significant sequence homology with other genes. The sequences of the target survivin mRNA and siRNA are listed below:

Survivin mRNA: 5' GUCUGGCGUAAGAUGAUGG 3'

siRNA: 5' GUCUGGCGUAAGAUGAUGGUU 3' (Sense)

3' UUCAGACCGCAUUCUACUACC 5' (Antisense)

MTT assay for cell proliferation

The effect of siRNA targeted to survivin in combination with 5-FU on the proliferation of MCF-7 cells was evaluated by MTT assay. Briefly, 5×10^3 cells were seeded in 96-well microtiter plate and allowed to grow till substratum attachment overnight. Lipofectamine™ 2000-mediated siRNA transfection of the cells was performed following the manufacturer's instructions with a final siRNA concentration of 5 nmol/L. After transfection of the cells by incubation for 6 h at 37 ℃, 100 μl of 5-FU diluted to 1.25, 2.5, 5.0, 10, and 20 μg/ml (which were around IC_{50} of 5-FU), respectively, were added into the wells, with the cells treated with siRNA or 5-FU alone as the controls and those without either treatment as the blank control. After a 48-hour incubation, 20 μl of MTT was added into each well, followed by further incubation for 4 h at 37 ℃. The absorbance (A) was determined at 490 nm with a MR 600 Microplate reader (Wallac 1420 Multilable counter). Each assay was performed in quadruplicate. The cell proliferation inhibition rate was calculated according the formula: Inhibition rate =$[(A_{control} - A_{sample})/(A_{control} - A_{blank})]\times100\%$.

Statistical analysis

Statistical analysis was performed with factorial analysis of variance design using SAS software .

Synergism analysis

The "Q" Method of Jin Zhenjun was used to evaluate the interaction between siRNA and 5-FU [10,11] on MCF-7 cells according to the equation $Q=E_{a+b}/(E_a+E_b-E_a\times E_b)$, where E_{a+b} is the synergism inhibition rate, Ea and Eb the inhibition rate of drug A and drug B used alone, respectively. Synergism of the two drugs is defined as a significantly greater effect when used in combination than the sum of the effects of the two drugs used alone. $Q<0.85$ suggests antagonism between the two drugs, $0.85\leqslant Q<1.15$ addition of their effect, and $Q\geqslant1.15$ synergism between them.

RESULTS

Inhibition of MCF-7 cell proliferation

Treatment of the MCF-7 cells with 5 nmol/L survivin siRNA reduced the IC_{50} of 5-FU from 4.42 to 1.18 μg/ml, and the inhibitory effect on MCF-7 cell proliferation was greater with combined treatment than with 5-FU alone (F=26.74, P<0.01). With the increase of 5-FU concentration, the increment of the inhibitory effect of the combined treatment was gradually lowered (Fig.1).

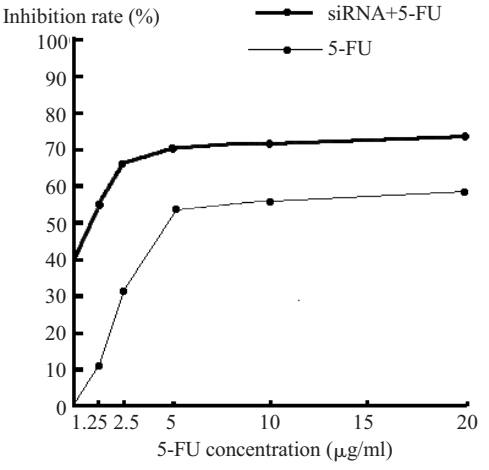

Fig.1 Inhibition of MCF-7 cell proliferation by 5-FU alone and in combination with survivin siRNA

Analysis of variance of the effect of 5-FU used in combination with survivin siRNA by factorial design

Analysis of variance was performed using SAS software with factorial design. The F value of siRNA*5-Fu was 26.74 (P<0.01), suggesting significant synergism between survinin siRNA and 5-FU.

Synergism between survivin siRNA and 5-FU

Tab.1 demonstrates obvious synergism between survivin siRNA and 5-FU at lower concentrations (1.25 and 2.5 μg/ml, $Q\geqslant1.15$) in inhibiting the proliferation of MCF-7 cells. As the concentration of 5-FU increased, the synergetic effect appeared to be weakened, but no antagonism was noted between the two agents.

DISCUSSION

During the last few years, several approaches have been attempted to improve the long-term results of chemotherapy for cancer, including the combination of conventional chemotherapy with gene therapeutic

Tab.1 Synergism between survivin siRNA and 5-FU in inhibiting proliferation of MCF-7 cells (Inhibition rate, %)

Group	5-FU concentration (μg/ml)				
	1.25	2.5	5.0	10	20
siRNA	40.8	40.8	40.8	40.8	40.8
5-FU	10.8	30.4	53.6	57.2	58.6
siRNA＋5-FU	56.7	67.8	70.2	72.2	74
Q	1.2	1.153	0.968	0.966	0.981

agents which enhance the cytotoxic effects of chemotherapy. Such gene therapeutic agents are expected, ideally, to selectively target the proteins or pathways that function inappropriately in cancer cells or play important roles in cell survival and proliferation after chemotherapy, but without affecting normal tissues. From this point of view, we conducted this *in vitro* study to test whether survivin expression inhibition, by means of siRNA in this case, may enhance the cytotoxic effects of chemotherapy, and obtained an affirmative result. We therefore believe that combination of chemotherapy with survivin inhibitors may potentially provide a useful approach for treating tumors with survivin overexpression.

This experiment was designed in anticipation of future clinical use of an antiapoptosis inhibitor as the chemosensitizer for cancer chemotherapy. An ideal candidate chemosensitizer should by itself have no or little cytotoxic effect at its therapeutive doses to minimize possible damage to the normal tissues. Upon this consideration, we performed the experiments combining survivin siRNA and chemotherapy using low concentrations of 5-FU which show minimal cytotoxicity when used alone. It was found that even at lower IC_{50} which may be of clinical significance, the inhibitory effect of combined treatment on MCF-7 cells was greater than that of 5-FU alone (F=26.74, P<0.01). Synergetic effect of survivin siRNA and 5-FU at lower concentrations was observed ($Q \geqslant 1.15$), suggesting that the combination of chemotherapy and survivin siRNA is a practical alternative for human cancer treatment.

Chemotherapy almost inevitably causes toxicity to normal somatic cells, and often induces drug tolerance of tumor cells. The mechanisms of multidrug resistance (MDR) in innate or acquired resistant tumor cell lines have proved to be associated with the resistance of the drug-induced apoptosis, and plerosis of the apoptosis mechanism might be a useful strategy in increasing chemosensitivity of the drug-resistant tumor cells. Asanuma [12] found an inverse relationship between survivin mRNA expression and radiosensitivity of 5 pancreatic cancer cell lines as well as between survivin expression and the clinical outcome of advanced esophageal cancer [13]. Ikeguchi [14] demonstrated in a gastric cancer cell line (MKN-45) that the expression rates of survivin mRNA 48 h after cell treatment with 0.1 and 1 mg/ml cisplatin (CDDP) were 2 to 6 folds higher than that in untreated cells, and the relative expression level of survivin protein 24 h after CDDP treatment increased by 2 to 5.5 folds, suggesting that survinin may mediate the resistance of the cancer cells to CDDP. These evidences strongly suggest that survinin may control microtubule stability and assembly of a normal mitotic spindle, preservation of cell ploidy, timing of cytokinesis, and helps malignant tumor cells escape chemotherapy/radiotherapy-induced G_2/M apoptotic checkpoint, possibly in correlation with their chemo/radiotherapy resistance, which can be reversed by inhibiting survivin expression.

As the cancer cells have an extraordinary capacity of adaptation and can easily acquire resistance to virtually any drug due to the redundancy of survival pathways, chemotherapy combined with gene therapy can be an attractive approach. However, these new combinations will have to be based on solid scientific and preclinical evidences, and the timing and sequence of their administration can be also of crucial importance.

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Survivin siRNA 协同 5-FU 抑制 MCF-7 细胞的增殖

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摘要:目的 研究以 survivin 为靶标的小干扰 RNA(siRNA)与化疗药 5-FU 联合应用抑制 MCF-7 细胞增殖的作用。**方法** 利用 T_7 RNA 聚合酶体外转录合成 siRNA 并筛选出一条 RNA 干扰 survivin 基因的靶序列。以脂质体为载体,将 survivin siRNA 转染至人乳腺癌 MCF-7 细胞中,用四氮唑盐(MTT)法染色并计算 siRNA 联用 5-FU 对 MCF-7 细胞的抑制率,用 SAS 统计软件及金正均 Q 值法进行统计分析。**结果** 单用 5-FU 处理细胞,其 IC_{50} 为 4.42 μg/ml,而加入 5 nmol/L siRNA 后, 其 IC_{50} 降为 1.18 μg/ml,siRNA 与 5-FU 联用对 MCF-7 细胞的抑制作用较单用 5-FU 明显增强(F=26.74,P<0.01);Q 值分析表明 survivin siRNA 与中低浓度的 5-FU 联用,有较好的协同作用(Q≥1.15)。**结论** survivin siRNA 可显著增强 5-FU 对 MCF-7 细胞增殖的抑制,提高肿瘤细胞对化疗药物的敏感性,克服耐药性的产生。

关键词:RNA 干扰;化学疗法;联合治疗;MCF-7 细胞
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