Antitumor activity of a novel survivin siRNA

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Abstract: Breast cancer resistance to therapy can result from expression of antiapoptotic genes. Survivin is an antiapoptotic gene that is over expressed in most human tumors. RNA interference using short interfering RNA (siRNA) can be used to specifically inhibit survivin expression. A novel siRNA targeting survivin was used to process MCF-7 cells. Cellular survivin mRNA and protein levels were determined by real-time qRT-PCR and Western blot, respectively. Cellular morphology and cell cycle were determined by fluorescence microscopy and flow cytometry. Cell proliferation was measured by MTT assay. Our data showed that the novel survivin-targeted siRNA could efficiently knockdown the expression of survivin, inhibit cell proliferation and cell cycle, especially at the G2/M checkpoint. These data suggest that the siRNA has potential for therapeutic applications.

Keywords: Survivin; siRNA; breast cancer; cell proliferation; cell cycle.

INTRODUCTION

Survivin, an inhibitor of apoptosis gene(Srinivasula and Ashwell 2008), has drawn many scientific researchers' attention because of its role in negative regulation of apoptosis and programmed cell death (Mobahat, Narendran, and Riabowol 2014). This means the disruption of survivin can lead to increase in apoptosis and decrease in tumor growth (Mita *et al*. 2008). Importantly, survivin protein is shown to be highly expressed in most human tumors and absent in normal tissues (Altieri 2003). Accordingly, inhibition of the survivin can be a new strategy for cancer therapy.

RNAi, a gene silencing technology, can be a powerful tool for cancer therapy (Deng *et al*. 2005). It is, however, challenging for siRNA to be translated in clinical therapy due to its poor stability and inefficient cellular delivery (Hendruschk *et al*. 2011). In recent years, many researchers have developed various strategies to address the problem of siRNA delivery, such as novel formulations and chemical modifications (Wu *et al*. 2014). It is important to understand that, in addition to the delivery system, the design of sequence of the siRNA determines its potency.

In the present study, a new siRNA sequence with improved potency was developed and evaluated in MCF-7 breast cancer cells.

MATERIALS AND METHODS

SiRNA design

SiRNA sequences were synthesized by Guangzhou RiboBio Co. (Guangzhou China) (table. 1). siRNA purification was conducted by HPLC.

Cell lines and cell culture

MCF-7, a human breast adenocarcinoma cell line, was purchased from the ATCC (Rockville MD). Cells were grown in DMEM (Gibco NY USA) containing 10% FBS and 1% antibiotics/antimycotics (Sigma-Aldrich, St. Louis MO) at 37˚C with 5% CO2 in air.

Cell transfection

Cells ($1.4\sim1.6\times10^5$cells/well) were seeded onto 6-well plates and growing at 37˚C. Before transfection, cell supernatant was removed and added the fresh serum-free Opti-MEMI medium. Tumor cells were transfected with survivin siRNA or negative-control siRNA using Lipofectamine 2000 (Invitrogen NY USA). The culture medium was replaced after 4h with fresh medium containing 10% FBS. The cells were harvested and evaluated after 48h.

Real-time rt-PCR for determination of survivin mRNA

Survivin gene expression was determined by real-time RT-PCR. TRIZOL reagent was used to extract total RNA (Takara Dalian China). Complementary deoxyribonucleic acids (cDNAs) were synthesized by reverse transcription from 1μg of total RNA. Forward and reverse primers used were as follows: 5'-CAGTGTTTCTTCTGCTTCAAGG-3' and 5'-CTTATT GTTGGTTTCCTTTGCAT-3' (Sangon Biotech Shanghai China). SYBR Green PCR kit (Takara) was used for the PCR reaction and each sample was analyzed in triplicate. The relative expression of each mRNA was detected and normalized to that of GAPDH mRNA as described (Chakrabarti *et al*., 2013).

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Proliferation assay

The proliferation assay was measured using a quantitative colorimetric assay with MTT (Sigma-Aldrich), as described previously (Wang Huang *et al*. 2014). Briefly, cells (1×10^4 cells/well) were grown in 96-well plates overnight. The effect of a series of siRNA concentration (0-50nM) to cells was investigated after the replacement of culture medium with serum free medium. The medium was removed after 4h and the cells were cultured in fresh medium for a period of time. Treated cells were subsequently incubated with 20µl MTT solution (0.5 mg/ml) for 4h at 37˚C in the dark. After that, 150µl/well DMSO was added to dissolve form azancrystals that formed and the absorbance at 490nm was measured by an automatic micro plate reader (Biotek USA) (Wang Huang *et al*. 2014, Li and Huang 2006).

Table 1: siRNAs sequences

Negative control siRNA	Sense	UUC UCC GAA CGU GUC ACG UTT
	Antisense	ACG UGA CAC GUU CGG AGA ATT
Survivin siRNA	Sense	CAA ACU GCU UCU UGA CAG AAAGdTdT
	Antisense	CUU UCU GUC AAG AAG CAG UUUGdTdT

Morphological variations of the nucleus

Cells ($0.8\sim1.0\times10^5$ cells/well) were seeded onto 6-well plates and treated with 50nM negative control siRNA or survivin siRNA. At 48h of post-transfection, cells were fixed with ice-cold methanol and washed with PBS. Nucleus were labeled by DAPI (0.01µg/µl) (Kedinger *et al*. 2013). For nuclei morphology analysis, cells were observed using a Nikon inverted microscope (Nikon Eclipse TE 2000-S, Amsterdam, Netherland).

Cell cycle and apoptosis analysis

Cells were fixed with ice-cold ethanol for 15min. And then, cells were incubated on ice with PBS containing 0.1% Triton X-100 and 5% BSA. After centrifugation, cells were resuspended in PBS containing RNase A and stained with PI (0.1mg/ml) for 10min at room temperature. (Kedinger *et al*. 2013). Cells were resuspended in PBS with 5mM EDTA and analyzed by a flow cytometer (BD Biosciences, San Jose, CA). DNA content was analyzed by FlowJo software.

STATISTICAL ANALYSIS

The data were analyzed using SPSS 16.0 software (IBM, Armonk, New York). The difference between two independent samples was analyzed by Student's t test. A statistically significant difference was considered to be presenting in $p<0.05$.

RESULTS

Down regulation of survivin in mcf cell lines

We designed a new siRNA sequence against survivin and invested the silence effects on MCF-7 cells. The mRNA and protein levels of survivin were determined by real-time qRT-PCR and Western blot at 48h after transfection with siRNA. Lipofectamine 2000 (Lipo2000) was used as the siRNA carrier.

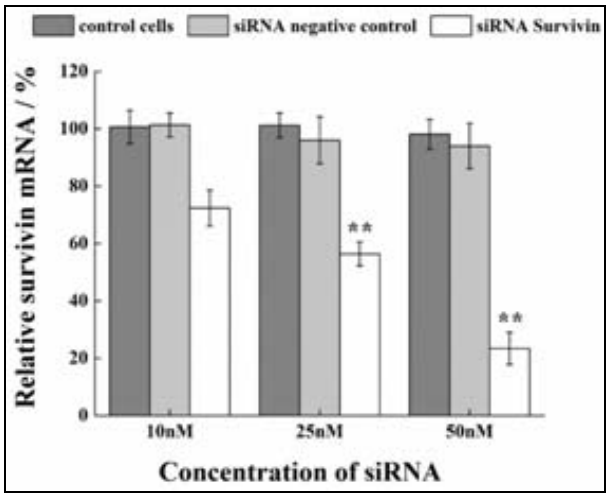

Fig. 1: Inhibition of survivin mRNA expression in MCF-7 cells analyzed by qPCR after transfection with siRNAs and lipo 2000. Survivin levels are expressed relative to GAPDH. **Statistically significant at $p<0.01$.

Fig. 2: Protein level of survivin analyzed by Western blot 24, 48 and 72 h after transfection with a negative control siRNA (2) or siRNA against survivin (3) (50 nM). Non-transfected cells (1) were also loaded. GAPDH was used as a loading control.

Different concentrations of survivin siRNA ranging from 10 to 50nM were added intoMCF-7 cells. After transfection, we observed a significant reduction in survivin mRNA level (fig. 1). The potency of new siRNA was optimal at 50nM and induced a 70 to 80%gene silencing compared to control cells. In addition, protein levels of survivin were analyzed by Western blot at 24, 48 and 72h after transfection. At 24h after transfection, survivin protein was already significantly reduced. Survivin expression inhibition reached 80% after 72h (fig. 2).

Effect on cellular morphology and cell proliferation

Our results (fig.3) showed that siRNA had concentration-dependent activity. When the concentration of siRNA reached 50nM, the viability of the cells was about 50 to

70% of the control. Cell viability decreased with increase in incubation time. Overall, the siRNA targeting survivin induced significant inhibitory effect on cell proliferation compared to non-treated and negative control-treated cells.

Treatment of MCF-7 cells with 50nM siRNA over 48 h caused marked multilobed nuclei and chromosomal aberrations under fluorescent microscope (fig. 4).

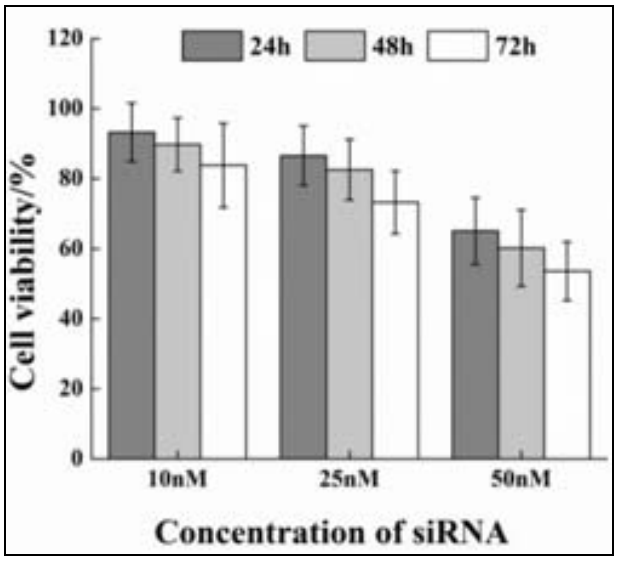

Fig. 3: Inhibitory effect on cell proliferation by siRNA against survivin. MTT assay analysis of viability of MCF-7 cells following 24-72 h of treatment with different doses of siRNA. The proliferation rate is represented as a percentage of cell growth relative to untransfected cells.

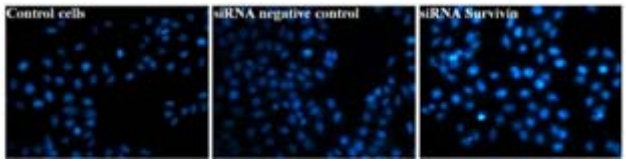

Fig. 4: Effect of survivin inhibition on MCF-7 cell nucleus. DAPI staining, 48h after transfection with siRNA negative control, survivin siRNA with Lipo 2000 (50 nM) showing the resence of mega-nuclei and chromosomal aberrations in cells transfected with survivin siRNA.

SiRNA induced cell cycle arrest

Cell proliferation inhibition and nucleus increase, chromosomal aberrations, characteristics of cell cycle arrest, were observed. The transfection of survivin siRNA causing a transient blockage of cell cycle progression may contribute to this phenomenon. In order to validate the cell cycle arrest, we analyzed by flow cytometry MCF-7 DNA content 48 h after transfection of siRNAs (fig. 5). Flow cytometry results showed MCF-7 cells of transfected object gene had G2/M phase arrest

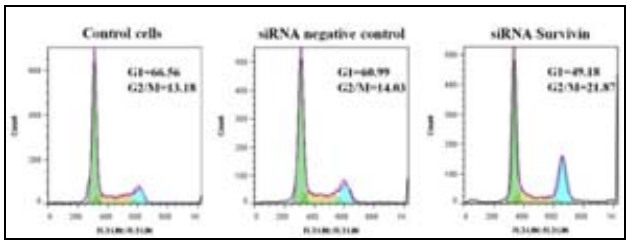

Fig. 5: Effect of survivin inhibition on MCF-7 cell cycle progression. Flow cytometer analysis of the cell cycle distribution of untransfected MCF-7 cells or 48h after transfection with negative control, survivin siRNA with Lipo 2000 (50nM).

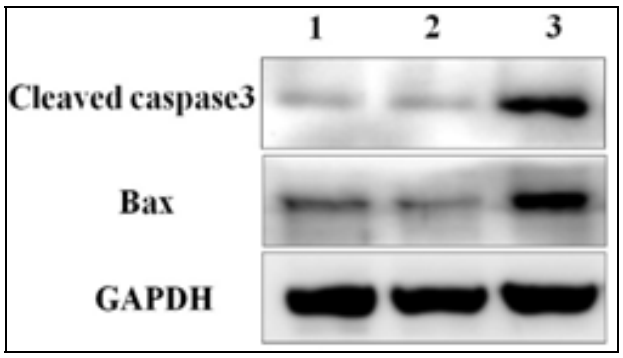

Fig. 6: The expression levels of the apoptosis signaling proteins cleaved caspase 3 and Bax examined using Western blot.

Caspase3 and bax activation contributes to cell apoptosis

Apoptosis-inducing proteinscleaved-caspase3 and Bax activation were detected in MCF-7 cells using Western blot at 48h after transfection. They were both increased in response to the down regulation of survivin (fig. 6). This showed that the new survivin siRNA promoted cell apoptosis via caspase 3 and Bax.

DISCUSSION

RNA interference is emerging as a promising strategy for cancer treatment (Wang Rao *et al*. 2014, Zhu *et al*. 2013). In recent years, several siRNA sequences targeting survivin have been studied (Karami *et al*. 2013, Zhang *et al*. 2010, Miao Lu and Zhang 2007). Although targeting survivin by siRNA has shown some promise in cancer, the potency of siRNA needs further improvement (Wiedemuth *et al*. 2014).

The present study evaluated a novel siRNA sequence targeting survivin. We have shown that it has remarkable silencing efficiency the levels of mRNA and protein. In addition, the siRNA exerted a significant effect on cell proliferation and cell cycle by inducing the G2/M phase arrest. Mean while, it was shown that cell apoptosis were also regulated by signaling pathways, especially through caspase 3 and Bax. The expression levels of caspase 3 and

Bax in cancer cells will increase when the cells were treated with siRNA. The results of this study suggest that the novel siRNA sequence has high potency and warrants further evaluation as a breast cancer therapeutic agent.

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