Inhibition Effect of shRNA on VEGF-C in Breast Cancer Cells

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CLC number: R737.9 Document code: A Article ID: 1000-9604(2009)03-0202-05
DOI: 10.1007/s11670-009-0202-9

ABSTRACT

Objective: To investigate the effect of RNA interfering on VEGF-C in MDA-MB-231 cells.

Methods: Three small interfering RNAs (siRNAa, siRNAb, siRNAc) were prepared. The most efficient one was screened and short hairpin (shRNA) was designed, the recombinant plasmid pGenesil-1/VEGF-C was constructed, and transfected into MDA-MB-231 cells by Lipofectamine TM 2000. RT-PCR, Western-blot an immunohistochemical methods were performed to detect the expression of VEGF-C.

Results: RT-PCR results showed that siRNAa, siRNAb, siRNAc could inhibit the growth of MDA-MB-231 cells, among which, siRNAa was the most significant, with an inhibition rate of 72.1%. The recombinant plasmid pGenesil-1/VEGF-C was successfully constructed using shRNA and pGenesil-1. VEGF-C expression was significantly inhibited as determined by RT-PCR, immunocytochemistry staining and Western blot (P<0.05).

Conclusion: shRNA RNAi technology could silence the expression of VEGF-C in MDA-MB-231 cells, which suggested that the technology may be one of the effective methods for inhibiting lymphangiogenesis in breast cancer.

Key words: siRNA; shRNA; MDA-MB-231 Cells; VEGF-C; pGenesil-1

INTRODUCTION

Lymphatic metastasis is an important way in breast cancer spread. Studies showed that vascular endothelia growth factor-C (VEGF-C) and its receptor VEGFR-3 signaling pathway may be involved in lymphangiogenesis and lymphatic metastasis. Up-regulation of VEGF-C was considered as an initiating factor of tumor lymphangiogenesis[1]. High expression of VEGF-C could promote lymphatic metastasis. Its mechanism was that VEGF-C induced the growth of lymphatic vessel around the tumor and the mesenchymal, as well as hyperplasia of lymphatic vessel in the tumor[2−4]. In our study, short hairpin RNA (shRNA) against VEGF-C was designed and transfected into breast cancer cells MDA-MB-231 by liposome. We observed the inhibition of shRNA on VEGF-C, which may provide a new theory for further clinical application.

Received: Jan. 15, 2009; **Accepted:** Apr. 16, 2009
This work was supported by a grant from the Key Project of Chongqing Science and Technology Committee (No.2005AC0076).
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MATERIALS AND METHODS

Design of VEGF-C siRNA

Human VEGF-C gene sequence is from Genbank (GI: 4885653). A few siRNAs were designed and screened with software Blast Research. Three siRNA sequences were chosen and a random sequence was set up as a control. The sequences were as following: siRNAa sense: CGA CAA ACA CCU UCU UUA ATT, antisense: UUA AAG AAG GUG UUU GUC GCG; siRNAb sense: GAC GUU AUU UGA AAU UAC ATT, antisense: UGU AAU UUC AAA UAA CGU CTT; siRNAc sense: CCA AUU ACA UGU GGA AUA ATT, antisense: UUA UUC CAC AUG UAA UUG GTG; siRNA negative sense: UUC UCC GAA CGU GUC ACG UTT, antisense: ACG UGA CAC GUU

CGG AGA ATT.

Cell Culture and Transfection

Breast cancer cells MDA-MB-231 were cultured in RPMI-1640 supplemented 10% fetal bovine serum, 2 mmol/L glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. All cells were maintained at 37°C in an incubator containing 5% CO_2. For transfection, cells were grown to approximately 80% confluence and transfected with siRNA by 30 μl of Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions.

Screening of siRNA (RT-PCR)

Total RNA was isolated from cells using Biozol (from BioFlux com) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into single-stranded cDNA according to the manufacturer's instructions (Takara). Amplification of growth factor RDNA and β-actin RNA as an internal control was carried out by PCR with the following primer pairs for VEGF-C, 5-CTT CTT CTC TGT GGC GTG TTG T-3, 5-GTC TCT TCT GCT CTT GAG TTG AGG-3, and for β-actin, 5-CAC CAC ACC TCC TAC AAT GAG-3, 5-GTG ATC TCC TCC TGC ATC CTG-3. The reaction mixture was first denatured at 95°C for 5 min and went through 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min and followed by 72°C for 10 min. The lengths of the amplification products were 303 bp and 717 bp respectively. The PCR products were visualized by ethidium bromide staining after 2% agarose gel electrophoresis, then a semi-quantitative analysis was carried on with Quantity One software.

Construction of the Recombinant Plasmid

A pair of complementary VEGF-C shRNA was synthesized according to the design principle. Its two terminals had BamHI and HindIII (the solid line designated), in order to connect with pGenesil. Meanwhile, for excision and determination, we designed SalI after transcriptional termination signal (the dotted line in the following sequence). The sequences are as following: sense, 5'-GAT CCG CGA CAA ACA CCT TCT TTA ATT CAA GAC GTT AAA GAA GGT GTT TGT CGT TTT TTG TCG ACA-3', antisense, 5-AGC TTG TCG ACA AAA AAC GAC AAA CAC CTT CTT TAA CGT CTT GAA TTA AAG AAG GTG TTT GTC GCG -3'. We inserted the shRNA into BamHI and HindIII sites of vector pGenesil-1. The constructed recombinant plasmids were confirmed by DNA sequencing and named pGenesil-1/VEGF-C.

Transfection of Recombinant Plasmid

pGenesil-1 and pGenesil-1/VEGF-C were transfected into MDA-MB-231 cells by Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions, and maintained in an incubator containing 5% CO_2 at 37°C for 48 h. RT-PCR were performed to detect the VEGF-C mRNA expression. Immunocyto-chemistry and Western blot method were performed to detect the VEGF-C protein expression. We used Image-Pro Plus 6.0 image analysis software to analyze the results of immunocytochemistry and protein expression of VEGF-C. The average optical density (AOD) = cumulative optical density (IOD) /the total area (Area).

RT-PCR and Western blot were semi-quantitative analyzed by using Quantity One software.

Statistical Analysis

All the data were presented as mean value±standardized deviate ($\bar{x}\pm s$) and homogeneity test for variance was checked with SPSS13.0 software. Comparison of groups was adopted by ANOVA with SNK-q test for post hoc analysis. Thresholded P value was set at a P value of <0.05.

RESULTS

Determination and Screening of the Most Effective siRNA by RT-PCR

There was a clear band at 303 bp in the blank control, liposome and siRNA negative groups. But, in the siRNA a, b, c group, the band became weaker, among which, siRNAa was the most significant (Figure 1). The relative density value was analyzed by Quantity One Software, the ratio of siRNAa was 0.243±0.018, the inhibition rate was 72.41%, there was significant difference compared with other groups (Table 1).

Expression of VEGF-C in the Transfected MDA-MB- 231 Cells

The production of RT-PCR was getting weaker in the siRNA transfected group than that in the empty plasmid and blank control group. The