

Down-Regulation of CXCR4 Expression by siRNA Inhibits Invasive Ability of Breast Cancer Cells

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OBJECTIVE To investigate the efficiency of gene silencing by CXCR4-siRNAs (small interfering RNA), and to examine the invasive ability and the expression of other metastatic-associated genes in siRNA-treated breast cancer cells.

METHODS Three siRNAs were designed and cloned into the pSilencTM 3.1-H1 neo vector. The reconstructed plasmids were purified and transfected into the T47D breast cancer cell line, which highly expressed CXCR4. The amount of CXCR4 expression in the transfected cells was measured by flow cytometry and Real-time PCR. Cell invasive ability was evaluated using 24-well Matrigel invasion chambers. In addition, the expression of other metastatic-associated genes, such as E-cad, IGFBP-5, FN and MMP-2, was assessed by Real-time PCR.

RESULTS The suppression rates of CXCR4 mRNA expression reached 95.7%, 85.9% and 98.3% compared with control-siRNA cells in the 3 CXCR4-siRNA T47D cells respectively. FCM assays for CXCR4 protein expression showed a similar inhibitory effect. The invasion indexes of these CXCR4-siRNA cells were 0.037, 0.290 and 0.188 respectively compared with control-siRNA cells. After treatment of the cells with CXCR4-siRNA, the expression of E-cad showed an upward tendency and that of IGFBP-5 had a downward trend, while alteration in expression of FN and MMP2 varied without a consistent effect.

CONCLUSION CXCR4 plays an important role in modulating migration of human breast cancer cells. Small interfering RNA can significantly silence the CXCR4 gene in the human T47D breast cancer cell line. The results of this study strengthen the need for further research on novel gene therapy against breast cancer metastasis.

KEYWORDS: breast cancer, CXCR4, siRNA, metastasis.

INTRODUCTION

Morbidity and mortality from cancers are mainly determined by organ-specific metastasis and the failure of chemotherapeutic drugs to selectively kill cancer cells at metastatic sites. Metastasis is a non-random process, and each cancer type has its own characteristic metastasis sites^[1]. For instance, breast cancer cells preferentially metastasize to the regional lymph nodes, lungs, liver, and bone. Prostate cancers usually metastasize to bone.

While there has been considerable progress in identifying genes that promote the metastasis of cancer cells, little is known concerning the genes that enable cancer cells to seed, survive, and proliferate at sites of metastasis. Muller and colleagues have shown that metastatic breast cancer cells overexpress the CXCR4 chemokine receptor^[2]. Additionally, sites to which breast cancer

cells metastasize express abundant amounts of a stromal derived factor-1 (SDF-1, recently renamed CXCL12), the ligand for CXCR4. Moreover functional blockade of CXCR4 by antibodies significantly inhibited lymph node and lung metastasis in xenograft models of breast cancer^[2]. Several studies also have reported on the crucial role of CXCR4 in different cancers, such as colorectal cancer, pancreatic cancer, oral squamous cell carcinoma, and osteosarcoma^[3-6]. These results suggest that SDF-1 serves as a homing factor for cancer cells, and that the signaling pathways activated upon interaction of SDF-1 with its exclusive CXCR4 receptor play an important role in the survival and proliferation of cancer cells once they are localized in a specific organ.

RNA interference technology, silencing targeted genes specifically with high efficiency in mammalian cells, has become a powerful tool for studying gene function^[7,8]. In the present study, the human breast cancer cell T47D line with CXCR4 overexpression was selected and transfected with a stable siRNA expressing system. Then, we tested the invasive ability of the transfected T47D cells in vitro. This study was aimed to exploit a novel approach to anti-metastasis.

MATERIALS AND METHODS

Reagents

The PSilencer™ 3.1-H1 neo vector was provided by the Ambion Co.; the reagents for transfection were produced by Eppendorff. G418 and Trizol were obtained from Invitrogen. Recombinant Homo SDF-1 alpha was obtained from PeproTechEC. Anti-CXCR4 McAb labeled with FITC and Matrigel invasion chambers were purchased from BD Biosciences. A Quantiteck SYBR Green PCR Kit and EndoFree Plasmid Maxi kit was provided by Qiagen.

Cell culture

Human breast carcinoma cell lines T47 and MCF-7, obtained from American Type Culture Collection, were cultured in 5% CO₂ at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO), 50 units/ml penicillin, and 50 g/ml streptomycin.

Selection of the CXCR4-overexpressed breast cancer cells with Real-time PCR

The total RNAs of cultured T47D cells and MCF-7 cells were extracted using the Trizol reagent. The quality of RNA was analyzed by electrophoresis. One µg of RNA was used as an initiation template for re-

verse transcription to synthesize cDNA. The PCR was performed by GeneAmp5700 (ABI) using a Real-time Quantitative PCR Kit. The primers for CXCR4 and GAPDH were designed by the OLIGO6.0 software with amplification lengths of 86 bp and 113 bp, respectively (Table1). The PCR reactions conditions were as follows: an initial hot denaturation at 94°C for 15 min and 35 cycles of PCR amplification were performed, with each cycle consisting of a denaturing step of 94°C for 15 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, followed by a final step at 72°C for 10 min. The results were analyzed by SDS software (USA).

Construction of the RNAi vectors

The PSilencer™ 3.1-H1 neo plasmid was linear with BamH I and Hind III overhangs. The 3 siRNAs, CXCR4-siRNA1/2/3 (Table 2), were designed according to the principles of the Ambion Co., synthesized and phosphorylated at 5' terminals by the Augct Co. Templates of siRNAs were annealed and ligated into pSilencer™ 3.1-H1 neo vector under the control of the H1 promoter. These plasmids were transformed into competent cells (E.coli DH 5 alpha Takara). The colony with recombinant plasmids was picked for sequencing. Negative control-siRNA vectors were provided by the kit with limited homology to any known human sequences.

Cell transfection

CXCR4-siRNAs (20 µg) were extracted using an EndoFree Plasmid Maxi kit, and 1×10⁶ cells mixed with 800 µl hypo-osmolar electroporation buffer and electrotransfected in 100 µs employing 1000 V, followed by transfer into 24-well plates. Following incubation for 24 h at 37°C, the cells were treated with G418 (400 µg/ml) for screening.

CXCR4 Real-time PCR

The expression of CXCR4 mRNA in the transfected cells was examined by Real-time PCR (See the above procedure).

FCM assay of CXCR4 expression

The transfected cells were digested with 0.25% trypsin, washed with PBS, and divided into two portions. One portion was added to 10 µl anti-CXCR4 McAb labeled with FITC, and the other was added to mouse FITC IgG2a. Each was incubated approximately 45 min at room temperature in the dark. After washing 2 times with PBS, the cells were resuspended in 500 µl PBS and analyzed by flow cytometry.

Cell proliferation

In vitro cell colony formation was conducted for a cell proliferation assay. A total of 200 transfected cells were plated in 6-well plates, resulting in 20-50 colonies per well after approximately 2 weeks of culture. Colonies that consisted of more than 50 cells were scored. Each experiment was repeated at least 3 times.

Tumor cell invasion assay

The most commonly used in vitro invasion assay is a modified Boyden chamber assay using a basement membrane matrix preparation, Matrigel, as the matrix barrier. The siRNA transfected cells were collected by trypsinization in the usual manner and resuspended in fresh medium at approximately 5×10^4 cells/ml. The cells were seeded into the upper chamber coated with Matrigel. SDF-1 was diluted to 400 ng/ml with RPMI 1640 and added to the bottom chamber. The chambers were incubated 22 h in a humidified incubator. Then the inserts were fixed in methanol and stained with H&E.

Tumor metastasis-associated gene assay

Expression of their tumor metastasis-associated genes, such as E-cad, IGFBP-5, FN and MMP2, was measured by Real-time PCR in the CXCR4-siRNA transfected cells.

Statistical analysis

All statistical significances were determined by the Student's *t* test.

RESULTS

Selection of the CXCR4-overexpressed cell line

Real-time PCR revealed that the expression of CXCR4 mRNA in the T47D cell line was higher than the MCF-7 cells (Fig.1). Therefore, T47D cells were chosen for our research model.

Table1. The sequences of primers.

Gene	Primer sequences	Fragment lengths (bp)
CXCR4	5'-CCTATGCAAGGCAGTCCATGT-3' 5'-GGTAGCGGTCCAGACTGATGA-3'	86
GAPDH	5'-CATGAGAAGTATGACAACAGCCT-3' 5'-AGTCCTTCCACGATACCAAAGT-3'	113

Table 2. The sequences of CXCR4-siRNA.

	Sequence	Changed into siRNA templates
siRNA1	AACCCTGTTTCCGTGAAGAAAAT	5'-GATCCGCCCTGTTTCCGTGAAGAAAATCAAGAGATTTCTTACGGAAA-CAGGGTTTTTGGAAA-3'
siRNA2	AAGGGTGTGAGTTTGAGAACACT	5'-GATCCGGGTGTGAGTTTGAGAACATTCAAGAGATGTTCTCAAACCTCA-CACCCTTTTTGGAAA-3'
siRNA3	AAGCGAGGTGGACATTCATCTGT	5'-GATCCGCGAGGTGGACATTCATCTTTCAAGAGAAGATGAATGTCCACCTC-GCTTTTTTGGAAA-3'

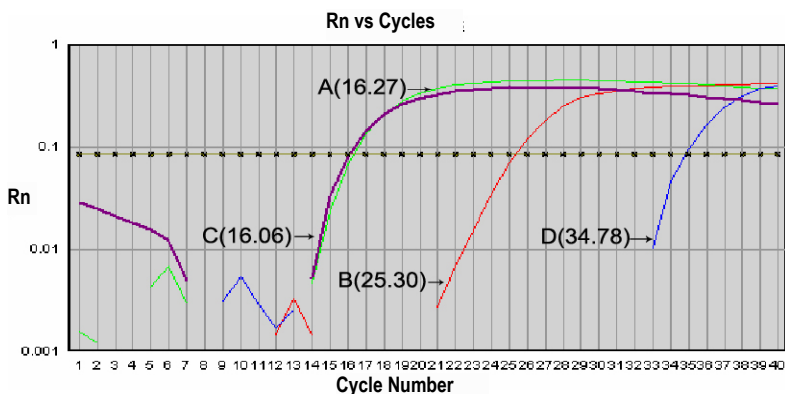


Fig.1. Selection of cells with high CXCR4 expression by Real-time PCR A, GAPDH /T47D; B, CXCR4/T47D; C, GAPDH/MCF-7; D, CXCR4/MCF-7. The values in the parentheses are Ct of amplification.

Cell transfection

The reconstructed CXCR4-siRNA vectors were identified by the restriction enzyme digest (BamH I and Hind III). Agarose gel electrophoresis showed that the location of 60 bp was the insert digested from the recombined plasmids (Fig.2). The T47D cell line was transfected by the siRNAs. After 4 days of G418 selection, the dead cells appeared, and cell colonies were monitored on the tenth day. After 21 days, the biggest cell clone was chosen (data not shown).

Detection of CXCR4 expression

The results of Real-time PCR showed that there was a significant CXCR4 suppression after transfecting siRNA into the T47D cells (Table 3). The suppression rate of the three siRNAs reached 95.7%, 85.9% and 98.3% respectively, compared with control-siRNA. The CXCR4 protein assay by FCM yielded similar results. The three CXCR4-siRNA cells had a positive protein expression rate of 4.9%, 11.1% and 5.5% respectively, whereas the rate in control-siRNA cells was 69.0%. The CXCR4 silence efficiency of siRNA1 and siRNA3 was approximate (Fig.3).

Cell proliferation and invasion assays

There was no significant alteration of cell growth be-

tween the CXCR4-siRNA and control-siRNA cells as determined by the cell-colony formation assay (Fig. 4). For an in vitro model system for invasion, we used a Matrigel invasion chamber. SDF-1 was added to the lower chamber to induce CXCR4-positive breast cancer cell invasion through the Matrigel. Fig. 5 and Table 4 show that the invasive capability of the 3 CXCR4-siRNA cells was significantly reduced compared with the control-siRNA cells ($P < 0.01$), as indicated by their invasion indices of 0.037, 0.290 and 0.188 respectively.

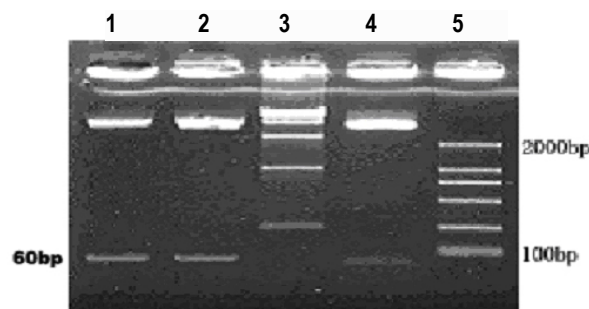


Fig.2. Restriction enzyme digest by BamH I and Hind III 1. siRNA1; 2. siRNA2; 3. Marker DL15000; 4. siRNA3; 5. Marker DL2000.

Table 3. The detection of mRNA of CXCR4 by Real-time PCR.

The type of cells	Ct (CXCR4)	Ct (GAPDH)	ΔCt	$\Delta\Delta Ct$	$2^{-\Delta\Delta Ct}$	Inhibition rate (%)
Control-siRNA	27.29	20.16	7.13	0	1	0
CXCR4-siRNA1	34.32	22.67	11.65	4.52	0.043285	95.7
CXCR4-siRNA2	32.12	22.16	9.96	2.83	0.140632	85.9
CXCR4-siRNA3	35.12	22.15	12.97	5.84	0.017458	98.3

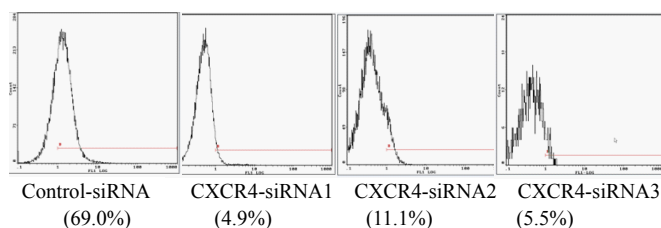


Fig.3. Flow cytometry evaluation of CXCR4 expression.

Table 4. Invasion assay analysis.

Cell type	Invading cell population ($\bar{x} \pm s$)	Invasion index
Control-siRNA	120.00 \pm 7.18*	1.000
CXCR4-siRNA1	4.40 \pm 2.07*	0.037
CXCR4-siRNA2	34.80 \pm 6.69*	0.290
CXCR4-siRNA3	22.60 \pm 3.58*	0.188

*Statistical significance compared with the control. $P < 0.01$ (Student's *t* test).

Expression of tumor metastatic-associated genes

Down regulation of CXCR4 by the 3 CXCR4-siRNAs resulted in enhanced E-cad expression, while

IGFBP-5 expression decreased (Fig.6) as measured by the Real-time PCR assay. Changes in FN and MMP2 expression were not consistent in the CXCR4-siRNA cells.

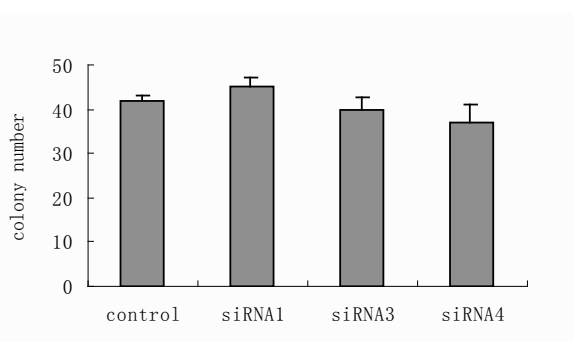


Fig.4. Cell-colony formation assay.

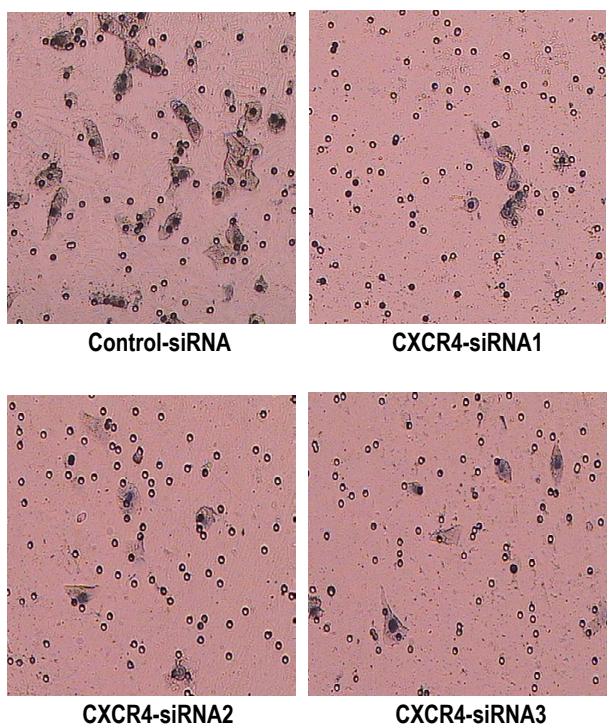


Fig.5. Matrigel invasion-chamber assay (40x).

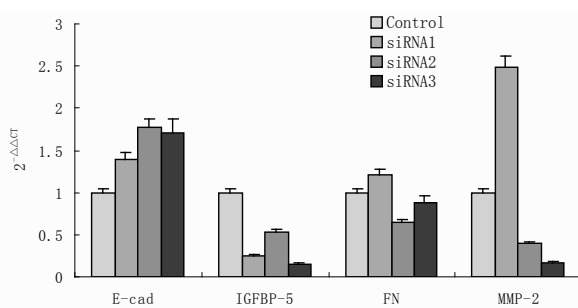


Fig. 6. The expression of tumor metastatic-associated genes.

DISCUSSION

Chemokines are a superfamily of chemotactic polypeptides that induce, through their interaction

with G-protein-coupled receptors, cytoskeletal rearrangement, firm adhesion to endothelial cells and directional migration^[9,10]. Cancer cell migration and metastasis seem to share similarities with chemokine-induced leukocyte trafficking and homing. It has been reported that expression of CXCR4 can be detected in many malignancies, such as prostate cancer^[11], breast cancer and melanoma^[12]. These tumors have similar metastatic patterns in terms of organ selectivity that involve lymph nodes, bone marrow, the lungs and liver. Therefore, CXCR4 and its ligand SDF-1 play a critical role in the organ-selective process of tumor metastasis. Down-regulation of CXCR4 may be useful in interfering with tumor invasion and metastasis.

RNA interference (RNAi) is the biological process of sequence-specific, post-transcriptional gene silencing in nature from prokaryotes to humans, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the target gene^[13]. Tuschl and colleagues^[14] first showed that short RNA duplexes can induce silencing in numerous mammalian cell lines. After that, alternative strategies to RNA delivery, such as plasmid-based or viral vector-based expression of siRNAs or short hairpin RNAs (shRNAs), were developed. These techniques provided the ability to create permanent cell lines with a desired loss-of-function phenotype, thereby extending the utility of RNAi as method for probing gene function in mammalian cells^[15,16]. In the present study we selected pSilencerTM3.1-H1 neo (Ambion Cop.) as a siRNA expression system, by which short hairpin RNAs (shRNAs) could be induced in the cancer cells under the control of the H1 promoter. In addition it incorporated a neo-resistance gene accessible for us to separate the stable-expressed cell line.

In the present study, the siRNAs against CXCR4 were designed according to Whitehead’s designing program online. In order to investigate the efficiency of gene silencing caused by siRNAs, we chose three fragments at different sites of the full sequence of the CXCR4 gene, among which the siRNA1 was located before the reading frame, the siRNA2 was in the reading frame and the siRNA3 was behind the reading frame. As the results show, the expressions of CXCR4 in the T47D cells were decreased significantly by each of the siRNAs, showing the substantial ability of gene knockdown using the RNAi technique.

The most commonly used in vitro invasion assay is a modified Boyden chamber assay using Matrigel as the matrix barrier^[17,18]. Matrigel is a mixture of basement membrane ECM (extracellular matrix) proteins, primarily laminins, and collagen IV, which are isolated from the Englebreth-Holm-Swarm mouse sar-

coma. Matrigel will form a gel that is similar in composition to a natural basement membrane when it is reconstituted in a culture medium. Cells can not freely migrate through it unless they are able to secrete proteases or have amoeboid movement. Our study indicated that after the CXCR4 gene of the T47D cells was silenced, their invasion capability was markedly inhibited, suggesting that CXCR4 plays a crucial role in breast cancer metastasis.

Metastasis is a complicated biological process as the spread and growth of tumor cells to distant organs is attributed to alterations of multiple factors. Metastatic-associated genes such as E-cad, IGFBP-5, FN and MMP-2, recently have become of great interest in this field^[19-22]. It has been accepted that lower expression of E-cad and higher expression of IGFBP-5, FN and MMP-2 might enforce the capacity of malignant metastasis. The results of our study demonstrated that the expression of E-cad in CXCR4-siRNA cells showed an upward tendency, and that expression of IGFBP-5 had a downward trend. Thus, we suggest that changes in E-cad and IGFBP-5 expression are induced by down-regulation of CXCR4, resulting in the deminished invasiveness of the cancer cells.

In conclusion, we have shown that siRNA can powerfully silence the CXCR4 gene in the human T47D breast cancer cell line, resulting in significant suppression of invasive ability of these cells. The alterations of expression of E-cad and IGFBP-5 were likely to be induced by the down-regulation of CXCR4 with consequent synergy affecting the invasiveness of the cancer cells. The correlation of CXCR4 expression with the metastatic-associated genes needs further study. Use of small interfering RNAs may provide us with a novel and powerful gene-silencing approach for future tumor gene therapy.

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