Inhibition of $C35$ gene expression by small interfering RNA induces apoptosis of breast cancer cells

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Summary C35 was reported to be a new biomarker and therapeutic target for breast cancer. To explore the functional importance of C35, we constructed small interfering RNA (siRNA) targeting $C35$ and investigated the effects of the siRNAs on $C35$ expression and apoptosis of T47D cells. $C35$ siRNAs were constructed and named psiRNA-$C35$-1 and psiRNA-$C35$-2. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blots were used to detect the effects of the siRNAs on mRNA and protein expression of $C35$ in T47D cells. The effects of the two siRNAs on apoptosis of T47D cells were detected by flow cytometry and terminal dUTP nicked-end labelling assays. Also, the apoptosis related molecule caspase-3 was detected using Western blots. The psiRNA-$C35$-1 and psiRNA-$C35$-2 siRNAs were verified by both EcoR I/Hind III digestion analysis and automated DNA sequencing. RT-PCR and Western blots showed that $C35$ mRNA and protein expression in T47D cells were obviously inhibited after psiRNA-$C35$-1 and psiRNA-$C35$-2 transfection. Flow cytometry and terminal dUTP nicked-end labelling assays showed that apoptosis of T47D cells was significantly induced after transfection with psiRNA-$C35$-1 and psiRNA-$C35$-2 ($p < 0.05$). Also, caspase-3 expression in the psiRNA-$C35$-1 and psiRNA-$C35$-2 transfected cells was obviously higher than that of the Lipofectamine and pTZU6+1 transfected cells. This study showed that apoptosis of T47D cells can be significantly induced by inhibiting $C35$ expression using siRNAs, which may be caused by activating caspase-3. $C35$ might play an important role in apoptosis of breast cancer cells, and therapeutic strategies targeting $C35$ may be useful for breast cancer treatment.

Keywords: $C35$, apoptosis, small interfering RNA (siRNA), breast cancer

1. Introduction

Breast cancer is the most common cancer among women except for non-melanoma skin cancer and is the second leading cause of cancer-related deaths in women today (1). Although early diagnosis approaches and proper management, including various options of evidence-based treatment have not only reduced mortality but also enhanced patients' quality of life, the mortality rate due to breast cancer in the world has continued to increase, and the number of patients is also increasing rapidly (2). Identification of phenotypic and genetic changes that are conserved throughout breast cancer disease progression may help to illuminate key events necessary to transform healthy cells, maintain malignancy, and facilitate development of metastases (3).

$C35$, a newly discovered gene, is located on chromosome 17q12 adjacent to the oncogene that encodes human epidermal growth factor receptor 2 (HER2/neu) (4). The $C35$ gene encodes a small 115 amino acid protein with a molecular weight of ~ 12 kDa that has no sequence similarity with any known genes or proteins, and its functional importance is presently unknown (3,6). Immunohistochemical studies in breast cancer lumpectomy samples showed that the $C35$ gene was over-expressed in more than 60% of breast cancer tissues, and was not evident in any normal tissues in

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women (5). They also confirmed the overexpression of C35 in HER2/neu-negative breast cancer patients and breast tumor cell lines, suggesting independent transcriptional control mechanisms for C35 (5). These studies suggested that C35 protein might be an important indicator of breast cancer. However, to establish C35 as a biomarker and therapeutic target for breast cancer treatment, it is of the utmost importance to explore the function of this novel protein (7).

RNA interference (RNAi) is a potent gene silencing mechanism conserved in most eukaryotic organisms for RNA-guided regulation of gene expression, in which double stranded ribonucleic acid inhibits the expression of specific genes with complementary nucleotide sequences (8). RNAi has been applied for functional genomic studies in a variety of areas, including cancer research, by facilitating a better understanding of the mechanisms that underlie tumorigenicity and the identification of novel factors that either promote or inhibit oncogenic transformation (9). RNAi mediated by small interfering RNAs (siRNAs) is a powerful technology allowing the silencing of mammalian genes with great specificity and potency (10,11). In this study, to explore the functional importance of C35, we constructed siRNAs targeting C35 and investigated the effects of siRNA constructs on C35 expression, as well as apoptosis of T47D cells.

2. Materials and Methods

2.1. Materials

Plasmid pTZU6+1 was received as a gift from Dr. David Engelke, University of Michigan, Ann Arbor, MI, USA. RNA oligonucleotides were synthesized by Biological Engineering Co., Shanghai, China. Human breast cancer cell line T47D, was obtained from Institute of Basic Medicine, Shandong Academy of Medical Sciences, Jì’nan, China. Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences, San Jose, CA, USA. TdT-mediated dUTP-biotin nick end labeling (TUNEL) Apoptosis Detection Kit was purchased from Roche, Madison, WI, USA. Rabbit anti-human C35 monoclonal antibody was purchased from Invitrogen, Grand Island, NY, USA.

2.2. Cells and cell culture conditions

T47D cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% of fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 U/mL of streptomycin in a humidified atmosphere with 5% CO2 at 37°C. For maintenance and subculture, T47D cells in exponential phase were treated with 0.25% trypsin solution containing 0.02% EDTA. After reaching 80% confluence, the cells were collected.

2.3. siRNAs

Two pairs of oligonucleotides targeting two different regions of C35 were designed according to C35 sequence in GenBank (NM_032339) following the rules of Tuschl T (12). The two regions within the C35 gene were as follows: siRNA-1 (position 259-278), sense: 5'-TCTGAGATCTCATGAGGCCATCTTCG GATGCCCTCAATGAGATCTTCTTTT-3'; antisense: 5'- CTAGAAAAAGATCTCATGAGGCCATCCGAA GATGCCCTCAATGAGATCTCTC-3'; siRNA-2 (position 282-300), sense: 5'-TGAGGAGCCAGTAATGGAG AACCTTCGGTTTCTCCATTACTGGCTCTTTTTT-3'; antisense: 5'-CTAGAAAAAGAGCCAGTAATGGAG AAACCGAGTTTTCTCCATTACTGGCTCC-3'. The underlined parts were the Xba I and Xho I digestion sites and the stem-loop structures. The four oligodeoxyribonucleotides encoding two siRNAs were dissolved in annealing buffer, 95°C for 5 min, and cooled gradually to room temperature to anneal.

2.4. Construction

siRNA expression vectors were prepared using the pTZU6+1 vector. The pTZU6+1 was digested with Sal I and Xba I and then ligated with the annealed oligodeoxyribonucleotides, yielding the pTZU6+1-C35 constructs, named, psiRNA-C35-1 and psiRNA-C35-2. Ligation mixtures were then transformed into E. coli DH5α. Ampicillin-resistant clones were picked and expanded. The recombinant plasmids were extracted according to the manufacturer’s protocol of QIAGEN EndoFree plasmid Maxi Kit (Qiagen, Hilden, Germany). After double digestion with EcoRI and Hind III, the fragments were verified using 1% agarose gel analysis. The constructed plasmids were also confirmed by DNA sequencing (Biological Engineering Co., Shanghai, China).

2.5. In vitro transfection

On the day before transfection, cells were collected and reseeded in 6-well plates at a density of 4 × 10^4/mL in serum-free DMEM medium. After 24 h, the cells were transfected using Lipofectamine™ 2000 according to previous studies (11). In brief, the cells were incubated with a mixture of 4 μg of DNA (pTZU6+1 control, psiRNA-C35-1, or psiRNA-C35-1) and diluted with Lipofectamine 2000 reagent, or only Lipofectamine 2000 for 4 h at 37°C. Transfection medium was then replaced with DMEM medium including 10% FBS and incubated for 48 h at 37°C. The transfection rates were measured using fluorescence microscopy.

2.6. Determination of C35 mRNA expression levels by reverse transcription-polymerase chain reaction (RT-PCR)
Semi-quantitative RT-PCR was used to detect the effects of psiRNA-C35-1 and psiRNA-C35-2 on the expression of C35 mRNA (13). Briefly, 72 h after transfection, total RNA was isolated using TRIZOL reagents according to the manufacturer’s protocol. Isolated total RNA was first reverse transcribed into cDNA using random primers and SuperScript™ II reverse transcriptase. Then cDNA was used as templates for amplification in PCR. Sequences of primers were as follows: for C35, sense, 5’-CGGAATTCATGAGCGGGGAGGAG-3’; antisense, 5’-GTGACGGCTCTTTTTACAACGTCGTGACTGGTTTTGCTACGACCTGCAGGCATGCAAGCTTGG-3’. For Western blots, 72 h after transfection, cells were lysed with RIPA buffer for 30 min at 4°C, and debris was removed by centrifugation at 20,000 × g for 10 min. Equal amounts of protein (20 μg) in the cell extracts were fractionated on 10% sodium dodecyl sulfate polyacrylamide gels followed by transfer to polyvinylidene difluoride membranes. Membranes were incubated for 1 h in 5% skim milk in PBS with monoclonal antibodies (C35: 1:1,000, capase-3: 1:1,000, or β-actin: 1:2,000) followed by incubation with secondary antibodies (1:4,000) for 1 h. Proteins were visualized with enhanced chemiluminescence using the ECL Western Blotting Starter Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and photographed with a Photometrics Cool Snap ES camera.

2.7. Determination of C35 protein expression levels by Western blots

For Western blots, 72 h after transfection, cells were lysed with RIPA buffer for 30 min at 4°C, and debris was removed by centrifugation at 20,000 × g for 10 min. Equal amounts of protein (20 μg) in the cell extracts were fractionated on 10% sodium dodecyl sulfate polyacrylamide gels followed by transfer to polyvinylidene difluoride membranes. Membranes were incubated for 1 h in 5% skim milk in PBS with monoclonal antibodies (C35: 1:1,000, capase-3: 1:1,000, or β-actin: 1:2,000) followed by incubation with secondary antibodies (1:4,000) for 1 h. Proteins were visualized with enhanced chemiluminescence using the ECL Western Blotting Starter Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

2.8. Apoptosis assays

Apoptosis was quantified by detecting surface exposure of phosphatidylserine in cells using an Annexin V-FITC kit (14). Briefly, after transfection for 72 h, cells were stained with 3 mL of Annexin V and 1 mL of propidium iodide (PI; 1 mg/mL) in 100 mL of Annexin V binding buffer (10 mM HEPES, pH 7.4, containing 140 mM NaCl and 5 mM CaCl2) and incubated for 15 min at room temperature in the dark. Subsequently, 400 mL of binding buffer was added and mixed gently. Cells were then observed using flow cytometry.

TUNEL assay was used to observe the cell morphology in culture. Briefly, cells (fixed in 4% paraformaldehyde for 20 min) were blocked with 3% H2O2 in methanol for 10 min at room temperature and permeabilized in ice-cold 0.1% Triton X-100, 0.1% sodium citrate for 2 min. TUNEL reaction mix was added and incubated for 1 h at 37°C in the dark under humidified conditions. TUNEL-Peroxidase (POD) converter was added and incubated for an additional 30 min in the dark under humidified conditions. Cells were then visualized with a Nikon Eclipse E600 microscope and photographed with a Photometrics Cool Snap ES camera.

3. Results

3.1. Construction of psiRNA-C35

The recombinant plasmids, named psiRNA-C35-1 and psiRNA-C35-2, were purified from transformed E. coli, and verified by EcoRI/HindIII digestion analysis and automated DNA sequencing. Recombinant psiRNA-C35 vectors which contain the siRNA coding sequences should yield new 400-bp fragments after double digestion with EcoRI/HindIII. This was verified by agarose gel analysis. As shown in Figure 1, the positive psiRNA-C35 constructs can yield new 400-bp fragments, while the negative control vectors can only yield 350-bp fragments. The inserted sequences in psiRNA-C35-1 and psiRNA-C35-2 were verified by automated DNA sequencing. The sequences were: psiRNA-C35-1, 5’-CGGAACACCGTCTCAGAGATCTTACATTGAGGCCATCTTCGAGTGCCTCAAT GAGATCCTTTTTTCATAGAGCGGACTTCGGTCCGC TTGGACTAGGACCCTGCAGGACTTCGGTCCG TAATGAGGCGCATGCAAGCTTGG-3’; psiRNA-C35-2, 5’-TCTTGGCTTATATATATCCTTGGAAAGGACGCAACCGTCTGAGAGATCTTACATTGAGGCCATCTTCGAGTGCCTCAAT GAGATCCTTTTTTCATAGAGCGGACTTCGGTCCGC TTGGACTAGGACCCTGCAGGACTTCGGTCCG TAATGAGGCGCATGCAAGCTTGG-3’; psiRNA-C35-3, 5’-CTTGGCTTATATATATCCTTGGAAAGGACGCAACCGTCTGAGAGATCTTACATTGAGGCCATCTTCGAGTGCCTCAAT GAGATCCTTTTTTCATAGAGCGGACTTCGGTCCGC TTGGACTAGGACCCTGCAGGACTTCGGTCCG TAATGAGGCGCATGCAAGCTTGG-3’; psiRNA-C35-4, 5’-CTTGGCTTATATATATCCTTGGAAAGGACGCAACCGTCTGAGAGATCTTACATTGAGGCCATCTTCGAGTGCCTCAAT GAGATCCTTTTTTCATAGAGCGGACTTCGGTCCGC TTGGACTAGGACCCTGCAGGACTTCGGTCCG TAATGAGGCGCATGCAAGCTTGG-3’; psiRNA-C35-5, 5’-CTTGGCTTATATATATCCTTGGAAAGGACGCAACCGTCTGAGAGATCTTACATTGAGGCCATCTTCGAGTGCCTCAAT GAGATCCTTTTTTCATAGAGCGGACTTCGGTCCGC TTGGACTAGGACCCTGCAGGACTTCGGTCCG TAATGAGGCGCATGCAAGCTTGG-3’; psiRNA-C35-6, 5’-CTTGGCTTATATATATCCTTGGAAAGGACGCAACCGTCTGAGAGATCTTACATTGAGGCCATCTTCGAGTGCCTCAAT GAGATCCTTTTTTCATAGAGCGGACTTCGGTCCGC TTGGACTAGGACCCTGCAGGACTTCGGTCCG TAATGAGGCGCATGCAAGCTTGG-3’; psiRNA-C35-7, 5’-CTTGGCTTATATATATCCTTGGAAAGGACGCAACCGTCTGAGAGATCTTACATTGAGGCCATCTTCGAGTGCCTCAAT GAGATCCTTTTTTCATAGAGCGGACTTCGGTCCGC TTGGACTAGGACCCTGCAGGACTTCGGTCCG TAATGAGGCGCATGCAAGCTTGG-3’.

Figure 1. Restriction enzyme digestion analysis of recombinant plasmids psiRNA-C35-1, psiRNA-C35-2, and pTZU6+1 control. 1, Marker; 2, pTZU6+1; 3, pTZU6+1 with EcoRI and HindIII; 4, psiRNA-C35-1; 5, psiRNA-C35-1 with EcoRI and HindIII; 6, psiRNA-C35-2; 7, psiRNA-C35-2 with EcoRI and HindIII.

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CACTGGCCGTCGTTCCTACGCTGACTGG
GAAACACCTGCGGTACCAACTTAACTC-3'. The
underlined parts are where the DNA sequences were
inserted. These results showed that both psiRNA-C35-1
and psiRNA-C35-2 were successfully constructed.

3.2. Effects of psiRNA-C35 constructs on C35 mRNA
expression in T47D cells

The effects of psiRNA-C35-1 and psiRNA-C35-2 on
the expression of C35 mRNA were detected by semi-
quantitative RT-PCR. As shown in Figure 2, the bands
of pTZU6+1 and Lipofectamine-transfected cells were
much more obvious than that of the psiRNA-C35-1
and psiRNA-C35-2-transfected cells. Normalized C35
mRNA levels of T47D cells transfected with pTZU6+1,
psiRNA-C35-1, and psiRNA-C35-2 were 95.3%,
40.0%, and 28.4%, respectively, as compared with
that of T47D cells transfected with Lipofectamine
(100.0%). This result suggested that siRNAs expressed
by psiRNA-C35-1 and psiRNA-C35-2 could effectively
inhibit C35 protein expression. Additionally, the effect
of psiRNA-C35-2 on C35 mRNA expression was more
obvious than that of psiRNA-C35-1.

3.3. Effects of psiRNA-C35 constructs on C35 protein
expression in T47D cells

Effects of psiRNA-C35-1 and psiRNA-C35-2 on
expression of C35 protein were detected by Western
blots. As shown in Figure 3, the bands of pTZU6+1
and Lipofectamine-transfected cells were much more
obvious than that of the psiRNA-C35-1 and psiRNA-
C35-2-transfected cells. Normalized C35 protein
levels of T47D cells transfected with pTZU6+1,
psiRNA-C35-1, and psiRNA-C35-2 were 94.9%,
29.6%, and 32.2%, respectively, as compared with
that of T47D cells transfected with Lipofectamine
(100.0%). This result suggested that siRNAs expressed
by psiRNA-C35-1 and psiRNA-C35-2 could effectively
inhibit C35 protein expression. Additionally, the effect
of psiRNA-C35-2 on C35 protein expression was more
obvious than that of psiRNA-C35-1.

3.4. Apoptosis assays

To evaluate the impact of silenced C35 expression
on apoptosis of T47D cells, apoptosis rates were detected
using an Annexin V-FITC kit. The result showed that
the apoptosis rates of the Lipofectamine, pTZU6+1,
psiRNA-C35-1, and psiRNA-C35-2 transfected cells
were 18.5%, 21.6%, 33.7%, and 44.9%, respectively.
The apoptosis rates of the psiRNA-C35-1 and
psiRNA-C35-2 transfected cells were significantly
higher than that of the Lipofectamine and pTZU6+1
transfected cells ($p < 0.05$) (Figure 4). The apoptosis
of T47D cells might be induced by the decreased
expression of C35 mRNA and protein.

Cell morphology in culture was observed using a
TUNEL assay. In the Lipofectamine and pTZU6+1
transfected cells, most of the cells with normal
morphology tightly attached to the culture dish,
and only a few cells were stained in the nucleus
or weakly in the cytoplasm. In the psiRNA-C35-1
and psiRNA-C35-2 transfected cells, concentrated
and brown nucleus staining and lightly distributed
cytoplasm staining was observed (Figure 5). This
result showed that apoptotic cells in the psiRNA-C35-1
and psiRNA-C35-2 transfected groups were much
greater than that of the Lipofectamine and pTZU6+1
transfected groups.

3.5. Effect of psiRNA-C35 constructs on caspase-3
expression in T47D cells

To investigate the mechanism by which siRNAs
expressed by psiRNA-C35 induces apoptosis of T47D
cells, caspase activity was assessed by measuring
the level of caspase-3 using Western blots. The
results showed that expression of caspase-3 in the
psiRNA-C35-1 and psiRNA-C35-2 transfected cells
was much higher than that of the Lipofectamine
and pTZU6+1 transfected cells (Figure 6). Also,
the expression of caspase-3 in the psiRNA-C35-2
transfected cells was higher than that of the
psiRNA-C35-1 transfected cells. The result suggested
that siRNA expressed by psiRNA-C35 might induce
apoptosis of T47D cells by activating caspase-3.
Further studies are needed to investigate the effect
of psiRNA-C35, especially psiRNA-C35-2, on the

A B C D
C35
β-actin

Figure 2. Semi-quantitative RT-PCR of C35 and β-actin
expression in T47D cells transfected with a Lipofectamine
control, pTZU6+1, psiRNA-C35-1, and psiRNA-C35-2. A,
Lipofectamine control; B, pTZU6+1; C, psiRNA-C35-1; D,
psiRNA-C35-2.

A B C D
C35
β-actin

Figure 3. Western blot analysis of C35 expression in
T47D cells infected with a Lipofectamine control,
pTZU6+1, psiRNA-C35-1, and psiRNA-C35-2. A,
Lipofectamine control; B, pTZU6+1; C, psiRNA-C35-1; D,
psiRNA-C35-2.
expression of other apoptosis related molecules.

4. Discussion

Breast cancer has emerged as the most frequent malignant neoplasm in the world in recent years, raising awareness in society of the issue of breast cancer. Identification of cancer-specific biomarkers has enormous potential to enhance detection, treatment, and prognosis of breast cancer (15). In addition, understanding the role of such biomarkers in the process of transformation could reveal opportunities to target cancer-specific proteins therapeutically and increase treatment options for breast cancer.

C35, a newly reported biomarker for breast cancer, was found in overabundance in more than 60% of breast cancer cases (5). The gene is closely linked with a previously identified breast cancer gene, HER2 (also spelled HER2/neu). HER2 is a gene on the surface of cells that, when functioning normally, has been found to be a key component in regulating cell growth (16,17). It was shown that 34% of breast cancer patients were found to have an overabundance of both C35 and HER2, while 31% tested positive for C35 and negative for HER2. Interestingly, all of the breast cancer patients who over-expressed HER2 also over-expressed C35 (5). Overexpression of C35 in HER2/neu-negative breast cancer patients and breast tumor cell lines was confirmed, suggesting independent transcriptional control mechanisms for C35 (5). Thus, C35 might be a more effective biomarker for breast cancer, while the functional importance is presently unknown.

To explore the functional importance of C35, we constructed siRNAs targeting C35 in this study. Due to its high efficiency and specificity, RNAi is now being widely used as a method to knockdown target genes, to study gene function or to be used in experimental treatment of some diseases (18,19). One problem in using siRNA to knockdown gene expression is target sequence selection. siRNAs that target different sites of the same gene can vary from strong to no inhibition of gene expression. In the present study, two siRNAs targeting different regions of the C35 gene were chosen and their ability to silence the expression of the C35
that apoptosis was significantly induced when C35 expression was silenced. These results suggested that inhibiting C35 expression by siRNAs could significantly induce apoptosis of breast cancer cells. Since induction of apoptosis stimulates a cascade of events that ultimately leads to cell death, caspases are the main executioners of Fas-mediated apoptosis, irrespective of the ceramide signalling pathway (20,21). Caspase-3 is thought to be a key apoptotic "executioner" enzyme in mammalian cells because its activation triggers the cascade of enzymatic events that culminates in the death of the cells (22). Thus, we detected caspase activity by measuring the level of caspase-3 using Western blots in this study. The result showed that psiRNA-C35 might induce apoptosis of T47D cells via activating caspase-3, which will be confirmed by further studies.

In conclusion, this study showed that apoptosis of T47D cells can be significantly induced by inhibiting C35 expression using siRNAs, which may be caused by activating caspase-3. C35 might play an important role in apoptosis of breast cancer cells, and therapeutic strategies targeting C35 may be useful for breast cancer treatment.

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