

Knockdown of Dicer in MCF-7 human breast carcinoma cells results in G1 arrest and increased sensitivity to cisplatin

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Abstract. Aberrant expression of microRNAs (miRNAs) in various human cancers suggests a role for miRNAs in tumorigenesis. Dicer is an essential component of the miRNA machinery, which mediates the processing of miRNAs. However, little is known about the role of Dicer in tumor proliferation and drug resistance. In this study, we found that knockdown of Dicer by siRNA led to significant G1 arrest and increased sensitivity to the DNA damaging agent, cisplatin, in breast cancer cell line MCF-7. Moreover, we found down-regulation of miR-21, a well-recognized miRNA frequently involved in a wide variety of cancers and up-regulation of cell cycle-dependent kinase inhibitor (CKI) p21 and p27. These data demonstrate that knockdown of Dicer inhibits human breast carcinoma cell growth and suggests a promising combination of anti-Dicer strategy and traditional chemotherapy to improve cancer treatment efficiency.

Introduction

MicroRNAs (miRNAs) are ~22-nucleotide non-coding RNA. A recent study demonstrates that aberrant expression of miRNAs in various human cancers plays a role in tumorigenesis (1-4). Dicer is an essential member of the RNase III family which controls maturation of miRNAs in the cytoplasm from microRNA precursors (pre-miRNAs) (5). Mature miRNAs then possess the ability to inhibit their targeting genes (6).

Up-regulation of Dicer in prostate cancer (7) and lung adenocarcinoma (8) have been reported. As the upstream modulator of miRNAs, Dicer up-regulation may result in a global increase of miRNA expression in cancer. However, it remains largely unclear as to the effect of Dicer depletion on chemosensitivity and proliferation of cancer cells. Breast cancer is one of the most common malignant tumors in women and severely threatens their health and life. It is reported that aberrant expression of miRNAs plays important roles in tumor formation, progression and chemosensitivity (4). It is thus of significance to explore the potential therapeutic effects of Dicer depletion in breast cancer cells.

In our present study, we found that the knockdown of expression of Dicer by RNA interference in MCF-7 cells induced changes in cell cycle and chemosensitivity to cisplatin and these changes correlated with the down-regulation of particular miRNAs. Considering the fundamental and multiple biological roles of Dicer in various cellular processes, our results suggest modulation of Dicer in breast cancer might become a promising therapeutic candidate for further clinical application.

Materials and methods

Cell culture. Human breast carcinoma cell line, MCF-7, was cultured in Minimum Eagle Medium (MEM, Gibco Life Technologies) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified incubator with 5% CO₂ at 37°C.

Transfection. FAM-labeled siRNA targeting Dicer and mock siRNA as negative control were chemically synthesized (Invitrogen) and stored at a concentration of 20 µM before use. Sequence information used in this study is provided below:

i) Dicer siRNA: siDicer-top: 5'-CAGUGUUAGUUGUC CGGUdTdT-3'; siDicer-bottom 5'-ACCGGCACAACUA ACACUGdTdT-3'; ii) Mock siRNA: simock-top 5'-ACGUG CACCGUUCGAGAAAdTdT-3'; simock-bottom: 5'-UUCU CCGAACGGUGCACGUdTdT-3'. The day before transfection, MCF-7 cells were replated in a 6-well plate in order to reach 70-80% confluence on the day of transfection. siRNA

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Table I. Nucleotide sequences of the primers used in RT-PCR.

Gene	Sense primer	Antisense primer	Length (bp)
Dicer	5'-TCTCTTTCCCAACTGGCATC-3'	5'-GGTGGTTCGTTTTGATTTGC-3'	556
GAPDH	5'-GAAGGTGAAGGTCGGAGTC-3'	5'-GAAGATGGTGATGGGATTTTC-3'	226
miR-21	5'-ACACTCCAGCTGGGTAGCTTATCAGACTG-3'	5'-GGTGTCTGGGAGTCG-3'	65
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'	94

(200 nM) complexed with 2 μ l/ml Lipofectamine 2000 reagent (Invitrogen) was added into each well according to the manufacturer's instructions. Transfection efficiency was detected by fluorescent microscopy 9 h later. Transfections were performed in triplicate for each treatment.

Cell cycle analysis. MCF-7 cells were harvested after transfection with siRNAs for 48 h. Cells were fixed overnight with 70% cold ethanol, washed twice with cold phosphate-buffered saline (PBS) and then incubated in PBS buffer 100 μ g/ml RNase A for 30 min at 37°C. Propidium iodide (4) 5 μ g/ml was added and cells detected with a flow cytometer (FACSCalibur, Beckton-Dickinson, USA).

MTT assay. Drug sensitivity of transfected MCF-7 cells was evaluated using the MTT assay. MCF-7 cells were seeded in flat-bottom 96-well plates at a density of 3,000 cells per well in 100 μ l of culture medium. After their adherence to the culture dish, cells were transfected for 24 h (according to the steps above) and then treated with cisplatin at different concentrations from 0.1 to 100 μ M. Then cells were cultured for an additional 24 h and cell survival was measured by (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl] (2H-tetrazolium hydroxide; MTT) (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. The colorimetric readout in this assay reflects the number of metabolically active mitochondria and hence viable cells in a given well. The resulting absorbance was measured at 490 nm with a spectrophotometer. MCF-7 cells without transfection and cisplatin treatment were defined as control. Inhibition ratio = (OD value in control group - OD value in drug group) / (OD value in control group - OD value in blank group) \times 100%. Data shown are representative of three independent experiments.

Semi-quantitative RT-PCR. The expression of genes was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). After 48 h transfection of MCF-7 cells with siRNA, total cellular RNA was extracted with TRIzol (Invitrogen) as described by the manufacturer. Total RNA was treated with MMLV reverse transcriptase (Takara) to generate cDNA using an oligo (dT) adaptor primer (42°C for 1 h, 75°C for 15 min) for mRNA or a specific stem-loop adaptor primer (42°C for 5 min, 75°C for 10 min) for miR-21 (5'-CTCAACT GGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAAC ATC-3') and U6 (5'-AACGCTTCACGAATTTGCGT-3'). Then, PCR amplification was performed in 20 μ l reaction solution. PCR reactions for Dicer and GAPDH gene were: 94°C for 5 min, 94°C for 45 sec, 56°C for 30 sec, 72°C for

1 min (30 cycles), 72°C for 5 min. PCR reactions for miR-21 and U6 RNAs were: 95°C for 10 min, 95°C for 15 sec, 60°C for 1 min (30 cycles). Triplicate wells were averaged and GAPDH and U6 were used as internal controls to normalize for loading differences between samples. The sequences of primers are available in Table I. The semi-quantitative RT-PCR products were analyzed by 2% agarose gel electrophoresis and visualized with Golden View.

Western blot analysis. After transfection of siRNA for 48 h in 3-mm culture dishes, MCF-7 cells were washed three times with PBS before being lysed on ice in 100 μ l freshly prepared extraction buffer (50 mM Tris-HCl pH 7.4; 1% NP-40; 150 mM NaCl; 1 mg/ml aprotinin; 1 mg/ml leupeptin; 1 mM Na_3VO_4 ; 1 mM NaF). Protein detection was performed using the Micro BCA™ Protein Assay Reagent kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Proteins were resolved at 40 μ g/lane in 10% SDS-polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Bedford, MA) for 2 h. Membranes were blocked for 1 h in PBS-Tween (0.1%) containing 5% nonfat milk and probed for 1 h with primary antibodies, including Dicer, p21, p27, CDK2, CDK4, Actin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Membranes then were incubated with respective secondary antibodies (anti-mouse IgG or anti-rabbit IgG, Amersham Biosciences, Piscataway, NJ) and were visualized by enhanced chemiluminescence (Millipore) according to the manufacturer's instructions. Shown are representative data from individual experiments that were repeated at least twice.

Statistical analysis. Data are expressed as mean \pm standard deviations (SD) for separate experiments. Statistical significance was estimated by ANOVA and by Student's t-test as appropriate. The difference was considered statistically significant at $p < 0.05$.

Results

Knockdown of Dicer by RNA interference in MCF-7 cells. FAM-labeled siRNA was transfected into MCF-7 cells. Nine hours later, transfection efficiency was determined by fluorescence microscopy. Almost 90% of MCF-7 cells emitted green fluorescence upon excitation, shown in Fig. 1A. Total cellular RNAs of control and transfected MCF-7 cells were extracted at 24, 48, 60 and 72 h after transfection, respectively. mRNA level of Dicer in the Dicer-siRNA group was significantly repressed compared with the Mock-siRNA group at 48 h after transfection. These data indicate that Dicer

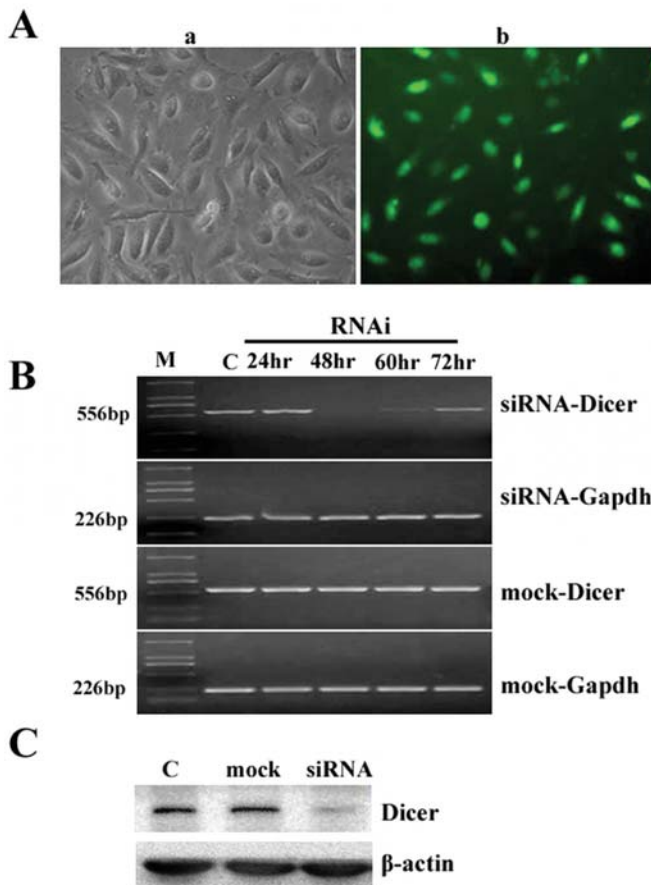


Figure 1. siRNA reduces expression of Dicer in MCF-7 cells. (A) Fluorescent microscope analysis of transfected MCF-7 cells (x400). (a) Transfected MCF-7 cells in bright field; (b) transfected MCF-7 cells in excitation light field. (B) mRNA expression of Dicer and GAPDH detected by semi-quantitative RT-PCR in MCF-7 cells at different times after transfection. (M, DNA marker D2000; C, untransfected MCF-7 cells; siRNA, MCF-7 cells transfected with Dicer siRNA; mock, MCF-7 cells transfected with Mock siRNA). (C) Western blot analysis of Dicer protein level in MCF-7 cells 48 h after transfection. (C, untransfected MCF-7 cells; mock, MCF-7 cells transfected with Mock siRNA for 48 h; siRNA, MCF-7 cells transfected with Dicer siRNA for 48 h).

siRNA could effectively suppress Dicer expression at 48 h but began to return at 60 h. Thus, all following experiments were performed at 48 h after transfection. The efficiency of Dicer knockdown was analyzed by semi-quantitative RT-PCR and Western blot analysis as shown in Fig. 1B and C.

Knockdown of Dicer led to G1 arrest in MCF-7 cells. To examine whether Dicer knockdown had an effect on cell growth, we employed a cell cycle assay for MCF-7 cells at 48 h after transfection with Dicer siRNA or Mock siRNA. Cell cycle distribution was assessed by flow cytometry. Compared to the Mock-siRNA, Dicer-siRNA induced a marked G1 arrest in MCF-7 cells (Fig. 2). As shown in Table II, the percentage of G1 phase increased from 68.9 ± 1.5 to 81.5 ± 1.2 .

Knockdown of Dicer increased the chemosensitivity of MCF-7 cells to cisplatin. In order to investigate whether Dicer depletion predisposes MCF-7 to apoptosis induction by the DNA damaging agent cisplatin, we performed a cell chemosensitivity assay. MCF-7 cells transfected with Dicer

Table II. Cell cycle analysis in different groups of MCF-7 cells.

Cell cycle/group	Control	Mock	siRNA
G0/G1	67.0 ± 1.3	68.9 ± 1.5	81.5 ± 1.2^a
S	22.5 ± 0.9	19.7 ± 1.4	14.3 ± 1.1^a
G2/M	10.5 ± 1.2	11.4 ± 1.3	4.2 ± 0.8^a

Statistical significance was determined using the unpaired t-test. For (siRNA, Mock), $^aP < 0.01$. Control, untransfected MCF-7 cells; Mock, MCF-7 cells transfected with Mock siRNA for 48 h; siRNA, MCF-7 cells transfected with Dicer siRNA for 48 h.

Table III. Growth inhibitory rate to cisplatin in different groups of MCF-7 cells.

Cisplatin/group	Control	Mock	siRNA
2.5 μ M	14.8 ± 1.4	10.7 ± 4.2	30.6 ± 3.7^a
5.0 μ M	39.0 ± 1.5	46.4 ± 4.3	71.5 ± 2.4^a
10 μ M	89.8 ± 1.0	90.3 ± 3.9	97.3 ± 1.4^a

Statistical significance was determined using the two-way ANOVA for (siRNA, Mock, control), $^aP < 0.05$. Control, untransfected MCF-7 cells; Mock, MCF-7 cells transfected with Mock siRNA for 48 h; siRNA, MCF-7 cells transfected with Dicer siRNA for 48 h.

siRNA or Mock siRNA were treated with cisplatin at various concentrations for 24 h. As shown in Fig. 3, cisplatin induced significant and dose-dependent decreases in cell viability. At concentrations of 2.5, 5.0 and 10 μ M, knockdown of Dicer in MCF-7 cells strikingly elevated sensitivity to cisplatin (Table III). Thus, we speculate that Dicer might exert some impact on drug sensitivity.

Knockdown of Dicer in MCF-7 cells upregulates expression of p21 and p27. To elucidate the mechanism of G1 arrest induced by knockdown of Dicer, we tested the effect of microRNA reduction on CDK2, CDK4 and cell cycle-dependent kinase inhibitors (CKI) p21 and p27; expression changes were detected by Western blot analysis. As shown in Fig. 4A, transfection of Dicer siRNA into MCF-7 cells upregulated levels of p21 and p27, but had no influence on either CDK2 or CDK4.

Coordinate decreases in miR-21 expression by knockdown of Dicer in MCF-7 cells. miR-21 is aberrantly upregulated in breast cancer cells (4), which can promote the proliferation of cancer cells through inhibition of apoptosis-related genes (1). To determine whether miR-21 expression was influenced by Dicer inhibition, total RNA was collected and analyzed by semi-quantitative RT-PCR. As shown in Fig. 4B, the level of

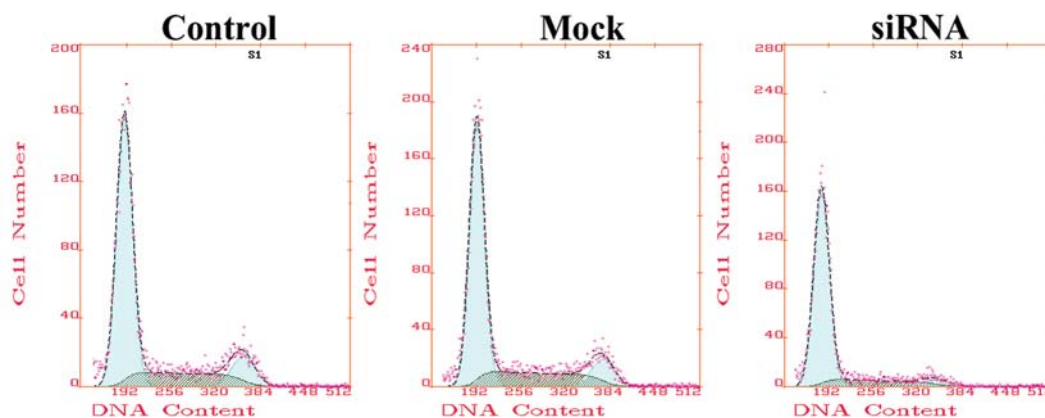


Figure 2. Dicer knockdown leads to G1 arrest in MCF-7 cells. A representative cell cycle analysis determined by flow cytometry with MCF-7 cells 48 h after transfection. Control, untransfected MCF-7 cells; Mock, MCF-7 cells transfected with Mock siRNA for 48 h; siRNA, MCF-7 cells transfected with Dicer siRNA for 48 h.

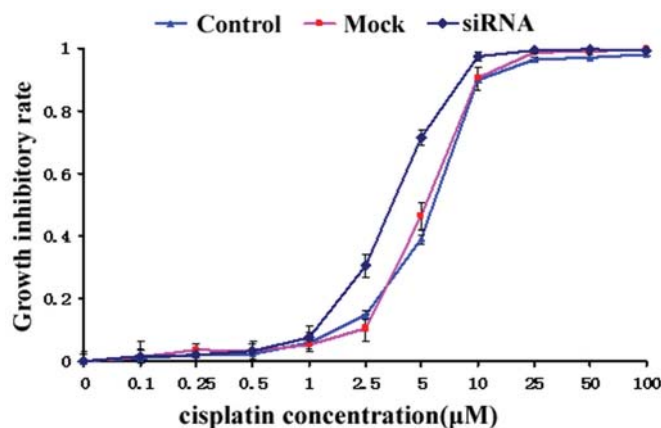


Figure 3. Dicer-directed RNAi sensitizes MCF-7 cells to inhibition with cisplatin. The growth inhibitory rate determined by MTT assay in transfected MCF-7 cells at different concentrations of cisplatin. Results are represented as mean \pm SD in dose-response plots. Control, untransfected MCF-7 cells; Mock, MCF-7 cells transfected with Mock siRNA for 48 h; siRNA, MCF-7 cells transfected with Dicer siRNA for 48 h.

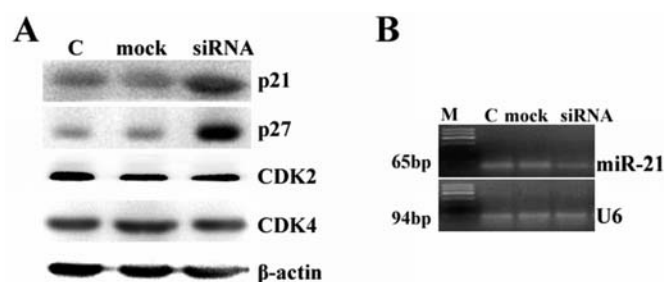


Figure 4. Suppression of Dicer in MCF-7 cells elevates p21 and p27 and decreases miR-21. (A) Western blot analyses of cell cycle-related proteins in MCF-7 cells 48 h after transfection. (B) miR-21 expression was determined by semi-quantitative RT-PCR with MCF-7 cells 48 h after transfection. (M, DNA marker I; control, untransfected MCF-7 cells; Mock, MCF-7 cells transfected with Mock siRNA for 48 h; siRNA, MCF-7 cells transfected with Dicer siRNA for 48 h).

miR-21 in MCF-7 cells transfected with Dicer-siRNA was down-regulated significantly in contrast to Mock siRNA-transfected cells.

Discussion

In this study, we investigated the effect of miRNA processing on tumor proliferation and drug resistance by knockdown of Dicer in breast cancer cell line MCF-7. We found that depletion of Dicer led to significant G1 arrest and increased sensitivity to cisplatin in MCF-7 cells and these changes correlated with the modulation of particular miRNAs.

After a stepwise process of converting pri-miRNAs into pre-miRNAs by Drosha in the nucleus and efficient nuclear export of pre-miRNAs (9,10), Dicer, a member of the RNase III superfamily of bidentate nucleases, executes cytoplasmic processing of pre-miRNAs into mature miRNAs (5). It has shown that miRNAs are aberrantly expressed or mutated in cancers such as breast cancer, hepatocellular carcinoma, lung cancer and colon cancer (1-4), suggesting that they may play a role as a novel class of oncogenes or tumor suppressor genes. miRNA signatures in breast cancer are associated with specific biological features such as estrogen and progesterone receptor expression, tumor stage, vascular invasion, or proliferation index (4). As an upstream modulator of miRNAs, Dicer up-regulation may in part explain aberrant expression of miRNAs in cancer. However, the function of Dicer in tumor cell proliferation and chemosensitivity is still not clearly understood.

Dicer depletion in MCF-7 cells results in defects in cell cycle control. A significant G1 phase arrest was induced by Dicer RNAi in MCF-7 cells in this study. After further Western blot analysis of p21 and p27, which can induce cell G1-arrest, we found that they were upregulated in protein level. A recent study showed that miR-106b modulates expression of p21/CDKN1A (11) and miR-221/miR-222 modulates p27/Kip1 (12,13). Thus, G1 arrest might be induced by down-regulation of miR-106b and miR-221/miR-222 after Dicer knockdown. Hatfield *et al* (14) found that germline stem cells with mutant Dicer were delayed in the G1 to S transition, which was dependent on the CDKI Dacapo, suggesting that miRNAs are required for stem cells to bypass the normal G1/S checkpoint.

RNA interference of Dicer sensitized MCF-7 cells to cisplatin significantly. Cisplatin, the most commonly used chemotherapeutic agent, can inhibit cell proliferation, induce

apoptosis and upregulate p53-targeted genes including p21 and p27 (15,16). Exogenous p21(WAF1/CIP1) expression resulted in significant cell growth inhibition and significantly enhanced chemosensitivity to cisplatin as well. In addition, apoptosis occurred earlier in cells transfected with p21 (WAF1/CIP1) after cisplatin treatment (17). Hence, up-regulation of p21 may explain increased chemosensitivity of MCF-7 cells to cisplatin after knockdown of Dicer. miRNAs can also substantially modulate sensitivity and resistance to anti-cancer drugs. For example, transfection of precursors or inhibitors to increase or suppress, respectively, expression of individual miRNAs affected the potencies of a number of anti-cancer agents up to 4-fold and the effect was most prominent with miR-21 (18). Furthermore, increased cell proliferation mediated by miR-21 was associated with decreased anti-apoptotic Bcl-2 (1). Potential targets of miR-21 include tumor suppressor genes such as programmed cell death 4 (PDCD4), PTEN and TPM1 (19-21). In our present study, knockdown of Dicer repressed expression of miRNAs, including miR-21, which may affect critical molecular events involved in tumor progression. This might be another explanation for increased drug sensitivity of MCF-7 cells to cisplatin after Dicer RNAi.

Taken together, transfection of chemically-synthesized Dicer siRNA in MCF-7 cells induced G1 arrest and high sensitization to cisplatin. In combination with standard chemotherapy, siRNA therapy might also reduce the chemoresistance of certain cancers, demonstrating the potential of siRNA therapy for treating malignant diseases (22,23). Further investigation of Dicer and miRNAs to evaluate their function in carcinogenesis will promote our exploitation of novel anti-cancer targets. They also represent potential approaches for improvement of chemotherapy.

Acknowledgements

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