RESEARCH ARTICLE

Effects of miR-155 Antisense Oligonucleotide on Breast Carcinoma Cell Line MDA-MB-157 and Implanted Tumors

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Abstract

Diverse studies have shown that miR-155 is overexpressed in different tumor types. However, the precise molecular mechanism of the ectopic expression of miR-155 in breast cancer is still poorly understood. To further explore the role of miR-155 in breast tumorigenesis, we here assessed the influence of miR-155 antisense oligonucleotide (miR-155 ASO) on MDA-MB-157 cell viability and apoptosis in vitro. Furthermore, the effects of inhibitory effects of miR-155 on the growth of xenograft tumors in vivo were determined with performance of immunohistochemistry to detect expression of caspase-3, a pivotal apoptosis regulatory factor, in xenografts. Transfection efficiency detected by laser confocal microscope was higher than 80%. The level of miR-155 expression was significantly decreased (P<0.05) in the cells transfected with miR-155 ASO, compared with that in cells transfected with a negative control. After being transfected with miR-155 ASO, the viability of MDA-MB-157 cells was reduced greatly (P<0.05) and the number of apoptotic cells was increased significantly. Additionally, miR-155 ASO inhibited the growth of transplanted tumor in vivo and significantly increased the expression of caspase-3. Taken together, our study revealed that miR-155 ASO can induce cell apoptosis and inhibit cell proliferation in vitro. Moreover, miR-155 ASO could significantly repress tumor growth in vivo, presumably by inducing apoptosis via caspase-3 up-regulation. These findings provide experimental evidence for using miR-155 as a therapeutic target of breast carcinoma.

Keywords: Breast neoplasms - microRNA-155 - antisense oligonucleotide - MDA-MB-157 cells - nude mice

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Introduction

Breast cancer is by far the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008 (Jemal et al., 2011). Despite previous research and resources dedicated to elucidating the mechanisms of breast cancer, the precise molecular mechanisms of its initiation and progression remain poorly understood.

MiRNAs are small (20-24 nucleotides [nt]) noncoding RNA gene products that have become known as important regulators of various cellular processes by post-transcriptionally modulate gene expression (Ambros, 2003; Bartel., 2009). There are now over 600 miRNAs estimated to play roles in humans (Griffiths-Jones., 2006), and about 30% of all genes are regulated by miRNAs (Yu, 2006). MiRNAs are key regulators of cellular differentiation (Lee et al., 1993; Chen et al., 2004), proliferation (Hayashita et al., 2005; He et al., 2005), cell survival and apoptosis (Ambros., 2003; Brennecke et al., 2003), and have been reported to be associated with tumorigenesis acting as oncogenes (He et al., 2005; Tam and Dahlberg., 2006; Papagiannakopoulos et al., 2008) or suppressors (Hammond., 2007; Dong and Lou., 2012). In particular, miR-155 has emerged as a key onco-mir, since it is one of the most consistently up-regulated miRNAs in a wide range of cancers (Yin et al., 2010; Yip et al., 2011; Donnem et al., 2012; Zheng et al., 2012).

Although miR-155 has been found to be up-regulated in breast cancer, its role in breast tumorigenesis has not yet been clarified. Therefore we chose MDA-MB-157 as a breast carcinoma cell line highly expressing miR-155 (Kong et al., 2010), as our primary experimental material. In this study, we first aimed to confirm that the effects of synthesized miR-155 ASO on MDA-MB-157 cells growth and proliferation in vitro. Next, we evaluated the role of miR-155 ASO in tumor formation in immunocompromised mice inoculated sc with MDA-MB-157 cells. Finally, we detected the expression of caspase-3 in tumor xenografts by immunochemistry to further explore the effects and the precise molecular mechanisms of the intervening measure targeting miR-155 tumorigenesis in vivo.

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Materials and Methods

Design and synthesis of miR-155 ASO sequences

The mature miRNA sequences are available from the miRNA Registry. The sequences of miRNA ASO were designed, according to the principle of sequences complementary to the mature mRNA. The ASO and the scrambled negative control (SCR) sequences used in this study are listed in Table 1. Both of them were chemically synthesized and 2’-OMe modified by Shanghai GenePharma Co., Ltd (Shanghai, China) and stored at -20°C.

Cell lines and transfection

Breast cancer cell line (MDA-MB-157) was obtained from ATCC and grown according to ATCC recommended culture conditions. Twenty four hours before transfection, MDA-MB-157 cells in the exponential phase of growth were seeded in 96- or 6-well plates (Costar) and allowed to grow overnight. The cells were then transfected with oligonucleotides using LipofectamineTM2000 reagent (Invitrogen) in OPTI MEM for 6 hours. Transfection complexes were prepared according to the manufacturer’s instructions. At the end of transfection, the cells were incubated in medium containing 10% fetal calf serum (FCS). Transfection efficiency was detected by laser confocal microscope.

Real-time PCR for quantitative analysis of miR-155

MDA-MB-157 cells were incubated in 6-well plates and transfected with 75nM oligonucleotides using the LipofectamineTM2000 reagent for 48 hours. The process was followed as described above. Briefly, miRNAs were isolated by miRcute miRNA isolation kit (Tiangen, China). The extracted products were then reverse transcription (RT) reaction using miRcute miRNA first-strand cDNA synthesis kit (Tiangen, China). The synthesized first-strand cDNA was kept at -20°C. All procedures were performed according to the instructions provided by the manufacturer. The miR-155 level was quantified by quantitative reverse transcription-PCR (qRT-PCR) with 5s small nuclear RNA as an internal normalized reference. The qPCR was performed on ABI 7500 Real-Time PCR System (ABI, USA) with miRcute miRNA qPCR detection kit (Tiangen, China), which included specific reverse primer for miRNAs. The qPCR reaction system contained 10ul 2xmiRcute miRNA premix, 0.4ul miR-155 primer, 0.4ul reverse primer, 2ul ten fold serial dilution of miRNA first-strand cDNA and 7.2ul RNase-free water. The procedure for PCR was 94°C 2 min; 94°C 20s, 60°C 30s, 72°C 30 s, 45 cycles. The sequences of the forward primers are listed in Table 1.

Table 1. The Sequences of Oligonucleotide and Primers for the Analysis of miR-155 Expression

| Gene name | Sequence (5’→3’) |
|-----------|-----------------|
| miR-155 ASO | ACCCCCUAUCAAGAUAUGACAUAA |
| FAM SCR | CAGUACUUUGUGAGUACAA |
| miR-155-F | GTTAAATGCTAATCGTGATAGGGGTAA |
| 5S rRNA-F | GTCTACGGCCATACCACCCTGAAC |

(F) Forward primer

Cell Viability and Apoptosis Assays

The effect of miR-155 ASO on MDA-MB-157 cells viability was determined by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt (WST-8) assay kit (CCK-8, Dojindo, Kumamoto, Japan). Twenty four hours before transfection, 1×10^4 MDA-MB-157 cells were seeded per well in 96-well plates and allowed to grow overnight. The cells were then transfected with three different concentration of miR-155 ASO (25 nM, 50 nM and 75 nM, respectively) and highest concentration of SCR siRNA (75 nM) using lipofectamineTM2000 according to the manufacturer’s protocol. After 48h, WST-8 was added into each well for 1 hour before the measurement according to the manufacturer’s instructions. The absorbance at 450 nm was measured by a microplate reader. Inhibition rate = (1-absorbance of treated cells/control cells)×100% (Zhang et al., 2002). Apoptosis assay was detected with Annexin-V-FITC/PI Apoptosis Detection Kit (Roche). MDA-MB-157 cells were transfected with miR-155 ASO or SCR as previously described for 6 hours, and incubated in medium containing 10% FCS for another 48 hours in 6-well plates. Cells were collected and double stained with FITC conjugated annexin V and propidium iodide (PI). For each sample, data from approximately 1×10^4 cells were recorded in the list mode on logarithmic scales. Apoptosis and necrosis were analysed by quadrant statistics on PI-negative, annexin V-positive cells and both positive cells, respectively.

Xenograft assays in nude mice

To evaluate in vivo tumorigenesis, breast carcinoma xenografting mouse model was used. 4-week-old male BALB/c athymic nude mice were obtained from the SLAC Laboratory Animals Co Ltd (Shanghai, China) and prepared for tumor implantation. All experimental procedures involving animals were performed in accordance with animal protocols approved by the Institutional Animal Use and Care Committee of Wenzhou Medical College and performed according to the institutional ethical guidelines for animal experiment. After resuspension in PBS, MDA-MB-157 cells (5×10^6 /mouse) were injected subcutaneously into the dorsal flanks of the nude mice. Ten days after implantation when the tumor became palpable at the size of ~5mm in diameter, intratumor injection with 50 μg of miR-155 ASO dissolved in 100 μL of DMEM mixed with 3μL of LipofectamineTM2000 was done twice a week (Li et al., 2009). The size of the tumor was measured every 5 days by a Vernier calliper along two perpendicular axes for a month. The volume of the tumor was calculated with the formula: volume (mm^3) = 0.5×width^2×length.

Immunohistochemistry

For immunohistochemistry, rabbit polyclonal antibody to caspase-3 (Blue Gene, Shanghai) was used as primary anti-body for overnight incubation at 4°C. The sections were subsequently treated with biotinylated secondary antibody, followed by further incubation with streptavidin-
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Figure 1. Transfection Efficiency Detected by Laser Confocal Microscope and miR-155 Expression in the Treated MDA-MB-157 Cells by Real-time PCR. (A) The bright field image (original magnification ×200). (B) The dark field image showed most of the cells (more than 80%) were stained green fluorescent, indicating that transfection efficiency was higher than 80% (original magnification ×200). (C) Representative amplification curves showed the CT value of miR-155 ASO group was higher than that of SCR group, indicating that miR-155 ASO down-regulated miR-155 levels in MDA-MB-157 cells. Reactions were done in triplicate. (a) 5s rRNA expression in MDA-MB-157 cells; (b) miR-155 of SCR group; (c) miR-155 of ASO group

miR-155 ASO down-regulation of miRNA expression

To validate whether miR-155 ASO decreased miR-155 levels in treated MDA-MB-157 cells, miR-155 and 5s rRNA expression was determined by real-time RT-PCR as anteriorly described. As shown in the amplification curves (Figure 1C), the CT value of miR-155 ASO group was higher than that of SCR group, and the Δ CT value was 4.81±0.20 and 3.04±0.09, separately. There was a statistical significance between the two groups (t=11.1, P=0.00), which showed that the level of miR-155 in MDA-MB-157 cells was down-regulated by miR-155 ASO.

miR-155 ASO inhibition of MDA-MB-157 cells Viability

In this study, we determined the influence of miR-155 ASO on cell viability by WST-8 assay. Optical densities at 450 nm were obtained for 4 groups: 1.23±0.08 for control group, 1.16±0.07 for liposomes group, 1.15±0.09 for SCR group and 1.15±0.10, 0.97±0.14 and 0.65±0.07, respectively for 25 nM, 50 nM and 75 nM concentrations of miR-155 ASO (Figure 2A). These results demonstrated that optical density and therefore cell viability was similar in control, SCR, 25 nM miR-155 ASO and lipidosome groups (P=0.31, one-way ANOVA test). However, there were significant differences of optical density in 50nM miR-155 ASO and 75 nM miR-155 ASO group compared with control group (P=0.00, P=0.00, one-way ANOVA test, respectively). Optical density and cell viability gradually decrease with the increase of miR-155 ASO concentration (1.15±0.10, 0.97±0.14, 0.65±0.07, respectively). At 75nM concentration of miR-155 ASO,
optical density and cell viability were nearly half of these parameters in control group. These data indicated that a higher concentration of miR-155 ASO had a higher toxicity effect on MDA-MB-157 cells and could decrease the cell viability and proliferation.

**miR-155 ASO promotion of MDA-MB-157 cells apoptosis**

To explore the effects of miR-155 ASO on cells apoptosis, miR-155 ASO treatment was investigated in MDA-MB-157 cells. Apoptotic MDA-MB-157 cells were detected by double staining with annexin V and PI. The results demonstrated that miR-155 ASO could induce cell apoptosis. Along with the increase of concentration of miR-155 ASO, the apoptosis rate of MDA-MB-157 cells gradually increased (Figure 2B). The double stained images are shown in Figure 2C.

**miR-155 ASO suppresses tumor growth in the nude mice**

To further investigate the role of miR-155 in tumor growth, we assessed the effects of inhibition of miR-155 on the growth of xenograft tumors in vivo. Excepted one nude mouse in saline group, all the other nude mice were successfully transplanted and had tumor growth. In nude mice, tumor treated with miR-155 ASO (biweekly intratumor injection), but not with miR-155 SCR, LipofectamineTM2000 or normal saline, significantly reduced tumor size (Figure 3). When dissected at the end of the study (day 30), the average tumor weight of the miR-155 group and saline group were 0.79±0.09 and 1.68±0.12, respectively, which indicated that the tumor suppressor rate was 52.9%.

**miR-155 ASO up-regulation of caspase-3 in xenograft tumors**

To further validate whether miR-155 ASO inhibits tumorigenesis of breast cancer xenografts by up-regulating a pivotal apoptosis regulatory factor, caspase-3, we performed immunohistochemistry for it. As expected, there were hardly any positive cells in xenograft tumors of saline group, while some positive cells were observed in liposomes group and 75 nM SCR group. However, in 75 nM ASO group, there were plenty of cells stained sepias, which indicated that most of the tumor cells were caspase-3-positive cells (Figure 4).

The differences of expression of caspase-3 among the four groups were statistically significant ($\chi^2=15.2$, $P=0.00$), the subsequent Nemenyi test indicated that the score of 75 nM ASO group was markedly higher than the other three groups (saline group: $\chi^2=34.5$, $P<0.05$; liposomes group: $\chi^2=9.55$, $P<0.05$; 75 nM SCR group: $\chi^2=8.17$, $P<0.05$).

**Discussion**

As previous research indicated, miR-155 acts as one of multifunctional miRNAs in many pathophysiological process, such as immunology (Louafi et al., 2010; Oertli et al., 2011; Ghorpade et al., 2012), inflammation (O’Connell et al., 2010; Bhattacharyya et al., 2011; Busch and Zernecke, 2012), hematopoiesis (Vasilatou et al., 2010), angiocardiopathy (Urbich et al., 2008; Yao et al., 2011) and carcinogenesis. It has been implicated in the promotion of tumor growth, proliferation, antiapoptosis, and response to chemotherapy (Kong et al., 2010). Diverse studies have shown that miR-155 is overexpressed in different tumor types. Taken together with the results presented here, a number of studies support the hypothesis that miR-155 might be one of the most relevant oncogene-like factors among the class of miRNAs.

In the present study, we utilized antisense-based technology, one of the most efficient tools with characteristics of high degree of specificity, high efficiency and low toxic side effects, for down-regulating the expression of the miRNA. Antisense oligonucleotides of DNA and RNA both inhibit miRNA function by pairing miRNA. However, because of DNA oligonucleotide degradation of pre-miRNAs, pre-miRNAs, and mature miRNAs through an RNase H cleavage, it leads to the alteration of miRNA levels in cells (Li et al., 2010). This change is easily and effectively validated by real-
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