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Combined inhibition of EGFR and c-ABL suppresses the growth of fulvestrant-resistant breast cancer cells through miR-375-autophagy axis

Lunming Liu [1], Weifeng Shen [1], Zhihui Zhu, Jinxiong Lin, Qingxia Fang, Yeping Ruan**, Huajun Zhao*

Institute of Pharmacology, College of Pharmaceutical Sciences, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, 311400, China

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ABSTRACT

Fulvestrant is the FDA-approved "pure anti-estrogen" agent for malignant breast cancer therapy. But endocrine resistance causes drug failure. A new approach is desired for fulvestrant-resistant breast cancer (FRBC) therapy. This study aims to find an effective approach to inhibit FRBC for patients with advanced breast cancer. MTT assay was first performed to detect the effect of inhibitors of c-ABL (imatinib) and EGFR (lapatinib) on FRBC cells. Microarray analysis was carried out to identify microRNA which is significantly changed between parental and FRBC cells. The related mechanisms were analyzed by qRT-PCR, MTT, AO staining and western blotting. Dual treatment significantly inhibited cell growth of FRBC and upregulated microRNA-375 (miR-375). Overexpression of miR-375 inhibited growth of FRBC cells, reduced autophagy, and decreased expression of ATG7 and LC3-II. Dual treatment elevated expression of miR-375 more than any single one of these two inhibitors. Overexpression of miR-375 increased cell growth inhibition induced by dual treatment, and the effect was attenuated when miR-375 was inhibited.

In conclusion, we identified that combined inhibition of EGFR and c-ABL can suppress the growth of FRBC cells and elucidated a mechanism within FRBC cells involving regulation of miR-375 and autophagy. Dual treatment may be useful for inhibiting fulvestrant-resistant breast cancer.

1. Introduction

Although most early-stage estrogen receptor (ER)-positive breast cancer responds to conventional anti-estrogens, including tamoxifen and aromatase inhibitors [1], resistant tumors eventually develop with prolonged treatment [2–5]. Approved as a second-line anti-estrogen therapy since 2002 [6], fulvestrant (ICI 182, 780; Faslodex) is a new mainstay of anti-estrogen therapy [7–10]. However, numerous laboratory studies [11–13] and clinical observations [2,5,14,15] suggest that breast cancer cells stop responding to fulvestrant treatment, and a replacement therapy does not exist.

Epidermal growth factor receptor (EGFR) is a cell surface ErbB-family receptor tyrosine kinase that includes ErbB2. EGFR is required for growth, survival, and metastasis of cancer cells and as such is a primary target for cancer therapy [16]. Small molecule kinase inhibitors, such as lapatinib and gefitinib, have been used to treat breast cancer as EGFR-targeted inhibitors [17], but the efficacies of lapatinib and gefitinib as single-agent inhibitors in breast cancer cells is poor.

The c-ABL proto-oncogene is a multi-functional non-receptor tyrosine kinase that regulates cell migration, responses to oxidative stress, and apoptosis [18,19]. Recent studies suggest that c-ABL plays an important role in cell growth, invasion, and resistance to chemotherapy in breast cancer [20–24]. The most common c-ABL inhibitor is imatinib [25].

MicroRNAs (miRNAs) are small (20–24 nucleotides) nucleotide non-coding RNAs that regulate expression of various genes. Evidence suggests that fundamental functions of miRNAs are associated with gene regulation tied to human diseases, including cancer [26,27]. Notably, miRNAs were found to function in the regulation

* Corresponding author.
** Corresponding author.
E-mail addresses: 372739049@qq.com (Y. Ruan), zhj@zcmu.edu.cn (H. Zhao).
[1] These authors contributed equally to this work.

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of autophagy. MiR-375 was one of the most significantly changed microRNAs in MCF-7 and fulvestrant-resistant breast cancer (FRBC) cells according to microarray analysis, but whether miR-375 plays a role in FRBC cells is unknown.

Here, we report that combined treatment with imatinib and lapatinib inhibits growth of FRBC cells, and we identified miR-375 as a downstream target. We demonstrated that miR-375 expression was transcriptionally upregulated by the combined treatment, and the effect of co-treatment was attenuated when miR-375 was knocked down. Finally, we report that ATG7 and LC3-II were targets of miR-375 and miR-375 may act as a downstream target by downregulating autophagy.

2. Materials and methods

2.1. Cell culture and chemicals

Human MCF-7 breast cancer cells were purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), stored in liquid nitrogen, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) culture medium (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin G, 2.5 μg/ml amphotericin B and 100 μg/ml streptomycin (complete medium) at 37 °C with 5% CO_2 in a humidified atmosphere.

FRBC cells were developed by culturing parental cells (MCF-7) in the presence of 0.5 μM fulvestrant (Sigma-Aldrich, St,Louis, MO) for ~1 year. Stable monoclonal resistant cells (FRBC1–5) were obtained and amplified for subsequent studies. MTT, DMSO, 3-methyladenine (3-Ma), acridine orange (AO), imatinib and lapatinib were purchased from Sigma-Aldrich.

2.2. Microarray analysis

Total RNAs were collected from parental MCF-7 cells and five monoclonal resistant cells (FRBC1-5) using TRIZOL reagent (Invitrogen, Carlsbad, CA). All samples were subjected to microarray assay performed at Novogene Bioinformatics Technology Co. Ltd, Beijing, China.

2.3. Cell proliferation assay

Cells viability was measured using MTT assay. Briefly, exponentially growing FRBC cells were seeded at a density of 3×10^3 cells/well in 96-well plates. After treatment (or no treatment) with lapatinib (5 μM), imatinib (20 μM), or lapatinib (5 μM) plus imatinib (20 μM), MTT solution was added into each well (final concentration 0.5 mg/ml), and cells were incubated for another 4 h at 37 °C in the dark. Formed formazan crystals were dissolved in 150 μl DMSO and relative cell viability was measured using a cell Imaging Multi-mode Reader (BioTek, CA) at 570 nm.

2.4. Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA) and then reverse-transcribed into cDNA by PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's instructions. After cDNA was mixed with SYBR Green (Bio-Rad, Berkeley, CA), qRT-PCR was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). PCR was performed as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Relative expression was measured using the $^{\Delta\Delta}$Ct method with U6 as an endogenous control. Nucleotide sequences of the primers were as follows:

miR-375-F: ACACTCCAGCTGGGTTTGTTCGTTCGGCTC
miR-375-R: TGGTGTCGTGGAGTCG
U6-F: CTCGCTTCGGCAGCACA
U6-R: AACGCTTCACGAATTTGCGT

2.5. Vector constructs, lentivirus production, and cell transfection

Commercially available lentiviral vectors were used to construct the LV-has-miR-375 vector and the LV-has-miR-375-inhibition vector (Shanghai Genechem Co., Ltd, China). The LV empty lentiviral construct pre-NC (Ubi-MCS-SV40-puromycin) and NC-inhibitor (Hu6-MCS-Ubiquitin-EGFP-IRES-puromycin) were negative controls. The lentiviral vectors were used at an appropriate multiplicity of infection (MOI) to infect FRBC1 and FRBC2 cells which grew to 40–50% confluence with polybrene (Shanghai Genechem Co., Ltd) and enhanced infection solution (Shanghai Genechem Co., Ltd). Stable cell lines were generated by selecting transfected cells in cultures containing 1 μg/ml puromycin (Sigma-Aldrich) for 5 days. Transfection efficiency was confirmed by observing green fluorescence and expression of miR-375 was analyzed using qRT-PCR.

2.6. Acridine orange (AO) staining

MCF-7 and FRBC cells were seeded into 24-well plates and cultured for 24 h. Cells were washed with PBS and stained with AO (final concentration 1 μg/ml) for 15 min. Slides were observed under a fluorescent microscope (Nikon, Tokyo, Japan).

2.7. Western blotting

Lysates of treated cells were isolated by incubation with RIPA lysis buffer containing PMSF and protease inhibitors cocktail (1 mM phenylmethanesulfonyl fluoride and 1 μg/ml leupeptin) and phosphatase inhibitors cocktail (1 mM sodium fluoride and 1 mM sodium orthovanadate). Protein was measured using a BCA protein assay kit (Beyotime, China). Proteins were separated on acrylamide gels and transferred onto PVDF membranes (Millipore, Burlington, MA). Membranes were then blocked with 5% nonfat milk, incubated with primary antibodies overnight at 4 °C and then with the appropriate secondary antibodies. Her2 (#4290), EGFR (#4267), ATG-7 (#8558), Crkl (#32H4), p-Crkl (#3181), LC3 (#3868s), p62 (#5114) and GAPDH (#5174) antibodies were purchased from Cell Signaling Technology (Boston, MA). ER (#1115-1) and p-EGFR (#1124-1) antibodies were purchased from abcam (Cambridge, UK). PR (sc-538) and C-Abl (sc-23) antibodies were purchased from Santa Cruz (Dallas, TX). HRP-conjugated secondary antibodies were purchased from Bio-Rad. Immunoblotting signals were detected using ECL (Bio-Rad).

2.8. Statistical analysis

Data are expressed as means ± SD of three independent experiments. Statistical analysis was performed using a two tailed Student's *t*-test. Criteria for statistical significance were $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results

3.1. Combined treatment inhibits the growth of FRBC cells

Fulvestrant-resistant breast cancer cell lines generated from MCF-7 cells were much less sensitive to fulvestrant compared with parental cell line (Fig. 1A). Characterization of FRBC cells

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