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Cross-talk between ER and HER2 regulates c-MYC-mediated glutamine metabolism in aromatase inhibitor resistant breast cancer cells

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ABSTRACT

Resistance to endocrine therapies in hormone receptor (HR)-positive breast cancer is a significant clinical problem for a considerable number of patients. The oncogenic transcription factor c-MYC (hereafter referred to as MYC), which regulates glutamine metabolism in cancer cells, has been linked to endocrine resistance. We were interested in whether MYC-mediated glutamine metabolism is also associated with aromatase inhibitor (AI) resistant breast cancer. We studied the expression and regulation of MYC and the effects of inhibition of MYC expression in both AI sensitive and resistant breast cancer cells. Considering the role of MYC in glutamine metabolism, we evaluated the contribution of glutamine to the proliferation of AI sensitive and resistant cells, and performed RNA-sequencing to investigate mechanisms of MYC-mediated glutamine utilization in AI resistance. We found that glutamine metabolism was independent of estrogen but still required estrogen receptor (ER) in AI resistant breast cancer cells. The expression of *MYC* oncogene was up-regulated through the cross-talk between ER and human epidermal growth factor receptor 2 (HER2) in AI resistant breast cancer cells. Moreover, the glutamine transporter solute carrier family (SLC) 1A5 was significantly up-regulated in AI resistant breast cancer cells. ER down-regulator fulvestrant inhibited MYC, SLC1A5, glutaminase (GLS) and glutamine consumption in AI resistant breast cancer cells. Inhibition of MYC, SLC1A5 and GLS decreased AI resistant breast cancer cell proliferation. Our study has uncovered that MYC expression is up-regulated by the cross-talk between ER and HER2 in AI resistant breast cancer cells. MYC-mediated glutamine metabolism is associated with AI resistance of breast cancer.

1. Introduction

Breast cancer is the most common cancer and the second leading cause of cancer death among women worldwide [1]. Hormone receptors (HRs) – the estrogen receptor (ER) and/or the progesterone receptor (PR) are expressed in approximately 70% of breast cancer. The growth of breast cancer cells in such patients is dependent on estrogen and ER. Estrogen-mediated ER signaling can be targeted by blocking estrogen biosynthesis with aromatase inhibitors (AIs) (i.e., letrozole, anastrozole, and exemestane), antagonizing the binding of estrogens to the ER with tamoxifen, and down-regulating ER with fulvestrant. Endocrine therapies play an essential role in the treatment of patients with HR-positive breast cancer in postmenopausal women [2]. Unfortunately, a considerable number of patients have *de novo* resistance or ultimately develop acquired resistance to endocrine therapies [2].

Cross-talk between ER and human epidermal growth factor receptor 2 (HER2) signaling pathways has been implicated in endocrine therapy resistance [3–5]. HER2 signaling is up-regulated in breast tumors of patients treated with AIs [6]. Up-regulation of HER2 signaling pathways, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3′-kinase (PI3K)/AKT, can phosphorylate and activate ER in a ligand-independent manner [7,8]. Many groups, including us, have showed that ER is up-regulated and constitutively activated in endocrine resistant breast cancer cells [9–11]. The *MYC* oncogene, which encodes c-MYC (MYC) protein, is a well-known ER-regulated gene [12,13]. MYC is a transcription factor and plays a critical role in cell proliferation,

Abbreviations: AI, aromatase inhibitor; DMEM, Dulbecco's Modified Eagle Medium; ER, estrogen receptor; FBS, fetal bovine serum; Gln, glutamine; GLS, glutaminase; GPNA, L-glutamic acid γ-(*p*-nitroanilide); HER2, human epidermal growth factor receptor 2; HR, hormone-receptor; IPA, ingenuity pathway analysis; LTED, long-term estrogen deprivatioin; MAPK, mitogen-activated protein kinase; MEM, minimal Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PI3K, phosphatidylinositol 3′-kinase; siRNA, short interfering RNA; SLC1A5, solute carrier family 1A5; TCA, tricarboxylic acid.

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growth, survival, differentiation and apoptosis [14]. Interestingly, MYC has been linked to endocrine resistance of breast cancer [15–18].

Glutamine is the most abundant amino acids in the body and plays an important role in cell proliferation. It is first converted to glutamate through the enzyme glutaminase (GLS), and then catabolized to α-ketoglutarate, an intermediate of the tricarboxylic acid (TCA) cycle [19]. Glutamine can be a source of both carbon and nitrogen for the synthesis of lipid, protein and nucleotide [20]. Although the growth and survival of most cancers depend on a high rate of aerobic glycolysis, some cancer cells cannot survive in the absence of exogenous glutamine, termed "glutamine addiction" [20]. Estrogen stimulation has been found to increase glutamine consumption in ER-positive breast cancer MCF7 cells [21], suggesting that glutamine metabolism is essential for estrogen-dependent cell proliferation. Oncogenic levels of MYC have also been linked to elevated glutamine uptake and metabolism in human cancers [22,23].

Given the association between MYC and endocrine resistance [15,16] as well as the regulation of glutamine metabolism by MYC in cancer cells [22,23], we hypothesized that MYC-mediated glutamine metabolism is also associated with AI resistance. We studied the expression and regulation of MYC and the effects of inhibition of MYC expression in both AI sensitive and resistant breast cancer cells. We evaluated the contribution of glutamine to cell proliferation, and the association between glutamine consumption and hormone in breast cancer cells. Finally, we performed RNA-sequencing and investigated mechanisms of MYC-mediated glutamine metabolism in AI resistance.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell line MCF7 derived cell lines MCF7aro and LTEDaro were generated in this laboratory and have been characterized and described previously [9,24]. MCF7aro was routinely cultured in minimal Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin-streptomycin, and 0.1 mg/mL G418. LTEDaro was maintained in phenol red-free MEM containing 10% charcoal/dextran-treated FBS with identical supplements as parental MCF7aro cells. For experiments under testosterone treatment, MCF7aro cells were cultured in phenol red-free MEM medium containing 10% charcoal/dextran-treated FBS for 72 h before treatment. Experiments under deprived glutamine or glucose culture conditions were performed by using phenol red-free Dulbecco's Modified Eagle medium (DMEM) without glucose and glutamine (GIBCO A14430-01) containing 10% charcoal/dextran-treated FBS.

2.2. Antibodies and reagents

Antihuman MYC (#5605), p-MAPK (#9101), MAPK (#9102), p-AKT (Ser473) (#9271), AKT (#9272), p-ER (Ser167) (#5587), GAPDH (#2118) antibodies were obtained from Cell Signaling Technology. Antihuman HER2 (#06-562) and p-ERα (Ser118) (ab32396) antibodies were from Abcam Inc., and antihuman ERα (HC-20) antibody (sc-543) was from Santa Cruz Biotechnology. The ER antagonist fulvestrant (#14409) and the SLC1A5 inhibitor L-glutamic acid γ-(*p*-nitroanilide) (GPNA) (G6133) were obtained from Sigma-Aldrich. The glutaminase inhibitor compound 968 (#352010) was from EMD Millipore. The AKT inhibitor MK-2206 (S1078) was from Selleck Chemicals. The nontargeting control siRNA (sc-37007) and MYC siRNA (sc-29226) were obtained from Santa Cruz Biotechnology. The HER2 siRNA (L-003126-00) was from Dharmacon. The p44/42 MAPK siRNA (#6560) was from Cell Signaling Technology.

2.3. Real-time PCR

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen) and quantified with a Nanodrop spectrophotometer. Reverse transcription was performed with SuperScript VILO cDNA synthesis kit (Invitrogen) from 2.5 μg total RNA. Real time PCR was performed using iQ5 multicolor real-time PCR detection system (Bio-Rad). The human *MYC* gene was amplified using the forward primer 5′-GGCTCCTGGCAAAAGGTCA-3′ and the reverse primer 5′-CTGCGTAGTTGTGCTGATGT-3′ (PrimerBank ID 239582723c1) [25]. The human *SLC1A5* gene was amplified using the forward primer 5′- GAGCTGCTTATCCGCTTCTTC- 3′ and the reverse primer 5′-GGGGCGTACCACATGATCC-3′ (PrimerBank ID 223468565c1) [25]. The human *GLS* gene was amplified using the forward primer 5′-AGGGTCTGTTACCTAGCTTGG-3′ and the reverse primer 5′-ACGTTCGCAATCCTGTAGATTT-3′ (PrimerBank ID 373251163c1) [25]. The *ACTB* (*β*-actin) gene was amplified using the forward primer 5′-CACCAACTGGGACGACAT-3′ and the reverse primer 5′-GCACAGCCTGGATAGCAAC-3′. The real time PCR was established with the PerfeCTa SYBR Green SuperMix (Quanta Biosciences). The PCR results were normalized with *β*-actin as an internal control and then expressed as relative expression compared with reference samples. Each experiment was performed in triplicate. The data are expressed as means ± SE.

2.4. Transfection

Transfection of MCF7aro and LTEDaro with control siRNA or siRNAs against MYC, HER2 or MAPK was performed using the siPORT NeoFX transfection agent (Ambion) according to the manufacturer's protocol. For immunoblotting, transfection was performed in 60-mm dishes. For the MTT assay, 96-well plates were used. Briefly, cells were trypsinized and diluted in the medium at 1×10^5 cells/mL. siPORT NeoFX transfection agent and siRNA were diluted in OPTI-MEM I medium (Invitrogen), respectively. After being mixed and incubated for 10 min, the mixtures of siRNA and transfection agent were dispersed into a culture plate or dish. Cell suspensions (1×10^5 cells/mL) were overlaid onto the transfection complexes. The final concentration of siRNA was 30 nM.

2.5. Western blotting

Western blotting was performed as previously described [26]. In brief, cells were lysed in RIPA buffer (Cell Signaling) on ice for 5 min and then sonicated for 60 s. The protein concentration was determined by protein assay kit (Bio-Rad), and the samples were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). After probing with a primary antibody, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody. Finally; signal intensity was determined with the SuperSignal West Pico Chemiluminescent (Thermo Scientific) substrate visualization. Relative expression of proteins was normalized to the internal control GAPDH.

2.6. Cell proliferation assay

Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At the indicated time, the media in each well of 96-well plates were removed from the cells and replaced with 0.1 mL of fresh phenol red-free medium containing 0.5 mg/mL MTT, and then the cells were incubated at 37 °C for 1 h. After discarding the medium, the