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Original Articles

Neratinib induces ErbB2 ubiquitylation and endocytic degradation via HSP90 dissociation in breast cancer cells

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ABSTRACT

Receptor tyrosine kinase ErbB2/HER2 is frequently observed to be overexpressed in human cancers, leading to over activation of downstream signaling modules. HER2 positive is a major type of breast cancer for which ErbB2 targeting is already proving to be an effective therapeutic strategy. Apart from antibodies against ErbB2, the small molecule tyrosine kinase inhibitor lapatinib has had successful clinical outcomes, and other inhibitors such as neratinib are currently undergoing clinical investigations. In this study we report the effects of lapatinib and neratinib on the mRNA and protein levels of the ErbB2 receptor. We provide evidence that neratinib-induced down regulation of ErbB2 occurs through ubiquitin-mediated endocytic sorting and lysosomal degradation. At the mechanistic level, neratinib treatment leads to HSP90 release from ErbB2 and its subsequent ubiquitylation and endocytic degradation. Our findings provide novel insights into the mechanism of ErbB2 inhibition by neratinib.

Introduction

ErbB2/HER2 belongs to the ErbB family of receptor tyrosine kinases, with amplification frequently observed in a number of malignancies [1]. ErbB2/HER2 amplification identifies a major subtype of breast cancer called HER2-positive breast cancer that accounts for approximately 25–30% of all breast cancer cases [2]. Targeting ErbB2 has proven to be an effective strategy in the clinical treatment of HER2-positive breast malignancies. Two approaches have been approved by the US Food and Drug Administration (FDA) for clinical use: humanized monoclonal antibodies targeting the extracellular domain of ErbB2 and small molecule tyrosine kinase inhibitors (TKI) that block the kinase activity of ErbB2 [3]. Antibody based therapies include trastuzumab, pertuzumab, and the antibody-drug conjugate ado-trastuzumab emtansine (T-DM1). Lapatinib is a tyrosine kinase inhibitor treatment and is reversible, targeting both ErbB2 and EGFR.

Neratinib (HKI-272) is another small molecule inhibitor that blocks the kinase activities of EGFR and ErbB2 [4]. Unlike lapatinib, neratinib irreversibly binds to the kinase domain of EGFR and ErbB2 via covalent interaction with conserved cysteine residues: C773 of EGFR and C805 of ErbB2 [5,6]. Multiple clinical investigations of neratinib treatment of HER2-positive breast cancer and other solid tumors are ongoing, with promising results for future treatment of HER2-positive malignancies [2].

The cell surface levels of many receptor tyrosine kinases are tightly regulated by endocytosis, which internalizes membrane receptors into the cells and subsequently sorts them into lysosomes for degradation via the endosome and multivesicular body systems [7]. ErbB2 is normally endocytosis impaired and requires the chaperone HSP90 for stability on the cell surface [8,9]. HSP90 inhibitors have been reported to induce ErbB2 degradation through either proteasomal or lysosomal pathways, which are regulated by CHIP-mediated post-translational modifications of ErbB2 by ubiquitin (ubiquitylation) [10–12].

The mechanistic studies of TKI lapatinib and neratinib mainly focus on the inhibition of receptor kinase activity and corresponding downstream signaling pathways, most prominently the RAS–MAPK and PI3K–AKT signaling cascades, both of which can be efficiently suppressed by lapatinib and neratinib. Conversely, the effects of lapatinib and neratinib on the expression levels of EGFR and ErbB2 receptors *per se* remain underexplored. In the present

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study, we report that treatment with lapatinib and neratinib leads to increased and decreased ErbB2 expression levels respectively, although ErbB2 mRNA is increased by both inhibitors. The two TKIs also induce the endocytosis of ErbB2. Mechanistically, neratinib triggers potent ubiquitylation and endocytic degradation of ErbB2 via HSP90 dissociation from ErbB2.

Materials and methods

Antibodies and other reagents

Mouse anti-ErbB2 (A-2), mouse anti-ErbB2 (9G6), mouse anti-LAMP1, rabbit anti-EEA1, and rabbit anti-GAPDH (FL-335) antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Mouse anti-GAPDH antibody was purchased from Proteintech (Wuhan, China). Goat anti-ErbB2 N-terminus antibody was purchased from R&D. Rabbit anti-HER2/ErbB2 (29D8), rabbit anti-phospho-S6RP, rabbit anti-phospho-Akt (Ser473), rabbit anti-phospho-MEK1/2 (Ser217/221), mouse anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), rabbit anti-phospho-mTOR, rabbit anti-MEK1/2, rabbit anti-ERK1/2, rabbit anti-AKT, and rabbit anti-phospho-ErbB2 antibodies were obtained from Cell Signaling Technologies. Mouse anti-EEA1 antibody was purchased from BD Transduction. Mouse anti-Ubiquitin (P4G7) antibody was purchased from Covance. Mouse anti-Tubulin antibody was obtained from Sigma. Secondary goat anti-mouse and anti-rabbit, donkey anti-goat antibodies were obtained from LICOR. Cycloheximide was purchased from MP Biologicals. Bortezomib, neratinib (HKI-272), lapatinib (GW-572016), GDC-0941, and trametinib were purchased from Selleck. Chloroquine, geldanamycin, and dynasore were obtained from Sigma.

Cell culture

Cell lines with ErbB2 overexpression were obtained from the American Type Culture Collection (ATCC) and maintained in a humidified incubator (Thermo) at 37 °C with 5% CO_2. SKBR3 cells were cultured in McCoy's 5A medium (Gibco, USA), while AU565 and HCC1954 were cultured in RPMI 1640 medium (Gibco, USA), all media were supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific).

Cell lysis and immunoblottings

Cultured cells were washed twice with ice-cold PBS before lysis using RIPA buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% w/v Triton X-100, 0.1% w/v SDS, 1% sodium deoxycholate) supplemented with mammalian protease and phosphatase inhibitors (Sigma). Lysates were cleared with centrifugation and protein concentrations were determined by a BCA protein assay (Thermo). Equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Merck Millipore, USA). Blots were incubated with 5% fat-free milk in PBS for an hour at room temperature and then incubated with primary antibodies at 4 °C overnight. Then, the blots were washed three times with PBS before incubation with LICOR 680 nm or 800 nm infrared secondary antibodies for 1 hour. Following the PBS washes, the blots were scanned on a LICOR Odyssey system. The acquired images were analyzed with Image Studio Version 4.0 according to manufacturer's instructions.

Immunofluorescence and confocal microscopy

ErbB2 overexpressing cells were cultured on glass coverslips in 6-well plates. Following treatment, cells on coverslips were fixed with 3% paraformaldehyde, then permeabilized with 0.2% Triton X100, and blocked in 10% goat serum. The cells were incubated with primary antibodies before addition of fluorescent secondary antibodies (Invitrogen, USA) at room temperature. Finally, the coverslips were washed and mounted. Cell staining was examined using a fluorescent microscope (Leica, Germany). To examine protein subcellular co-localization, Alexa Fluor® 488- and 594-conjugated secondary antibodies (Invitrogen, USA) were used together in immunofluorescence assays. Confocal images were captured with a Leica laser scanning confocal microscope (TCS SP5).

Quantitative PCR

ErbB2-overexpressing breast cancer cell lines were treated with the indicated inhibitors before total RNA extraction using Trizol (Invitrogen, USA). cDNA was generated, and quantitative PCR was performed as previously described [13]. The qPCR primers used were as follows: ErbB2 (forward 5′-GAGTGTCAGCCCCAGAATG-3′ and reverse 5′-GTAGGAGAGGTCAGGTTTCAC-3′), and beta-actin (forward 5′-CACCTTCTACAATGAGCTGCGTGTG-3′ and reverse 5′-ATAGCACAGCCTGGATAGCAACGTAC-3′).

Immunoprecipitation and co-immunoprecipitation assays

For endogenous immunoprecipitation experiments, SKBR3 cells were treated with 500 nM lapatinib or neratinib prior to lysis with RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Protein concentration was determined with BCA protein assays. One milligram of lysates was incubated with protein G-agarose and anti-ErbB2 antibody (Santa Cruz) for 4 hours at 4 °C. Beads were washed 3 times with YP-IP buffer (0.1% Nonidet P-40, 25 mM Tris-HCl pH 7.5, 150 mM NaCl), and proteins were eluted with 1.5X SDS PAGE sample buffer. Samples were analyzed by immunoblotting with anti-ErbB2 and anti-Ubiquitin antibodies. In co-immunoprecipitation assays, SKBR3 cells were treated as described above before lysis with NP40 lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40). Cleared lysates (1 mg per sample) were incubated with protein G-agarose and anti-ErbB2 antibody at 4 °C for 4 hours. Eluted protein samples were subjected to SDS PAGE analysis before immunoblotting assays with anti-ErbB2 and anti-HSP90 antibodies.

Flow cytometry

Cultured SKBR3 and AU565 cells were treated with 500 nM of lapatinib or neratinib (DMSO as control) before one million cells were harvested. For cell cycle analysis, the cells were washed with PBS and fixed using 70% ethanol, prior to staining with 50 μg/ml propidium iodide (PI). For apoptosis assays, the cells were processed for Annexin V and PI double staining using an apoptosis assay kit (KeyGEN Biotech, China) according to manufacturer instructions. The samples were analyzed using a flow cytometer (Accuri C6, BD Biosciences) and acquired data were analyzed with FlowJo version 7.6.1 (FlowJo, LLC, USA).

Statistical analysis

To determine significant differences, assays were carried out 3 times with independent biological repeats, and data were presented as the mean ± standard error of the mean (SEM). Significant differences were assessed via Student's t test using GraphPad Prism Version 5.01; $p < 0.05$ was considered statistically significant.

Results

Lapatinib and neratinib show opposite effects on ErbB2 levels

Given that breast cancer with ErbB2/HER2 amplification is the primary target for lapatinib and neratinib, we used three ErbB2-overexpressing breast cancer cell lines in this study: SKBR3, AU565, and HCC1954. We treated SKBR3 and AU565 cells with lapatinib and neratinib (both at 500 nM). As expected, the phosphorylation of ErbB2, mTOR, AKT, S6RP, MEK and ERK1/2 downstream of ErbB2 is potently inhibited, demonstrating the effectiveness of the inhibitors on ErbB2 kinase activity (Fig. 1A). However, ErbB2 expression levels were differently affected by lapatinib and neratinib, lapatinib increased ErbB2 abundance (1.72- and 1.63-fold in SKBR3 and AU565, respectively by 24 hours), whereas neratinib decreased ErbB2 expression (to 48.5% and 48.9% in SKBR3 and AU565, respectively), although both inhibitors showed similar effects on cell cycle distribution and apoptosis (Fig. 1B–D). We first wondered whether these inhibitors affect ErbB2 transcription, and we thus carried out quantitative PCR assays to measure ErbB2 mRNA levels following treatment. As described in Fig. 1E and F, ErbB2 mRNA levels increased following 6 hours of treatment with both lapatinib and neratinib. By 12 hours, lapatinib elevated ErbB2 mRNA to 249% and 216% relative to control samples in SKBR3 and AU565 cells, respectively, while neratinib led to increases of 159% (SKBR3) and 136% (AU565). It therefore seems that lapatinib-induced ErbB2 elevation can be attributed to enhanced transcription but that neratinib-triggered ErbB2 decreases cannot be explained at the mRNA level. Subsequently, we assessed the involvement of two major signaling cascades downstream of ErbB2, the RAS–MAPK and PI3K–AKT pathways, in lapatinib mediated regulation of ErbB2 expression. Using the MEK inhibitor trametinib and the PI3K inhibitor GDC0941, we specifically blocked the RAS–MAPK and PI3K–AKT cascades, respectively, and then compared ErbB2 levels to lapatinib treatment, which inhibits both pathways. In SKBR3 cells, both trametinib and GDC0941 treatment led to increases in ErbB2 expression, similar to lapatinib treatment (Fig. 1G); but in AU565 cells, GDC0941 treatment resulted in a partial increase of ErbB2 compared to lapatinib, while trametinib failed to alter ErbB2 levels (Fig. 1G).