Up-regulation of breast cancer resistance protein plays a role in HER2-mediated chemoresistance through PI3K/Akt and nuclear factor-kappa B signaling pathways in MCF7 breast cancer cells

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Human epidermal growth factor receptor 2 (HER2/neu, also known as ErbB2) overexpression is correlated with the poor prognosis and chemoresistance in cancer. Breast cancer resistance protein (BCRP and ABCG2) is a drug efflux pump responsible for multidrug resistance (MDR) in a variety of cancer cells. HER2 and BCRP are associated with poor treatment response in breast cancer patients, although the relationship between HER2 and BCRP expression is not clear. Here, we showed that transfection of HER2 into MCF7 breast cancer cells (MCF7/HER2) resulted in an up-regulation of BCRP via the phosphatidylinositol 3-kinase (PI3K)/Akt and nuclear factor-kappa B (NF-κB) signaling. Treatment of MCF/HER2 cells with the PI3K inhibitor LY294002, the IκB phosphorylation inhibitor Bay11-7082, and the dominant negative mutant of IκBα inhibited HER2-induced BCRP promoter activity. Furthermore, we found that HER2 overexpression led to an increased resistance of MCF7 cells to multiple antitumor drugs such as paclitaxel (Taxol), cisplatin (DDP), etoposide (VP-16), adriamycin (ADM), mitoxantrone (MX), and 5-fluorouracil (5-FU). Moreover, silencing the expression of BCRP or selectively inhibiting the activity of Akt or NF-κB sensitized the MCF7/HER2 cells to these chemotherapy agents at least in part. Taken together, up-regulation of BCRP through PI3K/AKT/NF-κB signaling pathway played an important role in HER2-mediated chemoresistance of MCF7 cells, and Akt, NF-κB, and BCRP pathways might serve as potential targets for therapeutic intervention.

Keywords BCRP; PI3K/AKT/NF-κB; chemoresistance

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resistance (MDR1), multidrug resistance-associated protein (MRP) and breast cancer resistance protein (BCRP and ABCG2) [11,12]. Among these ABC transporter proteins, BCRP is unique for its half-transporter property and functions as an energy-dependent efflux pump to extrude antitumor agents from the cytoplasm, thus reducing intracellular drug concentrations to sublethal levels [13]. Several studies have shown that BCRP may confer resistance to mitoxantrone (MX), anthracyclines, MTX, topoisomerase I inhibitors, gefitinib, doxorubicin, and 5-FU, which is particularly relevant to the problem of MDR in breast cancer [13–15]. Breast cancer resistance protein-mediated drug resistance and disposition may, therefore, be influenced by factors that can affect BCRP expression in breast cancer cell. Investigating the molecular mechanism by which BCRP expression is regulated would help us to reverse BCRP-mediated MDR.

In order to understand whether HER2 overexpression conferred resistance to chemotherapeutic agents and the molecular mechanisms involved, we therefore investigated the effect of HER2 on the expression and function of BCRP. Our data established partial molecular mechanism of chemoresistance in HER2-overexpression human breast cancer cell line MCF7 and provided evidence of a functional link between the HER2 and the BCRP signaling pathway.

Materials and Methods

Cell culture
The human breast cancer cell line MCF7 was purchased from American Type Culture Collection (ATCC, Manassas, USA) and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, USA). PA317 retroviral packaging cell line from ATCC was cultured in Dulbecco-modified eagle medium (DMEM, Gibco-BRL, Paisley, UK) supplemented with 10% FBS. Cells were incubated under standard conditions in a 37°C humidified 5% CO₂ atmosphere.

Plasmids, antibodies and reagents
The retroviral vector PLNSX and Plasmid pAVU6+27 were kindly provided by Dr. ML He at the University of Hong Kong (Hong Kong, China). pSV2/HER2 plasmid were a gift from Dr. ZQ Xiao (Xiangya School of Medicine, Changsha, China). The pAVU6+27/BCRP, pBabe and mutant plasmid pBabe-IκBα (IκBM) were described previously [16,17]. The pGL3-basic plasmid and the phRG-basic plasmid were purchased from Promega (Mannheim, Germany). Anti-HER2 antibody was purchased from NeoMarkers (Fremont, USA). The following antibodies were obtained from Cell Signaling (Beverly, USA): Akt, phospho-Akt (Ser473), IκBα, phospho-IκBα, p65 (Ser136), p44/p42 MAPK, phosphor-p44/p42 MAPK (Ser217/221). The following antibodies were obtained from Santa Cruz (California, USA): BCRP, Tubulin, histone H1 and horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibody. Anticancer drugs in research including Taxol, MX and 5-FU were purchased from Sigma Chemical Co., (St. Louis, USA) and cisplatin (DDP), etoposide (VP-16), and adriamycin (ADM) were from Shanghai Pharmacy Co., (Shanghai, China) LY294002, Bay11-7082, and PD98052 were purchased from EMD Biosciences (San Diego, USA).

Retroviral infection
A full-length HER2 gene was digested with HindIII and EcoRV from the pSV2/HER2 plasmid and subcloned into the retroviral vector PLNSX to generate pLNSX-HER2. For retroviral infection, the MCF7 cells at 70%–80% confluence were used. The culture medium was replaced with the virus-containing medium collected from the packaging cells (PA317) culture. After 5 h, the virus-containing medium was replaced with fresh RPMI 1640 medium supplemented with 10% FBS. The cells were selected with 700 μg/ml of G418 for 3 weeks. Three to five cell clones (MCF7/HER2) with resistance to G418 were picked, and added to culture medium containing 300 μg/ml G418. Control cells (MCF7/PLNSX) were transfected with an empty PLNSX vector.

Reverse transcription-polymerase chain reaction
Total RNA was isolated using Trizol (Invitrogen, Carlsbad, USA), and Reverse transcription-polymerase chain reaction (RT-PCR, MBI Fermentas, Vilnius, Lithuania) was used to analyze BCRP and HER2 expression. The 206 bp BCRP cDNA fragment was amplified with the primers 5’-CACCTTATGGCCTCAGGAA-3’ (sense) and 5’-CC TGCTTGGAAGGCTCTATG-3’ (antisense). The 176 bp HER2 cDNA fragment was amplified with the primers: 5’-ACATGCTCAGCCACCCTCTA-3’ (sense) and 5’-ACA TGCTCGGCACTCTA-3’ (antisense). As an internal control, the amplification of GAPDH mRNA was carried out with the primers 5’-ACCGTGGAGAAGGACTCAGA-3’ (sense) and 5’-GTACTTGCAGCTAGAGAG-3’ (antisense).

Western blot analysis
Cells were washed with cold phosphate-buffered saline (PBS) and kept on ice with RIPA buffer (Biyuntian, Shanghai, China) containing 1% phenylmethylsulfonyl. The cell debris was collected 30 min later by centrifugation at 13,000 rpm for 30 min at 4°C. Cytoplasmic and nuclear proteins were extracted with a commercially available kit (Active Motif, Carlsbad, USA). Protein concentrations...
were determined using BCA protein assay kit (Pierce, Rockford, USA). Equal amounts of protein lysate were electrophoretically separated on 8% or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, USA). The membranes were blocked by incubation in 3% non-fat dry milk for 1 h at room temperature, and then incubated with primary antibodies in PBS containing 0.01% Tween 20 overnight at 4°C. The membrane was washed three times with PBS and then incubated with an HRP-conjugated secondary antibody for 1 h at room temperature. The protein bands were detected using ECL detection system (Amersham Biosciences, Buckinghamshire, UK).

**Cytotoxicity assays**
The cells (4×10³ per well) were pre-cultured for 24 h in 96-well multiplates, then add the culture medium RPMI 1640 containing the different concentrations of Taxol, DDP, VP-16, ADM, MX or 5-FU. After treatment for 48 h, 5 mg/ml MTT (Sigma, St Louis, USA) solution was added to each well and incubated for 4 h at 37°C. After dissolving the resulting formazan product with acid-isopropanol, the absorbance was measured at 490 nm using ELISA microplate reader (Tecan, Mannedorf, Switzerland). Data represented the average absorbance of six wells in one experiment. The surviving rate of the cells was estimated via dividing the A490 nm of treated cells by the one experiment. The surviving rate of the cells was estimated from the drug concentration required to inhibit A490 to 50% of the control value. IC₅₀ values were estimated from the dose–response curve. Data were derived from at least three independent experiments.

**Luciferase reporter assay**
The construction of human wild-type and mutant of ABCG2 promoter vector have been described previously [16]. The ABCG2 promoter/firefly luciferase reporter vector was transfected in MCF7/HER2 and control cells using Lipofectamine 2000 (Invitrogen). The pGL3-basic plasmid, encoding firefly luciferase, was used to determine the basal level. In each experiment, the pHRG-Basic plasmid, encoding Renilla luciferase, was cotransfected for normalization purposes. Luminescence was measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega, Mannheim, Germany). Reporter activity was normalized by calculating the ratio of firefly/Renilla values. To assess the effects of the indicated inhibitors, MCF7/HER2 cells were pretreated with LY294002 or Bay 11-7082, or cotransfected with IκB-α or control plasmid pBabe. Each construct was tested in three independent transfections. Results were expressed as mean ± SD.

**Transfection of RNA interference plasmids**
The short-hairpin-RNA-encoding complementary single-stranded oligonucleotides corresponding to HER2 were designed according to the literature [16]. The oligonucleotides 5'-tcgacTGAACCTGACCTCCTCTCTAttcagaaTA GAGAGGTTCAGGTTTCTAattttt-3' and 5'-ctagaaaaaTGA AACCTGAACCTCCCTTcttctggaAGAGGTTCAG GTTTTGAG-3'; 5'-tcgagAGTCCTAAGGCCTCATGcttca agagGCATGCCTCCTTTAAAGAGTTTT-3' and 5'-ctagaaaa aaAGCTTCATAAGGCCTAGCttctggaGCATGCCTT TATGAAGCTG-3' were annealed and cloned into pAVU6+27 vector to generate HER2 (pAVU6+27/HER2) and control construction (pAVU6+27/control), respectively. Then, MCF7/HER2 cells were transfected with pAVU6+27/HER2, pAVU6+27/BCRP or pAVU6+27/ control using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. After being transfected for 48 h, the cells were collected to analyze the expression of the target protein.

**Statistical analysis**
Results were presented as the means ± SD from at least three independent experiments. Student’s t-test was used for the statistical analysis, and P values were determined using a two-tailed t-test assuming unequal variances (SPSS11.0, SPSS Inc., Chicago, USA). P < 0.05 was considered statistically significant.

**Results**
**HER2 overexpression leads to increased BCRP expression in MCF7 cells**
It is well known that HER2 activation enhances cancer progression and chemoresistance, and the BCRP is involved in the MDR in human breast cancer [3]. We hypothesized that BCRP played a role in HER2-mediated chemotherapy resistance. To test this hypothesis, we examined BCRP expression by RT-PCR and Western blot, and found that BCRP expression was higher in MCF7/HER2 cells than in the vector control cells (MCF7/PLNSX) both at the mRNA level and protein level (Fig. 1).

**Overexpression of HER2 induces PI3K/Akt/NF-κB signal transduction pathways’ activation**
Overexpression of HER2 has been reported to activate multiple signal transduction pathways including Ras-MAPK and PI3K/Akt/NF-κB, of which the latter has been shown to be critical for breast cancers development, progression and MDR [16]. We determined the state of these pathways in MCF7/HER2 cell, MCF7/PLNSX cell, and MCF7 cell. Western blot analysis showed that there was no change in the total levels of Akt, MAPK, and IκB among the cells, while the amounts of the pAkt and pIκB in MCF7/HER2...
cells were much higher than that in MCF7/PLNSX cells [Fig. 2(A)], which is in accordance with previous reports [2]. However, there was very little change in the levels of pMAPK. When NF-κB is activated, the nuclear p65 protein level is increased and cytosolic p65 pool is decreased, which is an indication of the nuclear translocation of NF-κB. Then, we detected the level of the most abundant subunit of NF-κB, p65, in nuclear and cytoplasmic extracts from MCF/HER2 cells and MCF7/PLNSX cells by Western blot. As shown in Fig. 2(B), the level of P65 in the nuclear increased in MCF7/HER2 cells, compared with MCF7/PLNSX cells. Taken together, these observations suggested that the PI3K/Akt/NF-κB signaling pathway was activated in HER2-overexpressing MCF7 cells.

Up-regulation of BCRP in MCF7/HER2 cells is via NF-κB activation and PI3K/AKT signaling

To determine whether HER2 was required for BCRP up-regulation, we used siRNA to knock down the HER2 expression in MCF/HER2 cells. Down-regulation of HER2 gene led to a significant decrease in BCRP expression in MCF7/HER2 cells compared with the pAVU6+27/control cells. In order to further explore the involvement of downstream processes, the MCF7/HER2 cells were treated with the PI3K/Akt inhibitor LY294002, the NF-κB inhibitor Bay11-7082 or MAPK inhibitor PD98052. As shown in Fig. 3, a decrease in the expression of BCRP was observed in the MCF7/HER2 cells treated with LY294002 and Bay11-7082. However, no significant change was observed by inhibition of MAPK by PD98052. These results suggested that HER2/AKT/NF-κB signaling pathways enhanced the expression of BCRP.

HER2 induces BCRP overexpression by increasing its promoter activity in MCF7 cells

BCRP expression can be regulated both at the levels of transcription (mRNA) and protein stability [18]. To further confirm whether HER2 regulates the promoter activity of the BCRP gene, and whether the PI3K/Akt/NF-κB signaling pathway mediates this effect, we performed reporter assays by transiently transfecting pGL3-BCRP reporter into MCF7/HER2 and MCF7/PLNSX cells. Relative luciferase activity was higher in MCF7/HER2 cells transfected with pGL3-BCRP than in MCF7/PLNSX control cells. Furthermore, the MCF7/HER2 cells were pretreated with PI3K inhibitor LY294002, NF-κB inhibitor Bay11-7082 or transfected with the dominant-negative IκBα expression plasmid. Consistent with decreased expression of BCRP in the protein level, we found that blocking HER2/Akt/NF-κB signaling pathway significantly downregulated the promoter activity. To verify the regulation of BCRP transcription by HER2 required an NF-κB binding site, we transfected an NF-κB-mutant construct to MCF7/HER2 cells.

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HER2 cells. The results showed that the luciferase activity of BCRP promoter was significantly reduced compared with the wild-type construct (Fig. 4).

**HER2 overexpression exhibits an increase in chemoresistance in MCF7 cells**

Since HER2 can induce BCRP expression, and BCRP plays an important role in chemoresistance, we then examined the sensitivity of MCF7/HER2 cell to a panel of six chemotherapeutic agents, including Taxol, DDP, VP-16, ADM, MX, and 5-Fu. These agents act with different mechanisms, and some of them are commonly used for breast cancer chemotherapy in clinics. As shown in

**Table 1 IC₅₀ values (mg/l) of different anticancer drugs in MCF7/HER2 cells and RI of the cells**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>MCF7</th>
<th>MCF7/PLNSX</th>
<th>MCF7/HER2</th>
<th>RI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxol</td>
<td>32.28 ± 5.19</td>
<td>33.67 ± 4.87</td>
<td>288.97 ± 5.58</td>
<td>8.58</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DDP</td>
<td>4.05 ± 0.94</td>
<td>4.01 ± 0.99</td>
<td>12.21 ± 1.35</td>
<td>3.04</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>VP-16</td>
<td>1.32 ± 0.06</td>
<td>1.28 ± 0.04</td>
<td>12.54 ± 1.18</td>
<td>9.79</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ADM</td>
<td>0.68 ± 0.03</td>
<td>0.66 ± 0.04</td>
<td>3.59 ± 0.16</td>
<td>5.44</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>5-Fu</td>
<td>12.79 ± 0.29</td>
<td>12.76 ± 0.31</td>
<td>158.85 ± 1.03</td>
<td>12.45</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MX</td>
<td>6.76 ± 0.18</td>
<td>6.68 ± 0.21</td>
<td>47.35 ± 0.86</td>
<td>7.08</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The MCF7, MCF/PLNSX and MCF7/HER2 cells were pulse-exposed to increasing concentrations of anticancer drugs for 72 h. The IC₅₀ values represent the mean ± SD (n = 3). RI means resistance index, representing IC₅₀(MCF7/HER2)/IC₅₀(MCF7/PLNSX).

The results demonstrated that HER2-overexpression could decrease the sensitivity of MCF7 breast cancer cells to toxic agents.

**BCRP depletion and suppressing Akt/NF-κB signaling pathways enhance the cytotoxicity of anticancer drugs against MCF/HER2 cells**

Finally, we investigated whether the increased resistance of MCF/HER2 cells to the panel of breast cancer drugs involved in the BCRP up-regulation, we silenced BCRP expression by RNAi [Fig. 5(A)] and then examined the effects of these chemotherapeutic agents on MCF7/HER2 cell vitality. As shown in Fig. 5(B), the inhibition of BCRP expression markedly sensitized MCF7/HER2 cells to VP-16, ADM, MX and 5-Fu, while the sensitivity to Taxol and DDP had no significant difference. Additionally, blocking activation of PI3K/AKT pathway by LY294002, and NF-κB signaling by Bay11-7082 could partially reverse the chemoresistance of MCF7/HER2 cells to these six drug tested, while the MAPK inhibitor PD98052 could not reverse HER2-mediated chemoresistance [Fig. 5(C)]. These results collectively indicated that BCRP, and the related PI3K/AKT/NF-κB signaling pathways play important roles in mediating the broad chemoresistance of MCF7/HER2 cells.

**Discussion**

This study was the first investigation into the regulation of BCRP gene expression by HER2 through the PI3K/Akt/NF-κB signaling pathway, which may be one of the mechanisms responsible for increased chemoresistance in HER2-overexpressing breast cancer cells.

Our results were consistent with previous reports, which showed that HER2 overexpression led to resistance to chemotherapeutic agents [3,6,7,19]. However, there are some discrepancies regarding chemosensitization by overexpression of HER2 in both laboratory and clinical studies. Some
studies suggested that HER2 overexpression had superior response to Taxanes, DDP and CMF (CTX, MTX, and 5-FU), whereas other studies showed no association between HER2-overexpression or HER2 amplification and resistance to CMF or to FEC (5-FU, Epirubicin, CTX) [10]. One possible reason for the controversy is that HER2 is frequently amplified in breast tumors as part of a wide region of amplification on chromosome 17q21. This amplicon contains many genes such as topoisomerase II-a and peroxisome proliferator-activated receptor binding protein, that are related to breast cancer susceptibility [20]. It is likely that the companion genes on the HER2 amplicon contribute to the reported drug sensitivity, while HER2 gene itself actually decreases chemosensitivity [9]. In addition, the comparison was made among different patients with different genetic background and fluorescence in situ hybridization or immunohistochemistry techniques for HER2 testing differed between these clinical studies. Nevertheless, there is no clear direct link between HER2 overexpression and chemosensitivity. In contrast, we compared chemosensitivity between HER2-low- and HER2-high-expressing cells with the same genetic background in our experimental model, which eliminated the multivariate in clinical studies. Taken together, further study is required to resolve the apparent contradictions between ErbB2 and chemotherapy sensitivity/resistance.

Indeed, the mechanism by which HER2-overexpression led to chemoresistance is very complex [21]. Recent work has often highlighted the importance of receptor kinases and cell survival/anti-apoptotic pathways in drug resistance [22], but very few connections have been made between receptor kinases and multidrug transporter expression or function. In a previous work, we found that a potential NF-kB binding motif located within the BCRP promoter region is probably involved in transcriptional regulation of BCRP gene [16,23]. The PI3K/AKT and consequently NF-kB signaling pathway was frequently activated in HER2 overexpressing cancer cells. Therefore, in this study, we aimed to investigate the possible interrelationships between NF-kB, HER2, and expression of BCRP in MCF7 human breast cancer cells. Using specific PI3K, MAPK, and NF-kB inhibitors, we showed that BCRP mRNA and protein were induced in MCF7/HER2 cells via PI3K/AKT/NF-kB pathways but not via MAPK pathways. However, it has been reported that regulation of P-gp expression and drug resistance via the PI3K/Akt/NF-kB signaling pathway [23]. Whether a similar association exists between the HER2 signaling pathway and other ABC transporters warrants further investigation.

In this study, we showed that the sensitivity to ADM, MX, and 5-FU of MCF7/HER2 cells was greatly increased by silencing BCRP expression, but it had no effect on the sensitivity to Taxol and DDP. Consistent with a previous study [24], this result indicated that the Taxol and DDP might not be substrates of BCRP pump. Moreover, this result also suggested that BCRP up-regulation was part of the mechanism of MCF7/HER2 cells against anticancer drugs, and multi-pathways might be involved in this process. Akt-mediated NF-kB activation that regulates several genes involved in cell survival, growth and anti-
apoptosis such as surviving, bcl-2, bcl-xl, X-linked inhibitor of apoptosis protein (XIAP), could also contribute to HER2-induced chemoresistance [25–27].

In summary, we demonstrated that BCRP was up-regulated by HER2 via the AKT/NF-κB signaling pathway, which may contribute at least partly to HER2-mediated chemoresistance. Our data suggested that there could be clinical benefits by combining appropriate conventional chemotherapeutic drugs with signaling transduction inhibitors that can block the HER2/AKT/NF-κB/BCRP pathway for the treatment of breast cancer. It should be pointed out that the MCF7 cell line, a well-characterized cell line, is not exactly the same as the breast cancer cells with respect to the expression of BCRP, and therefore, further studies are necessary to extrapolate data obtained in human subjects in vivo.

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**References**


