*Original Article*

# Effect of Trastuzumab on Notch-1 Signaling Pathway in Breast Cancer SK-BR3 Cells

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## ABSTRACT

**Objective:** To investigate the effects and mechanisms of trastuzumab on Notch-1 pathway in breast cancer cells, recognizing the significance of Notch-1 signaling pathway in trastuzumab resistance.

**Methods:** Immunocytochemistry staining and Western blotting were employed to justify the expression of Notch-1 protein in HER2-overexpressing SK-BR3 cells and HER2-non-overexpressing breast cancer MDA-MB-231 cells. Western blotting and reverse transcription PCR (RT-PCR) were used to detect the activated Notch-1 and Notch-1 target gene *HES-1* mRNA expression after SK-BR3 cells were treated with trastuzumab. Double immunofluorescence staining and co-immunoprecipitation were used to analyze the relationship of Notch-1 and HER2 proteins.

**Results:** The level of Notch-1 nuclear localization and activated Notch-1 proteins in HER2-overexpressing cells were significantly lower than in HER2-non-overexpressing cells ($P<0.01$), and the expressions of activated Notch-1 and *HES-1* mRNA were obviously increased after trastuzumab treatment ($P<0.05$), but HER2 expression did not change significantly for trastuzumab treating ($P>0.05$). Moreover, Notch-1 was discovered to co-localize and interact with HER2 in SK-BR3 cells.

**Conclusion:** Overexpression of HER2 decreased Notch-1 activity by the formation of a HER2-Notch1 complex, and trastuzumab can restore the activity of Notch-1 signaling pathway, which could be associated with cell resistance to trastuzumab.

**Key words:** Notch-1, HER2, Trastuzumab, Breast cancer

## INTRODUCTION

About 25%−30% of invasive breast cancer has been found overexpressing human epidermal growth factor receptor 2 (HER2/ErBb2/neu), a member of receptor tyrosine kinase[1]. HER2 overexpression has closely related to poor breast cancer prognosis and high invasiveness[2]. Numerous efforts have been directed at developing HER2-targeting cancer therapies. One successful example is trastuzumab (Herceptin; Genentech, San Francisco, CA), a humanized monoclonal antibody (mAb) against the extracellular domain of HER2. It showed a significantly survival benefit when combined with cytotoxic chemotherapy[3]. However, the objective response rate of trastuzumab was only 12%−34%. Most patients with metastatic breast cancer who responded initially to trastuzumab developed acquired resistance within the first year and the primary resistance to single-agent trastuzumab ranges from 66% to 88%[4]. Thus the primary and acquired resistance to trastuzumab therapy was significant clinical problem and has aroused considerable concern.

Notch proteins are highly conserved type I single transmembrane proteins. In mammals, there are four Notch receptors (Notch 1−4), and five ligands (Jagged 1 and 2, and Delta-like 1, 3, and 4). Notch receptors are composed of extracellular domain ($Notch^{EC}$), transmembrane domain ($Notch^{TM}$) and intracellular domain ($Notch^{IC}$/NIC), and the extracellular region consists of several tandem repeats of epidermal growth factor (EGF-like repeats), which mediate the interaction of receptors and ligands[5]. Notch pathway is triggered by ligand binding, inducing a cascade of proteolytic cleavages that release NIC. NIC as activated molecule then translocates to the nucleus to combine with transcription repressor CBF1 (also known as CSL or RBP-Jκ), and activates target gene transcription. The

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targets of Notch include HES and HEY family members[6], cell cycle regulators P21, cyclin D1, c-Myc, NF-κB2, and the factors that regulate apoptosis[7-11]. Numerous studies shown that *Notch-1* as oncogene in breast cancer inhibited tumor differentiation, and promoted proliferation and angiogenesis[12]. It was supposed that *Notch-1*, a tumor oncogene, maybe associated with trastuzumab resistance.

In this study, we detected the Notch-1 activity between breast cancer cells SK-BR3 and MDA-MB-231, and analyzed the expression of activated Notch-1 and HER2 proteins after trastuzumab treatment in SK-BR3 cells. Meanwhile, the interaction between Notch-1 and HER2 was detected by co-immunoprecipitation and immunofluorescence staining, so as to investigate the role of Notch-1 signaling pathway in trastuzumab resistance.

## MATERIALS AND METHODS

### Cell Lines

MDA-MB-231 cell line was maintained at Laboratory for Stem Cell and Tissue Engineering, Chongqing Medical University. SK-BR3 cell line was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). SK-BR3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA), MDA-MB-231 cells were cultured in RPMI-1640 (Gibco, USA), which were supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were maintained in an incubator at 37°C with a humidified atmosphere of 5% $CO_2$.

### Immunocytochemical Staining

Cell preparation and immunocytochemical staining were performed as described previously[13]. The goat anti-Notch-1 antibodies (Santa Cruz, CA) were added to cells and incubated overnight at 4°C. PBS was used to replace the first antibody as the negative control.

### Western Blotting

Cells were solubilized in radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mmol/L orthovanadate, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, pH 8.0). Western blotting was performed as described previously[14]. The primary antibodies were incubated overnight at 4°C. Signals were visualized and detected using ECL Plus Chemiluminescence Detection reagents (Bio-Rad, USA). Antibodies used were: HER2 (Santa Cruz, CA), and activated Notch-1 (Millipore, CA, USA ).

### Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cultured cells using the TRIzol method (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was reverse-transcribed to total cDNA using the PrimeScript™ RT reagent kit (TaKaRa Biotechnology, Co., Ltd. Japan). PCR was performed on cDNAs to detect relative expression of *HES-1* (forward primer: 5'-AAATGACAGTGAAGCACCTCCG-3', and reverse primer; 5'-GAAGCCTCCAAACACCTTAGCC-3') and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (forward primer; 5'-CGACAGTCAGCCGCATCTTCTT-3', and reverse primer; 5'-CATGAGTCCTTCCACGA-TACCAA-3'). The annealing temperatures for *HES-1* and *GAPDH* were 58°C and 60°C, respectively.

### Double Immunofluorescence Staining

Cell preparation and fixation were performed as immunocytochemistry staining. Goat anti-Notch-1 and mouse anti-HER2, as the primary antibodies, were incubated overnight at 4°C, followed by reaction with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (1:200) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-goat IgG at 37°C for 1 h. A laser scanning confocal microscope (LSCM; LSM510-Zeiss, Germany) was used to detect Notch-1 and HER2 fluorescence.

### Immunoprecipitations

All procedures were performed at 4°C unless otherwise specified. Approximately $10^7$ cells were collected after 48 h plated in 500 μl of cold radio-immunoprecipitation assay (RIPA) buffer. Cell lysates were added to protein G Agarose (Beyotime, China) and incubated for 1 h on a rocking platform to clarify the sample. After centrifuged, the primary antibodies (Notch-1 or HER2), or non-immune rabbit IgG were added to the supernatants, and rotated overnight at 4°C. The following day, the protein G Agarose (40 μl) was added to the mixture and rotated for 2 h, washed 5 times in RIPA buffer for 5 min each and resuspended and boiled in 40 μl sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Immunoprecipitated proteins were collected for Western blotting analyses.

### Statistical Analysis

All data were expressed as $\bar{x}\pm s$ from at least three independent experiments. Differences among four groups were determined by analysis of one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test for multiple comparisons, whereas differences between two groups were evaluated by the Student's *t*-test. $P<0.05$ was considered statistically significant. Statistical analysis was performed by the

SPSS 17.0 (SPSS Inc., Chicago, USA) statistical software programs.

## RESULTS

### Expression of Notch-1 in SK-BR3 and MDA-MB-231 Cells

The expression of Notch-1 can be seen from both cytoplasm and membrane in SK-BR3 and MDA-MB-231 cells, and there were no significant differences. But the nuclear localization of Notch-1 in SK-BR3 cells was significantly lower than that in MDA-MB-231 cells (Figure 1). For the important role of activated Notch-1 in Notch-1 signaling pathway, we detected the difference of expression of activated Notch-1 and HER2 by Western blotting in those two cell lines (Figure 2). The results showed that the protein expressing of activated Notch-1 was significantly decreased ($P$<0.01) in HER2 over-expressing cells (SK-BR3) by 2.27 fold as compared to HER2 lower-expressing breast cancer cells (MDA-MB-231). Those results suggested that overexpressing HER2 could suppress Notch-1 activity.

### Effect of Trastuzumab on the Expression Levels of Activated Notch-1 and HER2 Proteins and *HES-1* mRNA in SK-BR3 Cells

To detect the effect of trastuzumab on the Notch-1 pathway and HER2, the SK-BR3 cells were treated with trastuzumab (20 μg/ml) for 0, 24, 48 and 72 h, respectively. Comparing to the non-treated group, the results showed the levels of activated Notch-1 protein and *HES-1* mRNA significantly increased ($P$<0.01), and the peak time was 24 h. At 48 h, they tended to be stable (compared to 72 h, there was no remarkable difference, $P$>0.05, Figure 3). Meanwhile, compared with the

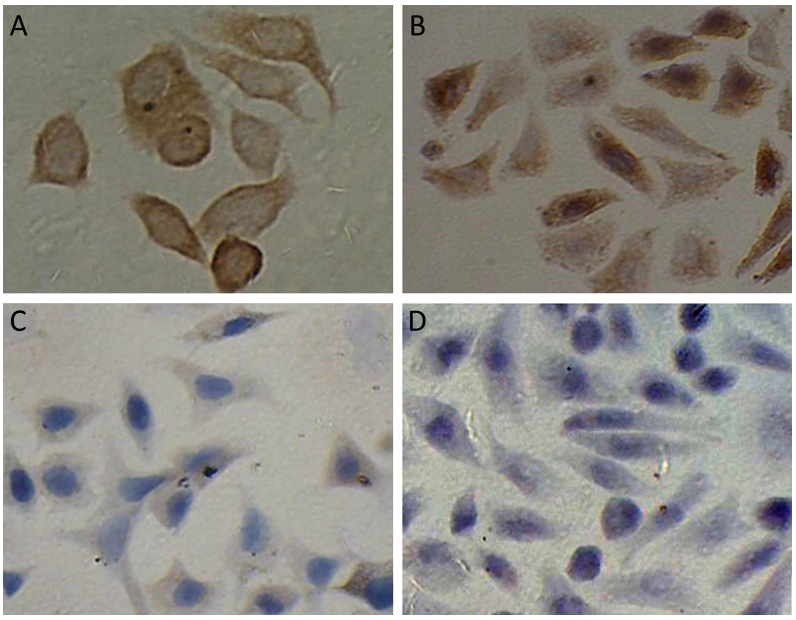


**Figure 1.** Immunohistochemical staining for Notch-1 in breast cancer cells (400×). **A:** Notch-1 expression was found in cell membrane and cytoplasm at various levels in SK-BR3 cells; **B:** Notch-1 expression was found in membrane and cytoplasm at various levels in MDA-MB-231 cells; **C, D:** Replacement of the anti-Notch-1 primary antibody with PBS in SK-BR3 cells and MDA-MB-231 cells showed the negative results. The results showed that the nuclear localization of Notch-1 in SK-BR3 cells was significantly lower than that in MDA-MB-231 cells.

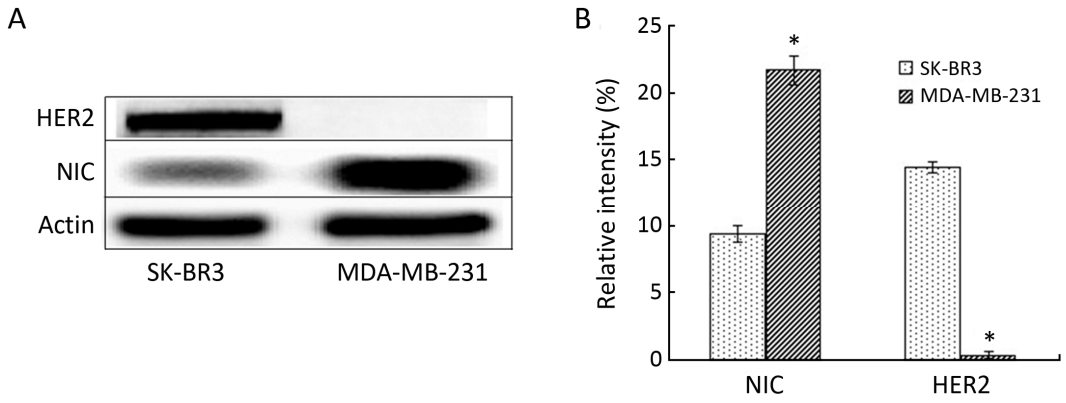


**Figure 2.** Expressions of activated Notch-1 and HER2 proteins in SK-BR3 and MDA-MB-231 cells by Western blotting. β-actin was used as a loading control. Results represent the $\bar{x}\pm s$ for three independent experiments at least. NIC represents activated Notch-1. *Significantly different from control value: $P$<0.01.

control group, the levels of HER2 were essentially not changed by the trastuzumab treatment (to compare with control group, there were no obviously differences, $P>0.05$, Figure 3A and B). Those observations suggest that trastuzumab could activate Notch signaling pathway through increasing the levels of activated Notch-1 proteins and its target gene *HES-1* mRNA, but can't influence on HER2 proteins, which is in agreement with the existing foundings[15].

### Co-localization and Interaction of Notch-1 with HER2 in SK-BR3 Cells

To identify the underlying molecular mechanism of the above findings, we tested the relationship between Notch-1 and HER2 by double immunofluorescence staining and co-immunoprecipitation (Figure 4). Double immunofluorescence staining showed co-localization of Notch-1 (red) and HER2 (green) in SK-BR3 cells. The yellow staining in dual-labeling experiments represented over-lapping areas of red and green fluorescent labels, suggesting co-localization of Notch-1 with HER2 in SK-BR3 cells. To further investigate the interaction of Notch-1 and HER2, immunoprecipitate of the anti-HER2 antibody was proved by the anti-Notch-1 antibody, and *vice versa*. Immunoreactive signals of HER2 and Notch-1 are clearly detected in the immunoprecipitates of anti-Notch-1 and anti-HER2, respectively. The results are shown as Figure 4, indicating the specificity of the Notch-1-HER2 interaction in SK-BR3 cells.

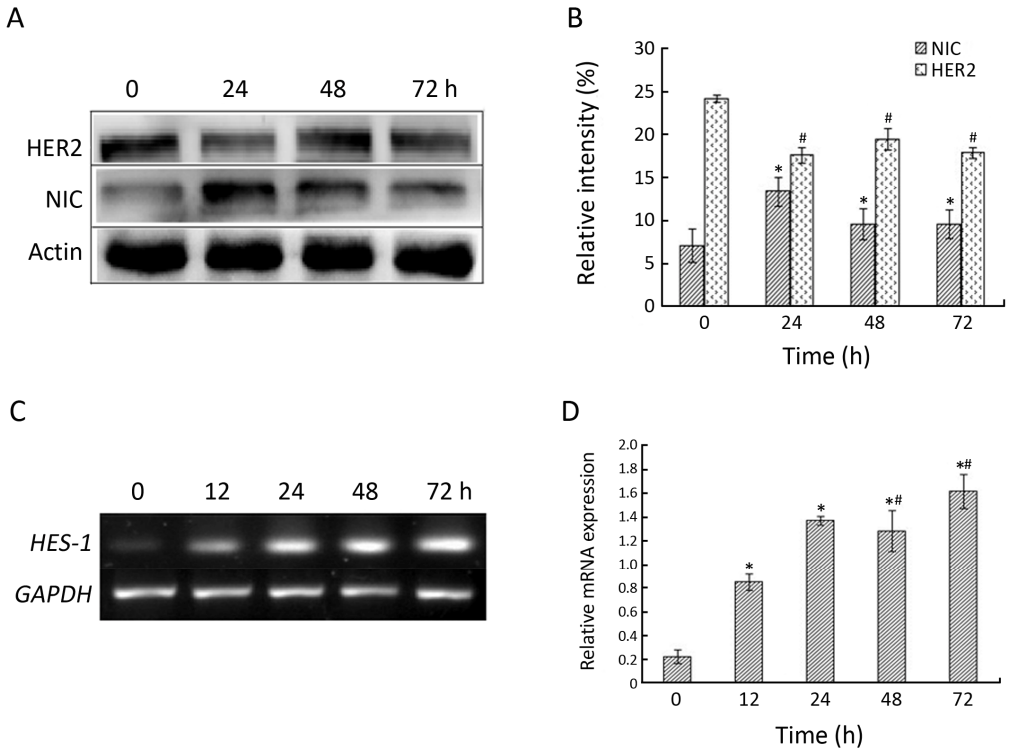


**Figure 3.** Effect of trastuzumab on the expression levels of activated Notch-1 and HER2 proteins and *HES-1* mRNA in SK-BR3 cells. **A, B:** SK-BR3 cells were treated with trastuzumab for 0, 24, 48 and 72 h, respectively. The expression levels of activated Notch-1 and HER2 were determined by Western blotting. NIC represents activated Notch-1. Significantly different from control value: $^{*}P<0.05$, $^{\#}P>0.05$. **C, D:** *HES-1* mRNA was detected by RT-PCR, in which SK-BR3 cells were treated with trastuzumab for 0, 12, 24, 48 and 72 h, respectively. Results represent the $\bar{x}\pm s$ for three independent experiments at least. $^{*}$Significantly different from control value: $P<0.05$; $^{\#}$Significantly different from 24 h group: $P>0.05$.

## DISCUSSION

The development of targeted therapies for breast cancer, such as trastuzumab (against HER2) and tamoxifen [against estrogen receptor (ER)], has a major effect on improving disease-free and overall survival for patients. However, it is known that the signaling pathways required for cell proliferation or invasion are related to multiple pathways in cross-talk. As a result, the specifically inhibition of HER2 leads to the signaling network in cancer cells to reset and respond by activating other pathways that can promote cancer cell proliferation and survival. In other words, cancer cells were no longer sensitive to the originally HER2 pathway targeted therapy and to enable therapeutic resistance.

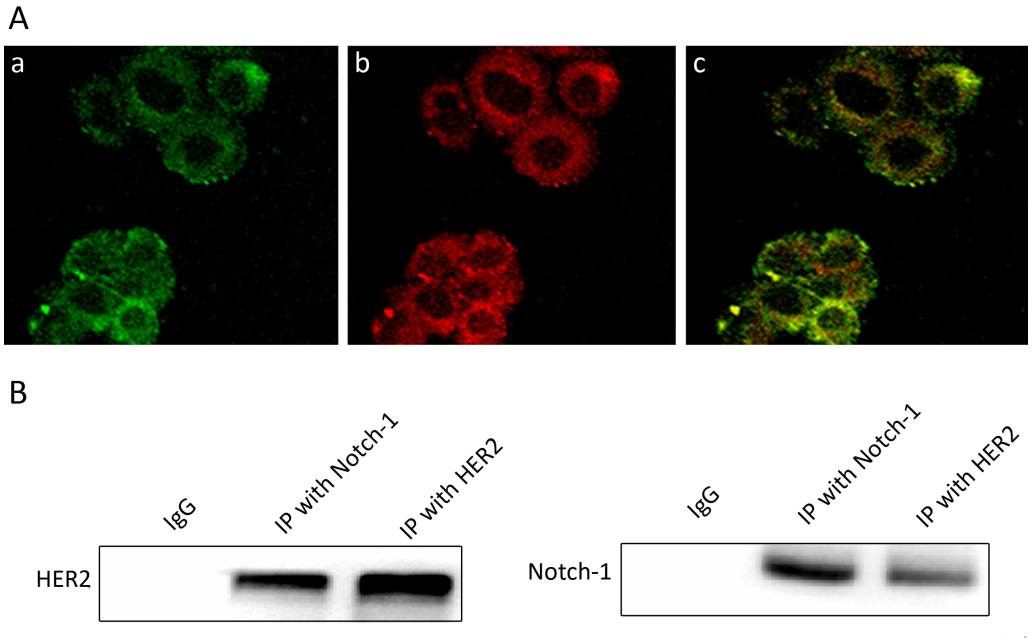


**Figure 4.** Co-localization and interaction of Notch-1 with HER2 in SK-BR3 cells. **A:** LSCM imaging of co-localization of Notch-1 with HER2 in SK-BR3 cells. Cells were stained with anti-Notch-1 pAb (a) and anti-HER2 mAb (b). The secondary antibody in (a) was FITC-conjugated rabbit anti-mouse IgG (green) and in (b) was TRITC-labeled rabbit anti-goat IgG (red). Co-localization of Notch-1 with HER2 results in a yellow staining (c) (400×); **B:** Co-immunoprecipitation assay of interaction of Notch-1 with HER2 in SK-BR3 cells. Co-immunoprecipitated proteins from anti-Notch-1 pAb, anti-HER2 mAb or non-immune rabbit IgG immunoprecipitating cells were analyzed by Western blotting using anti-Notch-1 pAb or anti-HER2 antibody, respectively. Specific interaction was found between Notch-1 and HER2 in SK-BR3 cells.

At present, a number of studies have led to the identification of factors associated with primary and acquired trastuzumab resistance. Loss or decreased expression of phosphatase and tensin homolog (PTEN), a phosphoinositide-3 kinase phosphatase (PI3K), or activation of the PI3K/protein kinase B (PI3K/AKT) pathway in cancer patients leads to trastuzumab resistance and an increased risk of progression to HER2-targeted therapy[16]. Moreover, the interaction between HER2 and trastuzumab may be sterically hindered. Increased mucin 4 (MUC4, cell surface associated) bound to HER2, which not only promoted HER2 phosphorylation, but interfered with trastuzumab to bind with HER2 through space occupying effect, finally decreased the susceptibility to drug[17]. Kang, et al.[18] held that up-regulated heat shock protein 27 (HSP27) in human breast cancer cells could reduce trastuzumab susceptibility by increasing HER2 protein stability. Moreover, they found that downregulation of HER2 by trastuzumab can be hindered by the structure of a HER2-HSP27 composite. Insulin-like growth factor 1 receptor (IGF-1R) could activate PI3K/AKT and mitogen-activated protein kinase (MAPK) signaling pathways by forming heterodimeration with HER2, eventually decreased the inhibition effect of trastuzumab[19]. Obviously, the resistance mechanisms of trastuzumab are linked of numerous signaling molecular, in which space occupying effect of HER2 may be an important type of action, thereby we engages the key molecular target for trastuzumab resistance lying on the presumption.

Notch receptors are a group of highly conserved transmembrane proteins, and closely related with embryonic development, cell differentiation and apoptosis. Notch signaling first linked to tumorigenesis was in pre-T-cell acute lymphoblastic leukemias (T-ALL)[20]. Dysregulation of Notch proteins has also been documented in breast, colon, pancreatic, lung, and head and neck carcinomas[21, 22]. Notch pathway could be either oncogenic or tumor-suppressive depending on the cell types and contexts. For example, in small cell lung cancer, activated Notch-1 and Notch-2 caused a potent G1 arrest accompanied by marked up regulation of $P21^{waf1/cip1}$, thereby Notch functions as a tumor suppressor in the type cells[23]. In contrast, in mouse mammary tumors, mouse mammary tumor virus (MMTV), which is widely used to identify oncogenes, frequently inserted into *Notch-1*[24] and *Notch-4*[25] genes, leading to the mammary tumor occurrence. Studies have shown that Notch-1 high-expression was

associated with poor overall survival, and can be considered as a prediction in breast cancer[26]. Notch signaling pathway plays a predominantly oncogenic role in breast tumor, which is largely through its interaction with other pathways, involving Ras/MAPK[27], transforming growth factor-β (TGF-β)[28], vascular endothelial growth factor (VEGF)[29] and so on. For the multiple cross-talk with other molecules, Notch-1 could be associated with resistance of drugs. The preliminary results from Nefedova Y, et al.[30] showed that the activation of Notch-1 resulted in protection of tumor cells from melphalan- and mitoxantrone-induced apoptosis and this protection was associated with up-regulation of $P21^{WAF/Cip}$. What's more, activated Notch-1 synergized with papillomavirus oncogenes in transformation of immortalized epithelial cells and led to the resistance to anoikis, an apoptotic response induced on matrix withdrawal, through the activation of PKB/AKT[31]. Osipo, et al.[32] reported that high level of HER2/neu suppressed Notch activity and HER2/neu inhibition with Trastzumab induced Notch activation, but the specific mechanisms were unclear. Those revealed that activated Notch-1 pathway played an important role in resistance of drugs.

In this study, we determined the expression of Notch-1 in breast cancer cells with immunohistochemical staining and Western blotting to explore the association between Notch-1 and HER2. As a result, Notch-1 positive expression has been observed on the cell membrane and cytoplasm of breast carcinoma cells as previously reported, and the activation levels of Notch-1 were significantly lower in HER2-over-expressing cells than in HER2-non-overexpressing cells, indicating that overexpression of HER2 inhibited Notch-1 transcriptional activity. Besides that, our research reveals a direct relationship between Notch-1 and HER2. Double immunofluorescence staining and co-immunoprecipitation results showed that Notch-1 co-localizes and interacts with HER2 in HER2-overexpressing breast cancer cells, which indicating that HER2 affected Notch-1 might result in reducing the binding between Notch-1 ligands and receptors, further decrease the activated Notch-1 levels. Then we used trastuzumab 20 μg/ml (the lowest blood concentration of patients *in vivo*[33]) to inhibit HER2, the results showed that the expression levels of activated Notch-1 and *HES-1* mRNA were up-regulated. We suspected the possible mechanism was trastuzumab competing with Notch-1 to bind ectodomain of HER2, which finally released Notch-1 and induced activation of Notch-1 pathway. Owing to Notch pathway activation, breast cancer cells could depend on Notch, but not HER2 to keep proliferation and/or survival, which ultimately interferences with the antitumor effect of trastuzumab. Meanwhile, we find that the expression of HER2 proteins do not change after trastuzumab treatment, perhaps because the compound of $Notch\text{-}1^{IC}$ and CSL could combine with the HER2 promoter, and further activate the transcription factor to maintain the expression of HER2[34].

In conclusion, our findings demonstrate that over-expressing HER2 decreased Notch-1 activity by the formation of HER2-Notch1 complex, and trastuzumab can restore the activity of Notch-1 signaling pathway, which could be associated with cell resistance to trastuzumab. Activation of Notch-1 signaling pathway could provide important clues for understanding the mechanism of trastuzumab resistance in breast cancer cells.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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