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

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