

Expression of Wnt3 Activates Wnt/ β -Catenin Pathway and Promotes EMT-like Phenotype in Trastuzumab-Resistant HER2-Overexpressing Breast Cancer Cells

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Abstract

To understand the mechanisms leading to trastuzumab resistance in HER2-overexpressing breast tumors, we created trastuzumab-insensitive cell lines (SKBR3/100-8 and BT474/100-2). The cell lines maintain HER2 receptor overexpression and show increase in EGF receptor (EGFR). Upon trastuzumab treatment, SKBR3/100-8 and BT474/100-2 cell lines displayed increased growth rate and invasiveness. The trastuzumab resistance in SKBR3/100-8 and BT474/100-2 was accompanied with activation of the Wnt/ β -catenin signaling pathway. Further investigation found that Wnt3 overexpression played a key role toward the development of trastuzumab resistance. The expression of Wnt3 in trastuzumab-resistant cells increased nuclear expression of β -catenin and transactivated expression of EGFR. The increased Wnt3 in the trastuzumab-resistant cells also promoted a partial EMT-like transition (epithelial-to-mesenchymal transition); increased N-cadherin, Twist, Slug; and decreased E-cadherin. Knockdown of Wnt3 by siRNA restored cytoplasmic expression of β -catenin and decreased EGFR expression in trastuzumab-resistant cells. Furthermore, the EMT markers were decreased, E-cadherin was increased, and the cell invasiveness was inhibited in response to the Wnt3 downregulation. Conversely, SKBR3 cells which had been stably transfected with full-length Wnt3 exhibited EMT-like transition. The Wnt3 transfectants, SKBR3/Wnt3-7 and SKBR3/Wnt3-9, showed a significant decrease in E-cadherin and increase in N-cadherin, Twist, and Slug. The cells were less sensitive to trastuzumab than parental SKBR3 and vector-transfected cells. In summary, our data suggest that Wnt3 overexpression activates Wnt/ β -catenin signaling pathway that leads to transactivation of EGFR and promotes EMT-like transition. This could be an important mechanism leading to trastuzumab resistance in HER2-overexpressing breast cancer cells. *Mol Cancer Res*; 10(12): 1597–606. ©2012 AACR.

Introduction

Trastuzumab treatment has improved the overall survival rate of patients with HER2-overexpressing breast cancer. However, some patients with HER2-overexpressing breast cancer do not respond to trastuzumab therapy, as a single agent or in combination with chemotherapy, and the mechanisms underlying the resistance phenotype are not well understood. Some studies have suggested increase in HER2 receptor homo-

and heterodimerization with other receptors of the ErbB family, such as HER1 (EGFR), HER3, and HER4 (1, 2). These interactions activate intracellular signaling via the mitogen-activated protein kinase (MAPK), or phosphoinositide 3-kinase (PI3K) pathways (3, 4). We have previously shown that activation of PI3K/Akt pathway inhibited the transcription factor FOXO1A, resulting in nuclear export of p27^{kip1} and reduced the inhibitory properties of trastuzumab (5). Patients with breast cancer with HER2-overexpressing tumors have increased active Akt (pAkt) in their tumors (6). The activation of PI3K/Akt and loss of PTEN may also result in accumulation of β -catenin, which suggests a crosstalk between the PI3K and Wnt signaling pathways (7–11).

The goal of this study is to understand the mechanisms leading to trastuzumab resistance in HER2-overexpressing breast tumors and which pathway-specific genes may contribute to the resistance.

Materials and Methods

Cell lines and cell cultures

The human breast cancer cell lines SKBR3 (ATCC: HTB-30) and BT474 (ATCC: HTB-20) were obtained from the

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American Type Culture Collection (ATCC). Unless otherwise stated, monolayer cultures of SKBR3 and BT474 cells were maintained in Dulbeccos' Modified Eagle's Media (DMEM):F12 medium with 10% FBS. The cell lines overexpressed the HER2/*c-erb-2* (HER2) gene product. The trastuzumab-resistant clones, SKBR3/100-8 and BT474/100-2, were generated from SKBR3 and BT474 cells, respectively. To select trastuzumab-resistant clones, SKBR3 and BT474 cells were plated in 24-well plates at low density and maintained in growth medium containing 10, 50, and 100 $\mu\text{g}/\text{mL}$ of trastuzumab. The SKBR3/100-8 and BT474/100-2 clones were maintained in growth medium containing 100 $\mu\text{g}/\text{mL}$ of trastuzumab for over 2 and 1 year, respectively. Both SKBR3/100-8 and BT474/100-2 were repeatedly confirmed as insensitive to trastuzumab. The SKBR3/Wnt3-7 and SKBR3/Wnt3-9 were generated by stable transfection of full-length Wnt genes into SKBR3 cells, as well as clonal selection.

Microarray analysis

Total RNA was isolated from SKBR3 and SKBR3/100-8 cultured cells by using RNeasy micro kit (#74004; Qiagen). The quality of RNA was determined by separation of the RNA via capillary electrophoresis using the Agilent 2100 Bioanalyzer. Whole Human Genome 4 \times 44K (Cat#: G4112F, Agilent) expression array was used to compare the gene profiles between SKBR3 and SKBR3/100-8. Microarray slides were read using Agilent Scanner. Agilent Feature Extraction software version 9.13 was used to calculate the gene expression values. Differences of $P < 0.01$ and ≥ 2 -fold expression were considered as significant.

The Wnt/ β -catenin pathway-regulated gene profiles in the cell lines were examined using Human Wnt/ β -catenin Regulated cDNA plate array (Cat# AP-0171, Signosis) according to manufacturer's instructions.

siRNA knockdown genes

Wnt3 siRNA, a pool of 3 target-specific 19–25 nt siRNA (sc-41106, Santa Cruz Biotechnology), was used to knockdown the Wnt3 gene. The EGFR siRNA (sc-29301, Santa Cruz Biotechnology) targeting specific 20–25 nt siRNA and the ErbB-3 siRNA (sc-35327, Santa Cruz Biotechnology) targeting specific 19–25 nt siRNA were used to knockdown EGFR and HER3 gene expression, respectively.

siRNA-A negative sequence (sc-37007, Santa Cruz Biotechnology) was used in parallel for each knockdown experiment served as control. Lipofectamine 2000 transfection reagent (Cat#: 11668-019, Invitrogen) was used for transfection following the manufacturer's instructions. Gene expression after siRNA knockdown was determined by PCR or quantitative reverse transcription PCR (qRT-PCR), with specific primers (Supplementary Table S1).

Overexpressing Wnt3 gene

Overexpressing Wnt3 was done by stable transfection of a full-length Wnt3 gene (RG21115, Origene) into the cell; the empty vector (PS100010, Origene) was also transfected into SKBR3 cells as a control. Lipofectamine PLUS reagent

(Invitrogen) was used for transfection following the manufacturer's instruction. The single clones from the Wnt3 transfected cells were selected by 400 $\mu\text{g}/\text{mL}$ G418 and confirmed by qRT-PCR and Western blot analysis.

Boyden Chamber invasion assay

The invasive assay was done in 24-well cell culture chambers using inserts with 8- μm pore membranes pre-coated with Matrigel (28 $\mu\text{g}/\text{insert}$; Sigma). Cell suspensions ($2 \times 10^5/\text{mL}$) were placed in the upper wells and fibroblast-conditioned medium was filled in the lower wells. The cells were cultured for 24 hours and then fixed by 0.5 mL of 0.5% glutaraldehyde in 1 \times PBS and stained by 0.5 mL of 0.5% Toluidine Blue. The numbers of invaded cells was counted with $\times 20$ objective of microscope from 3 files per membrane and then normalized with total numbers of cells. Each experiment was carried out twice and each condition was duplicated at each time.

Chromatin immunoprecipitation–qRT-PCR assay

The chromatin–protein complex was prepared from SKBR3 and SKBR3/100-8 cells by immunoprecipitation of the chromatin with β -catenin antibody using Magna-ChIP assay kit (Cat# 17-10085, Millipore) following the manufacturer's instruction and then qPCR was carried out. The primer sequences were designed to cover the LEF/TCF binding region of the promoter of EGFR (711–727): Lt: 5'-GCCTGGTCCCTCCTCCTC-3' and Rt: 5'-GCTCTCCCGATCAATACTGG-3'. Fold enrichment in the β -catenin precipitated samples was calculated relative to the mock samples (precipitated with normal IgG). The data were also calculated as % input to ensure consistent results.

Immunoblotting analysis

The NE-PER Nuclear and Cytoplasmic Extraction reagents (Cat#: 78833, Thermo Scientific) were used to extract the nuclear and cytoplasmic protein following the manufacturer's instructions. Immunoblotting was conducted with antibodies specific to HER2, E-cadherin, β -catenin, and histone from Cell Signaling; and EGFR, Wnt3, MMP-7, VEGF, α -tubulin, and β -actin from Santa Cruz Biotechnology.

Statistical analysis

The statistical significances of mean values among different cell lines were determined by 1-way ANOVA first, then by Student *t* test, and the fold changes were analyzed by χ^2 test. $P \leq 0.05$ was considered statistically significant.

Results

Characterization of trastuzumab-resistant cell lines, SKBR3/100-8 and BT474/100-2

Data from MTT assay showed that the growth of SKBR3 and BT474 parental cells was significantly inhibited by fresh treatment with 10 and 100 $\mu\text{g}/\text{mL}$ of trastuzumab, whereas the growth of SKBR3/100-8 and BT474/100-2 cells which

were maintained in media containing 100 $\mu\text{g}/\text{mL}$ trastuzumab were not inhibited in 5 days (Fig. 1A). The number of cells overexpressing HER2 receptors was reduced in SKBR3 and BT474 cells upon fresh treatment with trastuzumab (10 $\mu\text{g}/\text{mL}$) for 72 hours. However, SKBR3/100-8 and BT474/100-2 cells maintained in growth media containing 100 $\mu\text{g}/\text{mL}$ trastuzumab for a long period of time did not exhibit any significant difference in the overall number of HER2 receptor overexpression compared with the untreated parental cells (Fig. 1B, left). There was no change in phosphorylated HER2 receptors between the trastuzumab-resistant and -sensitive cells (Supplementary Fig. S1). Both SKBR3/100-8 and BT474/100-2 showed increased EGFR compared with parental cell lines (Fig. 1B, right). The HER2/HER3 dimerization was increased in SKBR3/100-8 (Supplementary Fig. S1) but no changes were found in BT474/100-2 (data not shown). Both SKBR3/100-8 and BT474/100-2 cells displayed significant enhanced invasive capacity (Fig. 1C).

Compared with parental SKBR3 and BT474 cells, the SKBR3/100-8 and BT474/100-2 cells had decreased E-cadherin and increased mesenchymal markers, N-cadherin, Slug, and Twist (Fig. 1D). Vimentin expression increased in BT474/100-2 compared with BT474, but there was no difference between SKBR3/100-8 and SKBR3 cells (Fig. 1D).

Deregulated Wnt pathway genes in the resistant clone, SKBR3/100-8, analyzed by Agilent chip microarray

Using the Agilent chip array ($4 \times 44\text{K}$), we have identified more than 3,000 genes. Many of these genes either increased or decreased in expression by ≥ 2 -fold ($P < 0.01$) in SKBR3/100-8 compared with parental cells, SKBR3.

The differentially expressed molecules included 42 transcriptional factors (TF) and 14 were analyzed by Ingenuity Pathway Analysis (IPA) as either activated (regulation z -score ≥ 2) or inhibited (regulation z -score ≤ -2) SKBR3/100-8 compared with SKBR3 based on regulation z -score (Data

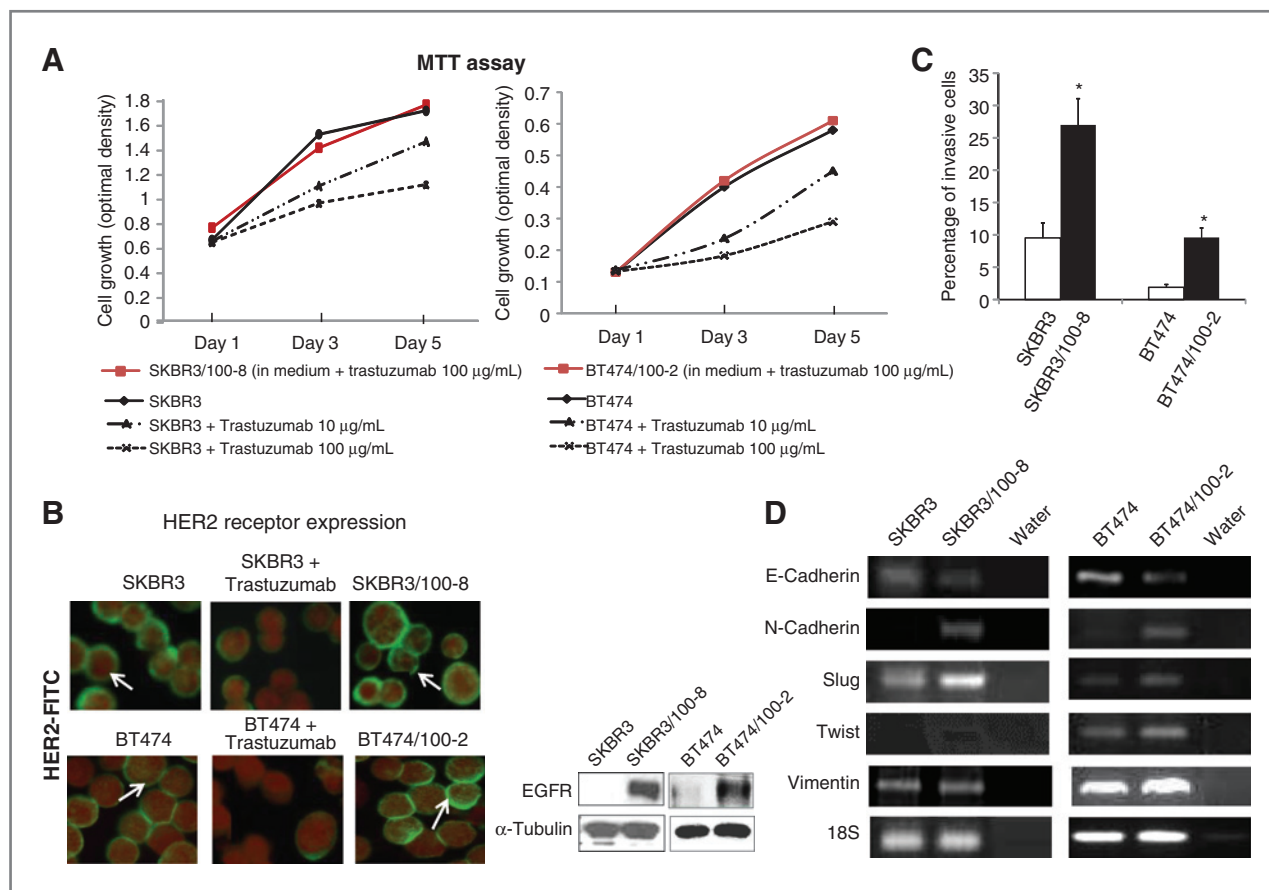


Figure 1. Characterization of trastuzumab-resistant clones. A, SKBR3/100-8 and BT474/100-2 were maintained in growth medium containing 100 $\mu\text{g}/\text{mL}$ of trastuzumab. SKBR3 and BT474 were treated with trastuzumab at 0, 10, and 100 $\mu\text{g}/\text{mL}$, and MTT assay was conducted at indicated days. B, SKBR3 and BT474 were treated with or without trastuzumab (10 $\mu\text{g}/\text{mL}$) for 3 days. HER2 receptors were assessed by immunofluorescence analysis with fluorescein isothiocyanate (FITC)-labeled anti-HER2 antibody (green), and the cell nuclei were labeled by propidium iodide (red). The arrows indicate positive HER2 staining (right). The EGFR protein expression in indicated cell lines was measured by Western blot analysis and α -tubulin was used as loading control (left). C, cell invasiveness in the indicated cell lines was measured by Boyden Chamber invasion assay as described in Materials and Methods. The invasive cells (mean \pm SD from 5 different areas) were counted. *, $P < 0.05$ compared with untreated cells. D, mRNA levels of the indicated genes in SKBR3, SKBR3/100-8, BT474, and BT474/100-2 were analyzed by RT-PCR and "water" was used as vehicle control.

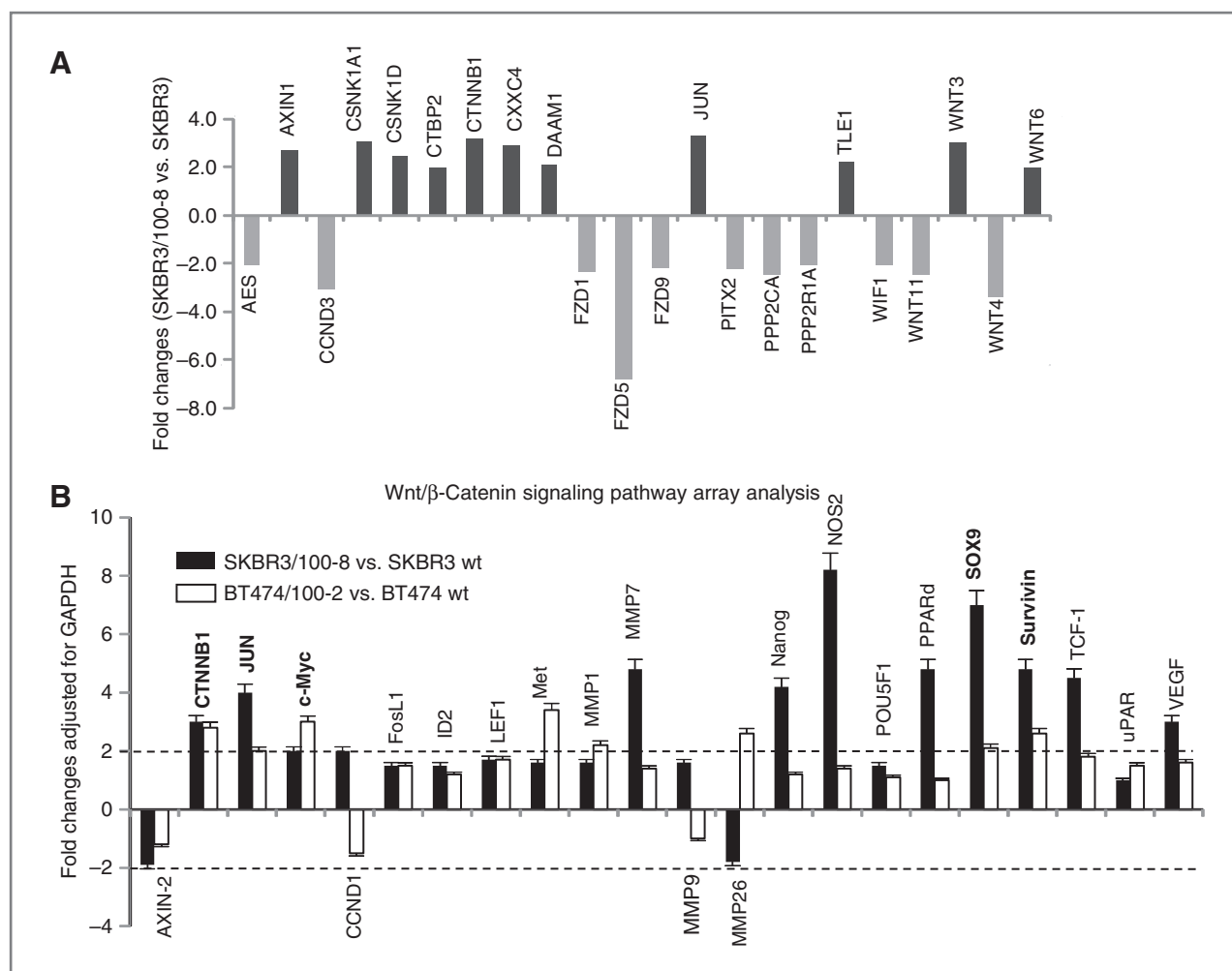


Figure 2. Activation of Wnt signaling pathway in trastuzumab-resistant cell lines. A, bar graph representing significantly upregulated (dark bars) or downregulated (gray bars) mRNA of Wnt pathway genes in SKBR3/100-8 compared with SKBR3 analyzed by microarray analysis. B, bar graph of regulated Wnt/β-catenin pathway signaling in SKBR3/100-8 (dark bars) and BT474/100-2 (open bars) compared with SKBR3 and BT474 analyzed by specific Wnt/β-catenin mRNA array. The relative level of each indicated gene was adjusted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and each bar indicates mean fold change and SD from 4 determinations.

not shown). Each transcriptional factor is networking with a number of molecules (between 5 and 319 molecules). The differentially expressed genes in the profile were associated with more than 200 signaling pathways and composed of 25 networks. Supplementary Figure S2 shows breast cancer-related signaling pathways that were regulated in the profile revealed by IPA. Approximately 95% of Wnt/β-catenin signaling genes were regulated (Supplementary Fig. S2).

Compared with SKBR3 cells, 22 genes in the Wnt pathway were significantly deregulated in SKBR3/100-8. Eleven genes were upregulated by ≥ 2 -fold and 11 genes were downregulated by ≥ 2 -fold in SKBR3/100-8 cells compared with SKBR3 cells (Fig. 2A). There were 19 Wnt ligands and 10 FZD receptors in the Agilent chip array. Among the 19 Wnt ligands, Wnt3 and Wnt6 were upregulated by 3- and 2.1-fold ($P < 0.001$), respectively, whereas Wnt4 and Wnt11 were downregulated significantly in SKBR3/100-8 cells compared with SKBR3 cells (Table 1). Of the 10 FZD receptors, FZD5,

FZD1, and FZD9 were significantly downregulated more than 2-fold in SKBR3/100-8 cells (Table 1). Except Wnt6 and FZD1, other Wnt ligands and FZDs were all confirmed having been regulated significantly in SKBR3/100-8 cells by qRT-PCR (Supplementary Fig. S3A). The Wnt3 and Wnt6 were also significantly upregulated in BT474/100-2 compared with BT474 (Supplementary Fig. S3B).

Activation of Wnt/β-catenin signaling pathway promotes EMT phenotype and transcriptionally regulates EGFR in trastuzumab-resistant cell lines

To further verify the activation of the Wnt/β-catenin signaling pathway in trastuzumab-resistant cells, a human Wnt/β-catenin-regulated cDNA plate array was used. The array consisted of 22 Wnt/β-catenin pathway-regulated genes; most genes in the array were upregulated in the resistant clones, SKBR3/100-8 and BT474/100-2 cells (Fig. 2B). Twelve genes were upregulated ≥ 2 -fold in SKBR3/

Table 1. Wnt ligands and Frizzled receptors expression in SKBR3/100-8 versus SKBR3 analyzed by gene array analysis

Name of genes	SKBR3/100-8 vs. SKBR3 (fold changes)	P
<i>Wnt1</i>	1.1	0.5
<i>Wnt2</i>	1.0	0.8
<i>Wnt2B</i>	1.1	0.3
<i>Wnt3</i>	3.0	<0.001
<i>Wnt3A</i>	1.0	0.8
<i>Wnt4</i>	-2.8	<0.001
<i>Wnt5A</i>	1.7	<0.001
<i>Wnt5B</i>	-1.3	0.03
<i>Wnt6</i>	2.1	<0.001
<i>Wnt7A</i>	-1.2	0.1
<i>Wnt7B</i>	-1.5	0.005
<i>Wnt8A</i>	-1.1	0.4
<i>Wnt8B</i>	-1.0	0.7
<i>Wnt9A</i>	-1.5	0.005
<i>Wnt9B</i>	-1.0	0.7
<i>Wnt10A</i>	-1.2	0.1
<i>Wnt10B</i>	-1.0	0.6
<i>Wnt11</i>	-2.4	<0.001
<i>Wnt16</i>	1.1	0.4
<i>FZD1</i>	-2.3	<0.001
<i>FZD2</i>	-1.4	0.03
<i>FZD3</i>	1.1	0.5
<i>FZD4</i>	-1.9	<0.001
<i>FZD5</i>	-6.7	<0.001
<i>FZD6</i>	-1.0	0.8
<i>FZD7</i>	-1.2	0.2
<i>FZD8</i>	-1.0	0.8
<i>FZD9</i>	-2.2	<0.001
<i>FZD10</i>	-1.0	0.8

NOTE: The characters and numbers in bold represent fold change > 2.0 and P value < 0.001.

100-8 compared with SKBR3, and 8 genes were upregulated ≥ 2 -fold in BT474/100-2 compared with BT474. The CTNNB1 was upregulated approximately 3-fold in both SKBR3/100-8 and BT474/100-2 clones. The c-Jun, c-Myc, Sox9, and survivin were also upregulated ≥ 2 -fold in both SKBR3/100-8 and BT474/100-2. The transcription factors, Nanog and TCF-1, were upregulated 4- to 5-fold in SKBR3/100-8 compared with SKBR3 (Fig. 2B). The matrix metalloprotease family gene, *MMP7*, was upregulated 5-fold in SKBR3/100-8 cells and *MMP26* was upregulated 2.8-fold in BT474/100-2 cells. The VEGF, NOS2, CCND1, and PPAR δ were also increased more than 2-fold in SKBR3/100-8 cells. Significant increases in the expression levels of several interesting genes in SKBR3/100-8 cells, including CTNNB1, TCF-1, Nanog, c-Jun, c-Myc, PPAR δ , and survivin, were further confirmed by qRT-PCR (Supplementary Fig. S4A). The protein levels of β -catenin, MMP7, and VEGF were confirmed and increased in SKBR3/100-8 cells

(Supplementary Fig. S4B). The upregulation of Wnt3 in trastuzumab-resistant cell lines, SKBR3/100-8 and BT474/100-2, was also verified by Western blot analysis (Fig. 3A). Figure 3A showed that protein level of E-cadherin was decreased and Twist was increased in both SKBR3/100-8 and BT474/100-2 cells. Even though the mRNA level of vimentin was similar between SKBR3 and SKBR3/100-8 (Fig. 1D), however, we found increased protein level of vimentin in SKBR3/100-8 compared with SKBR3. The SKBR3/100-8 cells also had increased N-cadherin protein expression (Fig. 3). The protein level of N-cadherin was undetectable in BT474 and BT474/100-2 cells. These results confirmed the PCR data of decreased E-cadherin and increased EMT markers in the trastuzumab-resistant cell lines, as shown in Fig. 1D. The decrease in E-cadherin and increase in N-cadherin were generally seen across most of SKBR3/100-8 cells compared with SKBR3 (Supplementary Fig. S5A and S5B). The results implied that activation of Wnt signaling might promote EMT or partial EMT-like transition in the trastuzumab-resistant cells.

We further examined the localization of β -catenin protein expression in SKBR3 and SKBR3/100-8 cells. Data in Fig. 3B showed that β -catenin was mainly present in the cytoplasm of SKBR3 cells. However, β -catenin was localized in both cytoplasm and nucleus of SKBR3/100-8 cells. Considering there may be heterogeneity within the cell line, immunofluorescent analysis was conducted to examine the β -catenin expression and localization in SKBR3/100-8 and SKBR3 cells. The data in Supplementary Fig. S5C confirmed the cell membrane and cytoplasmic expression of β -catenin in SKBR3. However, the β -catenin was observed in cell nuclei in most of the SKBR3/100-8 cells. The β -catenin translocated to cell nucleus could bind to T-cell factor/lymphoid enhancer factor (TCF/LEF) forming a β -catenin-TCF/LEF complex. Because of its transactivating ability, the β -catenin-TCF/LEF complex could bind to the promoter of genes and increase the activation of the gene expression that could be EGFR and/or HER2 in the trastuzumab-resistant cell lines. To test our hypothesis, chromatin immunoprecipitation (ChIP)-qPCR assay was conducted. Figure 3C showed enriched β -catenin binding to the promoter of EGFR through the LEF/TCF-binding region in SKBR3/100-8 compared with that in SKBR3. The data indicate that activation of Wnt/ β -catenin signaling leads to transcriptional upregulation of EGFR in trastuzumab-resistant cells. However, there was no difference in β -catenin binding to HER2 promoter between SKBR3 and SKBR3/100-8 cells (data not shown). It implies other mechanisms regulating the consistent overexpressing HER2 in SKBR3/100-8 cells.

Wnt3 knockdown restores the cytoplasmic expression of β -catenin, inhibits EMT-like transition, and reduces cell invasiveness

The siRNA knockdown of Wnt3 increased E-cadherin and decreased Slug and Twist in both SKBR3/100-8 and BT474/100-2 cells compared with SKBR3 and BT474 cells (Fig. 3D and E, left). The mRNA level of N-cadherin was decreased significantly in BT474/100-2 cells but not in

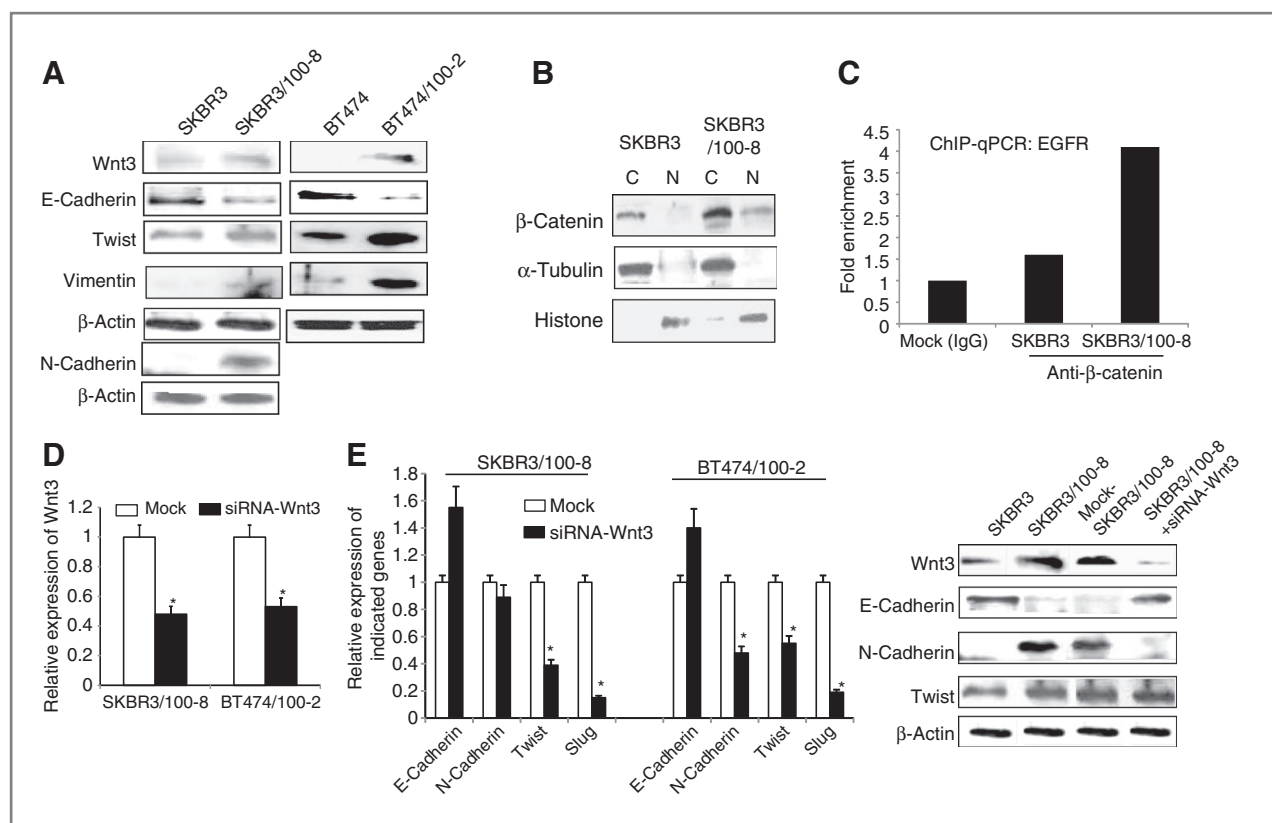


Figure 3. Wnt3 promotes EMT and nuclear translocation of β -catenin in trastuzumab-resistant cell lines. A, total protein levels of the indicated proteins were analyzed by Western blot analysis. B, nuclear (N) or cytoplasm (C) expressions of β -catenin were determined by Western blot analysis. The α -tubulin and histone were used for confirmation of cytoplasmic and nuclear protein extraction respectively. C, ChIP-qPCR assay was conducted as described in Materials and Methods. The bars indicated the fold enrichment of β -catenin binding to promoter of EGFR in SKBR3 and SKBR3/100-8 cells compared with mock cells (immunoprecipitated with IgG). D and E, left, the cells were treated with siRNA-Wnt3 or negative sequences (mock) for 72 hours and RT-PCR was carried out. The bars indicate relative expression of Wnt3 (D) and the indicated genes (E, left; mean \pm SD from 3 determinations). *, $P < 0.05$ compared with mock cells. E, right, cells were treated with siRNA-Wnt3 or negative sequence (mock) for 72 hours and the indicated protein expressions were analyzed by Western blot analysis. The β -actin was used as loading control.

SKBR3/100-8 cells (Fig. 3E, left). The protein level of N-cadherin, however, was down regulated significantly in SKBR3/100-8 cells treated with siRNA-Wnt3 (Fig. 3 right). Conversely, the Twist protein level was not changed in SKBR3/100-8 after knockdown of Wnt3 (Fig. 3E, right). The E-cadherin protein expression was upregulated in SKBR3/100-8 after Wnt3 knockdown which was consistent with its mRNA level.

Next, we examined whether Wnt3 knockdown could sensitize cells in response to trastuzumab. The SKBR3/100-8 and BT474/100-2 cells were treated with siRNA-Wnt3 or negative control sequence (siRNA-A) for 48 hours followed by fresh treatment with 20 to 200 μ g/mL of trastuzumab for 72 hours. The data in Fig. 4A show that 20 to 200 μ g/mL trastuzumab inhibited 35% to 45% cell growth in SKBR3 cells but only approximately 8% in SKBR3/100-8 cells or SKBR3/100-8 cells treated with siRNA-A (mock). In contrast, SKBR3/100-8 cells treated with Wnt3 siRNA, showed approximately 27% to 30% inhibitions with 20 to 200 μ g/mL trastuzumab. The cell growth inhibition was 25% to 30% in BT474 cells treated with 20 to 200 μ g/mL trastuzumab and a maximum of 10%

inhibition of cell growth in BT474/100-2 and BT474/100-2 treated with siRNA-A (Fig. 4A, right). While the cell growth was inhibited from 27% to 38% by 20 to 200 μ g/mL trastuzumab in BT474/100-2 cells treated with siRNA-Wnt3. The cell invasion was also significantly inhibited in SKBR3/100-8 and BT474/100-2 cells treated with siRNA-Wnt3 compared with the cells untreated with siRNA-Wnt3 or treated with siRNA-A (Fig. 4B).

The downregulation of Wnt3 by siRNA also resulted in decreased nuclear expression of β -catenin protein in SKBR3/100-8 cells and downregulation of mRNA level of EGFR by 30% and 26% in SKBR3/100-8 and BT474/100-2, respectively (Fig. 4C). In contrast, the mRNA level of Wnt3 was not changed after knockdown of EGFR (Supplementary Fig. S6).

Next, we pretreated the SKBR3/100-8 with siRNA-EGFR, followed by treatment with 10 μ g/mL of trastuzumab. We found that downregulation of EGFR by siRNA-EGFR had no effect on cell invasiveness of SKBR3/100-8 cells, however, increased their response to trastuzumab-induced inhibition of cell growth significantly (Fig. 4D). The data suggest that the increase in EGFR in SKBR3/100-8

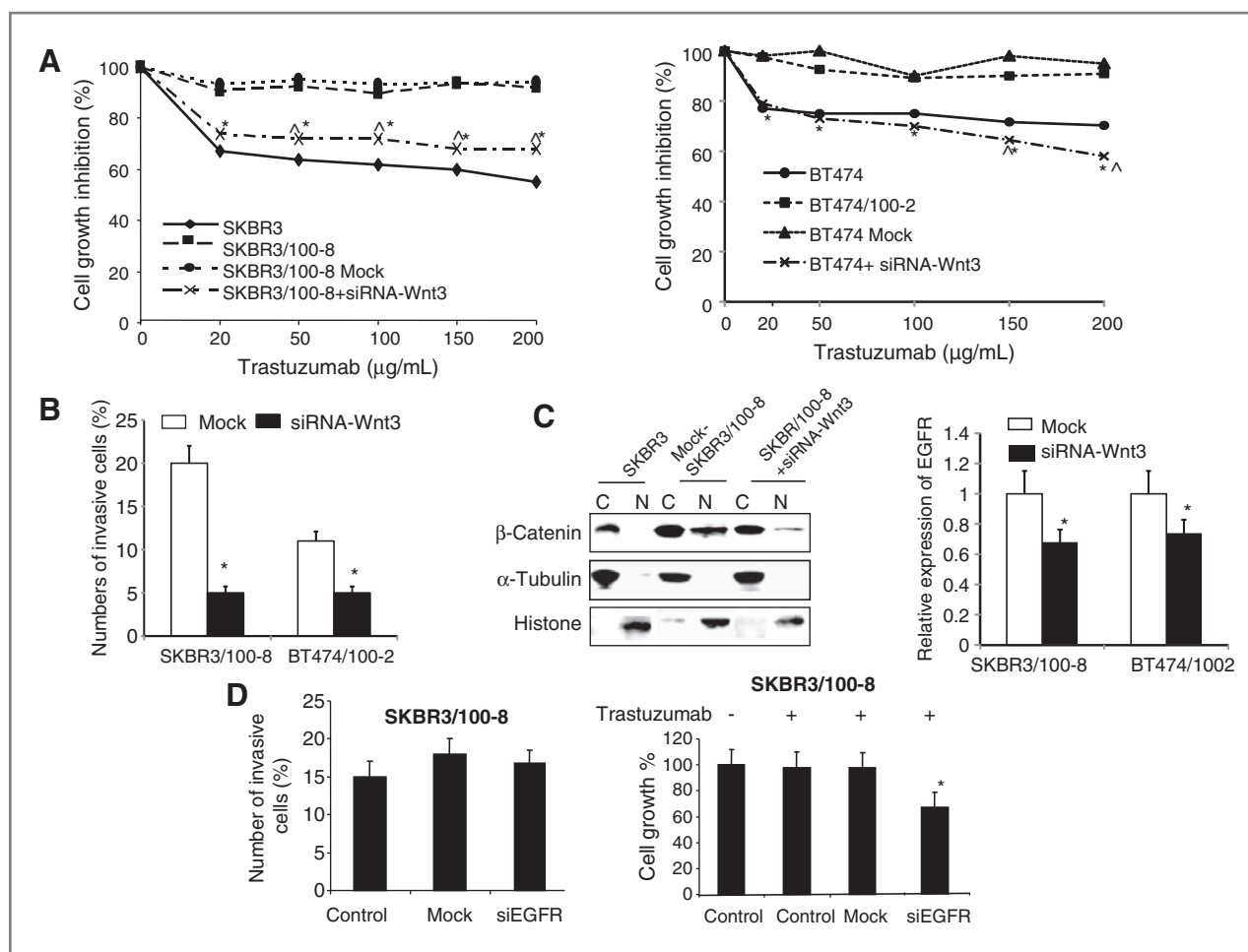


Figure 4. Knocking down Wnt3 increases the cells' response to trastuzumab and reduces cells' invasiveness. **A**, SKBR3/100-8 and BT474/100-2 were treated with siRNA-Wnt3 for 48 hours and then treated with trastuzumab at 0, 20, 50, 100, 150, and 200 $\mu\text{g/mL}$ for 3 days. The cell growth inhibitions by trastuzumab were determined by MTT assay. *, $P < 0.05$ compared with cells treated with siRNA negative sequence (mock), and each data point is the mean of 6 determinations. **B**, cells were treated with either siRNA-Wnt3 or negative sequence (mock) for 48 hours, and invasive assay was conducted as described in the Materials and Methods. The bars indicate percentage of the invasive cells (mean \pm SD from 5 different areas). *, $P < 0.05$. **C**, cells were either treated with siRNA-Wnt3 or negative sequence (mock) for 48 hours. The nuclear and cytoplasmic proteins were extracted and β -catenin expression was analyzed by Western blotting with antibody specific against β -catenin (left). α -Tubulin and histone were used for confirmation of cytoplasmic and nuclear protein extraction, respectively. The relative expression of EGFR in the cells was determined by qRT-PCR (right). The bars indicate mean \pm SD from 3 determinations. *, $P < 0.05$. **D**, SKBR3/100-8 was treated with either siRNA-EGFR or negative sequence (mock) for 48 hours, and invasive assay was conducted as described in the Materials and Methods (left). The bars indicate percentage of the invasive cells (mean \pm SD from 5 different areas). The cells were treated with or without trastuzumab (10 $\mu\text{g/mL}$) for 3 days after knockdown of EGFR. MTT assay was conducted (right), and the bars indicate percentage of cells growth (mean \pm SD) from 6 determinations. *, $P < 0.05$ compared with control and mock cells.

and BT474/100-2 leads to resistance to trastuzumab-induced inhibition of cell growth but may not be responsible for cell invasiveness directly. Because the SKBR3/100-8 showed increase in HER2/HER3 dimerization, similar experiments were carried out on SKBR3/100-8 cells pre-treated with siRNA-HER3. An apparent reduction in invasion by HER3 knockdown did not achieve significance (Supplementary Fig. S7A). However, the cell growth inhibition by trastuzumab was increased significantly after HER3 knockdown (Supplementary Fig. S7B).

Thus, the data suggested that Wnt3 acted as a key mediator in the localization of β -catenin and controls EMT-like transition resulting in increase in cell invasion,

proliferation, and transactivation of EGFR. These events contribute to the increase in resistance to trastuzumab in HER2-overexpressing breast cancer cells.

Overexpressing Wnt3 promotes EMT and reduces sensitivity to trastuzumab in SKBR3 wild-type cells

To ascertain the role of Wnt3 in EMT-like transition and trastuzumab resistance, a full-length Wnt3 gene was stably transfected into parental or wild-type SKBR3 cells. Figure 5A shows that the Wnt3 transfectants, SKBR3/Wnt3-7 and SKBR3/Wnt3-9 clones, had significant increase in the mRNA levels of Wnt3 compared with the vector transfectant, SKBR3/pGFPV3 cells. The mRNA level of E-cadherin

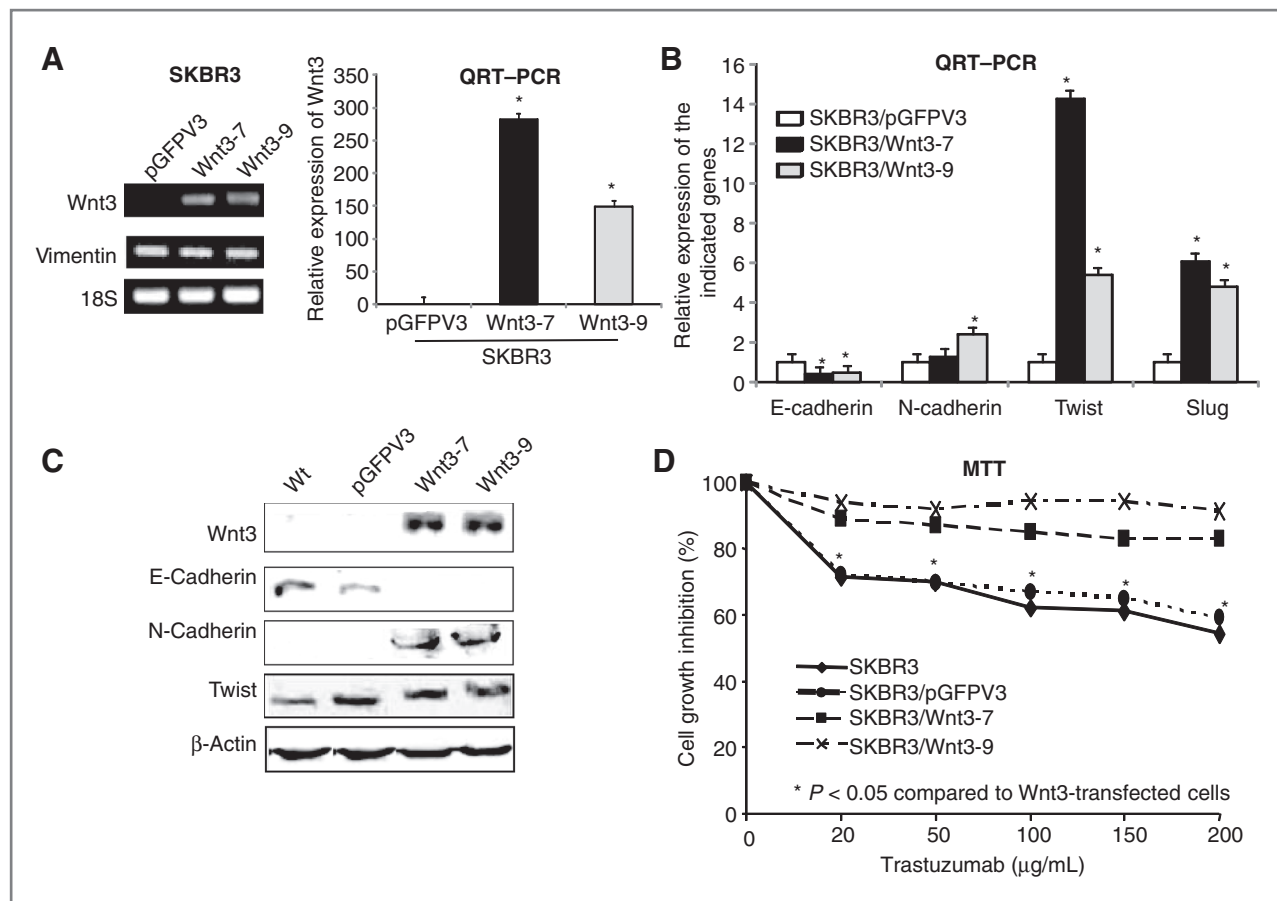


Figure 5. Overexpressing Wnt3 in SKBR3 cells promotes EMT and reduces sensitivity of trastuzumab. A, PCR (left) and qPCR (right) analyses confirmed the overexpressing Wnt3 in the full-length Wnt3 transfectants, Wnt3-7 and Wnt3-9, compared with empty vector-transfected SKBR3 cells (pGFPV3). Vimentin expression in the indicated cells was determined by PCR (left). B, relative expression of the indicated genes in SKBR3/pGFPV3 (open bar), SKBR3/Wnt3-7 (dark bar), and SKBR3/Wnt3-9 (gray bar) were determined by qRT-PCR. Each bar was the mean of 3 determinations with SD. *, $P < 0.05$ compared with SKBR3/pGFPV3. C, Western blot analysis was used to determine the protein levels of the indicated EMT marks in SKBR3 cells transfected with empty vector (pGFPV3), full-length Wnt3 (Wnt3-7 and Wnt3-9), or without transfection (wt). D, MTT assay was conducted in SKBR3, SKBR3/pGFPV3, SKBR3/Wnt3-7, and SKBR3/Wnt3-9 cells treated with the indicated doses of trastuzumab for 3 days. Each data point is the mean of 6 determinations. *, $P < 0.05$ compared with SKBR3/Wnt3-7 and SKBR3/Wnt3-9.

was downregulated and N-cadherin was upregulated in SKBR3/Wnt3-7 and SKBR3/Wnt3-9 compared with SKBR3/pGFPV3 (Fig. 5B). SKBR3/Wnt3-7 and SKBR3/Wnt3-9 also had significant increase in Twist and Slug (Fig. 5B) but no changes in vimentin (Fig. 5A). Data in Fig. 5C confirmed that the protein level of E-cadherin was downregulated and N-cadherin was upregulated following the upregulation of Wnt3 protein in both SKBR3/Wnt3-7 and SKBR3/Wnt3-9 cells. The Twist protein level, however, was not changed in these 2 Wnt3 transfectants (Fig. 5C). The increased Wnt3 expression reduced the growth-inhibiting effect of trastuzumab. Figure 5D shows that after 3 days of trastuzumab (20–200 μg/mL) treatment, cell growth was inhibited by 32% to 42% in the parental line, SKBR3, and 30% to 40% in SKBR3/pGFPV3. However, cells transfected with full-length Wnt3 showed significantly decreased sensitivity to trastuzumab. The cell growth inhibition by 20 to 200 μg/mL of trastuzumab was less than 10% in SKBR3/Wnt3-7 and SKBR3/Wnt3-9 (Fig. 5D). These data con-

firmed the role of Wnt3 in EMT-like transition on the cells that developed resistance to trastuzumab.

Discussion

The resistance to therapy is one of the biggest challenges in breast cancer treatment. Despite the success of trastuzumab as a monotherapy agent, a significant portion of HER2-positive breast cancers respond poorly to the treatment (12, 13). The underlying mechanisms responsible for the resistance are still not clear.

The EGFR/HER2 cross-talk has been suggested as one of the mechanisms of the development of trastuzumab resistance in HER2-overexpressing breast cancer cells (14, 15). In addition, Wnt1 can transcriptionally regulate EGFR in HER2-overexpressing breast cancer cells (16).

We conducted microarray analysis to identify the specific signaling pathway that mediated genes involved in HER2-overexpressing breast cancer cells' resistance to trastuzumab.

Twenty-two genes that are part of the Wnt pathway were either up- or downregulated ≥ 2 -fold in SKBR3/100-8 cells compared with parental SKBR3 cells. In addition, a series of Wnt/ β -catenin signaling molecules including β -catenin, c-Myc, c-Jun, Sox9, and survivin were upregulated in both SKBR3/100-8 and BT474/100-2 cells.

Deregulation of the Wnt signaling pathway has been associated with the development and progression of several types of human cancers including colorectal cancer, prostate cancer, breast cancer, and melanoma (11, 17–21). In colorectal cancer, the activation of the Wnt/ β -catenin pathway is mainly due to mutation or lost function of the *APC* gene or protein in a ligand-independent manner (11). The activation of the Wnt pathway in breast cancer, however, is more likely due to co-expression of Wnt ligands and FZD receptors (11, 22–25). The Wnt ligands, Wnt1, Wnt3a, Wnt4, Wnt5a, and Wnt7b, have been reported to mediate cell proliferation and migration through canonical or non-canonical Wnt pathways in breast cancer (11, 16, 24–26). Data from our current study showed that Wnt3 was upregulated upon the cells acquiring trastuzumab resistance in both SKBR3/100-8 and BT474/100-2 clones. The increased Wnt3 activated Wnt/ β -catenin signaling pathway and nuclear translocation of β -catenin. The β -catenin in nucleus interacted with TCF/LEF and transactivated expression of EGFR in trastuzumab-resistant cells. It may also reactivate Slug and induces EMT-like transition.

Converging evidence suggest that the canonical Wnt signaling pathway, through translocation of β -catenin, plays an important role in regulating EMT in different cancers including breast cancer (18, 27, 28). The EMT-like transition in cancer cells could promote tumor invasion and metastases, as well as mediate drug resistance (18, 27–29). Data from our current study concurred with those published observations.

The trastuzumab-resistant clone, SKBR3/100-8, also showed increased pAkt level compared with the parental cells, SKBR3 (data not shown). The activation of pAkt followed by inactivation of GSK3 β in SKBR3 cells has been shown in our previous study (5). The inactivation of GSK3 β may also be partially responsible for the regulation of β -catenin without involving Wnt3. To confirm the specific role of Wnt3 in the development of trastuzumab resistance, the Wnt3 in SKBR3/100-8 and BT474/100-2 cells was knocked down by siRNA. These Wnt3-knockdown cells showed upregulation of E-cadherin and downregulation of EMT markers, N-cadherin, twist, and Slug. Furthermore, the cell invasiveness was reduced and the growth-inhibitory effects of trastuzumab were restored.

The Wnt3 knockdown also restored cytoplasmic expression of β -catenin and decreased EGFR expression. Downregulation of EGFR sensitized the cells' response to trastuzumab-induced growth inhibition. However, the nuclear translocation of β -catenin by Wnt3 did not increase the β -catenin binding to HER2 promoter at the TCF/LEF-binding region (data not shown). Trastuzumab-resistant cells continue to overexpress HER2 even in the presence of high concentration of trastuzumab at 100 μ g/mL of

trastuzumab. Mechanism underlying this observation requires further investigations. Our current data confirm that expressing Wnt3 activates Wnt/ β -catenin signaling pathway, upregulates EGFR, and further leads to reduction of the growth-inhibiting effects of trastuzumab in HER2-overexpressing breast cancer cells. Hence, combination of trastuzumab and lapatinib targeting both HER2 and EGFR could overcome the trastuzumab resistance.

In contrast to the knockdown strategy, a full-length Wnt3 gene was stably transfected into a relatively sensitive to trastuzumab parental line, SKBR3 cells. The stably transfected Wnt3 clones tended to undergo EMT-like transition with downregulation of E-cadherin and upregulation of N-cadherin protein. The mRNA level of Twist and Slug were significantly increased again. The Wnt3 transfectants were less responsive to trastuzumab-induced growth inhibition.

It has been suggested that Wnt3 has a key role in human breast, rectal, lung, and gastric cancers through activation of the canonical Wnt/ β -catenin-TCF signaling pathway. The EMT-like phenotype has been observed in the development of resistance to tamoxifen in estrogen receptor-positive breast cancer cell line, MCF-7, and has been accompanied by a change in β -catenin phosphorylation (30). Our data from the current study provide an insight into the mechanism of HER2-overexpressing breast cancer cells that may become resistant to trastuzumab. This could be due to redundant expression of Wnt3.

Currently, Wnt signaling targeting includes small-molecule inhibitors of the β -catenin/TCF signaling activity, as well as antibodies targeting Wnt1 and Wnt2 (31). Data from our current study suggest that specifically targeting Wnt3 could overcome trastuzumab resistance and benefit patients with breast cancer with HER2-overexpressing tumors.

In conclusion, we have shown in the current study that the development of trastuzumab resistance in HER2-overexpressing breast cancer cells is accompanied by partial EMT-like transition. Expression of Wnt3 activation of the Wnt/ β -catenin signaling pathway is one of the underlying mechanisms for the development of resistance to trastuzumab.

Disclosure of Potential Conflicts of Interest

D.J. Slamon has honoraria from speakers bureau of Novartis, is a consultant/advisory board member of Novartis, GSK, Sanofi-Aventis. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Wu, N. Mosher, J.V. Vadgama
Writing, review, and/or revision of the manuscript: Y. Wu, J.V. Vadgama
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Wu
Study supervision: Y. Wu, J.V. Vadgama
Overall direction of the study and generated funding sources: J.V. Vadgama

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