Genes Affecting the Cell Cycle, Growth, Maintenance, and Drug Sensitivity Are Preferentially Regulated by Anti-HER2 Antibody through Phosphatidylinositol 3-Kinase-AKT Signaling*

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Xiao-Feng Le‡, Amy Lammayo‡, David Gold§, Yiling Lu¶, Weiquin Mao‡, Teresa Chang‡, Adarsh Patel‡, Gordon B. Mills‖, and Robert C. Bast, Jr.¶¶

From the Departments of ‡Experimental Therapeutics, ¶Biostatistics, and ‖Molecular Therapeutics, the University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

The molecular mechanisms by which the anti-HER2 antibodies trastuzumab and its murine equivalent 4D5 inhibit tumor growth and potentiates chemotherapy are not fully understood. Inhibition of signaling through the phosphatidylinositol 3-kinase (PI3K)-AKT pathway may be particularly important. Treatment of breast cancer cells that overexpress HER2 with trastuzumab inhibited HER2-HER3 association, decreased PDK1 activity, reduced Thr-308 and Ser-473 phosphorylation of AKT, and reduced AKT enzymatic activity. To place the role of PI3K-AKT in perspective, gene expression was studied by using Affymetrix microarrays and real time reverse transcription-PCR. Sixteen genes were consistently down-regulated 2.0–4.9-fold in two antibody-treated breast cancer cell lines. Fourteen of the 16 genes were involved in three major functional areas as follows: 7 in cell cycle regulation, particularly of the G2-M; 5 in DNA repair/replication; and 2 in modifying chromatin structure. Of the 16 antibody-regulated genes, 64% had roles in cell growth/maintenance and 52% contributed to the cell cycle. Direct inhibition of PI3K with an inhibitor markedly reduced expression of 14 genes that were also affected by the antibody. Constitutive activation of AKT1 blocked the effect of the anti-HER2 antibody on cell cycle arrest and on eight differentially expressed genes. The antibody enhanced docetaxel-induced growth inhibition but did not increase the fraction of apoptotic cells induced with docetaxel alone. In contrast, the antibody plus docetaxel markedly down-regulated two genes, HEC and DEEPEST, required for passage through G2-M. Thus, anti-HER2 antibody preferentially affects genes contributing to cell cycle progression and cell growth/maintenance, in part through the PI3K-AKT signaling. Transcriptional regulation by anti-HER2 antibody through PI3K-AKT pathway may potentiate the growth inhibitory activity of docetaxel by affecting cell cycle progression.

The human epidermal growth factor receptor 2 (HER2,1 also known as c-Neu or ErbB-2) encodes a 185-kDa transmembrane tyrosine kinase growth factor receptor. The ligand that binds to the homodimers of HER2 has not yet been identified. Rather, HER2 functions as a preferred co-receptor to form heterodimers with HER1 (epidermal growth factor receptor), HER3, or HER4. Of these heterodimers, HER2-HER3 is particularly important for intracellular signaling (1). HER2 signaling has been linked to a variety of cellular responses to growth factors under both normal and pathophysiological conditions. HER2 signaling is required not only during normal development of the mammary gland but also during development of the glia, neurons, and heart (1, 2). Amplification of the HER2 gene and overexpression of HER2 protein have been documented in ~30% of breast and 15% of ovarian cancers (3). In many (but not all) reports, HER2 overexpression has been associated with a more aggressive course of disease. Although the underlying mechanisms for this association are still not well characterized, HER2 overexpression has been linked to increased proliferation and invasiveness (4).

HER2 is currently one of the best defined targets for specific therapy. The substantially greater expression of HER2 on cancer cells than on normal epithelial tissues permits selective targeting of malignant cells. HER2 is expressed on the cell surface where it can interact with ligands and antibodies (1–4). Trastuzumab, a monoclonal antibody directed against the extracellular domain of HER2, is therapeutically active in HER2-positive breast carcinomas (3). Clinical trials in HER2-positive patients with breast cancer have demonstrated that targeted therapy with trastuzumab in conjunction with cytotoxic chemotherapy (such as platinum compounds, taxanes, and anthracyclines) improves time to disease progression and overall survival (3, 5). Clinical trials have also documented an increased risk for cardiotoxicity when trastuzumab is combined with anthracyclines, suggesting that HER2 signaling may contribute to normal heart function.

The mechanisms by which trastuzumab affects growth of HER2-positive cancer cells and enhances sensitivity to chemotherapy are not fully understood. Anti-HER2 antibody can down-regulate the HER2 receptor and prevent cleavage of the extracellular domain of the receptor (13). Receptors are, however, generally re-expressed within a matter of hours, and binding of anti-HER2 antibody can also alter intracellular signaling by enhancing kinase activity and preventing heterodimer formation. Our group and others have demonstrated that anti-HER2 monoclonal antibodies exert inhibitory effects on HER2; PI3K, phosphatidylinositol 3-kinase; RT, reverse transcription; PCNA, proliferating cell nuclear antigen; FC, fold change; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MES, 4-morpholinoethanesulfonic acid; PBS, phosphate-buffered saline; hIgG, human IgG; m, mammalian.

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† To whom correspondence should be addressed: University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 355, Houston, TX 77030-4009. Tel.: 713-792-7743; Fax: 713-792-7864; E-mail: rbast@mdanderson.org.

‡ The abbreviations used are: HER2, human epidermal growth factor receptor 2; PI3K, phosphatidylinositol 3-kinase; RT, reverse transcription; PCNA, proliferating cell nuclear antigen; FC, fold change; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MES, 4-morpholinoethanesulfonic acid; PBS, phosphate-buffered saline; hIgG, human IgG; m, mammalian.
on HER2-overexpressing breast cancer cells through induction of G1 cell cycle arrest associated with induction of p27Kip1 and reduction of CDK2 (6–11). We have further shown that post-translational regulation of p27Kip1 plays a critical role in the anti-HER2 antibody-mediated G1 cell cycle arrest and tumor growth inhibition (12). Of the post-translational mechanisms, we have shown that modulation of the phosphorylation of p27Kip1 protein is the mechanism by which anti-HER2 antibody up-regulates the protein (12). Anti-HER2 antibodies that inhibit tumor growth also prevent HER2-HER2 interaction and block the PI3K-AKT signaling pathway (6, 11, 16, 17). As the PI3K-AKT pathway is critically important to cell survival signaling, inhibition of the PI3K-AKT pathway may explain, in part, the ability of trastuzumab to enhance paclitaxel-induced apoptosis (20). Trastuzumab also suppresses DNA repair capacity (18) through as yet unknown pathways, contributing to the ability of the antibody to enhance the anti-tumor effect of DNA-damaging agents such as cisplatin (18) and radiotherapy (19). In vivo, trastuzumab inhibits angiogenesis and induces antibody-dependent cellular cytotoxicity (13, 14), potentially contributing to its activity. Loss or blockade of the FcγRIII receptor on leukocytes has been shown to severely impair the anti-tumor effect of trastuzumab in vivo (15), indicating involvement of Fc-receptor-dependent mechanisms.

Of the several mechanisms proposed for the action of anti-HER2 antibodies, interruption of the PI3K-AKT pathway may be critical for enhancing sensitivity to docetaxel and other cytotoxic drugs. Our current study explored the mechanisms of action of the anti-HER2 antibody and the impact of the antibody on activation of AKT as well as on sensitivity to docetaxel. Anti-HER2 antibody alone inhibited HER2-HER2 association, decreased PDK1 activity, reduced Thr-308 phosphorylation of AKT, and reduced AKT enzymatic activity. We have used a pharmacogenomic approach to compare the global changes that occur after treatment with anti-HER2 antibody and after treatment with the chemical inhibitor of PI3K. Treatment with anti-HER2 antibody decreased the expression of 16 genes. Fourteen of these 16 genes contribute to the following three different areas of cell function: cell cycle regulation, DNA repair/replication, and modification of chromatin structure. Direct inhibition of PI3K markedly decreased the expression of 14 genes regulated by the anti-HER2 antibody. Conversely, dominant active AKT prevented cell cycle G1 arrest and down-regulation of cell cycle genes induced by anti-HER2 antibody. A combination of anti-HER2 antibody and docetaxel exerted additive growth inhibition against breast cancer cell lines that overexpressed HER2. The combination did not increase the fraction of apoptotic cells induced with docetaxel alone but markedly down-regulated two genes that participate in cell cycle regulation, HEC and DEEPEST, required for passage through G2-M.

MATERIALS AND METHODS

Cell Culture—The human breast cancer cell lines, SKBr3 and BT474, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). SKBr3 cells were grown in complete medium that contained RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), 2 mM l-glutamine, 1 mM sodium pyruvate (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin in humidified air with 5% CO2 at 37 °C. BT474 cells were grown in complete medium containing Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 1 μM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. For all experiments, cells were detached with 0.25% trypsin, 0.02% EDTA. For cell culture, 2–6 × 105 cells were grown in complete medium containing Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 1 mM sodium pyruvate (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. For experiments, cells were detached with 0.25% trypsin, 0.02% EDTA. For cell culture, 2–6 × 105 exponentially growing cells were plated into 100-mm tissue culture dishes or 3 × 106 into 96-well plates in complete medium. After culture overnight in complete medium, cells were treated with anti-HER2 antibody 4D5 5–10 μg/ml (for SKBr3) or trastuzumab at 10 μg/ml (for BT474) in complete medium at 37 °C for 24 (for SKBr3) or 48 h (for BT474). Monoclonal antibody MOPC21 served as control antibody for 4D5 and was used at 5–10 μg/ml in SKBr3 cells. Human IgG served as control antibody for trastuzumab and was used at 10 μg/ml in BT474 cells.

Preparation of Fragmented cRNA for Affymetrix Analysis—A total of 15 μg of total RNA were used in the first-strand cDNA synthesis with T7(-dT)24 primer (GGCCAGTGAATTGTAATACGACTCACTATAGGG-CTCAATTCGAGGG-CGGCGG-dT24) (Proligo, Boulder, CO) by Superscript II (Invitrogen). The second-strand cDNA synthesis was carried out at 16 °C by adding Escherichia coli DNA ligase, E. coli DNA polymerase I, and RNase H into the reaction. This was followed by the addition of T4 DNA polymerase to blunt the ends of newly synthesized cDNA. Double-stranded cDNA was then purified by phase lock gel (Eppendorf, Westbury, NY) with phenol/chloroform extraction. The purified cDNA was then used as templates in an in vitro transcription to produce cRNA labeled with biotin using the BioArray High Yield RNA transcript labeling kit from Enzo Diagnostics, Inc. (Farmingdale, NY). The process of labeling was carried out according to the manufacturer’s recommendation, and cRNA was further purified with a Qiagen RNeasy mini kit (Valencia, CA). Approximately 15 μg of cRNA was fragmented by incubating in a buffer containing 200 mM Tris acetate (pH 8.1), 500 mM potassium acetate, and 150 mM magnesium acetate at 95 °C for 30 min. Agarose gel electrophoresis was performed before the synthesis of cRNA and after the fragmentation of cRNA to ensure the quality of the samples. Only intact, high quality cRNA samples were used for subsequent array hybridization.

Affymetrix Oligonucleotide Array Hybridization and Data Acquisition—cRNA hybridization to the human U95A arrays was performed at the M. D. Anderson Cancer Center Gene Microarray Facility by using an Affymetrix GeneChip System (Affymetrix, Santa Clara, CA). The fragmented cRNA was hybridized with pre-equilibrated Affymetrix arrays at 45 °C for 16 h. Hybridized chips were then washed in a fluidic station with nonstringent buffer (6× SSPE (1× SSPE is 0.18 mol/liter NaCl, plus 0.015 mol of sodium citrate), 0.01% Tween 20, and 0.005% antifoam) for 10 cycles and with stringent buffer (100 mmol/liter MES, 0.1 mol/liter NaCl, and 0.01% Tween 20) for 4 cycles, and stained with streptavidin-phycocerythrin. This was followed by incubation with biotinylated anti-avidin antibody. The cDNA array was placed on an Agilent ChipScanner to detect hybridization signals. Average target intensity was set at 500 arbitrary units. Each array was assessed for quality and stability by examining replicate copies of the same gene at different locations on the array. To ensure the quality of the cRNA samples and of the Affymetrix GeneChips, quality control experiments were performed using test chips, and the same cRNA sample before the test samples were processed. Further details are available from the

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CCSG shared resources web site (www.mdanderson.org/departments/dnmicroarray). The raw data (hybridization data) generated by MAS 5.0 were imported into Microsoft Excel and transferred to our Department of Biostatistics for analysis.

**Statistical Analysis of Affymetrix Array Data**—Before analyzing the Affymetrix array data, every Affymetrix Hu55a2 Gene Chip from our experiments underwent the following quality control checks: 1) scanner alignment and the proper dicing of images into correct cells; 2) overall chip brightness; and 3) spatial variation. Scanner alignment was checked by using the alternating pattern of positive and negative control cells on the border of each GeneChip (Affymetrix, Santa Clara, CA). The intensities of positive and negative controls were plotted as a function of border position to obtain visual confirmation that each image had been correctly aligned. Brightness was examined by looking at the histograms of detection $p$ values (provided by Affymetrix MAS 5.0) for each array. Detection $p$ values measure how likely a transcript was present at a level to be called present on the array. The $p$-value rule, chips are flagged if less than 10% of probe sets are detected at the $p = 0.01$ level. Spatial variation is not easily detected one chip at a time, so we compared the log transformed median corrected ratio ($Z$) for each cell between each combination of chips $A$ and $B$ ($Z = \log(a/B) - \text{median} (\log(a/B))$. The range in $Z$ was additionally constrained to enhance visual artifacts on the slide. All checks were passed for each chip.

A fundamental assumption of DNA microarray analysis is that there should be no differential expression. A standard Affymetrix MAS 5.0 statistical analysis tool package was used for each probe set to measure fold change (FC) on the log$_2$ scale, a 95% confidence interval for FC on the log$_2$ scale, a $p$ value for detection used to make the presence or absence calls for each gene, and a $p$ value for detecting change or differential expression between samples. The default standard Affymetrix change calls were not used. Here a probe set was considered differentially expressed if the $p$ value was very small, the detection $p$ value in at least one sample being compared was small, and the absolute lower bound of log$_2$(FC), the log$_2$ ratio, exceeded 0.8, corresponding to at least a 1.75-fold change. A second method designated the position-dependent nearest neighbor model was developed by Dr. Li Zhang at the Department of Biostatistics, M.D. Anderson Cancer Center (17). The position-dependent nearest neighbor model was available at the website (http://depts.washington.edu/csggroup/projects/DNAmicroarray). The raw data (hybridization data) generated by MAS 5.0 were imported into Microsoft Excel and transferred to our Department of Biostatistics for analysis.

**Reverse Transcription-PCR Analysis**—To verify the analysis results (Table 1) from Affymetrix chip hybridization, total RNA was reverse-transcribed with a random hexamer or TT-dT$_{12}$ primer (Invitrogen). An aliquot (50 ng of total RNA) of the first strand cDNA was used as a template for PCR. Two genes (DEEPET and H4FG listed in Table 1) that lacked the validated primer sets and probes for real time PCR were examined in this study with regular RT-PCR. Oligonucleotide sequences of the primer sets used in this study are as follows: mitotic spindle coiled-coil protein (DEEPET), sense (S)-AGCTTGGACAGCAGGATCGCA and antisense (AS)-TCTGGTAAAGTGGCGAGCT; H4 histone family, member G (H4FG), S-TAAAGGTTGCCTGGCGATGAAACA and AS-CCCTGAGCTTTGAGGGCA; and glyceraldehyde phosphate dehydrogenase (GAPDH), S-GAGGTCCAGGGATTCATGCA and AS-TTATGATTTGGAGGGATCTCG. To monitor better the amplification efficiency and to control experimental errors, a duplex PCR that simultaneously amplified two genes, one internal control GAPDH and one gene of interest (DEEPET or H4FG) in the same tube, was adopted. Duplex PCR was carried out in 50 µl containing 50 ng of cDNA, 50 pmol of each primer (25 pmol for H4FG primer set), 20 mM (NH$_4$)$_2$SO$_4$, 75 mM Tris-HCl (pH 8.8), 1.5 mM MgCl$_2$, 0.01% (v/v) Tween 20, and 0.2 mM each of dATP, dCTP, dGTP, and dTTP, using 1.25 units of Taq polymerase (Invitrogen). The following conditions were used: 95 °C, 3 min followed by 25 (H4FG) or 35 (DEEPET) cycles of denaturation (95 °C, 30 s), annealing (59 °C (DEEPET) or 62 °C (H4FG), 30 s), and extension (72 °C (DEEPET) or 68 °C (H4FG), 45 s). The reaction was incubated at 72 °C for 10 min at the conclusion of the PCR cycle. The resulting PCR product was analyzed by ethidium bromide-agarose gel electrophoresis. Bands were subjected to densitometric analysis and were normalized to expression of the internal control GAPDH. All validation experiments using duplex PCR were performed by two independent technicians and confirmed in both SKBR3 and BT474 cell lines. Two end cycle numbers were used for DEEPET (35 (better) and 40) and H4FG (25 (better) and 30). To exclude any possible contamination or errors, a positive control and a negative control were included in each experiment.

**Quantitative Real Time RT-PCR Analysis**—To validate gene expression in DEEPET, quantitative real time RT-PCR analysis was performed with an Applied Biosystems Prism 7900HT Sequence Detection System using TaqMan® universal PCR master mix according to the manufacturer’s specifications (Applied Biosystems Inc., Foster City, CA) for the 14 genes listed in Table I for which validated TaqMan Gene Expression Assays are available. The TaqMan probes and primers for CKS2 (assay identification number Hs00829071_s1), HEC (assay identification number Hs01691611_m1), and Hs01716169_m1, STK5 (assay identification number Hs00629912_m1), UBE2C (assay identification number Hs00853610_g1), ZWINT (assay identification number Hs01999552_m1), FEN1 (assay identification number Hs00748727_s1), PCNA (assay identification number Hs0247214_g1), RFC1 (assay identification number Hs00427469_g1), TOP2A (assay identification number Hs00172214_m1), TMMS (assay identification number Hs00403461_m1), Hs001736_m1), Hs01761691_m1), Hs00357789_g1), KIAA186 (assay identification number Hs00221421_m1), and PHLDA2 (assay identification number Hs01693968_m1) were assay-on-demand gene expression products (Applied Biosystems). Human GAPDH gene was used as endogenous control (Applied Biosystems, catalog number 4326317E). The gene-specific probes were labeled by using reporter dye FAM, and the GAPDH probe was labeled with a nonfluorescent quencher and the minor groove binder were linked at the 3’ end of probe as quenchers. The thermal cycling conditions were as follows: hold for 10 min at 95 °C, followed by two-step PCR for 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. All samples were performed in triplicate. Amplification data were analyzed with an Applied Biosystems Prism Sequence Detection Software version 2.1 (Applied Biosystems). Relative gene expression was calculated by using the comparative $C_{\text{t}}$ method (21) recommended by the manufacturer was used to compare the expression of the test sample versus an internal control (assay identification number Hs00357789_g1). $C_{\text{t}}$ values (provided by Affymetrix MAS 5.0) were additionally constrained to enhance sensitivity of gene expression detection.

**Preparation of Total Cell Lysate, Immunoprecipitation, and Immunoblot Analysis**—These procedures were performed as described previously (16).

**Immunohistochemical Staining**—The method was basically performed as described previously (22). Briefly, 4D5- or trastuzumab-treated cells were deposited on glass slides using Cytospin 3 (Thermo Electron Corp., Waltham, MA). The slides were air-dried and fixed with 4% paraformaldehyde at room temperature for 30 min. Slides were then washed in PBS and incubated with 3% H$_2$O$_2$ in methanol for 30 min to eliminate endogenous peroxidase activity. Nonspecific binding of primary antibodies was blocked by incubation with 5% skim milk for 30 min, and slides were then incubated with the PCNA monoclonal antibody (dilution of 1:2000) at room temperature for 2 h. The slides were then washed three times with PBS, incubated for 30 min with DAB (3,3′-diaminobenzidine tetrahydrochloride) (1:30 dilution in TPBS) and then incubated for 30 min with a biotinylated goat anti-mouse immunoglobulins link (BioGenex, San Ramon, CA), and followed by incubation in label reagent (BioGenex) for 30 min at room temperature. After applying the substrate solution (BioGenex), the slides were washed thoroughly with distilled water and mounted with aqueous mounting oil.

**Generation of Dominant Positive AKT1 Construct and Stable Transfection**—The human full-length AKT1 sequence coupled with an N-
Anti-HER2 Antibody Significantly Inhibits PI3K-AKT Signaling activity. A, 4D5 decreased HER2-HER3 interaction. SKBr3 cells were treated with 4D5 (10 μg/ml), MOPC21 (10 μg/ml), or heregulin (30 ng/ml) for 24 h and subjected to immunoprecipitation using anti-HER2 antibody ID5 as described under “Materials and Methods.” Immunoprecipitates (IP) were analyzed by Western blotting (WB) with an anti-HER3 antibody. The blot was stripped and reprobed with anti-e-Neu antibody. The numbers below the gel figure show the relative expression of HER3, which was obtained by densitometry after normalization with HER2 expression. B, anti-HER2 antibody trastuzumab inhibited PDK1 activity. BT474 cells were treated with trastuzumab at different concentrations or control hIgG (10 μg/ml) for 48 h and subjected to PDK1 assay as described under “Materials and Methods.” C, 4D5 decreased both serine and threonine phosphorylation of AKT at 473 and 308 sites in SKBr3 cells. SKBr3 cells were treated with 4D5 at different concentrations or control antibody (MOPC21 at 10 μg/ml) for 24 h and subjected to total protein extraction and Western blotting. The blot was probed with two phospho-AKT antibodies. The blot was then stripped and reprobed with anti-total AKT antibody.

RESULTS

Anti-HER2 Antibody Significantly Inhibits PI3K-AKT Signaling—In a previous report (16), we demonstrated that treatment with anti-HER2 antibody decreased PI3K activity and reduced phosphorylation of AKT on Ser-473 (Ser-473 AKT). In this report, we have extended these studies to determine the mechanisms by which anti-HER2 antibody mediates these actions by investigating the effect of anti-HER2 antibody on HER2-HER3 association, phosphoinositide-dependent kinase-1 (PDK1) kinase activity, phosphorylation of AKT at threonine 308 (Thr-308 AKT), and AKT enzymatic activity. As demonstrated previously (23, 24), the signaling from HER2 to PI3K-AKT pathway depends on the formation of HER2-HER3 heterodimers because HER3 has multiple consensus binding sites for the p85 PI3K subunit. To measure association of HER2 and HER3, complexes were immunoprecipitated with the ID5 anti-HER2 antibody (6), and Western blots were probed with anti-HER2 and anti-HER3 antibodies. Treatment of SKBr3 cells with 4D5 decreased the association of HER2 and HER3 by 70% when compared with MOPC21 control, whereas the association was enhanced by 3.1-fold under treatment with the ligand heregulin (Fig. 1A). PDK1 kinase, which is recruited to the membrane by the PtdIns products of PI3K, is an immediate downstream target of PI3K and is responsible for the phosphorylation of Thr-308 on AKT (25). The PDK1 enzymatic activity was inhibited by trastuzumab in a concentration-dependent manner (Fig. 1B). Consistent with our previous observations (16), phosphorylation of Ser-473 AKT was down-regulated by 4D5 or trastuzumab treatment in both SKBr3
and BT474 cells (Fig. 1, C and D). As shown in Fig. 1, C and D, phosphorylation of Thr-308 AKT was also down-regulated by 4D5 or trastuzumab treatment in concentration- and time-dependent manners in both SKBr3 and BT474 cell lines. Enzymatic activity of AKT was also significantly inhibited by trastuzumab in a concentration-dependent manner (Fig. 1E). Thus, anti-HER2 antibodies significantly inhibited cellular signaling through the PI3K-AKT pathway.

Treatment with Anti-HER2 Antibody Down-regulates Genes That Participate in Regulation of the Cell Cycle, Cell Growth, Cell Maintenance, and Chromatin Structure—To elucidate the mechanism by which inhibition of PI3K signaling contributes to the effects of anti-HER2 antibody, we have evaluated the impact of anti-HER2 antibody and of direct PI3K inhibition on gene expression by SKBr3 and BT474 breast cancer cells that overexpress HER2. Cells were treated with anti-HER2 antibody (4D5 or trastuzumab) or control antibody (MOPC21 or hIgG). Total RNA was isolated to permit synthesis of cRNA for Affymetrix array hybridization. The U95Av2 gene chip was used to measure expression of 12,000 known human genes. Every hybridized chip underwent quality control checks as described under “Materials and Methods.” Based on the two methods of array analysis describe above, anti-HER2 antibody down-regulated expression of 30 genes in SKBr3 and 123 genes in BT474. Treatment with anti-HER2 antibody up-regulated expression of 19 genes in SKBr3 and 16 genes in BT474 cells. When changes in both cell lines were considered, 24 genes were down-regulated and 4 genes were up-regulated. Attempts to validate these differences, however, suggested that more stringent criteria would be required to eliminate false positives. When genes were selected that exhibited a log ratio >1.0, corresponding to a 2-fold change in both cell lines, 16 genes were down-regulated, and no genes were up-regulated (Table I). RT-PCR, real time PCR, Western blotting, or immunohistochemistry subsequently confirmed the differential expression of all 16 genes. Thus, anti-HER2 antibody exerted a predominantly inhibitory effect on gene expression in breast cancer cells that overexpressed HER2.

Among the 16 differentially expressed genes, 15 had previously been identified, and one gene (KIAA0186) had not been characterized. Of the 15 known genes, 14 fell into three functional areas as follows: cell cycle regulation, DNA repair/repllication, and chromatin structure (Table I). With the aid of Affymetrix functional annotation software, we have compared the fraction of differentially expressed genes in each of 20 functional categories relative to the fraction in each category for all of the genes on the Hu95Av2 chip. Of the 16 antibody-regulated genes, 64% had roles in cell growth/maintenance, and 52% contributed to cell cycle progression. On the U95Av2 chip, only 5 and 35% of genes belonged to cell growth/maintenance and to the cell cycle, respectively. The data suggest that the genes involved in cell cycle and cell growth/maintenance were preferentially regulated by anti-HER2 antibody.

Anti-HER2 Antibody Preferentially Down-regulates Genes That Participate in the G2-M Phase of the Cell Cycle—Seven genes (STK15, CKS2, DEEPEST, UBE2C, MAD3L, ZWINT, and HEC) that relate to cell cycle control were down-regulated by 2.0–4.7-fold by anti-HER2 antibody (Table I). One notable feature of these seven cell cycle genes is that all participate in the G2-M phase, especially spindle formation, which could relate to the synergistic anti-tumor activity observed between taxanes and anti-HER2 antibody. To confirm these seven cell cycle genes were down-regulated by anti-HER2 antibody treatment, real time RT-PCR (six genes) and duplex RT-PCR (DEEPEST) were performed as described under “Materials and Methods.” SKBr3 cells were treated with 4D5 or MOPC21 control antibody for 24 h before extraction of total RNA. As shown in Fig. 2A, six genes were down-regulated by anti-HER2 antibody treatment in a concentration-dependent manner as detected by real time PCR. Consistent with the Affymetrix array data, all six genes were down-regulated 35–80% at the highest concentration of 4D5 in SKBr3 cells (Fig. 2A). To validate further these gene expression changes, BT474 cells were treated with trastuzumab or control hIgG antibody for 48 h before isolation of total RNA. Similar to the 4D5-treated SKBr3 cells, concentration-dependent inhibition of all six genes was confirmed by real time PCR in trastuzumab-treated BT474 cells (Fig. 2B). The level of DEEPEST expression was also shown by duplex PCR to decrease after trastuzumab treatment in BT474 cells (Fig. 2, C and D). DEEPEST expression was also inhibited by 4D5 treatment in SKBr3 cells (data not shown). The availability of a specific antibody permitted further confirmation of the down-regulation of the STK15 protein level by Western blot analysis as shown in Fig. 2E. Thus, anti-HER2 antibody significantly down-regulates cell cycle genes that particularly impact on the cell cycle G2-M transition.

Anti-HER2 Antibody Down-regulates Genes Involved in Control of DNA Repair and Replication—Five genes (PCNA, TOP2A, RFC4, TYMS, and FEN1) involved in control of DNA repair and replication were down-regulated 2.0–3.8-fold by anti-HER2 antibody (Table I). PCNA down-regulation was not only confirmed by real time RT-PCR (Fig. 3A) but also confirmed at protein level by Western blotting (Fig. 3B) and im-

### Table I

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold change</th>
<th>Function</th>
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<tr>
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<td>KIAA0186 gene product</td>
<td>KIAA0186</td>
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<td>CKS2</td>
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<td>Thymidylate synthetase</td>
<td>TYMS</td>
<td>SKBr3: -2.21, BT474: -3.77</td>
</tr>
<tr>
<td>ZW10 interactant</td>
<td>ZWINT</td>
<td>SKBr3: -2.32, BT474: -3.92</td>
</tr>
<tr>
<td>Serine/threonine kinase 15</td>
<td>STK15</td>
<td>SKBr3: -2.42, BT474: -4.28</td>
</tr>
<tr>
<td>Highly expressed in cancer</td>
<td>HEC</td>
<td>SKBr3: -2.56, BT474: -4.72</td>
</tr>
<tr>
<td>H4 histone family, member G</td>
<td>H4FG</td>
<td>SKBr3: -2.60, BT474: -4.88</td>
</tr>
</tbody>
</table>

* Fold change represents expression ratio of anti-HER2 antibody-treated sample over control antibody-treated sample in average sample.
munohistochemical staining (Fig. 3C) in both SKBr3 and BT474 cell lines. The decrease in PCNA RNA level was more dramatic in trastuzumab-treated BT474 cells than that in 4D5-treated SKBr3 cells (Fig. 3A). The decrease in expression of PCNA protein was more significant than that of PCNA RNA in SKBr3 cells (Fig. 3B). The other anti-HER2 regulated genes that participate in DNA repair and replication control, RFC4, TOP2A, TYMS, and FEN1, were also confirmed by real time RT-PCR in both SKBr3 cells (Fig. 4A) and BT474 cells (Fig. 4B). Down-regulation of RFC4, TOP2A, TYMS, and FEN1 expression by the antibodies was concentration-dependent (Fig. 4). FEN1, RFC4, and TOP2A were all down-regulated by more than 50% by the antibody treatment. The magnitude of down-regulation of TYMS was relatively modest, whereas inhibition of TOP2A expression was more prominent (Fig. 4). Thus, anti-HER2 antibody is able to negatively affect genes involved in DNA replication and repair.

**Anti-HER2 Antibody Down-regulates Genes That Modify Chromatin Structure**—The two genes H4FG and HMG2 that regulate chromatin structure and gene transcription were down-regulated 2.5–4.9-fold by the anti-HER2 antibody. Duplex RT-PCR (for H4FG shown in Fig. 5, A–C) and real time RT-PCR (for HMG2 shown in Fig. 5, D and E) confirmed the concentration-dependent down-regulation of these two chromatin-associated genes in both SKBr3 (Fig. 5, A, B and D) and BT474 (Fig. 5, C and E) cell lines treated with the anti-HER2 antibodies. These results revealed, for the first time, that chromatin-associated genes are one of the targets of the anti-HER2 antibody.

**Direct PI3K Inhibition Down-regulates Genes That Are Also Decreased by the Anti-HER2 Antibody**—Treatment with anti-HER2 antibody inhibits cancer cell growth, blocks signaling through PI3K-AKT, and down-regulates genes that control cell cycle, DNA repair/replication, and modify chromatin structure. To test the hypothesis that at least some genes down-regulated by treatment with anti-HER2 antibody are regulated by PI3K signaling, breast cancer cells that overexpress HER2 were treated with chemical inhibitors of PI3K to determine whether or not the genes down-regulated by the antibody are down-regulated by direct inhibition of PI3K. Real time RT-PCR and duplex RT-PCR (for DEEPEST and H4FG) were performed to assess the effects of PI3K inhibition. As shown in Fig. 6A, inhibition of PI3K with LY294002 suppressed expression of six cell cycle-related genes (CKS2, HEC, MAD3L, STK15, UBE2C, and ZWINT) in a concentration-dependent manner as demonstrated by real time PCR, resembling the activity of the anti-HER2 antibody treatments.
HER2 antibody shown in Fig. 2. Results from duplex RT-PCR indicated another cell cycle-related gene DEEPEST was also dose-dependently inhibited by LY294002 treatment (Fig. 6B). Five DNA replication and repair-related genes (FEN1, PCNA, RFC4, TOP2A, and TYMS) were suppressed by LY294002 treatment as detected by real time PCR (Fig. 6C). LY294002 treatment also significantly reduced the expression of two chromatin structure-related genes HMG2 and H4FG as demonstrated by real time PCR (Fig. 6D) and duplex PCR (Fig. 6E), respectively. These data suggest that LY294002 and anti-HER2 antibody exert similar effects on the above-mentioned 14 differentially expressed genes, consistent with the possibility that PI3K signaling mediates some of the transcriptional effects of anti-HER2 antibody.

Constitutive Activation of AKT1 Blocks the Ability of Anti-HER2 Antibody to Down-regulate Genes and to Induce Cell Cycle G1 Arrest—If signaling through the PI3K pathways, particularly the PI3K-AKT pathway, mediates down-regulation of the relevant genes, then expression of a dominant positive AKT could blunt the response to anti-HER2 antibodies. Consequently, we constructed a mammalian expression vector for dominant positive AKT1 (mAKT1) and stably transfected it into SKBr3 cells. As shown in Fig. 7A, the established mAKT1 clone expressed a high level of AKT1 protein that was functionally active as illustrated by strong phosphorylation of both Ser-473 and Thr-308. The effects of anti-HER2 antibody on the differentially expressed genes and cell cycle were tested in two active mAKT1 clones. The expression of active mAKT1 blocked the down-regulation of CKS2, HEC, UBE2C, and ZWINT genes (cell cycle-related, Fig. 7B); of RFC4 and TYMS genes (DNA replication/repair related, Fig. 7E); and of HMG2 and H4FG genes (chromatin-related, Fig. 7, B and C) induced by the anti-HER2 antibody. Most interestingly, active mAKT1 did not block the down-regulation of DEEPEST, MAD3L, STK15 (cell cycle-related, Fig. 7, D and E), FEN1, PCNA, and TOP2A (DNA replication/repair related, Fig. 7E) by the anti-HER2 antibody. Anti-HER2 antibody did down-regulate the expression of these 14 genes in the control clones that contained the empty vector pcDNA 3.0 (data not shown). As expected, expression of active mAKT1 (Fig. 7F) stimulated the cell cycle pro-
Regression evidenced by fewer cells in the G₁ phase (36.2–45.1%) when compared with the control clone (61.9%). Expression of active mAKT₁ decreased 4D₅-mediated G₁ accumulation from control 78 to 41.4 or to 51% in the two clones (Fig. 7F), indicating mAKT₁ severely impaired anti-HER2 antibody-induced cell cycle G₁ arrest. Taken together, these data demonstrate that the differentially expressed genes were, at least in part, regulated by PI3K-AKT signaling, and inhibition of the PI3K-AKT pathway is an important mechanism for the activity of anti-HER2 antibody.

Anti-HER2 Antibody Enhances Docetaxel-induced Growth Inhibition Associated with Enhanced Down-regulation of HEC and DEEPEST Expression—Anti-HER2 antibody was shown to enhance the toxicity of paclitaxel in pre-clinical and clinical settings (3, 26). Taxane derivatives, including paclitaxel and docetaxel, are anti-mitotic agents that target microtubules and thus spindle function and arrest cell cycling in G₂-M (27). Treatment of breast cancer cells with anti-HER2 antibodies reduced expression of the genes that particularly regulate spindle in the G₂-M phase of the cell cycle (Fig. 2 and Table 1). These data prompted us to investigate the effects of combined treatment with anti-HER2 antibody and docetaxel on these seven cell cycle genes identified in this study. Inhibition of anchorage-dependent cell growth of SKBr3 cells with a combination of 4D₅ anti-HER2 antibody and docetaxel was confirmed using a crystal violet mitogenic assay (12, 26). As shown in Fig. 8A, 4 nM docetaxel inhibited SKBr3 cell growth by 62.5%, whereas anti-HER2 antibody 4D₅ (5 µg/ml) alone inhibited growth by 51%. A combination of 4D₅ and docetaxel produced significantly greater inhibition of 81%, whereas control antibody and docetaxel produced 60% growth inhibition (Fig. 8A). Most surprisingly, the combination of 4D₅ and docetaxel did not induce more apoptotic cells (25.6%) than did MOPC and docetaxel (30.4%) (Fig. 8B). Compared with the control group MOPC21 plus docetaxel, more cells accumulated in the G₁ phase and fewer cells in the G₂-M phase in combination treatment with 4D₅ and docetaxel (Fig. 8B). These results suggested that the effect of anti-HER2 antibody on cell cycle regulation might contribute to its ability to potentiate the growth inhibition of docetaxel. When the expression of cell cycle-related genes was assayed, a combination of 4D₅ antibody and docetaxel additively down-regulated the expression level of HEC (by real time PCR) and DEEPEST (by duplex PCR), whereas docetaxel alone and control antibody plus docetaxel did not alter or slightly increased the expression of HEC and DEEPEST (Fig. 8, C and D). The other five cell cycle genes that were regulated by anti-HER2 antibody (CKS2, STK15, UBE2C, MAD3L, and ZWINT) were not further down-regulated by treatment with a combination of 4D₅ and docetaxel (data not shown). Thus, enhanced down-regulation of HEC and DEEPEST expression by anti-HER2 antibody and docetaxel was associated with greater inhibition of tumor growth in breast cancer cells that overexpress HER2.

**FIG. 4.** Validation of DNA repair and replication-related genes. The four DNA repair and replication-related genes RFC4, TOP2A, TYMS, and FEN1 were assessed by real time RT-PCR as described under “Materials and Methods” in SKBr3 cells (A) and in BT474 cells (B). * depicts results statistically significantly different relative to the control group (p < 0.05). ** depicts results statistically significantly different relative to the control group (p < 0.01).
DISCUSSION

Treatment of breast cancer cells that overexpress HER-2 with anti-HER2 antibody inhibited HER2-HER3 association, decreased PDK1 activity, reduced Thr-308 and Ser-473 phosphorylation of AKT, and reduced AKT enzymatic activity. Decreased signaling through AKT could result from interference with the formation of HER2-HER3 heterodimers, preventing phosphorylation of HER3 and docking of PI3K subunits. Crystal structure was recently resolved at 2.5 Å for the entire extracellular domain of HER2 complexed with trastuzumab (38). Trastuzumab binds to domain IV of the receptor on the C-terminal portion of the juxtamembrane region of HER2 at a site containing the binding pocket for an extended domain II loop that mediates formation of inter-receptor dimers (38). Thus, anti-HER2 antibodies 4D5 and trastuzumab could block the HER2-HER3 interaction, thus preventing activation of the PI3K-AKT pathway. Data in Fig. 1 support this possibility and confirm previous observations that anti-HER2 antibodies inhibit PI3K-AKT signaling (11, 16, 17).

To define the possible mechanism(s) of action of anti-HER2 antibody, gene expression in breast cancer cells treated with anti-HER2 antibody was compared with that in cells treated with control antibody. Sixteen genes were significantly down-regulated by anti-HER2 antibody, 15 with known function and 1 not yet characterized. Fourteen of the 15 known genes were classified into the following three major functional areas: 7 in cell cycle regulation largely related to the G2-M phase; 5 in DNA repair/replication; and 2 affecting chromatin structure (Table I). Anti-HER2 antibody had the greatest impact on genes affecting cell cycle and cell growth/maintenance (Table I). One pro-apoptotic gene (PHLDA2) was down-regulated by anti-HER2 antibody. PHLDA2 may enhance Fas expression (28), but decreased expression after treatment with anti-HER2 antibody suggests that transcriptional regulation of this gene does not contribute to antibody-mediated growth inhibition.
A combination of trastuzumab and paclitaxel additively inhibits growth of human breast cancer cell lines (26). Treatment of mice bearing human breast cancer xenografts using a combination of trastuzumab and paclitaxel rendered 59% of animals tumor free, compared with 17.3% of mice treated with paclitaxel alone (29). Clinical studies have demonstrated substantially greater benefit when trastuzumab and paclitaxel are used concurrently to treat patients with metastatic breast cancers that overexpress HER2 (30). The mechanisms underlying the interaction between paclitaxel and trastuzumab remain unclear. Recently, sensitization of paclitaxel-induced apoptosis by anti-HER2 antibody has been proposed as an important mechanism underlying additive growth inhibition (20). The combination of docetaxel and trastuzumab has also been shown to be active against breast cancers that overexpress HER2 (31). Most surprisingly, treatment with anti-HER2 antibody did not enhance docetaxel-induced apoptosis. Rather, the addition of anti-HER2 antibody increased growth inhibition without a further increase in the fraction of apoptotic cells. Enhanced growth inhibition was associated with enhanced down-regulation of two genes involved in the G2-M phase of the cell cycle, HEC and DEEPEST (Fig. 8). Although trastuzumab may increase paclitaxel-induced apoptosis in some cell lines (20), we believe that inhibition of expression of cell cycle G2-M genes by anti-HER2 antibody also may contribute to the molecular mechanisms by which anti-HER2 antibody enhances the anti-tumor activity of docetaxel.

Trastuzumab can also suppress DNA repair capacity (18), possibly contributing to the enhancement of the anti-tumor effect of cisplatin and of radiotherapy. Among the DNA repair/replcation genes, TOP2A, a key enzyme in DNA replication, is located adjacent to the HER2 oncogene at the chromosome location 17q12-q21 and is either amplified or deleted in almost 90% of HER2-amplified primary breast tumors (32). TOP2A expression was down-regulated by anti-HER2 antibody on Affymetrix arrays, and decreased gene expression was confirmed in both cell lines by real time PCR (Fig. 4). By decreasing TOP2A activity, treatment with anti-HER2 antibody might also enhance the chemosensitivity to topoisomerase II-inhibitors such as etoposide. Recently, Liang et al. (41) have reported that trastuzumab induces radiosensitization of breast cancer cell lines that overexpress HER2 through the PI3K-AKT pathway. Our data in Figs. 3, 4, and 7 demonstrated that trastuzumab down-regulated five genes that related to DNA repair/replcation control (FEN1, PCNA, RFC4, TOP2A, and TYMS) through the PI3K pathways. Therefore, this study suggests one mechanism by which trastuzumab can potentiate the efficacy of radiotherapy and DNA-damaging agents on breast cancer cells that overexpress HER2.

The negative effect of anti-HER2 antibody on genes associated with regulation of chromatin structure is a novel observation that suggests additional mechanisms of action for anti-HER2 antibody. Both H4FG and HMG2 are chromatin-associated proteins that are able to bend DNA into DNA circles and to facilitate cooperative interactions between cis-acting
Anti-HER2 Antibody Targets Cell Cycle and Growth Genes

Expression of active mAKT1 blocked anti-HER2 antibody-induced inhibition of eight genes but did not block expression of the other six genes (Fig. 7). This interesting observation suggests that the PI3K-AKT1 pathway is not the only signaling pathway involved in regulation of the cell cycle and DNA replication/repair. Two chromatin structure-related genes, *H4FG* and *HM2G*, may be primarily regulated through the PI3K-AKT pathway, based on the results shown in Fig. 7, B and C. Other AKT isoforms and/or PI3K pathways such as PI3K-SGK3, PI3K-p70S6K, PI3K-PTEN, PI3K-Raf, and others may also be involved in the regulation of these genes and in the mechanisms of action for the antibody. We suggest* that trastuzumab has a negative effect on SGK3 and p70S6K activities as well. Because the mAKT1 cells were resistant to treatment with anti-HER2 antibody (Fig. 7F), we believe that PI3K-AKT is a critical pathway but not the only pathway that mediates the mechanisms of action for the antibody.

As we have shown previously, anti-HER2 antibody blocks HER2-overexpressed breast cancer cells at the G1 phase of the cell cycle and reduces the number of cells at the S phase (6, 12). This work illustrates that anti-HER2 antibody markedly down-regulates the expression of seven G2-M phase genes (STK15, CKS2, DEEPEST, UBE2C, MAD3L, ZWINT, and HEC). We believe that this down-regulation of the G2-M phase genes could result from cell cycle G1 arrest. In consideration of the new role of PI3K-AKT in cell cycle G2-M phase, it is also

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2 X.-F. Le and R. C. Bast, Jr., unpublished data.
Anti-HER2 Antibody Targets Cell Cycle and Growth Genes

Anti-HER2 antibody primarily affects the genes involved in cell cycle and acts in concert with docetaxel to decrease tumor growth. The pathway by which anti-HER2 antibody inhibits proliferation of HER2-overexpressed breast cancer cells.

The data presented here provides new evidence related to the pathways by which anti-HER2 antibody inhibits proliferation and acts in concert with docetaxel to decrease tumor growth. Anti-HER2 antibody primarily affects the genes involved in cell cycle progression, particularly in the G2-M phase. Our results further demonstrated that inhibition of PI3K-AKT signaling is an important mechanism by which the anti-HER2 antibody induces cell cycle G1 arrest and down-regulates a number of target genes. These data show that the potentiation of growth inhibition with the combination of anti-HER2 antibody and docetaxel is associated with enhanced down-regulation of two G2-M phase genes, HEC and DEEPEST.

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REFERENCES


FIG. 8. Potentiation of docetaxel-induced growth inhibition by anti-HER2 antibody is associated with additive down-regulation of HEC and DEEPEST gene expression. A, anti-HER2 antibody enhances docetaxel-induced growth inhibition. The anchorage-dependent growth assay was carried out in 96-well microplates as stated under “Materials and Methods.” SKBr3 cells were treated with antibodies (MOPC21 or 4D5) and docetaxel (DTX) simultaneously or individual agent alone for 3 days. * indicates results statistically significantly different relative to the control group (p < 0.05). B, 4D5 plus docetaxel combination do not produce higher apoptosis but more cells in the cell cycle G1 phase. SKBr3 cells were treated as stated in A and harvested for cell cycle analysis as described under “Materials and Methods.” Shown is a representative of five results. C, 4D5 plus docetaxel combination is associated with additive down-regulation of HEC gene expression. SKBr3 cells were treated as stated in A and subjected to total RNA isolation and real time RT-PCR analysis. * depicts results statistically significantly different relative to the control group (p < 0.05). D, 4D5 plus docetaxel combination do not produce higher apoptosis but more cells in the cell cycle G1 phase. SKBr3 cells were treated as stated in A and subjected to total RNA isolation and duplex RT-PCR analysis. Shown is the relative expression of DEEPEST gene. Cells were treated as stated in A and subjected to total RNA isolation and real time RT-PCR analysis. * shows results statistically significantly different relative to the control group (p < 0.05).