Expression of p95HER2, a Truncated Form of the HER2 Receptor, and Response to Anti-HER2 Therapies in Breast Cancer

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Background Women with HER2–overexpressing breast cancers have poor prognosis, and many are resistant to the HER2 monoclonal antibody trastuzumab. A subgroup of HER2–overexpressing tumors also express p95HER2, an amino terminally truncated receptor that has kinase activity. Because p95HER2 cannot bind to trastuzumab but should be responsive to the HER2 tyrosine kinase inhibitor lapatinib, we compared the sensitivity of tumors expressing p95HER2 and tumors expressing the full-length HER2 receptor to these agents.

Methods MCF-7 and T47D breast cancer cells were stably transfected with either full-length HER2 or p95HER2. We studied the effects of trastuzumab and lapatinib on receptor signaling, cell proliferation, and the growth of xenograft tumors. A paraffin-based immunofluorescence assay was developed to study the association between p95HER2 expression and sensitivity to trastuzumab in patients with advanced breast cancer. All statistical tests were two-sided.

Results Treatment of p95HER2–expressing cells with lapatinib inhibited p95HER2 phosphorylation, reduced downstream phosphorylation of Akt and mitogen-activated protein kinases, inhibited cell growth (MCF-7p95HER2 clones, lapatinib versus control, mean growth inhibition = 57.6% versus 22.6%, difference = 35%, 95% confidence interval [CI] = 22.5% to 47.3%; $P<.001$; T47Dp95HER2 clones, lapatinib versus control, mean growth inhibition = 36.8% versus 20%, difference = 16.8%, 95% CI = 11.3% to 22.3%, $P<.001$), and inhibited growth of MCF-7p95HER2 xenograft tumors (lapatinib versus control, mean = 288.8 versus 435 mm^3, difference = 146.2 mm^3, CI = 73.8 to 218.5 mm^3, $P = .002$). By contrast, treatment with trastuzumab had no effect on any of these parameters. Of 46 patients with metastatic breast cancer who were treated with trastuzumab, only one of nine patients (11.1%) expressing p95HER2 responded to trastuzumab (with a partial response), whereas 19 of the 37 patients (51.4%) with tumors expressing full-length HER2 achieved either a complete (five patients) or a partial (14 patients) response ($P = .029$).

Conclusions Breast tumors that express p95HER2 are resistant to trastuzumab and may require alternative or additional anti-HER2–targeting strategies.

J Natl Cancer Inst 2007;99:628–38

HER2, also known as ErbB2, is one of four members (HER1–4) of the epidermal growth factor receptor or HER family. All HER receptors share a similar structure: an extracellular ligand-binding domain, a short hydrophobic transmembrane region, and a cytoplasmic tyrosine kinase domain (1,2). Hetero- or homodimerization of HER receptors, induced by ligand binding or receptor overexpression, leads to the activation of the receptor kinase and to the subsequent phosphorylation of several tyrosine residues. In turn, these phosphorylated tyrosine residues, located within the carboxyl terminus of the receptors, recruit mediators and activate signaling pathways that result in the modification of the cell growth, differentiation, and survival.

HER2 is overexpressed/amplified in approximately 15%–25% of human breast cancers, and overexpression/amplification is associated with an aggressive phenotype (3,4). Trastuzumab, a recombinant humanized monoclonal antibody that binds with high affinity to the extracellular domain of HER2 (5–7), provides substantial clinical benefits in patients with HER2–overexpressing advanced breast cancer (8–13) and improves survival when added

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DOI: 10.1093/jnci/djk134

to chemotherapy (12,13). In addition, trastuzumab has been recently shown to improve relapse-free survival and overall survival in patients with HER2–overexpressing early breast cancer (14–16). However, 70%–80% of patients with HER2–overexpressing breast cancer do not respond to trastuzumab given as single agent therapy due to either primary or acquired resistance.

There are several potential mechanisms for trastuzumab resistance. These include inactivation or loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (17) and activation of other tyrosine kinase receptors, including the insulin-like growth factor receptor (IGF-1R) (18). Another potential mechanism of resistance is the accumulation of truncated forms of the HER2 receptor that lack the extracellular trastuzumab-binding domain. Amino terminally truncated carboxyl terminal fragments of HER2, collectively known as p95HER2 or C-terminal fragments, are frequently found in HER2–expressing breast cancer cell lines and tumors (19). In fact, these fragments are the predominant HER2 forms in some tumors (19). These fragments arise through the proteolytic shedding of the extracellular domain of full-length HER2 (20,21) or by alternative initiation of translation from two methionine residues (611 and 687) that are located before and after the transmembrane domain, respectively (22). The biologic function of p95HER2 has not been fully characterized, although overexpression of p95HER2 has been shown to lead to growth of tumor xenografts in nude mice (22). The p95HER2 protein has kinase activity, and this activity is required for tumor growth (21); however, the mechanisms involved and their possible relationship with those used by full-length HER2 are unknown.

The fact that the truncated receptor p95HER2 has kinase activity in the absence of the trastuzumab-binding extracellular domain led us to hypothesize that p95HER2–expressing tumors may be resistant to trastuzumab but sensitive to the inhibitory effects of lapatinib, a low–molecular-weight tyrosine kinase inhibitor (TKI) of HER2 (23) that has activity in patients with HER2–expressing tumors that are resistant to trastuzumab (24). To test this hypothesis, we created breast cancer cells stably transfected with full-length HER2 or truncated p95HER2 and examined the effects of trastuzumab and lapatinib on receptor activation and on growth of these cells in vitro and of tumors derived from them in vivo. In addition, we analyzed the activity of trastuzumab in patients with p95HER2–expressing breast tumors using a newly developed immunofluorescence-based method to detect expression of p95HER2 in paraffin-embedded tumors.

CONTEXT AND CAVEATS

Prior knowledge

HER overexpression is associated with poor outcome in patients with breast cancer, and many of these tumors are resistant to the HER2 monoclonal antibody trastuzumab. A subgroup of HER2–overexpressing tumors also expresses p95HER2, a truncated form of the receptor that does not bind to trastuzumab but retains tyrosine kinase activity.

Study design

Response to trastuzumab and the tyrosine kinase inhibitor lapatinib in in vitro and in vivo models of breast cancer expressing full-length HER2 or truncated p95HER2. Response of advanced breast cancer to trastuzumab and p95HER2 expression was measured in patient samples using a new immunofluorescence technique.

Contributions

p95HER kinase activity and cell proliferation in cell lines and growth of xenograft tumors expressing p95HER2 was inhibited by lapatinib, whereas these cells and tumors were resistant to trastuzumab. p95HER2 expression was directly associated with trastuzumab resistance in human breast tumors.

Implications

p95HER2–overepxressing breast cancers may be resistant to trastuzumab and therefore may require secondary anti-HER2–targeting therapies.

Limitations

Because the studies were performed using cell lines, mouse models, and a limited number of human tumor samples, it is unknown how applicable the results are to human breast cancer.

Materials and Methods

Cell Lines and Tissue Samples

MCF-7 and T47D cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle medium/Ham F12 1:1 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Life Technologies, Inc, Paisley, UK) at 37 °C in 5% CO_2.

Breast tumors used in this study were from surgical resections at Vall d'Hebron University Hospital and were obtained following institutional guidelines. Written informed consent for the performance of tumor molecular studies was obtained from all patients who provided tissue.

Expression Vectors and Cell Transfection

Full-length HER2 cDNA (accession number X03363) from pcDNA 3.1 Zeo(+)-HER2 (22) was subcloned into the bicistronic vector pIRES-hyg1 (Clontech, Oxford, UK) (25). p95HER2 cDNA was isolated by digestion of the full-length HER2 cDNA with HindII and subcloned into pIRES-hyg1. Mutation of p95HER2 at lysine 753 was introduced into the p95HER2 cDNA (p95HER2 KD) using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the following primers: CCAGTGGCCATCGCAG TGTTGAGGG (forward) and CCCTCAACACTGCGATGGC CACTGG (reverse).

MCF-7 and T47D breast cancer cells were transfected with vectors using the nonliposomal reagent FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. For selection of stably transfected cells, hygromycin B (Life Technologies, Inc) was added to the culture media at a concentration of 100 μg/mL, and single colonies were isolated. Stable clones of both MCF-7 and T47D cells transfected with empty pIRES-hyg1 (only expressing hygromicin resistance) served as negative controls. Stable clones were characterized by both immunoblot and immunofluorescence as described below.

Cell Treatments

Trastuzumab (Herceptin; kindly provided by Genentech Inc, South San Francisco, CA) was dissolved in sterile apyrogen water (as a stock solution at 150 μM) and stored at 4 °C. Lapatinib

(Tykerb; kindly provided by GlaxoSmithKline, Research Triangle Park, NJ) was dissolved in dimethyl sulfoxide (DMSO as a stock solution at 10 mM) and stored at −20 °C. Cells to be analyzed by immunoblotting were treated with trastuzumab at a final concentration of 200 nM in the culture media and with lapatinib at a final concentration of 1 μM in the culture media. Cells subjected to proliferation assays were treated with trastuzumab at final concentrations of 0.2–1 μM in the culture media and lapatinib at a final concentration of 10 μM in the culture media. DMSO (equal volume to that of treated cells) was added to the culture media of the control (untreated) cells (for lapatinib only).

Immunofluorescence Analysis of HER2 and p95HER2

Untreated MCF-7 and T47D stable clones (HER2, p95HER2, and empty vector controls) cultured in DMEM/F12 with 10% FBS were analyzed for cellular localization of HER2 and p95HER2 by indirect immunofluorescence. Briefly, 4×10^4 cells were seeded on 14-mm diameter coverslips and fixed with 4% paraformaldehyde/0.1% Triton X-100 for 20 minutes. Cells were blocked (1 hour) and incubated (1 hour) with mouse monoclonal anti-HER2 CB11 (1:100, Biogenex, San Ramon, CA) in 1% bovine serum albumin (BSA) plus 0.1% saponin in phosphate-buffered saline (PBS; 0.025 M Na_2HPO_4, 0.025 M K_2PO_4 in 0.87% of NaCl). Cells were then washed three times with PBS and incubated with fluorescein isothiocyanate–conjugated Alexa Fluor 488 secondary antibody (1:1000, Molecular Probes, Eugene, OR) in 1% BSA/PBS for 45 minutes. Cells were then washed three times with PBS, mounted in Mowiol, and visualized by confocal microscopy. All procedures were performed at room temperature. All experiments were repeated three times.

HER2 and p95HER2 Protein Immunoprecipitation and Western Blot

For immunoprecipitation experiments, MCF-7 and T47D clones were grown in 100-mm dishes (at 60%–70% confluence) and treated with either trastuzumab or lapatinib at the indicated doses and time. Cells were washed twice with ice-cold PBS and scraped into ice-cold lysis buffer (50 mmol/L HEPES [pH 7.0], 10% glycerol, 1% Triton X-100, 5 mmol/L EDTA, 1 mmol/L $MgCl_2$, 25 mmol/L NaF, 50 μg/mL leupeptin, 50 μg/mL aprotinin, 0.5 mmol/L orthovanadate, and 1 mmol/L phenylmethylsulfonyl fluoride). Lysates were centrifuged at 15 000*g* for 20 minutes at 4 °C, and supernatants were removed and assayed for protein concentration using the Dc Protein assay (Bio-Rad, Hercules, CA). Volumes of 500 μL of lysis buffer containing equal amount of proteins were incubated with trastuzumab (for HER2) or anti-hemagglutinin (HA) antibody (anti-HA hybridome, 1:100, Babco, Richmond, CA, for p95HER2) overnight at 4 °C with gentle rotation. Protein A sepharose beads (Amersham Biosciences, Little Chafont, UK) were added for 2 hours and washed three times with lysis buffer before suspension in sodium dodecyl sulfate (SDS)–loading buffer.

For immunoblots, total lysates, and immunoprecipitation extracts from MCF-7 and T47D clones were resolved by SDS–polyacrylamide gel electrophoresis on 8% (for phosphotyrosine [p-Tyr], phospho–HER2 [p-HER2], and total HER2 detection) or 12% (for phospho-mitogen-activated protein kinases [MAPKs] [p-MAPKs], total MAPKs, phospho-Akt [p-Akt], and total Akt detection) acrylamide, and electrophoretically transferred to nitrocellulose membranes. Membranes were hybridized with the following primary antibodies: mouse monoclonal anti–p-Tyr (clone 4G10, 1:1000, Upstate Lake Placid, NY), rabbit polyclonal anti–p-HER2 (Y1248, 1:2000, Upstate), mouse monoclonal anti–total HER2 (CB11, 1:1000, Biogenex), rabbit polyclonal phospho-p44/42 MAPK (Thr202/Tyr204, 1:1000, Cell Signaling Technology, Beverly, MA), rabbit polyclonal total MAPKs (1:1000, Cell Signaling Technology), rabbit polyclonal p-Akt (Ser473) (1:1000, Cell Signaling Technology), and rabbit polyclonal total Akt (1:1000, Cell Signaling Technology). Anti–p-Tyr, anti–p-HER2, and anti–total HER2 antibodies were incubated in Tris-buffered saline–Tween buffer (T-TBS, 50mM Tris-HCl pH7.5, 150mM NaCl, 0.1% Tween 20)/5% non-fat dry milk. Anti–p-MAPKs, anti–total MAPKs, anti–p-Akt and anti–total Akt were incubated in T-TBS/5% BSA. Mouse and rabbit horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences) were used at 1:3000 in T-TBS/5% non-fat dry milk. Protein-antibody complexes were detected by chemiluminescence with the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL), and images were captured with a FUJIFILM LAS-3000 camera system. For protein staining, membranes were incubated in 0.1% Ponceau S solution (Sigma, St Louis, MI). Densitometric analyses for protein quantification were done using Image Gauge 4.2 software (Fuji Film, Tokyo, Japan). p-Akt and p-MAPK signals were normalized to total Akt and total MAPKs, respectively. The experiments were repeated four times.

In Vitro Proliferation Assays and Cell Cycle Analysis

Cell proliferation was assayed using the WST-1 reagent (Roche) according to the manufacturer's protocol. Briefly, 5.0×10^3 cells from MCF-7 and T47D clones were seeded in triplicate in 96-well plates and treated with either trastuzumab (0.2–1 μM for 72 hours) or lapatinib (10 μM for 72 hours). Numbers of viable cells were estimated on the basis of their ability to metabolize the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to formazan by mitochondrial dehydrogenases. Quantification of the formazan dye was performed using a scanning microplate reader (Mod 550, Bio-Rad). Cell growth inhibition was calculated [1 − (treated cells/untreated cells) × 100] and reported as percent growth inhibition. Assays were repeated three times.

For cell cycle analysis, clones of both MCF-7 and T47D cells were seeded at 40%–50% confluence in 100-mm culture dishes and treated with lapatinib (10 μM for 48 hours). Cells were incubated in 70% ethanol at −20 °C overnight, treated with RNAse A at 20 μg/mL, stained with 0.5 μg/mL propidium iodide, and evaluated by flow cytometry (Beckman Coulter Epics XL, Beckman Coulter, Fullerton, CA). The experiments were repeated three times.

Tumor Xenografts in Nude Mice

Mice were maintained and treated in accordance with institutional guidelines of Vall d'Hebron University Hospital Care and Use Committee. Six- to eight-week old female BALB/c athymic (nu+/nu+, n = 64) mice were purchased from Charles Rivers Laboratories (Paris, France). Mice were housed in air-filtered laminar flow cabinets

with a 12-hour light cycle and food and water ad libitum. Mice were handled with aseptic procedures and allowed to acclimatize to local conditions for 1 week before the experimental manipulations. A 17β-estradiol pellet (Innovative Research of America, Sarasota, FL) was inserted subcutaneously to each mouse 1 day before injection with MCF-7 cells stably expressing full-length HER2 or truncated p95HER2 and T47D cells stably expressing p95HER2 or the tyrosine kinase mutant p95HER2 KD. For MCF-7 clones, 1.5×10^7 cells were injected into the right flanks of 48 mice (24 per cell line), and treatment began when tumors reached an average size of 150 mm^3 (20 days after injection) and were considered as established growing xenografts. Trastuzumab (20 mg/kg in sterile PBS) or sterile PBS (control) was given intraperitoneally every 4 days. Lapatinib (100 mg/kg) was administered daily by oral gavage in 0.5% hydroxypropylmethylcellulose, 0.1% Tween 80. To allow for enough time to compare the treatment effects, it was predetermined that a minimum of 2 weeks of therapy was required before conducting a formal analysis. For T47D clones, 2.0×10^7 cells were injected into the right flanks of 16 mice (eight per cell line). Tumor xenografts were measured with calipers every 3 days, and tumor volume was determined using the formula: (length x width^2) × (π/6). Eight mice were used for each experimental condition, and results are presented as means and 95% confidence intervals (CIs). All the results were confirmed in independently conducted experiments. At the end of the experiments (38 days after injection for MCF-7 clones and 45 days after injection for T47D clones), the mice were anesthetized with 1.5% isofluorane-air mixture and killed by cervical dislocation.

Detection of p95HER2 in Breast Tumors by Immunofluorescence

Immunofluorescence analysis of p95HER2 was performed on two sequential formalin-fixed paraffin-embedded 4-μm tissue sections from 96 breast specimens (50 for the immunofluorescence protocol validation and 46 for the trastuzumab response study) placed on positively charged glass slides (two per specimen). After deparaffinization, antigen retrieval was performed by incubation in 10 mM citrate buffer (pH 6.0) (DAKO, Carpinteria, CA) in a heated (97 °C) water bath for 40 minutes. Nonspecific binding was blocked by immersing the sections in a TBS/5% BSA solution for 10 minutes. Sections of all tumors were incubated with a mouse monoclonal antibody to the intracellular domain of HER2 (clone CB11) diluted 1:20 for 60 minutes, and separate sections were incubated with a mouse monoclonal antibody to the anti–HER2 extracellular domain (clone CBE1, Novocastra, Newcastle Upon Tyne, UK) diluted 1:10 for 120 minutes, and both were incubated, subsequently, with a rabbit polyclonal anti–AE1/AE3 cytoplasmic cytokeratin (Biogenex, San Ramon, CA) diluted 1:50 for 30 minutes. Antibodies to HER2 were detected using Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes, Eugene, OR) diluted 1:700 for 30 minutes, and antibody to cytokeratin, using an Alexa Fluor 488 mouse anti-rabbit IgG. Sections were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (Vysis, Downers Grove, IL). All incubations were performed at room temperature.

Tumors were scored as positive for p95HER2 expression if any cytoplasmic staining was detected with the CB11 anti–HER2 antibody (26). Cytoplasmic staining was confirmed by colocalization of the CB11 anti–HER2 antibody with the anti-cytokeratin antibody, as observed by a yellow (red and green overlapping) signal. In addition, HER2 staining as detected with CB11 (that recognizes both membrane and cytoplasmic HER2) was compared with that of the pure membrane staining observed with the CBE1 antibody against the HER2 extracellular domain to further confirm the cytoplasmic staining (p95HER2 expression) detected by the CB11 antibody. All fluorescence assays were performed using a Dako Autostainer. Staining was evaluated using a FluoView FV1000 Olympus Confocal Microscope by a pathologist (Dr F. Rojo) who was blinded to clinical information.

Statistical Analysis

Data presented are the means and 95% confidence intervals (CIs) of three or more independent experiments. For in vitro assays and nude mice experiments, comparisons between groups were made

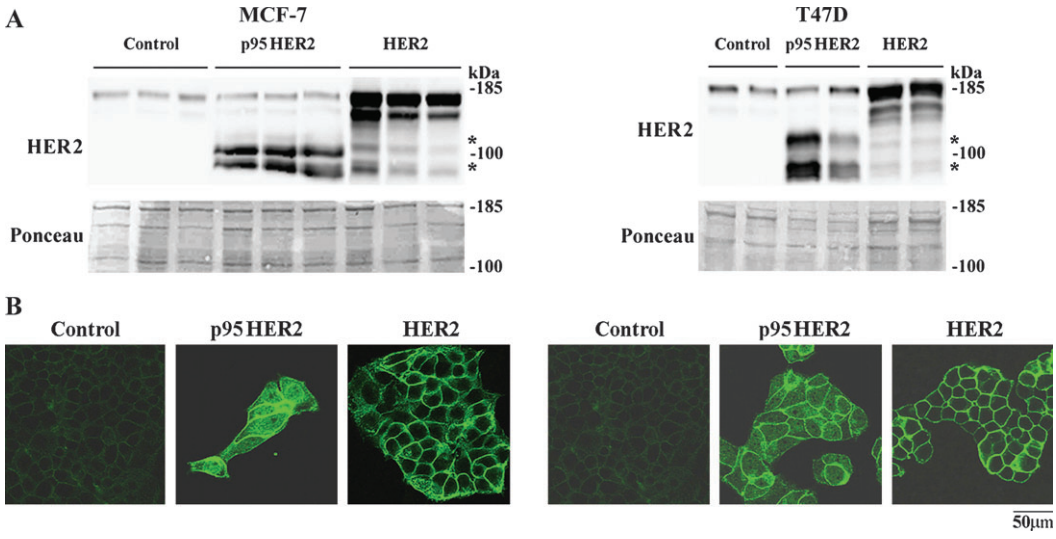

Fig. 1. HER2 and p95HER2 expression and subcellular localization in stably transfected breast cancer cells. **A**) Lysates from clones transfected with empty vector (control) from MCF-7 (**left**) and T47D (**right**), stably expressing full-length HER2 and truncated p95HER2, were subjected to western blotting using mouse monoclonal anti–total HER2 CB11 antibody. **Asterisks** indicate proteins similar in size to those expressed by cells stably expressing p95HER2. Protein loading and transfer are shown by Ponceau staining. Molecular weight marker sizes are indicated to the **right**. Data shown are from one of three experiments with similar results. **B**) Immunofluorescence staining of clones transfected with empty vector (control) of both MCF-7 (**left**) and T47D (**right**) cells and clones stably expressing full-length HER2 and p95HER2 using mouse monoclonal anti–total HER2 CB11. **Scale bar** = 50 μM. Data shown are from one of three experiments with similar results.

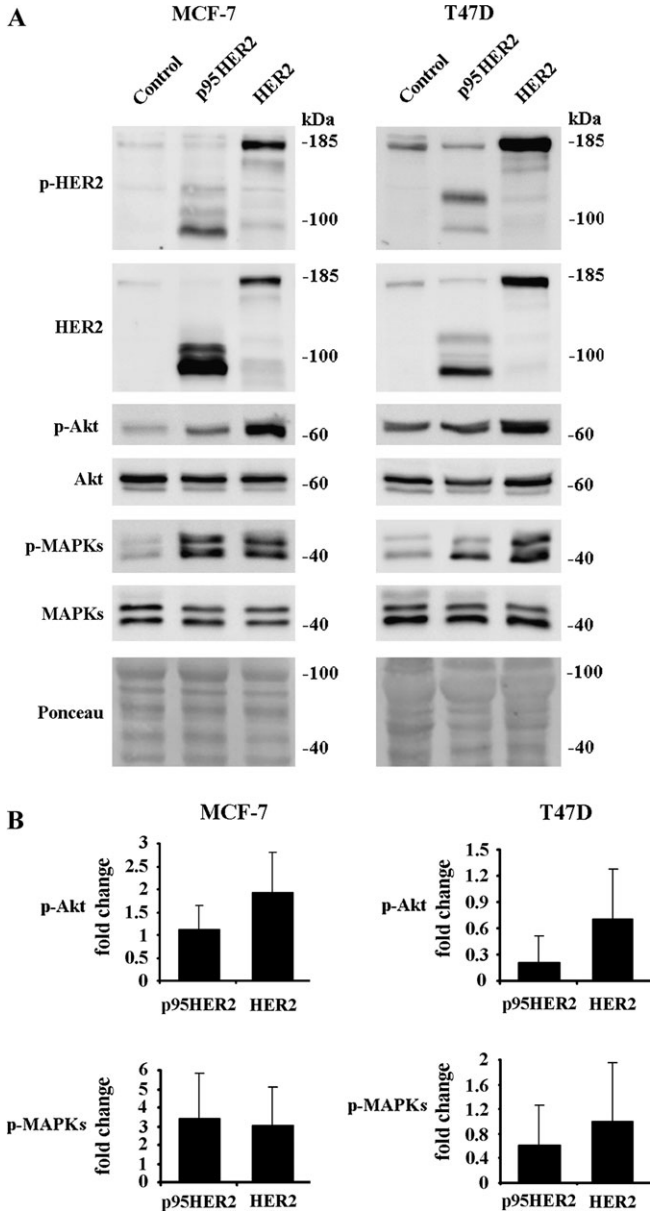

Fig. 2. Phosphorylation of HER2 and p95HER2 and Akt and activation of mitogen-activated protein kinases (MAPKs) in MCF-7 and T47D clones. **A**) Lysates from clones transfected with empty vector (control) of both MCF-7 and T47D breast cancer cells and clones stably expressing full-length HER2 and truncated p95HER2 were subjected to western blotting with anti-HER2, anti–phospho-HER2 (p-HER2), anti-Akt, anti–phospho-Akt (p-Akt), anti-MAPKs, and anti–phospho-MAPKs (p-MAPKs) antibodies. Protein loading and transfer are shown by Ponceau staining. Molecular weight markers shown at **right**. **B**) Densitometric analyses of the western blots in (**A**) for p-Akt, Akt, p-MAPKs, and MAPKs were performed using Image Gauge 4.2 software. For both p-Akt and p-MAPKs signals (normalized to Akt and MAPKs, respectively), ratios of p95HER2/control (p95HER2) and HER2/control (HER2) were calculated and are expressed as fold change (i.e., positive numbers indicate an increase). Means and 95% confidence intervals of four independent experiments are shown.

using a two-tailed Student's *t* test. Differences for which *P* was less than .05 were considered to be statistically significant.

Associations between trastuzumab response and p95HER2 expression in breast cancer patients were studied by contingency tables and analyzed by the chi-square test. Results were considered to be statistically significant when *P* was less than .05. Response rate was considered according to the response evaluation criteria in solid tumors (27) for patients who achieved either a complete or a partial remission. All statistical analyses were performed using the SPSS 12.0 statistical software (SPSS Inc, Chicago, IL).

Results

Generation of Cells Expressing p95HER2

To analyze the effect of the expression of p95HER2 on different signal transduction pathways, cell proliferation, and tumor development, we first transfected MCF-7 and T47D breast cancer cell lines and selected clones that stably expressed either full-length or truncated HER2. To express only p95HER2, we used a cDNA construct containing a deletion of the first 530 amino acids. As expected, cells transfected with this deletion construct expressed two HER2 fragments of approximately 95 and 110 kDa (Fig. 1, A). Cells that were transfected with the wild-type HER2 cDNA expressed both the full-length receptor at 185 kDa and truncated forms that were similar in size to those expressed from the deletion construct. In agreement with our previous report (22), we observed that the full-length HER2 receptor accumulated primarily at the plasma membrane, whereas p95HER2 was localized to the plasma membrane, cytoplasm, and nucleus (Fig. 1, B).

Effects of Anti-HER2 Therapies on Phosphorylation and Activation of Downstream Signaling Molecules by p95HER2

In cells transfected with full-length HER2, the levels of p-Akt and p-MAPKs were higher than in cells transfected with empty vector (p-Akt MCF-7HER2/p-Akt MCF-7 vector control, fold change = 1.92; 95% CI = 1.01 to 2.82; p-Akt T47DHER2/p-AktT47D vector control, fold change = 0.7; 95% CI = 0.1 to 1.3; p-MAPKs MCF-7HER2/p-MAPKs MCF-7 vector control, fold change = 3.03; 95% CI = 0.86 to 5.19; p-MAPKs T47DHER2/p-MAPKs T47D vector control, fold change = 1.01; 95% CI = –0.13 to 2.15) (Fig. 2, A and B), suggesting that, as expected, both MAPKs and Akt signaling pathways are constitutively active. In agreement with our previous reports that have shown that p95HER2 is biologically active (22,28,29), we also observed increased activation of Akt and MAPKs in p95HER2 transfectants compared with empty vector control clones (p-Akt MCF-7p95HER2/p-Akt MCF-7 vector control, fold change = 1.11; 95% CI = 0.57 to 1.65; p-Akt T47Dp95HER2/p-Akt T47D vector control, fold change = 0.21; 95% CI = –0.1 to 0.53; p-MAPKs MCF-7p95HER2/p-MAPKs MCF-7 vector control, fold change = 3.37; 95% CI = 0.82 to 5.92; p-MAPKs T47Dp95HER2/p-MAPKs T47D vector control, fold change = 0.62; 95% CI = –0.04 to 1.28).

To determine whether the activity of p95HER2 could be inhibited by the two main classes of anti-HER2 therapies currently being explored in the clinic—monoclonal antibodies against the extracellular domain of the receptor and low–molecular-weight TKIs (30)—we studied the effects of the monoclonal antibody trastuzumab and lapatinib, a low–molecular-weight TKI of HER2 (23) on full-length HER2– and p95HER2–expressing breast cancer cells. Lapatinib treatment of MCF-7 clones expressing either HER2 or p95HER2 resulted in a long-lasting (48 hours) inhibition of phosphorylation of both

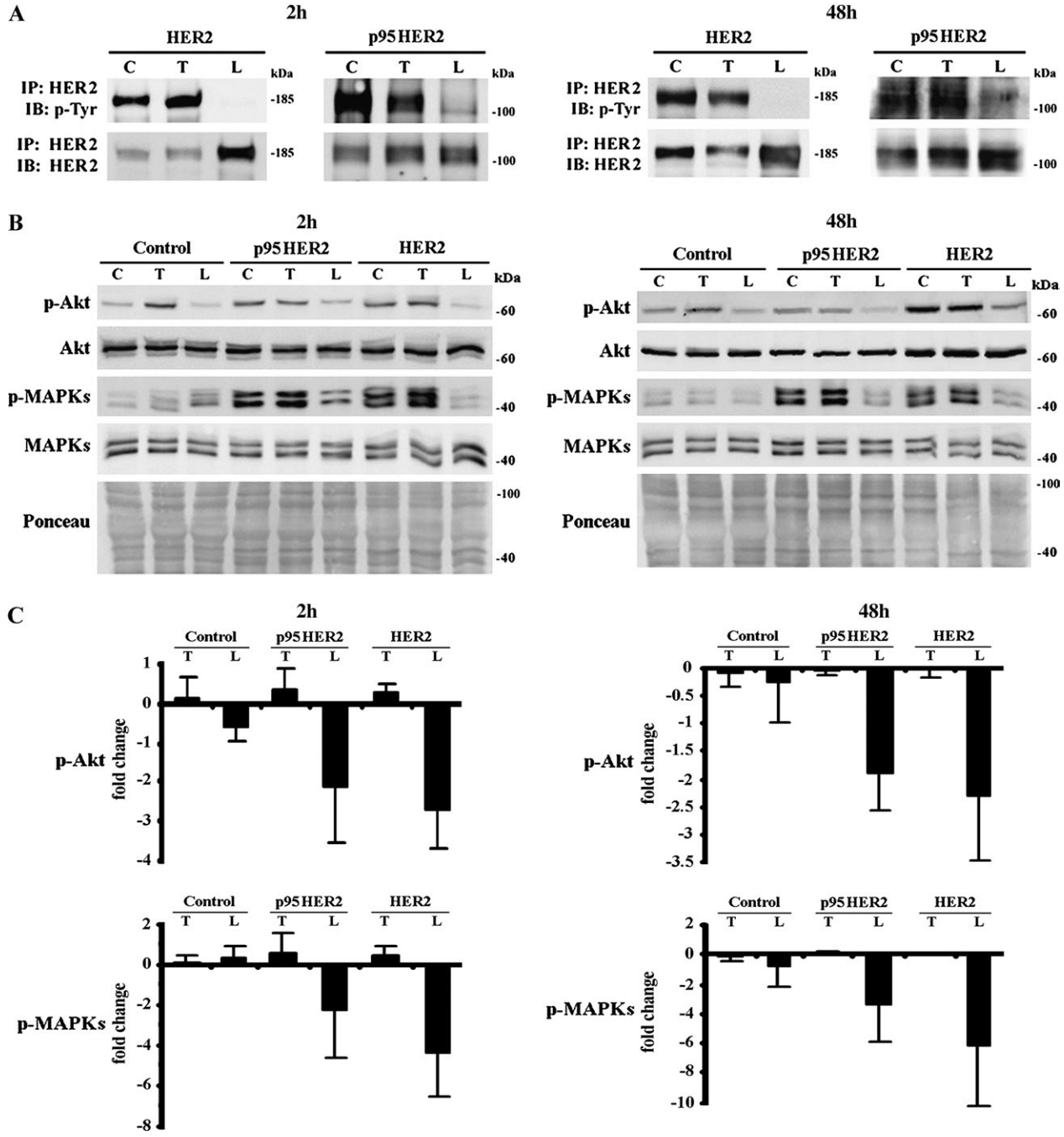

Fig. 3. Effects of lapatinib and trastuzumab on full-length HER2 and p95HER2 phosphorylation and downstream Akt and mitogen-activated protein kinases (MAPKs) signaling. **A**) MCF-7 breast cancer clones stably expressing full-length HER2 and truncated p95HER2 were treated with 200 nM trastuzumab (T), 1 μM lapatinib (L), or dimethyl sulfoxide (C) for 2 and 48 hours. Cell lysates were subjected to immunoprecipitation with anti-HER2 antibodies followed by western blotting with anti–phospho(p)-Tyr and anti-Her2 antibodies. Data shown are from one of four experiments with similar results. **B**) Clones of empty vector-transfected (control) MCF-7 and clones stably expressing full-length HER2 or p95HER2 were treated as in (**A**). Lysates were subjected to western blotting with anti–phospho(p)-Akt, anti-Akt, anti–p-MAPKs, and anti-MAPKs antibodies. Protein loading and transfer are shown by Ponceau staining. Molecular weight markers are shown to the **right**. **C**) Densitometric analyses of the western blots in (**B**) for p-Akt, Akt, p-MAPKs, and MAPKs were performed using Image Gauge 4.2 software. For both p-Akt and p-MAPKs signals (normalized to Akt and MAPKs, respectively), ratios T/C (T) and L/C (L) were calculated for either mock transfected (control), p95HER2 or HER2 clones and expressed as fold change (i.e., positive numbers indicate an increase). Means and 95% confidence intervals of four independent experiments are shown.

forms of HER2 (Fig. 3, A). Moreover, lapatinib consistently increased the levels of HER2 in immunoprecipitates (Fig. 3, A), a finding that has not yet been explained. By contrast, treatment with trastuzumab for 2 hours resulted in no inhibition or a slight increase in the phosphorylation levels of HER2 (Fig. 3, A). A slight activation of HER2 phosphorylation by short-term treatment with trastuzumab has been previously reported (17,31). Consistent with the known ability of trastuzumab to

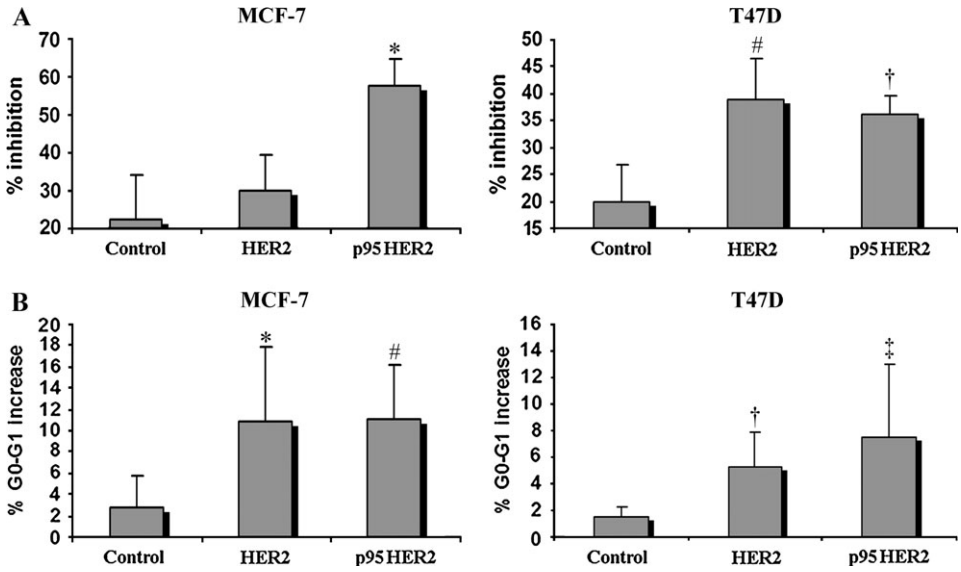

Fig. 4. Cell growth inhibition and G0–G1 phase accumulation of breast cancer cells stably expressing full-length HER2 or p95HER2 after treatment with lapatinib. **A**) Proliferation of mock transfected (control), p95HER2, and HER2 clones from both MCF-7 and T47D cells was analyzed using the WST-1 reagent after treatment for 72 hours with lapatinib (10 μM). Means and 95% confidence intervals of three independent experiments performed in triplicate are shown (Student's two-sided *t* test: *, *P*<.001 versus MCF-7 control; #, *P* = .15, †, *P*<.001 versus T47D control). **B**) Cell cycle analysis was evaluated by flow cytometry. Both MCF-7 and T47D clones were seeded in 100mm culture dishes, treated 48 hours with lapatinib (10 μM), and stained with 0.5μg/mL propidium iodide. Means and 95% confidence interval of three independent experiments are shown (Student's two-sided *t* test: *, *P* = .028 and #, *P* = .21 versus MCF-7 control; †, *P* = .022 and ‡, *P* = .035 versus T47D control).

induce HER2 internalization and a decrease in the levels of membrane-bound HER2 (32), at longer time points, a trastuzumab-induced decrease in total HER2 receptor levels was evident. Trastuzumab had no effect on p95HER2 phosphorylation (Fig. 3, A) as expected based on the lack of trastuzumab-binding domain.

The inhibition of tyrosine phosphorylation of HER2 and p95HER2 by lapatinib was accompanied by inhibition of phosphorylation of the downstream signaling proteins, Akt and MAPKs, in MCF-7 clones (Fig. 3, B and C). For example, at 2 hours (p-Akt: MCF-7HER2, lapatinib versus control treatment, fold change = –2.68; 95% CI = –1.49 to –3.88; MCF-7p95HER2, lapatinib versus control, fold change = –2.1; 95% CI = –0.56 to –3.63; p-MAPKs: MCF-7HER2, lapatinib versus control, fold change = –4.37; 95% CI = –2.07 to –6.66; MCF-7p95HER2, lapatinib versus control, fold change = –2.31; 95% CI = –0.15 to –4.78) and 48 hours (p-Akt: MCF-7HER2, lapatinib versus control, fold change = –2.32; 95% CI = –1.15 to –3.49; MCFp95HER2, lapatinib versus control, fold change = –1.89; 95% CI = –1.2 to –2.59; p-MAPKs: MCF-7HER2, lapatinib versus control, fold change = –6.1; 95% CI = –1.84 to –10.36; MCF-7p95HER2, lapatinib versus control, fold change = –3.44; 95% CI = –0.9 to –5.99) of treatment, indicating that cells expressing p95HER2 are sensitive to this TKI. Similar results were obtained in T47D cells (data not shown).

Antiproliferative Effects of Anti-HER2 Therapies on p95HER expressing cells

We next analyzed the antiproliferative effects of anti-HER2 therapies on MCF-7 and T47D cells expressing p95HER2. Cells expressing p95HER2 and full-length HER2 were both more sensitive to lapatinib treatment than controls expressing empty vector (MCF-7p95HER2 clones, lapatinib versus control, mean growth inhibition = 57.6% versus 22.6%, difference = 35%, 95% confidence interval [CI] = 22.5% to 47.3%; *P*<.001; T47Dp95HER2 clones, lapatinib versus control, mean growth inhibition = 36.8% versus 20%, difference = 16.8%, 95% CI = 11.3% to 22.3%; *P*<.001; T47DHER2 clones, lapatinib versus control, mean growth inhibition = 36.6% versus 20%, difference = 16.6%, 95% CI = 4.28% to 29.0%; *P* = .015; Fig. 4, A). Consistent with the effect on proliferation, progression through the G0–G1 phase of the cell cycle was slower in cells expressing full-length HER2 and p95HER2 than in vector control cells after treatment with lapatinib (cells in G0–G1: MCF-7HER2 clones, lapatinib versus control, mean = 11.0% versus 2.82%, difference = 8.18%, 95% CI = 1.17% to 15.3%; *P* = .028; MCF-7p95HER2 clones, lapatinib versus control mean = 10.6% versus 2.82%, difference = 7.78%, 95% CI = 1.48% to 14.2%, *P* = .021; T47DHER2 clones, lapatinib versus control, mean = 5.4% versus 1.5%, difference = 3.92%, 95% CI = 0.8% to 7.04%; *P* =.022; and T47Dp95HER2 clones, lapatinib versus control, mean = 7.35% versus 1.5%, difference = 5.85%, 95% CI = 0.6% to 11.1%; *P* = .035; Fig. 4, B). Although the lapatinib-induced growth inhibition of MCF-7HER2 clones did not reach statistical significance (Fig. 4), the increased number of cells in G0–G1 phase of the cell cycle suggests an inhibitory effect of lapatinib in these clones as well.

These results suggest that the consequences of tyrosine kinase inhibition on p95HER2 are similar to those on full-length HER2 and provide evidence that the proliferation of cells expressing p95HER2 is dependent of the kinase activity of these fragments. In contrast to lapatinib, trastuzumab did not influence the proliferation of any clone, even when used at a high concentration (up to 1 μM) in the culture media (data not shown). Cell proliferation and cell cycle studies were carried out using different clones isolated from independent transfections, suggesting that the observed results are not due to clonal variability.

We recently showed that T47D cells expressing p95HER2 are resistant to trastuzumab (22). To confirm and extend this result, we analyzed the effect of trastuzumab or lapatinib on the growth of xenografts derived from MCF-7 cells stably transfected with HER2 or p95HER2. When tumor volumes reached a mean size of 150 mm^3 (approximately at 20 days), mice were randomly assigned to one of three treatment groups: placebo, lapatinib at 100 mg/kg daily, and trastuzumab at 20 mg/kg twice a week (8 mice per

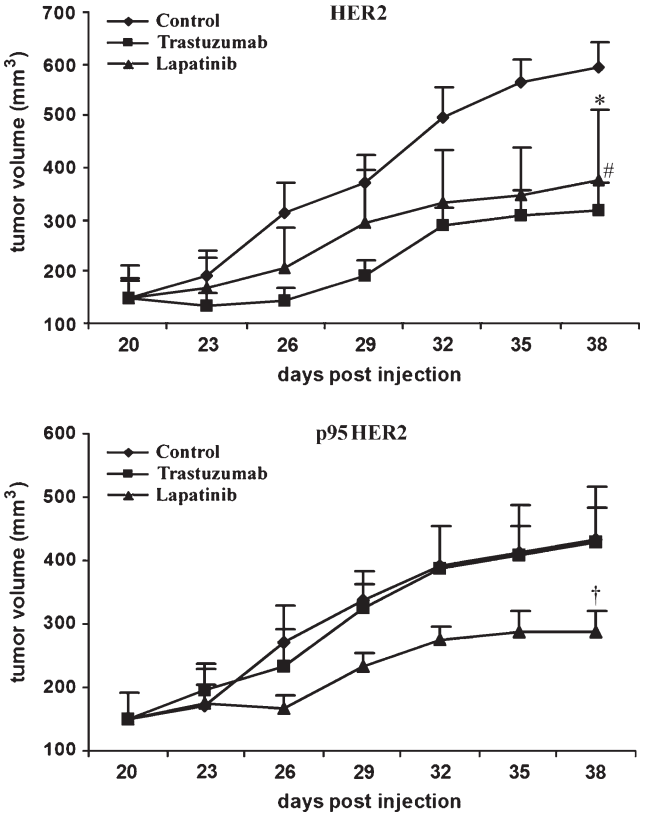

Fig. 5. Antitumor activity of lapatinib in HER2 and p95HER2 tumor xenografts. MCF-7 breast cancer cells stably expressing full-length HER2 or p95HER2 were injected into BALB/c athymic mice (n = 48). Treatment started after tumors reached 150 mm^3 (day 20). Mice were treated (n = 8 mice per treatment group) with trastuzumab (20 mg/kg, **squares**) or phosphate-buffered saline (control, **diamonds**) intraperitoneally every 4 days. Lapatinib (100 mg/kg) was administered daily by oral gavage (**triangles**). Mice were treated for 18 days and tumors measured every 3 days. Means and 95% confidence intervals are shown. Tumor sizes of control mice and those of mice treated with lapatinib and trastuzumab groups were compared at the end of treatment (day 38) using a two-sided student's *t* test. *, *P* = .003 and #, *P*<.001 versus HER2 control; †, *P* = .002 versus p95HER2 control.

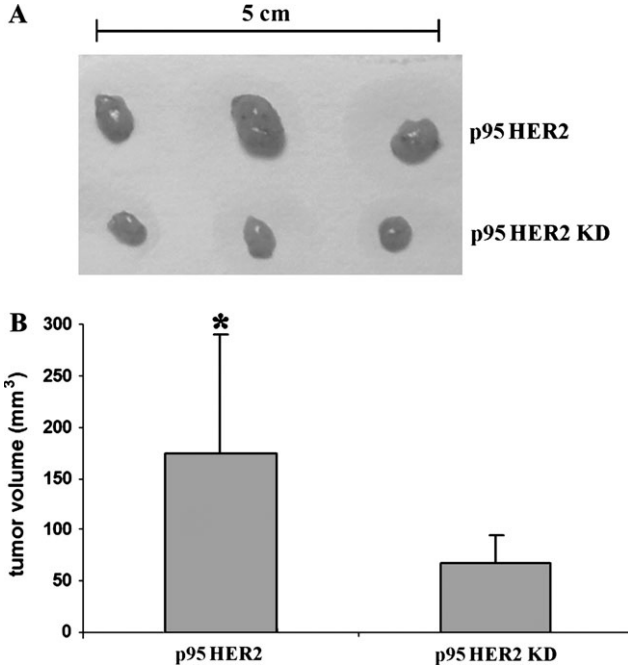

Fig. 6. Effect of p95HER2 kinase activity on growth of breast cancer xenografts in nude mice. T47D cells (2.0 × 10^7) stably expressing p95HER2 or the tyrosine kinase mutant p95HER2 KD were injected into BALB/c athymic mice (n = 16). Each group had eight mice, and tumor growth was measured every 3 days for 45 days. **A**) Representative p95HER2 and p95HER2 KD xenograft tumors. **B**) Quantification of tumor volumes 45 days after injection. Student's *t* test (two-sided) was used to compare tumor sizes between the groups, and means and 95% confidence intervals are shown *, *P* = .027, calculated using a two-sided Student's *t* test.

group). Full-length HER2–expressing xenografts responded to both trastuzumab and lapatinib, whereas p95HER2–expressing xenografts were completely resistant to trastuzumab but remained sensitive to lapatinib after 18 days of treatment (HER2: trastuzumab versus control, mean = 319.4 versus 595.2 mm^3, difference = 275.8 mm^3, 95% CI = 218 to 333.6 mm^3; *P*<.001; lapatinib versus control, mean = 378 versus 595.2mm^3, difference = 217.2 mm^3, 95% CI = 100.3 to 334.1 mm^3; *P* = .003; p95HER2: lapatinib versus control, mean = 288.8 versus 435 mm^3, difference = 146.2 mm^3, 95% CI = 73.9 to 218.5 mm^3; *P* = .002; Fig. 5).

To confirm the effects of lapatinib in silencing the activity of p95HER2 tyrosine kinase on tumor formation, we generated a T47D breast cancer cell line (T47D p95HER2 KD) that expresses p95HER2 with a mutation in lysine 753. This mutation prevents the binding of ATP and, thus, blocks its kinase activity (33). At day 45, tumors arising from T47D p95HER2 KD cells were substantially smaller than those from p95HER2 cells with intact kinase activity (T47Dp95HER2 KD versus T47Dp95HER2, mean = 67 versus 175 mm^3, difference = 108 mm^3, 95% CI = 16.9 to 199.1 mm^3; *P* = .027; Fig. 6, A and B). Collectively, these results show that inhibition of p95HER2 tyrosine kinase activity inhibits cellular proliferation and tumor formation.

Detection of p95HER2 Expression in Breast Tumors by Immunofluorescence

The presence of p95HER2 in human breast tumors has been detected so far only by western blot analysis (19,20,34). This technique requires a large amount of fresh-frozen tumor tissue, a serious limitation because such tissue is rarely available from clinical samples. To circumvent this problem, we developed an immunoflourescence-based p95HER2 detection assay that can be performed on clinical routine formalin-fixed paraffin-embedded tissue sections. This technique builds from the observation that p95HER2, but not full-length HER2, is localized both to the cytoplasm and the cell membrane (Fig. 1).

Tumors were scored as positive for p95HER2 expression if any degree of cytoplasmic staining was detected with the anti–HER2 antibody, which binds to the cytoplasmic domain of the receptor, and if colocalization of HER2 staining with that of a cytoplasmic cytokeratin was observed. To validate the assay, we analyzed 50 HER2–overexpressing breast cancer surgical specimens that had previously been analyzed by an immunoblot with the antibody against the cytoplasmic domain of HER2. These samples included 25 that were known from previous immunoblot analyses to express p95HER2 and 25 that express only the full-length receptor

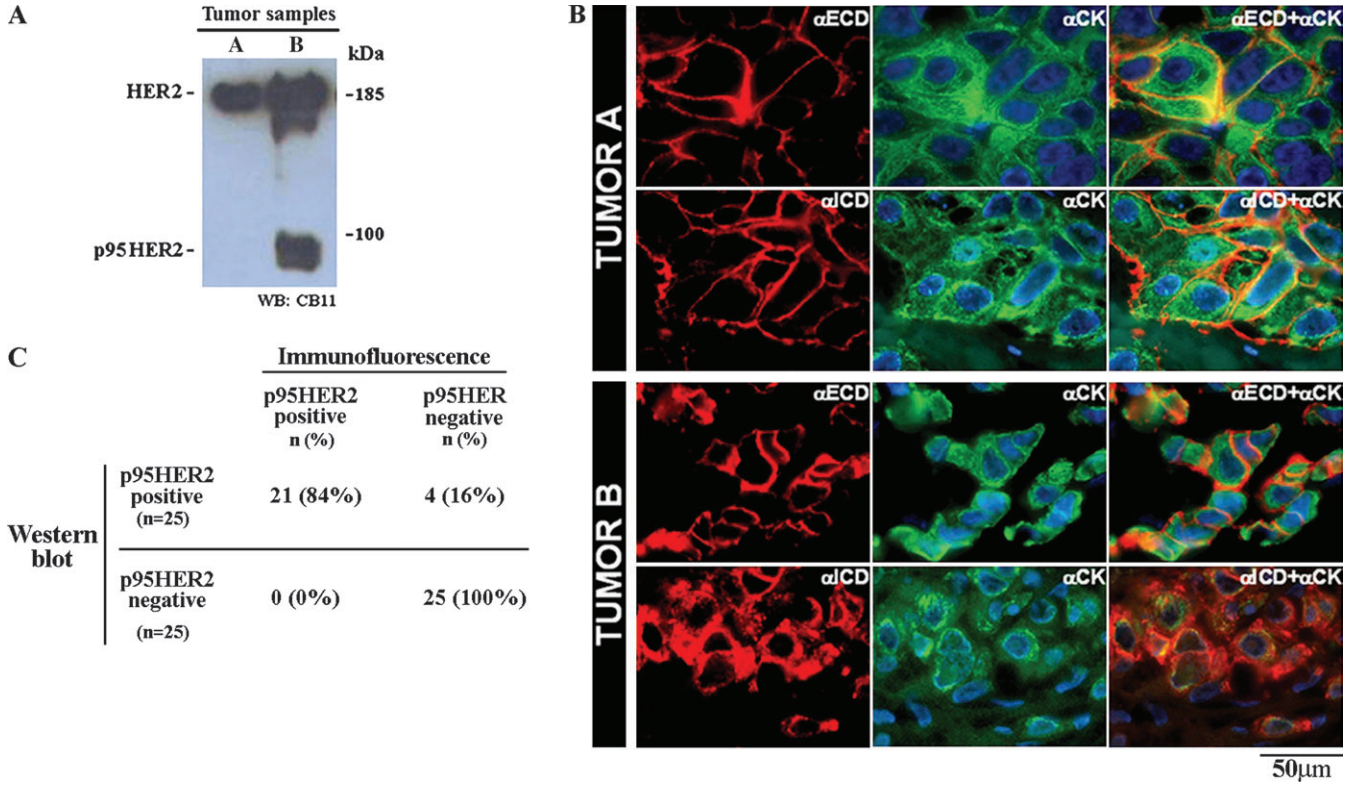

Fig. 7. HER2 full-length and p95HER2 expression in human breast tumor specimens. **A**) Two infiltrating ductal adenocarcinomas were subjected to western blot analysis using the CB11 anti-HER2 antibody. Tumor A expresses only the full-length HER2 receptor. Tumor B expresses both HER2 full-length receptor and p95HER2. **B**) Two serial sections from tumors in (**A**) were assayed by immunofluorescence using the CBE1 antibody (to the extracellular domain of HER2) and CB11 (to the intracellular domain of HER2) (**red**). Staining of the cytoplasm was performed with a polyclonal antibody to AE1–AE3 cytokeratins (**green**). Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (**blue**). Tumor A shows only membrane staining with both antibodies. Tumor B shows membrane staining with the CBE1 antibody but combined membrane and cytoplasmic staining with the CB11 antibody, which colocalizes with the anti-cytokeratin antibody (**yellow**). **Scale bar** = 50 μM. **C**) Concordance between western blot detection and immunofluorescence staining in 50 breast cancer tissue samples. Out of the 25 tumors with p95HER2 expression by western blot, 21 (84%) were found positive for p95HER2 staining by immunofluorescence. All 25 p95HER2–negative tumors (100%) detected by western blot were confirmed by immunofluorescence showing solely membrane staining.

(Fig. 7, A and B). Of the 25 tumors with p95HER2 expression detected by immunoblot, 21 were positive for p95HER2 expression in the immunofluorescence assay (Fig. 7, C). However, none of the tumors that expressed only full-length HER2 by immunoblot showed cytoplasmic staining of HER2 by immunofluoresence. Because immunoblot may be considered as the gold standard for p95HER2 detection, we calculated positive and negative predictive values of the immunofluorescence assay based on the previously reported prevalence of p95HER2 expression in HER2–overexpressing population (19,34) using Bayesian statistics. The positive predictive value for p95HER2 detection using immunofluorescence was 100%, and the negative predictive value was 94%.

p95HER2 Expression and Trastuzumab Resistance in Breast Cancer Patients

Using the immunofluorescence-based test, we next analyzed whether there was an association between the expression of p95HER2 in breast tumors and resistance to trastuzumab. We retrospectively analyzed samples that had been obtained from a total of 46 patients with HER2–overexpressing advanced breast tumors who had been treated with trastuzumab at the Vall d'Hebron University Hospital and at the Hospital Universitari Arnau de Villanova and from whom there were available tumor blocks. Of the 46 patients, 24 had been treated with trastuzumab alone and the rest with trastuzumab-containing combinations (11 with vinorelbine, nine with taxanes, and two with hormones). Of the 46 patients, nine expressed p95HER2 and the remaining 37 expressed only full-length HER2 as determined by the immunofluorescence assay. p95HER2 expression was strongly associated with trastuzumab resistance—only one of the nine (11.1%) patients with tumors expressing p95HER2 responded (with a partial response) to trastuzumab, whereas 19 of the 37 patients (51.4%) with tumors expressing full-length HER2 achieved either a complete (n = 5) or a partial (n = 14) response (P = .029) (Table 1).

Table 1. p95HER2 expression in breast tumors and trastuzumab resistance*

p95HER2 status	Response n (%)	No response n (%)
Negative (n = 37)	19 (51.4)	18 (48.6)
Positive (n = 9)	1 (11.1)	8 (88.9)

* Expression of p95HER2 was evaluated by immunofluorescence in samples from 46 patients with HER2–overexpressing advanced breast tumors who were treated with trastuzumab. Association between p95HER2 status and either complete or partial response to trastuzumab is shown (P = .029, calculated using a two-sided chi-square test).

Discussion

In this study, we have provided evidence that p95HER2 expression in breast tumors may result in differential sensitivity to anti–HER2 therapies. Cells stably expressing p95HER2 were resistant to the monoclonal antibody trastuzumab but remained sensitive to the antiproliferative effects of the TKI lapatinib, both in vitro and in vivo. Furthermore, in a series of patients with HER2–positive advanced breast cancer who were treated with trastuzumab, the presence of p95HER2 was associated with clinical resistance to trastuzumab, whereas tumors expressing only the full-length receptor exhibited a high response rate to trastuzumab. It should be noted that other several potential mechanisms responsible for trastuzumab resistance, such as PTEN inactivation or loss (17) and activation of IGF-1R (18), may be present in tumors expressing only the full-length receptor and not responding to trastuzumab.

Our findings support that further characterization of HER2–expressing breast tumors, based on the presence or absence of p95HER2, may assist in the selection of the appropriate anti–HER2 therapy. Hence, HER2-positive–p95-negative tumors may be highly sensitive to trastuzumab-containing therapy, whereas HER2-positive–p95-positive tumors may benefit from lapatinib-based therapy instead. We are planning to confirm this concept prospectively in a phase III neoadjuvant study that will compare the efficacy of trastuzumab versus lapatinib versus both agents given in combination. It is also likely that HER2-positive–p95-positive tumors will be sensitive to a second generation of TKIs, such as the irreversible HER2 inhibitor HKI-272 (30), an agent that has clinical activity in patients with advanced, trastuzumab-refractory breast cancer (35). In addition, HER2 is a client protein of the molecular chaperone Hsp90, and therapy with Hsp90 inhibitors has been shown to decrease HER2 expression in preclinical models (36). A phase 1 study has recently shown clinical activity of an Hsp90 inhibitor in patients who were refractory to trastuzumab treatment (37), and we are currently analyzing in preclinical models whether Hsp90 inhibition results in p95HER2 degradation.

An additional targeting approach may be contemplated depending on the mechanisms of p95HER2 expression in a given tumor. The p95HER2 protein may be generated by different, nonmutually exclusive, mechanisms, including alternative initiation of translation (22), extracellular domain shedding by a yet undefined metalloprotease (21), and alternative RNA processing (38). The mechanism(s) underlying p95HER2 expression in a given breast tumor are still unknown, but in those cases in which p95HER2 is generated predominantly by shedding of the extracellular domain, an alternative strategy to reverse trastuzumab resistance would be a combined therapy with a metalloprotease inhibitor and trastuzumab.

Our study has limitations, and as a result, several questions remain unanswered regarding the role of p95HER2 in breast tumors. It would have been desirable to have analyzed a larger number of patients who were treated with trastuzumab monotherapy, but the number of patients treated with trastuzumab as a single therapy either from clinical trials or from clinical practice is limited because trastuzumab is mostly given in combination with chemotherapy. It will also be important to determine in future studies whether p95HER2 status remains unchanged throughout the natural history of a given tumor or if it varies during tumorigenesis. In this regard, it could be speculated that p95HER2 expression may develop through a mechanism of acquired resistance to trastuzumab in a fashion similar to the development of secondary kinase mutations with acquired resistance to imatinib in patients with chronic myeloid leukemia and gastrointestinal stromal tumors (30).

Finally, our immunofluorescence method for detection of p95HER2 in tumors is easily performed in paraffin-embedded tumors, and in a test set, it was highly concordant with the western blot assay. This new methodology provides a unique tool for p95HER2 evaluation in tumor samples, and we are incorporating it in upcoming neoadjuvant and adjuvant studies of trastuzumab and lapatinib in patients with early HER2–overexpressing breast cancer. If the results of the p95HER2 studies are confirmatory, p95HER2 testing would be an important addition to our quest for improved patient selection of HER2-targeted therapies.

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Notes

Supported in full by a grant of the Breast Cancer Research Foundation. Dr Ocaña is a recipient of an American Society of Clinical Oncology Young Investigator Award. Dr Baselga is currently involved in conducting clinical trials that are sponsored by Hoffman-La Roche and GlaxoSmithKline. The sponsors are not responsible for the study design, data collection and analysis, interpretation of the data, or the preparation of the manuscript.

Manuscript received October 10, 2006; revised January 30, 2007; accepted March 2, 2007.