A Major Role of p95/611-CTF, a Carboxy-Terminal Fragment of HER2, in the Down-modulation of the Estrogen Receptor in HER2-Positive Breast Cancers

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Abstract

Current classification of breast cancers depends in great part on the expression of human epidermal growth factor receptor 2 (HER2), a cell surface tyrosine kinase receptor, and estrogen receptor (ER), the nuclear receptor for estrogen. In addition to reliable biomarkers, these receptors are targets of effective and widely used antitumor drugs. During malignant progression, HER2 and ER can establish an intricate cross-talk. In some cases, HER2 overexpression leads to the downregulation of ER and undermining of anti-ER therapies. A subgroup of HER2-positive breast cancer patients with poor prognosis expresses a heterogeneous collection of HER2 carboxy-terminal fragments (CTF) collectively known as p95HER2. One of these fragments, 611-CTF, is oncogenic in a variety of preclinical models. However, because of the lack of an appropriate tool to specifically analyze its levels in the clinical setting, the value of 611-CTF as a biomarker has not been established yet. Here, we show that 611-CTF induces resistance to anti-estrogen therapy and a more pronounced down-modulation of ER than that induced by full-length HER2. To validate this effect in breast cancer samples, we developed specific anti–611-CTF antibodies. With these antibodies, we showed that, whereas the frequency of ER positivity in HER2-positive/611-CTF-negative tumors (72.6%) is similar to that reported for HER2-negative tumors (70–80%), the number of ER-positive tumors in the 611-CTF-positive subgroup is very low (31.2%). These results reveal a mechanism of ER regulation mediated by HER2, which suggests a new strategy to improve responses to endocrine therapy in breast cancer.

Introduction

Breast cancer is a heterogenous disease; analysis of gene expression profiles has shown the existence of at least five types of breast cancers with different prognoses (1). However, in practical terms and because gene expression profiles are not routinely performed, the classification of breast cancer depends in great part on the expression of two receptors: human epidermal growth factor receptor 2 (HER2), a tyrosine kinase that belongs to the epidermal growth factor (EGFR) family, and estrogen receptor (ER), the nuclear receptor for the steroid hormone estrogen. These receptors are not only useful in the classification of breast cancers but they are also targets of effective antitumor drugs (2, 3).

HER2 becomes active on homodimerization or heterodimerization with other members of the EGFR family. Activated HER2 transduces intracellular signals through phosphorylation cascades that, in turn, regulate the expression of genes that coordinately control cell differentiation, proliferation, death, adhesion, and migration (4). HER2 expression is elevated in 20–30% of breast cancers. These tumors are currently treated with herceptin, a monoclonal antibody against the extracellular domain of HER2, and/or lapatinib, a small molecule that inhibits its tyrosine kinase activity (2).

Estrogen is a central regulator of normal female physiology; however, because it promotes cell proliferation and survival, continuous exposure to endogenous or exogenous estrogen may cause breast cancer (5). Estrogen binds and activates two nuclear receptors, ERα and ERβ. ERα (hereafter called ER) mediates several of the effects of estrogen on normal and cancerous breast tissue by directly regulating the transcription of many genes (genomic actions) and by participating in cytoplasmic signaling cascades (nongenomic actions; ref. 6). Current endocrine therapies targeting estrogen include antiestrogens that bind ER, such as tamoxifen,
and aromatase inhibitors, which interfere with the synthesis of estrogen (7).

The HER2 and ER signaling pathways establish intricate connections during the progression of breast cancer (reviewed in ref. 8). Activation of HER2 or related receptors down-modulates the ER through the activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling, which, in turn, leads to phosphorylation and negative regulation of FOXO3a (9, 10). This transcription factor controls the expression of ER in breast cancer cells (11). Additional mechanisms may include the upregulation of microRNA-206, which targets the ER mRNA (12), and the activation of mitogen-activated protein kinase (MAPK) and NF-κB pathways (13). The down-modulation of ER by HER2 overexpression likely explains the relative low percentage of HER2-overexpressing patients that express ER (~50%) compared with the percentage of HER2-negative patients that express the nuclear receptor (70–80%; ref. 14).

From a therapeutic point of view, overactivation of HER2 or its downstream effectors MAPK and PI3K elicits resistance to tamoxifen (15). One of the mechanisms proposed is the down-modulation of ER expression by HER2 signaling. In addition, HER2 can lead to activation of ER by phosphorylation in the absence of ER ligands and to the generation of mitogenic signals independent of ER ligands (reviewed in refs. 16, 17).

Approximately 30% of HER2-positive patients express a variety of receptor fragments of 90 to 115 kDa, collectively known as p95HER2 of carboxy-terminal fragments (CTF; refs. 18, 19). p95HER2/CTFs are generated by at least two mechanisms, proteolytic shedding of the extracellular domain (18, 20) and alternative initiation of translation from internal AUG codons (21). Compared with tumors expressing only the full-length receptor, p95HER2-positive tumors have worse prognosis (19); they are more likely to metastasize (22), and they are resistant to treatment with herceptin (23).

Whereas several p95HER2/CTFs are inactive, one of them, generated by alternative initiation of translation from the AUG codon at position 611 (611-CTF), is an oncogenic form of HER2 that drives breast cancer progression in vivo (24). The hyperactivity of this fragment depends on its ability to constitutively dimerize through intermolecular disulfide bonds (24). Thus, 611-CTF is likely causally involved in the progression of p95HER2-positive tumors.

Despite its likelihood, the functional interaction between 611-CTF and ER has not been analyzed to date. Here, we show that 611-CTF expression results in down-modulation of ER by phosphorylation in breast cancer cells. To confirm this down-modulation in the clinical setting, we developed specific antibodies as a tool to detect 611-CTF in breast cancer specimens by immunohistochemistry. This was possible because of the existence of epitopes exposed in 611-CTF but masked in full-length HER2. Using these antibodies, we showed that 611-CTF is present in the majority of samples classified as p95HER2-positive by Western blot. Supporting the results with cultured cells, we showed that only 31.2% of breast tumors expressing 611-CTF expressed ER. In contrast, the percentage of tumors expressing ER and HER2, but not 611-CTF, was 72.6%, similar to the percentage of HER2-negative tumors expressing ER. These results show the importance of 611-CTF in the downregulation of ER in HER2-positive tumors and illustrate the usefulness of the anti-611-CTF antibodies to conduct translational research.

**Materials and Methods**

**Cell lines, animal studies, and tissue samples**

All the cells used in this study were obtained from American Type Culture Collection and were cultured for fewer than 6 months after receipt. The generation and characterization of MCF7 Tet-Off (BD Biosciences) transfected with full-length HER2, 611-CTF, and 648-CTF have been recently published (25). Cells were obtained from BD Biosciences, cultured and maintained in DMEM/Ham F12 (1:1) supplemented with 10% fetal bovine serum and 2 mmol/L l-glutamine (Life Technologies, Inc.) at 37°C in 5% CO2.

**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. MCF7 Tet-Off cells, transfected with the cDNA encoding 611-CTF under the control of a Tet/Dox-responsive element, were injected into the right flanks of 6- to 8-week-old female BALB/c athymic mice purchased from Charles Rivers Laboratories. The expression of 611-CTF was repressed by adding doxycycline (1 g/L) to the drinking water until tumors were ~200 mm3. Then mice were randomized and treated with or without doxycycline (1 g/L) or tamoxifen (26 mg/L) freshly prepared daily and given via drinking water. Tumor xenografts were measured with calipers every 3 days, and tumor volume was determined using the formula: (length × width2) × (π/6). At the end of the experiment, the animals were anesthetized with a 1.5% isoflurane-air mixture and were killed by cervical dislocation. Results are presented as mean ± SD of tumor volume.

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

**Plasmids and reagents**

Expression vectors for 611-CTF, 613-CTF, and 616-CTF were made by cloning HindIII-EcoRI PCR products into pCDNA3.1(+). Lapatinib (Tykerb) was provided by GlaxoSmithKline.

**Antibodies**

Antibodies were from BioGenex [anti-HER2 (CB11)], Thermo Scientific [anti-ERα, Ab-10 (clone TE11.5D11) antibody, anti–progesterone receptor (PR), Ab-8 (hPRa2 + hPRa3)], Invitrogen (Alexa Fluor 488), GE Healthcare (ECL antimonouse IgG, ECL antimonouse IgG), and Novoceastra [anti-ER (NCL-L-ER-6F11) and anti-PR (RTU-PGR-AB)].

**Generation of antibodies against 611-CTF**

**Polyclonal antibodies.** Rabbits were maintained and treated in accordance with the institutional guidelines of Vall
d’Hebron University Hospital Care and Use Committee. A peptide with the 32 NH\(_2\) terminal amino acids of 611-CTF (MPIWKFEDDEGASQPSINSTHSVVLDDKGC) was used to immunize four New Zealand white rabbits, which were injected three times in a period of 3 months. To avoid nonspecific conjugation with keyhole limpet hemocyanin, the four cysteines in the middle of the peptide sequence of HER2 were replaced by serines. After the animal exsanguinations, the immunized serum was purified on a protein A column.

**Monoclonal antibodies.** The monoclonal antibodies were generated by the biotechnology company Abyntek using the same peptide as the one used to generate the polyclonal antibodies.

**Western blot**

Extracts for immunoblots were prepared in modified radioimmunoprecipitation buffer [20 mmol/L Na\(_2\)HPO\(_4\)/NaOH (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EDTA, 100 mmol/L phenylmethylsulfonyl fluoride, 25 mmol/L NaF, 16 μg/mL aprotinin, 10 μg/mL leupeptin, and 1.3 mmol/L Na\(_3\)VO\(_4\)]. Protein concentrations were determined with detergent-compatible protein assay reagents (Bio-Rad). Samples were mixed with loading buffer [final concentrations, 62 mmol/L Tris (pH 6.8), 12% glycerol, 2.5% -mercaptoethanol and incubated at 99°C for 5 minutes before fractionation of 15 μg of protein by SDS-PAGE. Where appropriate, signals in Western blots were quantified with the software ImageJ 1.38 (NIH). All experiments were repeated three times.

**Immunoprecipitation**

Cell lysates were incubated with different antibodies during 1 hour at 4°C. Then immunocomplexes were purified with protein A. Immunoprecipitates were washed three times with lysis buffer, mixed with loading buffer, and analyzed by Western blot. The experiments were repeated three times.

**Immunofluorescence microscopy**

Cells seeded on glass coverslips were washed with PBS, fixed with 4% paraformaldehyde for 20 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were blocked (1 hour) and incubated (1 hour) with mouse monoclonal anti-HER2 CB11 (1:100), 32H2 (2 μg/μL), and 20F4 (20 μg/μL) [in 1% bovine serum albumin (BSA)] plus 0.1% saponin in PBS (0.025 mol/L Na\(_2\)HPO\(_4\), 0.025 mol/L K\(_2\)HPO\(_4\) in 0.87% of NaCl). Cells were then washed three times with PBS and incubated with FITC-conjugated Alexa Fluor 488 secondary antibody (1:1,000) in 1% BSA/PBS for 45 minutes. Cells were then washed three times with PBS, mounted in Vectashield with 4',6-diamidino-2-phenylindole, and visualized by fluorescence or confocal microscopy. All procedures were performed at room temperature. All experiments were repeated three times.

**Flow cytometry**

MCF7 cells expressing HER2, 611-CTF, or 648-CTF were washed at 4°C with PBS and detached in PBS containing 10 mmol/L of EDTA. Detached cells were incubated with 10 μg/mL of anti-32H2 monoclonal antibody in PBS containing 5% BSA for 30 minutes at 4°C and washed and stained for 30 minutes at 4°C with FITC-conjugated antimouse IgG (Becton Dickinson) in PBS containing 5% BSA. Flow cytometry was done on a FACScan using FACScan research software (Becton Dickinson Immunocytometry Systems).

**Immunohistochemistry and evaluation**

For a tumor cell to get a positive score, membrane staining was required for both c-erbB2 and 611-CTF. HER2 expression was detected with two different tests, the Food and Drug Administration–approved HercepTest kit (polyclonal rabbit antibody, DAKO) and the monoclonal antibody anti-c-erbB2 CB11. 611-CTF expression was detected using the 32H2 antibody in a concentration of 20 μg/mL. After boiling the sections in an autoclave in citrate buffer (pH 7.8), the Envision System (DAKO) was used, for all three antibodies, to visualize immunostaining. In these samples, HER2 expression was scored exactly as described for the Food and Drugs Administration–approved HercepTest in four categories: 0, 1+, 2+, and 3+. Scores of below 2+ were considered as negatives, and scores of 3+ were considered as positives. In scores of 2+, fluorescence *in situ* hybridization (FISH) analysis was performed to verify HER2 amplification. In 32H2, tumors were scored positive when at least 10% of the tumor cells are with complete membrane staining.

MCF7 cells expressing HER2, 611-CTF, or 648-CTF were washed at 4°C with PBS and detached in PBS containing 5 mmol/L of EDTA. Then cells were centrifuged, and cell pellets were fixed in 10% neutral formalin, dehydrated, and embedded in paraffin. Sections from cell pellets or human tissues of a thickness of 3 μm were placed on polysilicone-coated glass slides. Immunohistochemical analyses were performed using the same protocol described above. The antibody concentration used was 20 μg/mL for 20F4 and 2 μg/mL for 32H2.

**FISH for HER2/neu**

FISH was done according to the PathVysion (Vysis, Inc.) guidelines described in the package insert as approved by the U.S. Food and Drug Administration.

**Statistical analysis**

Associations between hormone receptors, tumor size, biological activity, and 611-CTF expression in breast cancer patients were analyzed by Mann-Whitney *U* test. Results were considered to be statistically significant at *P* < 0.05. All statistical analyses were performed using the SPSS 12.0 statistical software (SPSS, Inc.).

**Results**

**p95/611-CTF induces down-modulation of ER and resistance to tamoxifen**

We have previously shown that 611-CTF, a hyperactive isoform of HER2 (Fig. 1A), is present in a subset of human
Figure 1. Expression of 611-CTF induces the downregulation of ER. A, schematic drawing showing the HER2 constructs used in these studies. The kinase domain is represented by a hatched box. The N and the C at the beginning and at the end of the rectangle representing HER2 identify the amino terminus and the carboxy terminus, respectively. The vertical line represents the plasma membrane, and the extracellular (out) and intracellular (in) regions are marked. The regions of HER2 recognized by the antibodies Herceptin and CB11 are shown. B, MCF7 Tet-Off clones stably transfected with the cDNA encoding HER2 or 611-CTF under the control of a Tet/Dox-responsive element were kept with or without doxycycline (Dox) for 24 h, lysed, and analyzed by Western blot with the anti-HER2 CB11 antibody. C and D, the same cells as in B were washed with media without doxycycline and cultured for different periods of time; then, cells were lysed. Cell lysates were analyzed by Western blot with an antibody against ERα (anti-ER, C) or the progesterone receptor (anti-PR, D). Results of three independent experiments were quantified, and the averages are presented. E, MCF7 Tet-Off clones stably transfected with empty vector or the same vector containing the cDNA of HER2 or 611-CTF under the control of a Tet/Dox-responsive element were kept without doxycycline (Dox) for 72 h. Then cells were fixed and immunostained with antibodies against the intracellular domain of HER2, ER, or PR. Bar, 30 μm (bottom right). F, BT474 cells were treated with the indicated concentrations of the tyrosine kinase inhibitor lapatinib for 24 h. Then cells were lysed, and cell lysates were analyzed by Western blot with the indicated antibodies. The levels of ER from three independent experiments were quantified, and the results are presented as averages ± SD.
breast tumors (24). Due to the importance of the cross-talk between HER2 and ER signaling during breast cancer progression and treatment, we analyzed the possible interaction between 611-CTF and ER. As a first approach, we used MCF7 cells, a classical model, to study estrogen signaling (25), transfected with cDNAs encoding full-length HER2 or 611-CTF under the control of a promoter repressible by doxycycline (Fig. 1B; ref. 24).

As expected, induction of the expression of HER2 resulted in down-modulation of ER as analyzed by Western blot (Supplementary Fig. S1; Fig. 1C). Analysis of cells expressing 611-CTF showed that the expression of this hyperactive HER2 isoform resulted in a more pronounced down-modulation of ER (Supplementary Fig. S1; Fig. 1C). Immunohistochemical analysis (Fig. 1E) confirmed the Western blot results.

The expression of PR is transcriptionally regulated by ER (26). In fact, the expression of PR is considered a reliable marker of ER activity (recently reviewed in ref. 27). Thus, to determine the functional consequences of the down-modulation of ER induced by 611-CTF, we also analyzed the expression of PR. As shown in Fig. 1D and E and confirming the results with ER, the expression of 611-CTF led to down-modulation of PR.

HER2 signaling can be specifically targeted by the small molecule tyrosine kinase inhibitor lapatinib. Given the fact that BT474 cells express high levels of HER2 and p95HER2/CTFs (20), we hypothesized that the expression of ER should be repressed in this cell line. Confirming this hypothesis, treatment of BT474 cells with lapatinib, in addition to effectively block HER2 signaling as judged by the decrease in the levels of p-Akt and p-Erk1/2, induced upregulation of the ER (Fig. 1F).

Expression of HER2 has been shown to elicit resistance to tamoxifen (16, 17). To analyze if 611-CTF also induces resistance to the antiestrogen, we xenografted MCF7/611-CTF cells into nude mice (Fig. 2A). As expected, in the presence of doxycycline, i.e., with the repressed expression of 611-CTF, the growth of MCF7 cells was inhibited by tamoxifen (Fig. 2A). Induction of the expression of 611-CTF led to the growth of larger tumors and to the down-modulation of the ER (Fig. 2A and B). The growth of MCF7 cells expressing 611-CTF was not inhibited by tamoxifen, showing that expression of the HER2 fragment induces resistance to the antiestrogen. Immunohistochemical analysis confirmed that expression of 611-CTF down-modulated that of ER (Fig. 2B).

The results in Figs. 1 and 2 revealed that, compared with HER2, 611-CTF induces a profound downregulation of ER and resistance to tamoxifen treatment, suggesting that this HER2 fragment plays a role in the regulation of ER in breast tumors.

**Generation and characterization of antibodies against 611-CTFs**

To determine whether the down-modulation of ER caused by the expression of 611-CTF in cultured cells also occurs in breast cancer patients, both proteins would have to be
analyzed in a large series of human HER2-positive samples. Whereas the determination of ER levels by immunohistochemistry is a routine technique in the clinic (28), a comparable method to analyze the levels of 611-CTF is not currently available. Thus, we aimed to develop new antibodies specific for 611-CTF.

The extracellular juxtamembrane region of HER2 is cysteine-rich (Fig. 3A). In the context of the full-length molecule, this region is highly structured and maintained by three intramolecular disulfide bonds established by six cysteines (Fig. 3A; ref. 29). In contrast, the same region is presumably unstructured and sterically more accessible in 611-CTF. Therefore, we hypothesized that the extracellular juxtamembrane domains of HER2 and 611-CTF are antigenically distinct and hence the existence of epitopes exposed at the NH2 terminus of 611-CTF while masked in full-length HER2.

To test this hypothesis, we generated a rabbit polyclonal antibody against a peptide corresponding to the 32 NH2-terminal amino acids of 611-CTF (Fig. 3A). This antibody recognized both full-length HER2 and 611-CTF by Western blot (i.e., under denaturing and reducing conditions; Supplementary Fig. S2). However, further characterization showed the existence of epitope(s) exposed under native conditions only in 611-CTF, as analyzed by immunoprecipitation and indirect immunofluorescence microscopy (Supplementary Fig. S2).

Next, we generated and characterized several mouse monoclonal antibodies against the same peptide; for simplicity, we will only present the results obtained with two representative specific antibodies. Western blot analysis of cells transfected with two 611-CTF NH2-terminal deletion constructs showed that the antibodies 32H2 and 20F4 recognized overlapping but distinct epitopes. Deletion of proline 612 completely prevented and did not affect the recognition of 611-CTF by 32H2 and 20F4, respectively (Fig. 3A and B), whereas the binding of 20F4 was abolished by deletion of the first five amino acids of the HER2 fragment (Fig. 3A and B).

As expected both antibodies bound to denatured and reduced HER2 in Western blots (Fig. 4A). As a negative control, we used cells expressing 648-CTF (Fig. 4A), a fragment that lacks the peptide sequence against which the antibodies were raised (Fig. 1A). Importantly, both antibodies showed a strong preference for 611-CTF over HER2 when assayed by immununoprecipitation (Fig. 4B), indirect immunofluorescence (Fig. 4C), flow cytometry (Fig. 4D; Supplementary Fig. S3), and immunohistochemistry on formalin-fixed paraffin sections (Fig. 4E). These results confirmed that the antibodies generated are a useful tool to specifically detect the presence of 611-CTF using a variety of techniques, including immunohistochemistry, a commonly used technique in the clinical setting. We chose the antibody 32H2 for further experiments because it showed a better performance than that of 20F4 by flow cytometry and immunohistochemistry (Supplementary Figs. S3 and S4).

**611-CTF levels in p95HER2-positive tumors**

HER2-positive breast cancers expressing p95HER2 have worse prognosis and are more likely to metastasize compared with those expressing only the full-length receptor (19, 22). This suggests that p95HER2 is causally involved in the progression of a subgroup of breast cancers. p95HER2 is a heterogeneous series of HER2 fragments that includes soluble as well as transmembrane fragments (21). Among these fragments, only 611-CTF seems to be hyperactive (24). Thus, it seemed reasonable to hypothesize that the majority of p95HER2-positive patients express 611-CTF. To test this and to further characterize the 32H2 antibody against 611-CTF, we analyzed the levels of this fragment by immunohistochemistry in a series of 35 HER2-positive samples previously analyzed for the presence of p95HER2 by Western blot (Fig. 5; Table 1; ref. 23).

As shown in Table 1, the majority (90.5%) of p95HER2-positive samples, as judged by Western blot, expressed 611-CTF as judged by immunohistochemistry with the 32H2 antibody (Fig. 4A and Table 1). Only 2 of 21 samples were positive by Western blot but negative by immunohistochemistry, and only 1 of 14 samples was negative by Western blot but stained positively with the 32H2 antibody (Table 1). Thus,
for the majority of samples, detection of p95HER2 fragments by Western blotting coincides with the expression of 611-CTF.

Next, using immunohistochemistry, we analyzed an independent collection of 56 formalin-fixed paraffin sections of breast tumors for which the lymph node status at the time of resection was available. In agreement with the previous reports on the value of p95HER2 as a maker of bad prognosis and metastasis (19, 22) and with the fact that 611-CTF is a component of p95HER2 in the majority of samples, we found that 611-CTF-positive patients also had a higher risk of

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**Figure 4.** Characterization of monoclonal antibodies specific for 611-CTF. A, MCF7 Tet-Off clones stably transfected with cDNA encoding HER2, 611-CTF, or 648-CTF under the control of a Tet/Dox-responsive element were grown without doxycycline for 48 h and lysed. Cell lysates were analyzed by Western blot with CB11, an antibody against the cytoplasmic domain of HER2, or with 32H2 and 20F4, two independent monoclonal antibodies raised against the NH2 terminus of 611-CTF (see Fig. 3). B, the lysates from MCF7 cells treated as in A were mixed (1:1:1) and subjected to immunoprecipitation with the indicated monoclonal antibodies. Eluted immunoprecipitates were analyzed by Western blot with the CB11 antibody. As a negative control, a mock immunoprecipitation with no antibody (−) was performed. C, the same cells as in A were analyzed in a confocal microscope by indirect immunofluorescence with the indicated antibodies. Bar, 30 μm (bottom right). D, the same cells as in A were analyzed by flow cytometry using different concentrations of the anti-611-CTF antibody 32H2 or only secondary antibody. E, the same cells as in A were fixed, paraffin-embedded, and immunostained with CB11, an antibody against the cytoplasmic domain of HER2, or with 32H2 and 20F4, two independent monoclonal antibodies raised against the NH2 terminus of 611-CTF (see Fig. 3). Bar, 30 μm (bottom right).
metastasis (Supplementary Fig. S5). These results further validated the antibody used to detect 611-CTF and indicate that this fragment may play a causal role in the progression of breast cancers expressing p95HER2.

**Expression of 611-CTF, but not of HER2, inversely correlates with ER**

Early reports showing that the expressions of ER and HER2 tend to be inversely correlated (30) have been confirmed recently. Whereas nearly 80% of HER2-negative breast tumors are positive for ER, only ~50% of HER2-positive breast tumors are ER positive (14).

To analyze the possible relationship between 611-CTF and ER expression in human breast cancers, we analyzed the expression of ER in a new cohort of 83 HER2-positive patients classified according to the expression of 611-CTF as judged by immunohistochemistry with the 32H2 antibody (Table 2). In agreement with the published studies, 56.6% of the samples expressed detectable levels of ER. However, in the 611-CTF–positive subgroup, only 31.2% of the samples expressed ER (Table 2). Notably, the percentage of ER-positive samples in patients expressing HER2, but not 611-CTF (72.6%, Table 2), did not differ significantly (exact binomial test, $P = 0.313$) from that reported for HER2-negative patients (78.2%) in a large series of samples (14). These results show that the expression of 611-CTF is inversely correlated with the expression of ER and indicate that 611-CTF plays a major role in the down-modulation of ER in HER2-positive patients.

Approximately 50% of HER2-positive patients express PR (14). In agreement with this result, 47% of the HER2-positive patients analyzed here were PR positive (Table 2). As expected, given the fact that ER promotes the expression of PR, among 611-CTF–positive patients, expression of PR was less frequent (25.0%). In a clear parallelism with the percentages observed for ER, the percentage of PR positivity in the group of patients positive for HER2 but negative for 611-CTF (60.8%) is clearly higher than in the group of 611-CTF–expressing patients.

These results confirm that 611-CTF plays a major role in the regulation of the ER expression and open the possibility that expression of this HER2 fragment conditions the response to endocrine therapy.

**Discussion**

Previous reports have shown that p95HER2 correlates with poor prognosis of breast cancer patients and confers...
Table 2. Association of 611-CTF and ER expression in a cohort of 83 HER2-positive breast cancer samples

<table>
<thead>
<tr>
<th>611-CTF</th>
<th>Total</th>
<th>ER+</th>
<th>ER−</th>
<th>PR+</th>
<th>PR−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive*</td>
<td>32 (38.5)</td>
<td>10 (31.2)</td>
<td>22 (68.8)</td>
<td>8 (25)</td>
<td>24 (75)</td>
</tr>
<tr>
<td>Negative</td>
<td>51 (61.5)</td>
<td>37 (72.6)</td>
<td>14 (27.4)</td>
<td>31 (60.8)</td>
<td>20 (39.2)</td>
</tr>
<tr>
<td>Total</td>
<td>83 (100)</td>
<td>47 (56.6)</td>
<td>36 (43.4)</td>
<td>39 (47)</td>
<td>44 (53)</td>
</tr>
</tbody>
</table>

*611-CTF positive was defined as >10% of tumor cells showing whole membrane staining and ER positive as >10% of tumor cells showing nuclear staining.

resistance to treatment with herceptin (19, 22, 23). Initially, p95HER2 was assumed to arise through the proteolytic processing of full-length HER2 by metalloproteases (18, 31, 32). In fact, it was named after the theoretical molecular size of the cell-associated fragment generated by proteolytic shedding of the ectodomain of HER2. Following this assumption and because the molecular weight of proteins varies with cell-specific posttranslational modifications, samples of breast tumors analyzed by Western blot were classified as p95HER2-positive if immunoreactivity was detected in the 90 to 115 kDa region. Additional fragments arise through proteolytic cleavage of the intracellular domain of HER2 by caspases (33) and calpains (34). However, the molecular size of these fragments is <50 kDa (33, 34), and therefore, they have not been considered within the p95HER2 group of fragments.

In contrast to the original hypothesis, we have shown recently that, in addition to proteolytic processing, alternative initiation of translation from different methionine codons can generate HER2 CTFs (21). In fact, p95HER2 is a heterogeneous collection of soluble and transmembrane fragments of different lengths (24). Whereas the soluble fragments are inactive and may represent degradation byproducts, one of the transmembrane fragments, 611-CTF, is hyperactive (24). Expression of 611-CTF leads to the activation of several intracellular signaling cascades, such as the MAPK, Akt, Src, and PLCγ signal transduction pathways, and promotes cell migration (35). In addition, expression of low levels of 611-CTF in the mammary gland of mice induces the growth of breast tumors far more aggressive than those generated by full-length HER2 (25). Furthermore, as shown here, 611-CTF plays a major role in the downregulation of the ER and resistance to tamoxifen in breast cancer cells, a remarkable result given the relevance of the interplay between the HER2 and ER signaling pathways in breast cancer progression and treatment. Therefore, all lines of evidence obtained with the cultured cells and animal models point to a major role of 611-CTF in the progression of a subgroup of HER2-positive breast cancers.

To confirm in specimens from breast cancer patients the down-modulation of ER by 611-CTF observed in preclinical models, we developed a specific tool to robustly and reproducibly analyze the expression of 611-CTF. Western blot was considered, in principle, a valid technique and has been used by several authors (18, 20, 23). However, it requires fresh-frozen tumor tissue, frequently not available. Furthermore, to identify 611-CTF from tumor samples, the migration of candidate bands has to be compared with that of the forms expressed in cells transfected with a cDNA construct encoding 611-CTF. This comparison results in ambiguous conclusions when the intensity of the candidate band is not comparable with that used as reference, frequently requiring repetition of the analysis. Thus, although Western blot has been a useful technique to confirm the existence of 611-CTF in a limited number of samples (24), it is not appropriate for the analysis of a large series of tumors.

The development of antibodies against epitopes masked in full-length HER2 but exposed in 611-CTF circumvented most of the problems posed by the use of Western blot. These antibodies allowed the detection of 611-CTF in formalin-fixed paraffin-embedded sections, the most common way to store samples from patients without the need of including internal controls.

Once available, we used these antibodies to classify HER2 tumors as 611-CTF positive or negative. Subsequent analysis of the expression of ER and PR in these subgroups confirmed the down-modulation of ER induced by 611-CTF in cultured cells. We showed that only a minor proportion of 611-CTF-positive tumors express ER and PR compared with HER2-positive tumors that do not express the fragment (Table 2). These results may have implications when tailoring a therapy for HER2-positive patients. Patients expressing 611-CTF are not likely to benefit from the treatment with monoclonal antibodies against the extracellular domain of HER2. In contrast, small molecule inhibitors, which block the activity of the hyperactive fragment, may be appropriate to treat this subgroup of HER2-positive patients. Interestingly, it has been shown recently that the small-molecule tyrosine kinase inhibitor lapatinib restores hormone sensitivity in cells with acquired endocrine resistance (36). Because p95HER2 exerted a potent, but reversible, down-modulation of ER expression, it is likely that its inhibition results in the upregulation of ER; thus, we propose that the p95HER2-positive subgroup of tumors may benefit from a dual treatment with lapatinib and anti-ER therapies.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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