Spontaneous and pronase-induced HER2 truncation increases the trastuzumab binding capacity of breast cancer tissues and cell lines

Running Title: HER2 truncation and trastuzumab binding in breast cancer

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Conflict of interest statement

No competing interests to declare.
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Abstract

A subgroup of HER2 overexpressing breast tumours co-expresses p95\textsuperscript{HER2}, a truncated HER2 receptor that retains a functional HER2 kinase domain but lacks the extracellular domain, thus impairing trastuzumab binding. We evaluated p95\textsuperscript{HER2} expression in 99 frozen breast carcinoma samples by western blot analysis. The HER2-positive cell line BT474 treated with pervanadate or pronase was used as positive control for p95\textsuperscript{HER2} expression. Immunohistochemistry was performed on parallel formalin-fixed paraffin-embedded sections of the same case series using antibodies directed against either the intra- or extra-cellular binding domain of HER2. In particular, biotinylated trastuzumab (BiotHER) was used to evaluate the binding capacity of the humanised antibody. To avoid a subjective evaluation of the score values and the percentage of immunostained cells, the slides were scanned and automatically analysed. The number of cases with HER2 overexpression (score 3+) and HER2 gene amplification was higher in the p185\textsuperscript{HER2} positive/p95\textsuperscript{HER2} positive samples than in the p185\textsuperscript{HER2} positive/p95\textsuperscript{HER2} negative group. Automated analysis confirmed a significantly higher percentage of 3+ scored cells in p95\textsuperscript{HER2} positive cases. Conversely, the percentage of 2+ scored cells was higher in p95\textsuperscript{HER2} negative cases. The status of the HER2 extracellular domain was then studied using flow cytometry on BT474 cells after pronase enzymatic digestion using trastuzumab and pertuzumab, while the presence of HER2-HER3 dimers was studied using a proximity-ligation assay. In vitro experiments showed that short-term pronase digestion of BT474 cells produced two HER2 fragments (of 95 and 150 kDa, detectable in tissue specimens as well), increased the binding affinity of trastuzumab, reduced the rate of HER2-HER3 dimers and did not interfere with pertuzumab-binding capacity. In conclusion, the presence of p95\textsuperscript{HER2} as detected by western blot analysis does not compromise the immunohistochemical detection of HER2. Our data suggest that a reduction of the receptor steric hindrance as induced by enzymatic shedding may facilitate the binding capacity of trastuzumab.

Keywords: Breast Cancer, HER2, p95\textsuperscript{HER2}, pronase, western blot, trastuzumab binding site.
Introduction

The human proto-oncogene HER2 encodes for the full-length membrane-spanning receptor p185HER2. The overexpression of p185HER2 plays a pathogenic role in approximately 20% of breast cancers[1]. The large 110 kDa extracellular domain (ECD) of HER2 consists of 621 amino acids and is subdivided into four domains (I-IV)[2, 3]. The HER2 ECD constitutively adopts an active extended conformation, which is acquired by other EGFRs only after ligand binding. This structural feature of ECD favours the homo- and hetero-dimerisation of HER2, thus encouraging activation of the intracellular tyrosine kinase domain and triggering downstream signalling events[4]. Pertuzumab, a humanised monoclonal antibody, binds to HER2 near the centre of domain II and inhibits HER2 dimerisation with other members of the EGFR family[5, 6]. In contrast, binding of trastuzumab to domain IV of HER2 does not interfere with heregulin-induced HER2-HER3 dimer formation but prevents HER2 shedding and inhibits intracellular signalling[7].

Approximately 30% of HER2-positive patients express a variety of receptor fragments sized between 90 and 115 kDa. These are collectively known as p95HER2 carboxy-terminal fragments (CTF)[8]. In breast cancer, HER2-CTFs may be synthesised[9, 10] or produced by an enzymatic sheddase[11]. A slow proteolytic cleavage performed by metalloproteases can occur at a site proximal to the transmembrane domain[12-14] thus generating a fragment of 95 kDa that retains kinase activity[15-19]. It is yet to be determined whether p95HER2 levels in breast tumours are directly linked to the HER2 concentration in the serum[20-22]. The rapid and potent shedding of HER2 ECD produced in vitro by pervanadate[23], a general inhibitor of protein-tyrosine phosphatases with a non-specific metalloprotease activation capacity, induces an increase in p95HER2 levels in BT474 breast cancer cells and delivers the trastuzumab epitope to the cell culture medium[16]. Scaltriti et al.[14] hypothesised that the tumours expressing p95HER2 in the absence of the trastuzumab-binding extracellular domain may be resistant to the humanised antibody. However, a translational investigation from the neoadjuvant GeparQuattro study (incorporating
chemotherapy plus trastuzumab treatment) showed that $p95^{\text{HER2}}$ expression, measured using a monoclonal antibody that specifically recognises the 611 CTF by immunohistochemistry (IHC), indicates a response to trastuzumab[24].

In previous studies using biotinylated trastuzumab (BiotHER) as the primary antibody for IHC[25, 26], we demonstrated that the trastuzumab binding site was only detectable in approximately 50% of $HER2$ amplified breast cancers, while the reported percentage of $HER2$ overexpression using anti-intracellular domain (ICD) antibodies in amplified breast cancers is approximately 90%[27]. Similarly, we previously showed a lower percentage of ECD overexpression using the TAB250 antibody compared to the ICD expression as evaluated by CB11 in amplified breast cancers[28].

Taken these data together, our hypothesis was that the expression of $HER2$ truncated forms could reduce the availability of the epitopes necessary for a reaction with specific anti-ECD antibodies. Therefore, we evaluated the expression of the $HER2$-truncated forms by western blot (WB) analysis in a series of breast carcinomas and compared the level of expression with the IHC results using antibodies directed against both the ICD and ECD of $HER2$. The results were compared and validated with those obtained in vitro in BT474 $HER2$-positive breast cancer cells after enzymatic induction of $HER2$ truncation.

**Material and Methods**

**Tissue Samples**

Paired FFPE tissue blocks and snap frozen specimens (stored at –80°C in frozen tissue matrix OCT) from 99 breast cancers were obtained from the pathology files of the authors’ institutions. The fixation was performed in buffered formalin at room temperature (RT) for 24-48 hr. Permission for the project was obtained from the Institutional Review Board, and the study was conducted in compliance with the ethical regulatory requirements for the handling of biological specimens from tumour banks of the participating institutions (i.e., samples were exclusively available for research
purposes in retrospective studies). The same institutional ethics review board agreed that written consent from the patients was not required, given that the study did not interfere with diagnosis or treatment decisions. The patient data were anonymised prior to analysis.

*Fluorescence in situ hybridisation (FISH)*

FISH was performed using probes for HER2 (17q12) and CEP17 (Vysis, Inc, Downers Grove, IL, USA) as previously reported[29]. The analysis was performed by selection and automated acquisition of six invasive areas on each slide using the Metafer Scanning System (Carl Zeiss MetaSystems GmbH) and AxioImager epifluorescence microscope. The PathVysion V2 software (FDA approved) was used to analyse the results. The cases were scored according to the ASCO guidelines[30].

*Immunostaining*

The expression of the ICD was evaluated using Herceptest™ (kit, DAKO, Glostrup, Denmark) on the Dako Autostainer and CB11 (diluted 1:200, Novocastra Laboratories, Newcastle-upon-Tyne, UK) using the Bond™ automated stainer (Menarini Diagnostics, Florence, Italy). To evaluate the effect of p95HER2 expression on ECD detection via IHC, the sections were processed using the TAB250 mouse monoclonal antibody (Zymed, S. Francisco, CA, USA). In addition, to evaluate the trastuzumab-specific binding site, we used BiotHER as previously reported[25, 26]. Briefly, 1 mg of trastuzumab was diluted in saline solution and dialysed overnight in 0.1 M Na2CO3, pH 8.5. To a 1 ml solution, 0.12 ml of Aminohexanoyl-Biotin-N-Hydroxysuccinimide ester (AH-BNHS, Biospa, Milan, Italy) was added, gently mixed for 4 hr at RT and dialysed in phosphate buffered saline. We tested the ECD overexpression with or without pre-treatment with pronase, diluted to 0.1% Endogenous peroxidases were inhibited by incubating sections with 6% H2O2 for 7 min. The sections were then incubated with the primary antibodies diluted in PBS with 1% BSA (TAB250 1:20, BiotHER 1:100) for 30 min at RT. After rinsing in PBS, EnVision+ System Labelled Polymer-HRP (Dako) anti-mouse and HRP-conjugated streptavidin (diluted 1:50, BioGenex, San Ramon, CA) were used to reveal TAB250 and BiotHER staining, respectively. The reaction was
developed using a solution of 3,3’-diaminobenzidine and H2O2 for 5 min. The nuclei were counterstained with Mayer’s haemalum.

To avoid a subjective evaluation of the percentage of immunostained cells in cancer tissues and to assess the intra-tumour heterogeneity of HER2 expression, BiotHER and CB11-stained slides were scanned and analysed using a ScanScope XT automated scanning system (Aperio Technologies, Vista, CA) and the integrated FDA-approved software. Each section was subdivided into 6 quadrants in which 8 to 10 tumour foci were randomly selected (Figure 1). The score results (percentage of cells scored as 3+, 2+ and 1+) of each quadrant were recorded. A variance analysis comparing the percentage of positive cells in each quadrant was performed. Then, the mean percentage of the different score values in each quadrant was compared to the p95HER2 status as defined by WB. Statistical analyses were performed using Student’s t-test assuming unequal variances (software freely available on http://studentsttest.com/). The sections obtained from BT474 blocks (control and test) were processed with the same IHC protocols reported above.

*In vitro induction of p95HER2*

To induce p95HER2 production in BT474, cells were treated with freshly prepared pervanadate (0.5 M sodium orthovanadate and 0.5 M H2O2)[16]. After overnight starvation (DMEM with 0.5% FCS), the cells were treated with 0.1 or 1 mM pervanadate for 30, 60, 90 and 180 min. At each time point, the level of soluble p97-115HER2 (the soluble circulating fragment of p185HER2) was analysed in the supernatant using the human sHER2 enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (BMS207, Bender MedSystems GmbH, Vienna, Austria). The results were normalised against non-conditioned medium. To test the effect on cell viability of H2O2 added to orthovanadate to obtain pervanadate, BT474 cells were incubated for 90 min with either 1 mM H2O2 alone or with the addition of 500 U/ml of catalase[31] after mixing orthovanadate and H2O2.

Alternatively, at confluence, BT474 cells were washed with PBS and treated with pronase (0.1% and 0.02% in PBS) at RT for 10 min. The cells were then collected and washed twice with PBS.
supplemented with 3X protease inhibitors cocktail (complete, EDTA-free, Roche Diagnostics S.p.A, 200900 Monza (MB), Italy).

The cell growth was monitored in flasks with an EVOS inverted microscope (Advanced Microscopy Group, Bothell, WA), and cell viability was assessed using trypan blue vital staining. Each experiment was performed in triplicate. The paraffin-embedded blocks were prepared from cell pellets obtained after fixation in buffered formalin.

**Western blot (WB) analysis**

After each treatment described above, the expression of HER2 was assessed by WB on BT474 cells. From the breast cancer specimens, 10 frozen sections containing approximately 1 cm² of viable tumour cells were analysed. The adequacy was verified on haematoxylin-eosin stained sections before and after cutting. One out of 99 cases showing only *in situ* carcinoma on the frozen section was discarded. The frozen sections were collected in tubes and washed with cold PBS to remove the OCT and blood residues. The samples were lysed at 4°C for 30 min in RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100 and 0.1% SDS) with protease inhibitors. The total protein extracts were quantified with the BCA protein assay (Thermo Fisher Scientific Inc, Rockford, IL, USA), divided into aliquots of 25 µg and stored at –80°C.

After the addition of 5X loading buffer, each sample was boiled for 5 min and loaded onto an 8% polyacrylamide gel. Each gel contained a control lane loaded with 25 µg of protein extracted from pervanadate-treated BT474 cells as marker for p95\(^{\text{HER2}}\). Electrophoresis was performed in TGS buffer; after separation, proteins were transferred to a nitrocellulose membrane. After saturation with TBS and 5% milk, the membrane was immunoblotted either with anti-HER2 (A0485, polyclonal antibody Dako, Glostrup, Denmark) at 1:1000 or with anti-actin at 1:800 as a loading control. Both antibodies were diluted in TBS with 5% BSA and incubated overnight at 4°C. The membrane was washed and incubated with an anti-rabbit peroxidase-labelled secondary antibody. Finally, the membrane was developed with ECL luminol (Bio-Rad, Hercules, CA, USA).
p185HER2 was considered overexpressed (++) if the WB band was $\geq p185^{\text{HER2}}$ level in 25 $\mu$g of BT474 cells. Lower p185HER2 expression levels were classified as p185HER2 (+). Specimens were scored as positive for p95HER2 (+) if a distinct band was present at the same molecular weight as the p95HER2 pervanadate-induced band in BT474 cell extracts.

**Flow cytometry**

To obtain a single cell suspension, BT474 cells were detached with trypsin/EDTA and collected into a 50 ml tube with 20 ml of DMEM medium at 37°C for one hr to allow the replacement of all the surface molecules eventually digested by trypsin. Afterwards, four tubes of $10^6$ cells each were centrifuged. Two cell pellets were incubated for 10 min at RT with 100 $\mu$l of 0.1% pronase, controls were kept in PBS. To ensure the complete pronase inactivation, the cells were washed once with DMEM and twice with PBS supplemented with 2X protease inhibitors. The cells were then incubated at RT for 30 min with trastuzumab or with pertuzumab (Genentech, Inc., South San Francisco, CA, USA), both at a final concentration of 100 ng/ml, followed by a FITC-conjugated anti-human secondary antibody. The stained cells were analysed with a Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA).

**Immunoprecipitation analyses**

The pronase-treated BT474 cells were lysed in RIPA buffer as described above. Total extract aliquots of 500 $\mu$g were incubated with 2 $\mu$g of trastuzumab or pertuzumab at 4°C overnight under gentle agitation. Twenty $\mu$l of sepharose-protein A and sepharose-protein G beads were added. After incubation for 2 hr at 4°C the sepharose beads were washed and rinsed with 1X loading buffer. The immunoprecipitated proteins and 25 $\mu$g of control total extracts were analysed by western blot using the same protocol described above.

**Proximity ligation assay (PLA)**

All reagents used for the PLA analysis were from Olink Bioscience (Uppsala science park, 75183 Uppsala, Sweden). The PLA reactions were performed following the manufacturer’s instructions using anti-HER2 (A0485 polyclonal diluted 1:300) and anti-HER3 (monoclonal antibody clone
6F12 diluted 1:100 Lab Vision, Thermo Fisher Scientific Inc, Kalamazoo, MI USA.) as primary antibodies. Student’s t-test was used assuming unequal variances.

Results

Correlation of $p95_{\text{HER2}}$ and IHC results in breast cancer specimens

By WB analysis, $p185_{\text{HER2}}$ was detectable in 50 cases. Forty samples showed $HER2$ amplification by FISH. Among these amplified cases, 20 were $p185_{\text{HER2}}^{++}$, and 12 of these also expressed $p95_{\text{HER2}}$ by WB (Figure 2). In the latter subgroup of cases, five showed a 150 kDa fragment as well. Twenty cases $p185_{\text{HER2}}^{++}$ were negative for $p95_{\text{HER2}}$. Two out of 7 cases with $HER2$ gene gain (less than six gene signals per cell) had high expression levels of $p185_{\text{HER2}}$, but not $p95_{\text{HER2}}$. Three cases with very low expression levels of $p185_{\text{HER2}}$ did not show any genetic alterations.

The effect of $p95_{\text{HER2}}$ expression on the $HER2$ IHC expression of breast cancer tissues is shown in table 1. The number of cases showing $HER2$ overexpression (score 3+; >10% of $HER2^{+}$ cells with strong and complete membrane staining) was higher in the series scored as $p185_{\text{HER2}}^{++}/p95_{\text{HER2}}^{++}$ by WB than in the series $p185_{\text{HER2}}^{++}/p95_{\text{HER2}}^{-}$. This result was confirmed using both the anti-ICD (HerceptestTM and CB11) and the anti-ECD (TAB250 and BiotHER) antibodies following antigen retrieval with pronase. Pronase increased the 3+ scored cells with BiotHER and TAB250 (Figure 3) antibodies as compared to not enzyme treated sections, regardless of the level of $p95_{\text{HER2}}$.

Automated analysis of CB11 and BiotHER immunostaining of breast cancer confirmed a significantly higher percentage of 3+ scored cells in $p95_{\text{HER2}}^{+}$ cases compared to the $p95_{\text{HER2}}^{-}$ (p value < 0.005; Figure 4A, D). In contrast, the percentage of 2+ scored cells was higher in $p95_{\text{HER2}}^{-}$ cases (p value < 0.005; Figure 4B, E). In both series, the immunostaining was homogeneously distributed. The percentage of cells scored 1+ was similar in the two groups (p value > 0.005; Figure 4C, F). No significant differences among the 3+ (p value >> 0.005), 2+ (p value >> 0.005) and 1+ (p value >> 0.005) quadrants were observed at variance analysis indicating uniform quadrant selection.
**p95\text{HER2} induction and cell viability in BT474**

Incubation of BT474 cells with pervanadate (1 mM) induced a shedding of HER2 ECD that increased in a time-dependent manner, as shown by the ELISA test (Figure 5A); this occurred in parallel to the p95\text{HER2} increase (Figure 5B). However, BT474 cells rapidly detached from the culture plate, and cell viability was highly compromised by the treatment with pervanadate even after 30 min of exposure (Figure 6A, B) with almost 80% cell death. The toxic effect of pervanadate was confirmed when catalase was used to block excess H2O2 (Figure 6C). In contrast, H2O2 alone did not induce any sign of cellular stress (Figure 6D). Lower concentrations of pervanadate (0.1 mM) did not induce p95\text{HER2} even after 3 hr of treatment (Figure 5C) but maintained the cytotoxic effect.

Pronase treatment digested p185\text{HER2} and generated two fragments (Figure 7), and did not compromise cell viability, as shown by EVOS examination (Figure 6E, F) and trypan blue assay. The cells treated with 0.02% pronase showed a thin p95\text{HER2} band, a clear and intense band at 150 kDa and maintained strong expression of p185\text{HER2}. Higher pronase concentrations (0.1%) led to a further increase in the 95 kDa and 150 kDa band signals coupled with an almost complete disappearance of the p185 band (Figure 7).

**Trastuzumab and pertuzumab binding**

Membrane immunostaining with BiotHER following pronase treatment increased in intensity (Figure 8). Flow cytometry on control BT474 cells showed 67% of cells with higher trastuzumab affinity and the remaining 33% with lower affinity for trastuzumab (Figure 9A). After 10 min of pronase exposition, 94% of cells showed high trastuzumab binding (Figure 9B). By immunoprecipitation we showed that the 150 kDa HER2 fragment induced by pronase treatment still bound trastuzumab (Figure 10). Pronase treatment did not affect the binding capacity of pertuzumab as shown by flow cytometry (Figure 9 C, D) and confirmed by immunoprecipitation assay (Figure 10).

**HER2-HER3 dimer dissociation**
The PLA performed using anti-HER2 and -HER3 antibodies allowed for the analysis of heterodimers in BT474. The mean number of signals per cell (mean = 20.30; mode = 18; standard deviation = 10.53; n = 315) in the control was significantly higher (p < 0.005) as determined by a t-test analysis than the mean number of signals per cell (mean = 13.76; mode = 5; standard deviation = 9.88; n = 384) in the BT474 pronase-treated cells (Figure 11).

**Discussion**

Our first objective was to evaluate whether p$^{HER2}$ may impair the trastuzumab-binding epitope. Using WB and IHC analyses, we confirmed the results by Molina et al.[18] demonstrating that p$^{HER2}$ fragments in breast cancer tissues are generally associated with high levels of p$^{HER2}$. However, in contrast to our expectations, we observed that the IHC expression of the trastuzumab epitope as detected by BiotHER in breast cancer tissue was higher in p$^{HER2+}$ cases compared to cases with similar high levels of p$^{HER2}$ but negative for p$^{HER2}$. This result could explain the response of tumours that expressed p$^{HER2}$ to trastuzumab therapy measured by IHC as shown in the neoadjuvant GeparQuattro study[24].

A possible explanation for this unexpected increase in immunoreactivity of the anti-HER2 antibodies in the presence of truncated HER2 may be the reduction of the antigen “steric hindrance”. It has been demonstrated that the number of antigen molecules that are bound to the cell surface could significantly affect the ability of antibodies to bind, causing a steric hindrance[32]. If the antigenic determinants are closely spaced on the cell surface, a spatial interference may result such that a smaller distance between the antigenic determinants leads to a greater likelihood of reduced antibody binding. In previous experiments Valabrega and colleagues[33] were able to modulate the expression of HER2 in SKBR3 HER2-overexpressing breast cancer cells by using small interfering RNAs at different concentrations. In agreement with our hypothesis, it was shown that a HER2 reduction up to 70%, as measured by WB analysis and flow cytometry, still
corresponded to an immunoreactivity of cells scored as 3+ and that HER2 reduction up to 40% rendered cancer cells more sensitive to trastuzumab treatment.

To study the effect of HER2 truncation in vitro, as suggested by others[16], we used pervanadate, a protein-tyrosine phosphatase inhibitor that induces HER2 shedding in BT474 cells through non-specific metalloprotease activity. Unfortunately, we found pervanadate to be highly toxic. An alternative method to test our hypothesis was to simulate proteolysis by treating cells directly with a proteases cocktail known as pronase, a mixture of at least 10 proteases[34] commonly used in IHC for antigen retrieval of FFPE tissues. Digestion using pronase-based solutions is designed to break the protein cross-links and unmask the antigens and epitopes in FFPE tissue sections, enhancing the antibody staining intensity[35]. In addition, pronase treatment of high molecular weight glycoproteins is a useful method for obtaining glycoprotein fragmentation without significant loss of biological activity[36]. We demonstrated that pronase treatment of BT474 cells led to a proteolytic cleavage of HER2 with formation of 95 kDa and 150 kDa fragments without affecting cell viability. In turn, this proteolysis increased trastuzumab affinity, did not affect pertuzumab-binding capacity and reduced HER2-HER3 dimer formation in breast cancer cells. The principal explanation for the triple effect of pronase could be the unmasking of the trastuzumab antigenic epitope and the shedding of domain I that leads to the formation of the 150 kDa fragment. This HER2 fragment is likely to maintain the integrity of the pertuzumab-binding domains, while the blocking of the interaction of domain I with domain III may inhibit the open conformation of HER2 and prevent dimer formation[5, 37-39]. By WB we showed that the 150 kDa fragments is present in human breast cancer tissues as well.

The size of the antibody molecule (150 kDa for IgG) may be another source of interference, particularly if the epitopes are closely spaced. Thus, steric hindrance is likely to be intensified when the antibodies have been labelled with other molecules such as biotin. In our IHC tissue protocol,
the effect of trastuzumab biotinylation on the avidin-biotin reaction was controlled by using aminocaproate, which limits the steric hindrance by providing a six-carbon spacer to biotin and thus improves accessibility to the binding site on avidin.

In conclusion, we have shown that the presence of p95^HER2 increases the trastuzumab-binding capacity in cancer tissues. The shedding phenomenon induced *in vivo* by metalloprotease and *in vitro* by pronase could thus improve the binding of antibodies with specific HER2 epitopes and allow trastuzumab to more efficiently reach its epitope by reducing the steric hindrance of cancer cells that may carry millions of p185^HER2 molecules on their surface.
Acknowledgments

We thank Dr. Alessandra Stacchini for the support with flow cytometry experiments. This study was funded by Ricerca Sanitaria Finalizzata Regione Piemonte 2009, PRIN Programmi di Ricerca Scientifica di rilevante interesse nazionale (co-funding 2008), Rete Oncologica Piemonte e Valle d’Aosta 2010 and AIRC Grants IG 10787.

Statement of author contributions

DR performed all the experiments, participated in the coordination of the study, and helped draft the manuscript; LD performed the statistical analysis; CM participated in the case collection, in FISH analysis and in revision of the manuscript; LM performed the slides scanning and analyses of the virtual slides; IC and PC revised the immunohistochemical data; AR participated in the case collection and revision of the immunohistochemical data; FM participated in the writing of the manuscript; PS and NB performed surgery and provided surgical specimens; GV and MR contributed to case series and histopathological revision; AS conceived and designed the study, analyzed the data and wrote the final manuscript.
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**Table 1:** Correlation of \( \text{p185}^{\text{HER2}} \) and \( \text{p95}^{\text{HER2}} \) expression by western blot and score 3+ immunohistochemical results (>10% of HER2 positive cells with strong and complete membrane expression) with anti-HER2 antibodies in 50 breast cancers.

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<th>Western blot</th>
<th>Anti-intracellular domain</th>
<th>Anti-extracellular domain</th>
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<tr>
<td>p185++/p95+</td>
<td>( CB11 ) (75)</td>
<td>( A048 ) (58)</td>
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<tr>
<td>p185++/p95-</td>
<td>( 3/10 ) (30)</td>
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FIGURE LEGENDS

**Figure 1: Automated IHC analysis.** Micrographs illustrate an example of quadrant selection (A), the APERIO software result layout (B) and table of percentage of positivity (C).

**Figure 2: Western blot analysis performed on breast cancer tissues.** p185\(^{HER2++}/p95^{HER2-}\) cases (Lanes 1, 2, 4, 6); p185\(^{HER2++}/p95^{HER2+}\) cases (lanes 3, 7); p185\(^{HER2+}/p95^{HER2-}\) cases (lanes 5, 8); pervanadate treated BT474 cells (lane 9); a subgroup of cases with high expression of p185\(^{HER2}\) and p95\(^{HER2}\) showed a 150 kDa fragment (lanes 3, 7).

**Figure 3: Pronase antigen retrieval.** A breast carcinoma sample immunostained with BiotHER following routine IHC procedure (no antigen retrieval) (A). Pronase antigen retrieval increases both percentage and intensity of immunostaining (B).

**Figure 4: Automated analysis of HER2 extracellular domain expression by IHC.** Distribution of mean percentage of BiotHER expression within 6 different fields (i.e.: section quadrants Mq1-6) (A, B, C). Differences between p95\(^{HER2}\) positive (red) and negative (green) cases are statistically significant in 3+ score (A, D) and +2 score (B, E) categories, whereas no statistically significant difference is observed in 1+ scored cases (C, F).

**Figure 5: Induction of HER2 shedding on BT474 cells by pervanadate.** HER2 ECD levels in culture medium as evaluated by ELISA increase after 1mM pervanadate treatment (A), which in parallel induce p95\(^{HER2}\) as shown by western blot (B). No p95\(^{HER2}\) induction was observed using 0.1mM pervanadate (C).

**Figure 6: Effects of proteolytic treatments on cell cultures.** Control BT474 cells (A, E). Following 1mM pervanadate alone (B) or pervanadate and catalase treatment (C) BT474 cells detach from the dish and die (B). Cell viability is not compromised in BT474 cells treated with 1mM hydrogen peroxide alone (D) and in BT474 cells treated with 0.1% pronase (F).

**Figure 7: Pronase induced HER2 cleavage.** Treatment with 0.1% pronase (lane 1) and with 0.02% pronase (lane 2) in BT474 cells leads to p185\(^{HER2}\) cleavage and formation of 150 kDa and 95kDa bands. Control BT474 cells (lane 3).
Figure 8: Effects of proteolytic treatments on the expression of trastuzumab binding site in BT474 cells. BiotHER staining in: control BT474 cells (A), in vitro pronase treated cells (B) or pronase antigen retrieval of sections cut from FFPE cell blocks (C). A stronger and more diffuse staining is observed in BT474 cells treated with pronase (B) and in sections following pronase antigen retrieval (C).

Figure 9: Flow cytometry analysis on BT474 cells. Flow cytometry analysis on control BT474 cells shows two populations, one (67% of cells) with high trastuzumab affinity compared to the remaining 33% with lower affinity (A). After 10 minutes of pronase exposition, there is a shift to 94% of cells with high affinity (B). The binding capacity of pertuzumab is not affected by pronase treatment (C, D).

Figure 10: Pertuzumab binding site. Immunoprecipitated HER2 protein using pertuzumab or trastuzumab shows a band at 180 kDa in control (lanes 1,3) and at 150 kDa in pronase treated (lanes 2,4) BT474 cells, confirming the presence of the antibody binding site.

Figure 11: PLA analysis on HER2/HER3 dimer. Dimers are identified by the fluorescent signals (red spots): a higher number of dimers is observed in control BT474 cells (A), as compared with pronase treated BT474 cells (B).
Figure 1
Figure 2
Figure 3
Figure 5
Figure 6
Figure 9
Figure 10