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*Chapter X*

# Role of Δ16HER2 Splice Variant in Breast Tumor Progression and Response to HER2-Targeted Therapy

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## Abstract

HER2 overexpression is necessary but not sufficient to induce malignant transformation, as clearly shown in mice transgenically expressing the rat protooncogene HER2/neu but only developing tumors when accompanied by alterations such as deletions in the HER2 extracellular domain. Interestingly, an alternative splice form of the human homologous HER2 gene with an in-frame deletion in the same region mutated in neu protooncogene transgenic mice has been detected in human breast carcinomas. This splice variant encodes a receptor lacking exon 16, which immediately precedes the transmembrane domain containing two cysteine residues. Loss of these cysteine residues causes a conformational change in the HER2 receptor extracellular domain that favors intermolecular disulfide bonding and, consequently, homodimerization leading to cell transformation. Real-time PCR analysis revealed that the Δ16HER2 variant represents about 9% of the HER2 transcripts in human primary breast tumors. Athymic mice

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develop mammary tumors after injection of cells ectopically expressing the Δ16HER2 variant, which forms stable dimers and couples to multiple oncogenic signaling through activated Src kinase pathways. Thus, the Δ16HER2 isoform appears to be one, if not the only, oncogenic variant of the human HER2 proto-oncogene. Furthermore, Δ16HER2 compromises trastuzumab action, strongly suggesting that trastuzumab-non-responsive patients express high levels of stable Δ16HER2 dimers on their HER2-amplified/overexpressed tumors that to some extent impair drug binding and therapeutic benefits. Such a hypothesis is consistent with clinical studies showing that increased HER2 FISH ratios (>8) decrease susceptibility to trastuzumab.

# Introduction

HER2 (epidermal growth factor receptor 2; EGFR2), discovered in 1985 by two independent laboratories [1;2], is a 185-kD transmembrane receptor that, together with its relatives HER1 (EGFR), HER3 and HER4, belongs to the HER family of receptor tyrosine kinases. Each of these receptors consists of an extracellular binding domain, a transmembrane lipophilic segment, and (except for HER3) a functional intracellular tyrosine kinase domain. The tyrosine kinase domains are activated by both homodimerization and heterodimerization, generally induced by a specific ligand. Unlike the extracellular domains of the three other HER receptors, that of HER2 can adopt a fixed conformation resembling a ligand-activated state, permitting it to dimerize in the absence of a ligand [3]. Once activated, HER2 signaling promotes cell proliferation and survival. HER2 overexpression has been described in a variety of human malignancies, e.g., breast, ovarian, gastric and lung carcinomas [4];HER2 gene amplification is rare except in breast cancer, in which both amplification of the gene, detected by FISH, and HER2 overexpression, identified by the IHC-based Hercep Test, occur in approximately the same proportion of cases (20-30%) [5]. Most clinical studies to evaluate the relationship between HER2 overexpression and breast cancer outcome show that women with HER2-positive tumors have a poorer prognosis than those with HER2-negative tumors [6-8]. Gene expression analyses have shown that HER2 overexpression identifies a subset of tumors [9] [10] characterized by the lack of expression of genes associated with hormone receptor signaling pathways and high-level expression of a cluster of genes associated with proliferation [11].

Differential levels of HER2 expression in normal breast tissue versus HER2-overexpressing breast carcinomas, together with the demonstration of a key role for HER2 in tumor progression, make HER2 an ideal target for specific therapeutic approaches. The benefit of anti-HER2 therapies demonstrated in clinical trials indicates that HER2 is, to date, one of the most promising molecules for targeted therapy [12]. Because of the survival benefit, trastuzumab, a recombinant humanized monoclonal antibody directed to the extracellular domain of the HER2 protein, was approved in 1998 by the FDA for clinical treatment of women with HER2-positive metastatic breast cancer (MBC) and, in 2006, for adjuvant treatment of patients with early breast cancer. While results thus far demonstrate the clinical benefit of trastuzumab, the fact remains that this antibody administered according to currently approved protocols cures only about 50% of patients with HER2-positive early breast carcinoma and cannot cure those with HER2-positive MBC. Improved treatment of these particularly aggressive breast carcinomas awaits further understanding of the

mechanisms by which HER2 overexpression exerts its functional role in increasing tumor aggressiveness and in conditioning the response to conventional anti-tumor therapies.

## Relevance of the Delta16HER2 Splice Variant for HER2-Driven Transformation

The genetic alteration underlying HER2 overexpression, primarily due to gene amplification in human primary breast cancers [13], is necessary but not sufficient to induce transformation. The development of transgenic mouse models that recapitulate the initial events of HER2-induced mammary tumorigenesis has been key in furthering the understanding of the molecular basis for HER2 transforming activity. In fact, transgenic studies provided the first direct evidence that the mammary-specific expression of the rat HER2/neu gene induces tumors only when accompanied by in-frame activating deletions or insertions of cysteine residues within the wild-type (WT) HER2/neu extracellular domain [14]. In these transgenic mice, tumors arose only when the oncoprotein carried mutations, in particular, small deletions in the extracellular domain that promote the HER2/neu transforming activity of this protein through formation of intermolecular disulfide bonds [15]. Interestingly, an alternative splice form of the human homologous HER2 gene, containing an in-frame deletion in the same region that is mutated in neu protooncogene transgenic mice has been detected in human breast carcinomas [16]. This splice variant, Δ16HER2, encodes a receptor lacking exon 16 (based on Ensembl database-ENSE00001121079), which immediately precedes the transmembrane domain containing two cysteine residues (Figure 1)

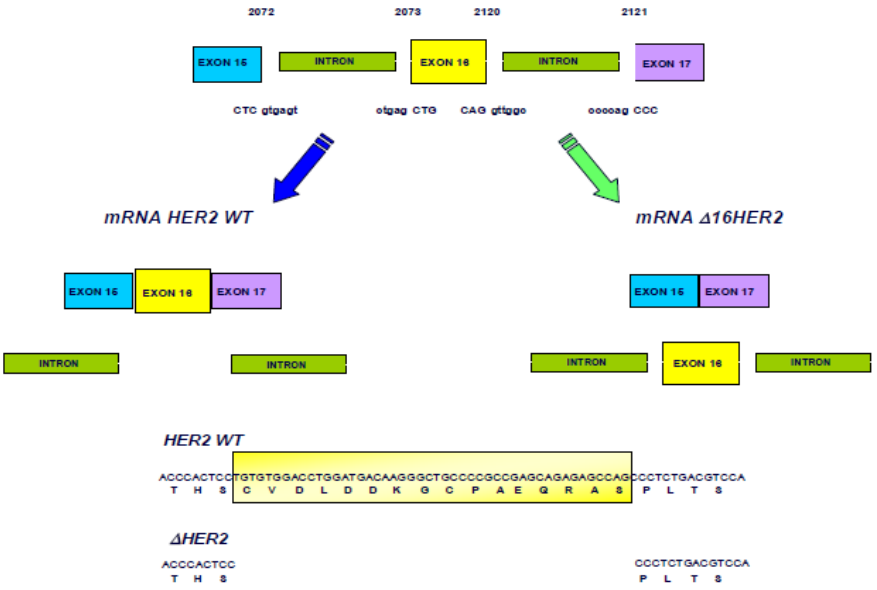



Figure 1. Alternative splice form of the human HER2 gene with an in-frame deletion of exon 16.

The loss of these cysteine residues appears to induce a conformational change in the HER2 receptor extracellular domain that promotes intermolecular disulfide bonding and, in

turn, constitutively active stable HER2 homodimers that activate mitogenic signaling from the transmembrane receptor. Several studies have reported the expression of an alternatively spliced human HER2 isoform encoding a protein with a 16-amino acid in-frame deletion in the cysteine-rich region of the HER2 extracellular domain (Δ16HER2) [15-17] as a proportion (4-9%) of the total HER2 transcript, raising the possibility of its role in HER2-driven transformation. In keeping, alternative splicing resulting in deletions within the extracellular domain of growth factor receptors such as Met [18], Ron [19] and fibroblast growth factor receptor 2 (FGFR2) [20;21] was shown to represent a unique mechanism for the generation of novel transcripts encoding constitutively activated molecules.

We recently demonstrated that the Δ16HER2 variant ectopically expressed in human embryonic kidney HEK293 and murine fibroblast NIH3T3 cells was constitutively active due to its ability to form disulfide-bridged homodimers. In that study, we also found that athymic mice injected s.c. with Δ16HER2/HEK293 transfectants developed tumors, whereas mice injected with HEK293 control cells ectopically overexpressing only WT HER2 did not [17], indicating that the Δ16HER2 variant is tumorigenic *"per se"*. Very recently, Mitra et al [22] observed *in vitro* that the Δ16HER2 variant ectopically expressed in MCF7 breast cancer cells forms stable dimers. Moreover, MCF7 cells transfected with WT HER2 or Δ16HER2 showed a slight but equivalent increase in EGFR expression and phosphorylation when compared with vector control cells, while HER3 was activated in the WT HER2- but not in the Δ16HER2-expressing cells. In addition, expression of HER4 was enhanced in the WT HER2 transfectants, whereas Δ16HER2-MCF7 cells lacked HER4 expression. Thus, it appears that unlikely additional EGFR family members significantly contribute to Δ16HER2 oncogenic activity. Ectopic Δ16HER2 expression led to increased activation of multiple oncogenic pathways, e.g., FAK, Src kinase, phosphatidylinositol 3-kinase/AKT, and mitogen-activated protein kinase, as compared to cells expressing WT HER2. In particular, Δ16HER2 was found to facilitate recruitment and activation of Src kinase through direct interaction at the cell membrane, as shown by coimmunoprecipitation assay demonstrating a physical interaction between Src kinase and Δ16HER2. Δ16HER2 also dramatically potentiated MCF7 cell migration and invasion. Consistent with these *in vitro* results, these authors showed that 89% of 27 patients with HER2-positive tumors coexpressing Δ16HER2 presented positive lymph nodes and also tended to have higher-grade estrogen receptor-negative tumors. The highly significant association of Δ16HER2 expression with positive lymph nodes ($P< 0.0001$) observed in this limited analysis strongly implicates Δ16HER2 as a clinically important and tumor-specific HER2 molecular alteration that promotes aggressive locally disseminated metastatic breast cancer.

## Δ16HER2 Expression and Response to HER2-Targeted Therapy

Not all patients with HER2-positive tumors respond to new HER2-targeted therapies, possibly reflecting inefficient targeting by the biodrug of the splice variant receptor, especially the disulfide-bonded HER2 dimers. In fact, we demonstrated lower reactivity of trastuzumab, which binds to the juxtamembrane region of HER2, with Δ16HER2-transfected HEK-293 cells compared to WT HER2/HEK-293 due to a lack of reactivity with disulfide-

bonded HER2 homodimers [17]. Since WT HER2 gene amplification in human primary breast cancers increases Δ16HER2 levels above a critical threshold, allowing the variant to contribute to breast cancer progression and resistance to trastuzumab, and that trastuzumab-non-responsive patients may be those whose HER2-amplified/overexpressing tumors express high levels of stable Δ16HER2, impairing drug binding and therapeutic benefits. Such hypotheses are consistent with clinical findings indicating that increased HER2 FISH ratios (>8) decrease susceptibility to trastuzumab[23]. Although Mitra and coworkers [22] observed equivalent levels of trastuzumab binding to both WT HER2 and Δ16HER2 at the cell surface of expressing MCF7 cells, the authors found no trastuzumab effect on either cell proliferation or invasion of Δ16HER2-expressing MCF-7 and NIH3T3 cells. The demonstration that direct coupling of Δ16HER2 to Src kinase mediated the potentiated metastatic and oncogenic properties of Δ16HER2 and that cotargeting of Δ16HER2 and Src kinase with the single-agent tyrosine kinase inhibitor dasatinib resulted in Src inactivation, destabilization of Δ16HER2, and suppressed tumorigenicity suggest that Src kinase inhibition might overcome Δ16HER2 refractoriness to trastuzumab.

In addition, in Δ16HER2 transiently transfected HEK-293 cells, we found that treatment with the tyrosine kinase inhibitor emodin, which affects the HER2 catalytic domain [24], completely abolished both HER2 monomeric and disulfide-bond HER2 phosphorylation [17]. Thus, the constitutive activation of disulfide-bond HER2 homodimers is sensitive to this tyrosine kinase inhibitor. These findings suggest the potential usefulness of tyrosine kinase inhibitors such as dasatinib and/or the HER2 tyrosine kinase inhibitor lapatinib, which is approved for clinical treatment of HER2-positive breast carcinoma patients, in combination with trastuzumab to improve the treatment of HER2-positive patients, especially those with tumors presenting elevated HER2 gene copies.

## Δ16HER2 Homodimers and Tumor Microenvironment

It is well-established that an intrinsic feature of the tumor phenotype compared to normal physiological conditions is a lower extracellular pH (pHe) caused by increased export of metabolic acids derived from a high glycolysis rate or by alterations in clearance of extracellular acid [25]. Furthermore, hypoxia, a pathological condition frequently associated with human solid tumors, induces additional upregulation of glycolysis, which can contribute in modulating the tumor microenvironment [26]. During pathological conditions such as cancer, the extracellular redox state may be altered to cause altered functions or activity of specific proteins such as proteases, soluble factors or extracellular matrix proteins. Recent studies strongly support an important relationship between unbalanced extracellular redox state, cancer cell aggressiveness and response to cancer therapy [27;28]. We previously found *in vitro* that fostering an extracellular redox state using increasing doses of the anti-oxidant agent 2-mercaptoethanol (ME) to break disulfide bridges restores trastuzumab binding to Δ16HER2 (Figure 2)[17].

Thus, a tumor acidic microenvironment representing a metabolic state that determines higher gradients between intra- and extracellular pH (ΔpH) might influence the formation of stable Δ16HER2 homodimers, thus affecting the tumorigenic potential of Δ16HER2-

overexpressing tumors and specific anti-HER2 drug susceptibility. Indeed, lower pHe has been associated with tumorigenic transformation, chromosomal rearrangements, and altered gene expression, and pre-clinical and clinical studies now consistently identify the reversed pH of the tumor microenvironment as an important cause of drug resistance in solid tumors [26;29]. In this context, it becomes important to determine whether a correlation exists among elevated levels of Δ16HER2 homodimers, acidic pHe signature, HER2 FISH scores, sensitivity to trastuzumab, and prognosis .Our preliminary genomic analysis of 44 HER2-overexpressing primary breast cancers (IHC 3+ or 2+/FISH-positive) identified 3 subgroups (HER2/A, HER2/B and HER2/C) based on their gene expression profiles, each characterized by the enrichment of the gene sets listed in Figure 3.

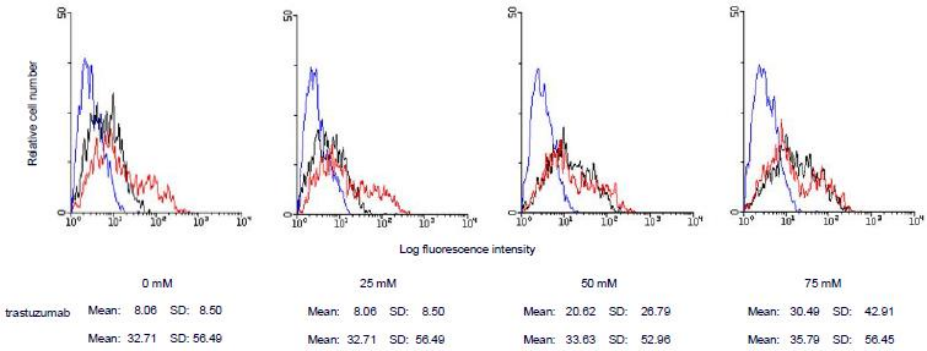
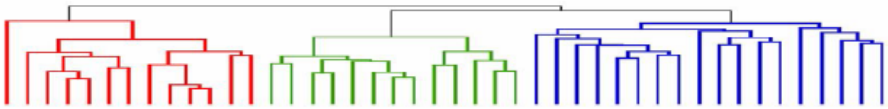



Figure 2. Indirect immunofluorescence reactivity of trastuzumab (black line) or an anti-HER2 monoclonal antibody that does not cross-react with Trastuzumab (MGR2) (red line) on Δ16HER2 expressing HEK-293 cells cultured in different concentration of 2-mercaptoethanol (ME), evaluated by flow cytometry.



| HER2/A | HER2/B | HER2/C |
|---|---|---|
| NITROGEN METABOLISM<br><br>G PROTEIN-COUPLED RECEPTOR | **Estrogen pathway**<br>ESTROGEN RECEPTOR SIGNALING PATHWAY<br><br>**Lymphoid infiltration**<br>ANTIGEN PROCESSING AND PRESENTATION<br>INFLAMMATORY CYTOKINES & RECEPTORS<br>T-CELL AND B-CELL ACTIVATION<br>TH1-TH2-TH3<br><br>**Stress response**<br>HYPOXIA SIGNALING PATHWAY<br>OXIDATIVE PHOSPHORYLATION<br>STRESS RESPONSE TO CELLULAR DAMAGE | **Proliferation**<br>CELL CYCLE<br>MAP KINASE SIGNALING PATHWAY<br>SIGNAL TRANSDUCTION PATHWAY<br>VV_70GENE_SIGNATURE<br>EGF_PDGF SIGNALING PATHWAY<br><br>**Lymphoid infiltration**<br>NATURAL KILLER CELL MEDIATED CYTOTOXICITY<br>INFLAMMATORY CYTOKINES & RECEPTORS<br>ANTIGEN PROCESSING AND PRESENTATION<br><br>**Adhesion**<br>YANG_H ET AL_CAFFEINE SIGNATURE<br>ECM3_BREAST CANCER SIGNATURE<br>TGF-BETA SIGNALING PATHWAY |

Figure 3. HER2 subgroups identified by unsupervised hierarchical clustering of 44 human HER2-overexpressing breast cancer cases and gene sets enriched in each subgroup by GSEA analysis using software v2.01 (32).

Genes related to the hypoxia signaling pathway, oxidative phosphorylation, and stress response to cellular damage were mainly found in subset HER2/B. Moreover, subset HER2/B overexpressed carbonic anhydrase genes, reported to be key pH regulators [30] and functionally linked to the regulation of the reversed tumor pH [31], as compared to the HER2/A and HER2/C subsets. These findings raise the possibility that an acidic pHe affects Δ16HER2 homodimerization in a subset of HER2-overexpressing breast tumors and, in turn, tumor aggressiveness and trastuzumab activity. Future analyses will include evaluation of Δ16HER2 homodimer levels in tumor extracts from profiled HER2-overexpressing breast cancer cases in correlation with disease outcome, as well as analysis of Δ16HER2 homodimers in additional HER2-overexpressing patients now receiving adjuvant trastuzumab treatment to help define the involvement of this HER2 spliced form in response to trastuzumab.

## Conclusion

The constitutive splice variant of human HER2 lacking exon 16 (Δ16HER2) is tumorigenic *"per se"*; however, not all molecules encoded by alternatively spliced HER2 mRNA are dimerized in transfected cells, suggesting that disulfide bond stabilization also depends on the redox conditions of the tumor microenvironment. Thus, the proliferation signal derived from dimerized HER2 receptors may change as a function of cellular metabolism as well as acidic pHe around tumor cells. Importantly, increasing evidence points to the role of Δ16HER2 in conditioning the response to trastuzumab. Thus, clinical data showing that not all patients with HER2-positive tumors respond to trastuzumab therapy might reflect, in part, the inefficient targeting of the splice variant receptor, particularly, the disulfide-bonded HER2dimers, by this drug. The finding that cultures of Δ16HER2 transfectants in the presence of a reducing agent increased the number of trastuzumab-positive cells points to the need for a better understanding of the relevance of redox conditions in the tumor microenvironment to activation of growth factor receptors such as Δ16HER2 and, consequently, to trastuzumab antitumor activity. On the other hand, activation of Δ16HER2 homodimers was completely blocked *in vitro* by a tyrosine kinase inhibitor of HER2, raising the possibility that a combination of trastuzumab and the clinically approved tyrosine kinase inhibitor lapatinib might improve the treatment of HER2-positive breast carcinomas, especially those with high levels of Δ16HER2 (HER2 FISH ratios >8) and presenting a peculiar microenvironment that facilitates formation of Δ16HER2 homodimers.

Overall, elucidation of the biological involvement of this oncogenic variant in human breast cancer progression and in specific anti-HER2 drug susceptibility/resistance awaits extensive further studies, including those in transgenic mouse models expressing human Δ16HER2 in the mammary glands and currently being developed.

## Conflict of Interest

The authors have declared that no conflict of interest exists.

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