Deep Insight Section

delta16HER2 splice variant and its role in HER2-overexpressing breast cancer

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Abstract

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HER2-positive overexpressing breast cancer and targeted therapy

HER2 is a tyrosine kinase receptor (RTK) belonging to the family of epidermal growth factor receptors which includes HER1 (EGFR), HER3 and HER4.
Members of this family are characterized by an extracellular binding domain (ECD), a transmembrane fragment and an intracellular tyrosine kinase domain (ICD) which is integral to downstream signaling, except in the case of HER3 (Yarden and Pines, 2012).
These RTKs are activated by both homo- and heterodimerization mainly induced by the binding of a specific ligand. The HER2ECD, unlike that of the three other HERs, can assume a fixed conformation resembling a ligand-activated state and permitting its dimerization in the absence of ligand (Cho et al., 2003; Yarden and Pines, 2012). HER2 is the preferred partner of dimerization of the other HER members and, together with HER3, enhances the most powerful mitogenic and survival signaling cascade through the activation of PI3K-Akt and MAPK downstream pathways (Yarden and Pines, 2012).
HER2 amplification and/or overexpression occurs in 20-25% of human breast cancers (BCs) and identifies a particularly aggressive BC subtype with enhanced proliferation, metastatic potential and subsequent decreased survival (Arteaga et al., 2011; Prat and Perou, 2011; Cancer Genome Atlas Network, 2012; Hurvitz et al., 2013). Based on HER2 overexpression in malignant breast tumor cells as compared to normal tissues, novel rationally designed targeted agents, such as trastuzumab (T), lapatinib and pertuzumab, have been introduced in the clinical management of BC patients (De et al., 2013; Krop, 2013). In particular, the humanized monoclonal antibody (MAb) T binds to the juxtamembrane region (subdomain IV) of HER2ECD and has been approved in combination with chemotherapy as the standard of care for HER2-positive metastatic BC, in early disease and also, very recently, in the neo-adjuvant setting (Arteaga et al., 2011; De et al., 2013). Although T administration induces significant clinical benefits in a consistent number of patients, tumors progress in a high percentage of cases after one year of treatment and develop *de novo* or acquired resistance (Rexer and Arteaga, 2012; Wong and Lee, 2012). Potential resistance mechanisms, including activation of compensatory signal transduction pathways (HER2 cross-talk with other RTKs and amplification of HER signaling and/or alterations of the PI3K/AKT pathway) or defects in apoptosis and cell cycle control, have been demonstrated in *in vitro* models (Rexer and Arteaga, 2012) but await clinical validation.

Generation mechanism	HER2 isoforms	References
Alternative splicing	delta16HER2 p100 herstatin	Jackson et al., 2013
Alternative initiation of translation	611-CTF (p110 or p95HER2) 687-CTF (p95cyto)	Ward et al., 2013
Proteolytic cleavage	648-CTF (p95m) HER2ECD (p110) H2NTF	Ghedini et al., 2010; Morancho et al., 2013; Ward et al., 2013

Table 1. HER2 isoforms.

Interestingly, recent preclinical studies suggest that HER2-overexpressing tumors able to evade T activity continue to exert HER2 oncogenic action as they retain HER2 amplification and overexpression (Hurvitz et al., 2013; Krop and Burstein, 2013). A significant contribution to T resistance could also derive from genetic or molecular events that lead to an increase in HER2 oncogenic potential and/or influence T binding to the receptor (Rexer and Arteaga, 2012). Indeed, different isoforms of the HER2 receptor have been identified in human BC deriving from alternative initiation of translation or splicing and proteolytic shedding (Table 1) and are not recognized by the humanized antibody (Arribas et al., 2011; Garrett and Arteaga, 2011; Rexer and Arteaga, 2012).
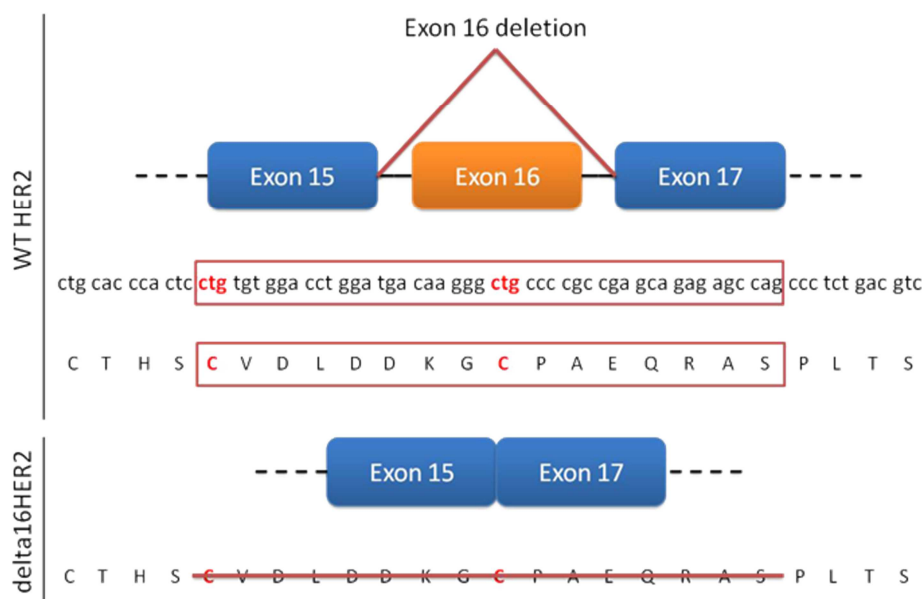
delta16HER2 splice variant

It is widely accepted that wild-type (WT) full-length HER2 gene amplification is necessary, but not sufficient, to induce transformation (Slamon et al., 1989). Indeed, transgenic expression of the mammary-specific rat proto-oncogene HER2/neu generates tumors only when the protein shows alterations in the ECD, such as in-frame deletions or insertions of cysteine residues inducing conformational changes in the juxtamembrane region of WT HER2/neu receptor (Siegel et al., 1994; Ursini-Siegel et al., 2007). The consequent cysteine residues imbalance plays a primary role in the regulation of HER2 catalytic activity because it induces receptor activation, promoting constitutive homodimerization through stable intermolecular disulfide bonds formation (Kwong and Hung, 1998; Chan et al., 1999; Siegel et al., 1999; Ursini-Siegel et al., 2007). In this context, different studies reported the constitutive expression of a human HER2 alternative splice isoform which carries an in-frame deletion in the same mutated region of the rat HER2/neu proto-oncogene (Kwong and Hung, 1998; Siegel et al., 1999). This splice variant produces an aberrant receptor that lacks exon 16 (delta16HER2) (Figure 1) and, in turn, 16 amino acids (634-649) in the HER2ECD (domain IV), including two relevant cysteine residues close to the T-binding epitope. The deletions result in stable constitutively active homodimer formation, enhanced multi-signaling activity and accelerated transformation (Kwong and Hung, 1998; Castiglioni et al., 2006; Mitra et al., 2009; Marchini et al., 2011; Alajati et al., 2013).

- delta16HER2 splice variant and HER2-driven transformation

In our previous analysis of 46 human HER2-positive BCs, we found that delta16HER2 was constitutively co-expressed with WT HER2 in ~90% of the BCs and its transcript represented 4-9% of total WT HER2 mRNA (Castiglioni et al., 2006), suggesting a possible role for this variant in WT HER2-driven tumorigenesis. Analysis of an additional 57 human primary BCs (Figure 2) showed that increasing WT HER2 transcript levels were correlated with a proportional enhancement of delta16HER2, thereby allowing it to contribute to BC progression. We also showed that delta16HER2-positive NIH3T3 and HEK293 transfectants, but not their counterparts ectopically expressing WT HER2, constitutively expressed active disulfide-bridged homodimers and that only mice xenotransplanted with delta16-transfectants developed tumors (Castiglioni et al., 2006). Thus, WT HER2 requires additional genetic alterations to induce neoplastic transformation. More recently, Mitra et al. (2009) showed that delta16HER2-overexpressing MCF7 human BC transfectants expressed stable dimers and were more invasive than WT HER2-transfected MCF7 cells.

In addition, exogenous expression of delta16HER2 in comparison to WT HER2 resulted in an increased activation of multiple downstream signaling pathways involving FAK, PI3K/Akt, MAPK and Src kinase.

Figure 1. Schematic representation of the alternative delta16HER2 splice form of the human HER2 gene with an in-frame deletion of exon 16.

Interestingly, those studies also revealed a direct correlation between node status and delta16HER2 mRNA expression (Mitra et al., 2009), implicating delta16HER2 in human BC progression. Further confirmation of delta16HER2 intrinsic oncogenicity came from our recent generation a mouse line transgenically expressing the human delta16HER2 variant using a bicistronic vector encoding both delta16HER2 and luciferase reporter genes under the MMTV promoter (Marchini et al., 2011). Our transgenic line was characterized by a higher tumor incidence as well as a shorter latency (about 8 weeks of age) compared to the MMTVhuHER2 transgenic model, which developed human WT HER2-overexpressing spontaneous mammary tumors after 28 weeks of age (Finkle et al., 2004; Marchini et al., 2011). The delta16HER2 variant was expressed on mammary tumor cells as cell surface-associated phosphorylated stable homodimers and monomers, and its downstream signals were significantly coupled to an activated Src kinase. In this transgenic model, about 5 copies of the delta16HER2 transgene were sufficient to drive early transformation in contrast to 30-50 copies of WT HER2 transgene needed to develop mammary tumors in the MMTVhuHER2 model (Finkle et al., 2004; Marchini et al., 2011). Very recently, Alajati et al. (2013) provided further *in vitro* evidence that delta16HER2 is able to increase invasion and proliferation and also to decrease apoptosis either in two- or three-dimensional conditions. Consistent with our previous findings (Castiglioni et al., 2006), orthotopically injected delta16HER2-transfected MCF10A and MCF7 cells

were shown to be more tumorigenic than WT HER2-transfected cells (Alajati et al., 2013). The same authors applied proteomic and genomic platforms finding a panel of phosphorylated signaling transducers and developed a delta16HER2-gene signature which was associated with ER-negative, high grade and metastatic BC patients. However, because the delta16HER2 signature was analyzed within a dataset including all BC subtypes, its usefulness in the clinical management of HER2 BC patients remains unclear.
- delta16HER2 susceptibility to anti-tumor therapies
We reported that engineered delta16HER2-HEK293 cells expressing constitutively active cell surface disulfide-bound homodimers bound T with low efficiency compared to other anti-HER2ECD MAbs (Castiglioni et al., 2006), suggesting potential delta16HER2-mediated interference with T clinical benefits (Sasso et al., 2011; Pupa et al., 2011). Moreover, Mitra et al. (2009) showed that delta16HER2-positive cells were resistant to *in vitro* T treatment compared to WT HER2-positive cells and, in a small cohort of HER2-overexpressing human BC cases, also observed that the aggressive features driven by delta16HER2 were mediated by activated Src, which is considered a common node of T-resistance mechanisms (Zhang et al., 2011; De et al., 2013). Indeed, *in vitro* treatment of delta16HER2-engineered tumor cells with dasatinib, a multi-BCR/Abl and Src family tyrosine kinase inhibitor, led to deactivation of delta16HER2 and its tumorigenic capacity (Mitra et al., 2009).
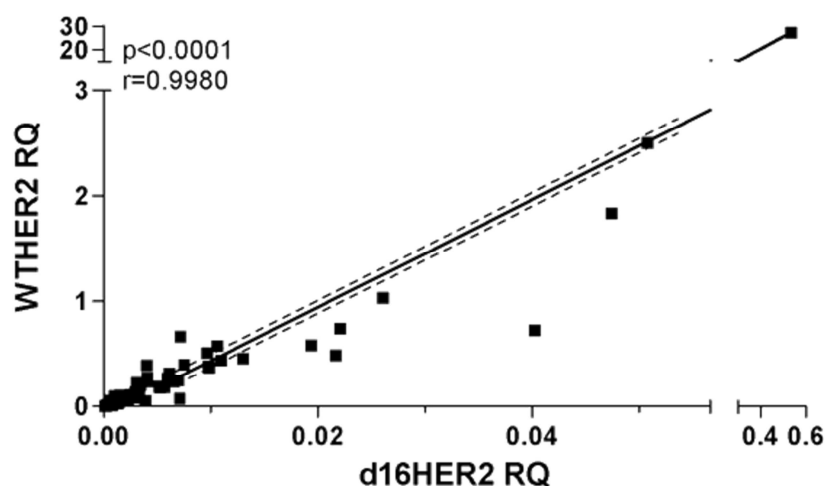
Figure 2. Correlation between quantitative real time-PCR threshold cycles obtained from cDNA amplification of delta16HER2 and WT HER2 genes in 57 human BC samples was analyzed by linear regression and Pearson correlation coefficient r.

Indeed, our studies to test the *in vitro* therapeutic effects of dasatinib on constitutively expressing delta16HER2-positive mammary tumor cells with highly activated Src kinase expression showed that delta16HER2 cells were significantly more sensitive to this agent than were WT HER2 mammary tumor cells (manuscript in preparation). We previously found that delta16HER2 monomers and homodimers were dephosphorylated after *in vitro* treatment with another tyrosine kinase inhibitor, emodin, which interacts with several molecular targets, including NF-κB, HIF-1α, AKT/mTOR, STAT3, CXCR4, topoisomerase II, p53, p21 and also HER2 (Castiglioni et al., 2006; Shrimali et al., 2013). Together, these results suggest the potential efficacy of including tyrosine kinase inhibitors in therapeutic regimens directed against delta16HER2.

On the contrary, in a pilot *in vivo* experiment with a xenograft model, Alajati et al. (2013) observed that delta16HER2-transfectants orthotopically injected in mammary glands of immunodeficient mice were able to metastasize to lungs and were found to be sensitive to T treatment.

Notably, delta16HER2 expression has also been reported in 30% of estrogen receptor (ER)-positive BC patients who are most commonly treated with the selective ER modulator tamoxifen (Cittelly et al., 2010a). Indeed, half of HER2-expressing tumors are also ER-positive and the majority of these encounter tamoxifen resistance and estrogen-independent growth (Sasso et al., 2011). Cittelly et al. (Cittelly et al., 2010a; Cittelly et al., 2010b) showed that in HER2- and ER-positive BCs, delta16HER2 promotes tamoxifen resistance in both *in vitro* and *in vivo* pre-clinical models. Specifically, they found that delta16HER2-transfected cells treated with tamoxifen upregulated Bcl-2 by downregulating miR-15 and 16, which

specifically target and repress Bcl-2, and that tumor growth of delta16HER2-xenografts was resistant to endocrine therapy and independent of estrogen (Cittelly et al., 2010a). In delta16HER2-transfected cells, they also observed the decrease in miR-342, which was also downregulated in primary BCs refractory to tamoxifen treatment (Cittelly et al., 2010b).

- delta16HER2 and the tumor microenvironment

The recent wealth of literature showing that not only deregulated cancer cell proliferation but also alterations in the tumor microenvironment can influence solid tumor growth has raised the possibility for new anti-cancer strategies including interfering with transformed cell proliferative processes and blocking of tumor cell-host microenvironment interactions (Eccles, 2010). Indeed, the existence of a significant relationship among unbalanced extracellular redox state, cancer cell aggressiveness and response to cancer therapy has been described (Pelicano et al., 2004; Trachootham et al., 2009), raising the potential efficacy of a novel anti-tumor therapeutic strategy based on the targeting of the acidic extracellular pH (pHe) (Neri and Supuran, 2011), since tumor pHe drives cell proliferation, promotes metastatic potential and supports chemoresistance (De Milito et al., 2010). In particular, proton pump inhibitors (PPIs), which are currently used in the anti-acid treatment of peptic disease, might inhibit acidification of the tumor microenvironment while increasing tumor cell sensitivity to cytotoxic agents in different oncotypes (Luciani et al., 2004; Spugnini et al., 2010). Targeting microenvironment conditions, we previously showed that delta16HER2-HEK293 cells treated with increasing doses of the anti-oxidant β2-mercaptoethanol restored T binding to the HER2 receptor (Castiglioni et al., 2006; Pupa et al., 2011).

Moreover, our preliminary data (manuscript in preparation) show that the treatment of constitutively expressing delta16HER2-positive mammary tumor cells with PPIs modulates delta16HER2 homodimer expression and activation levels. Together, the findings point to the promise of improved management of HER2-positive BC patients through novel therapeutic strategies that include both PPI and agents, such as phenylethylisothiocyanate, PEITC and a glutathione disulfide mimetic, NOV-002, which are under investigation in our delta16HER2-positive *in vitro* and *in vivo* models, able to interfere with exacerbated extracellular microenvironment conditions.

Conclusions

Expression of the constitutive delta16HER2 splice variant represents an important genetic event guiding HER2-driven neoplastic transformation. Indeed, tumor-related alternative splicing could be indicative of splicing patterns specific to malignant breast tissue and could induce oncogenic transformation or favor cancer cell survival through resistance to therapy (Jackson et al., 2013). *In vitro* data suggest that delta16HER2 represents one of the potential mechanisms of T resistance.

While it is tempting to speculate that delta16HER2-expressing BC patients will benefit from aggressive therapeutic regimens (Jackson et al., 2013), current clinical diagnostic techniques to identify patients with HER2 overexpression or amplification fail to distinguish between WT HER2 and delta16HER2 and all the other HER2 isoforms (Ward et al., 2013).

Moreover, screening assays and reagents to evaluate delta16HER2 expression levels in human BCs (Sasso et al., 2011; Pupa et al., 2011) in order to predict treatment response (Jackson et al., 2013) and reach the final goal of personalized cancer therapy remain to be developed. The availability of transgenic mouse models will enable further studies to elucidate the pathobiological roles of the constitutively activated HER2 splice variant both in mammary tumorigenesis and in the susceptibility to anti-HER2 targeted therapies and also as a novel target for the improved management of HER2-positive disease.

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