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Original Article

A critical role for HER3 in *HER2*-amplified and non-amplified breast cancers: function of a kinase-dead RTK

Nandini Dey[1,2], Casey Williams[1,2], Brain Leyland-Jones[1,2], Pradip De[1,2]

[1]Department of Molecular and Experimental Medicine, Avera Cancer Institute, Sioux Falls, SD; [2]Department of Internal Medicine, SSOM, University of South Dakota, SD

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Abstract: *ERBB3*/HER3 is the most intriguing RTK by virtue of its ability to transduce multiple cytosolic signals for the proliferation and growth of tumor cells in spite of being a "kinase dead" receptor that binds to its true ligand, heregulin. Although other members of the HER3 family like EGFR and HER2 have long been recognized to be associated with breast tumorigenesis and studied because of their predictive and prognostic value, the significance of HER3 as an irrefutable component of HER family signalosome is a relatively new development. The recent understanding of signals originating from the oncogenic partnership of HER3 with HER2 in the context of *HER2* amplification/overexpression showed the critical clinical value for the treatment of HER2+BC. The downstream signaling cascade (included but not limited to the PI3K signaling) associated with signals originating from HER2:HER3 dimers play a vital role in the tumorigenesis, drug-resistance and tumor progression of HER2+BC. *The upregulation of HER3 activity provides an alternate "escape route" via which tumor cells bypass either the inhibition of the HER family RTKs or the inhibition of the downstream PI3K-AKT-mTOR signaling pathway.* By understanding the signaling that provides this "escape route" for these tumor cells treated with a targeted therapy (HER2 inhibitors or inhibitors of downstream PI3K-AKT-mTOR signaling pathway), we are just beginning to appreciate the prognostic value of HER3 in breast cancer. In this review, we will discuss the relevance of HER3 signaling in the context of, (1) downstream oncogenic signals and (2) therapeutic options in *HER2* amplified BC.

Keywords: Breast cancer, PI3K, HER2, HER3, neuregulin

Introduction

Breast tumorigenesis is a multistep process proceeding first through hyperplasia, to non-invasive ductal carcinoma in situ (DCIS), invasive adenocarcinoma, and finally to the formation of distant organ metastases [1]. Transmembrane receptor tyrosine kinases (RTKs) bind to growth factors transmit signals essential for the growth and differentiation of tumor cells. Aberrant regulation of HER family RTKs is common in BC. Three members of this family of 4 related members, HER1, HER2, and HER4 exhibit dimerization-induced tyrosine phosphorylation and catalytic activation, with the exception of HER3 which is devoid of catalytic activity. Numerous (~13) soluble ligands of the HER family receptors have been identified having specific binding activities to one or more HER receptors [2].

HER3 is expressed in HER2+ tumors in mice and humans [3-5]. DCIS lesions frequently overexpress HER2 [6, 7] but the expression of HER3 in DCIS has not been reported to date. Recently, Vaught and group elegantly demonstrated the importance of HER3 in all stages of HER2-mediated breast transformation by using genetically engineered mouse models to impair HER3 expression within HER2+ mammary epithelial cells. Their studies revealed that the formation of HER2-mediated tumor progression and distant organ metastatic lesions were substantially reduced upon HER3 ablation [1]. It has been known that the central nervous system remains a sanctuary for HER2+ breast cancer [8] and a higher rate of "brain only" metastatic behavior is observed in co-overexpressed HER2/HER3 breast cancer population ($p = 0.042$) [5]. This is of critical therapeutic importance, as the therapeutic antibody trastuzumab, an approved ther-

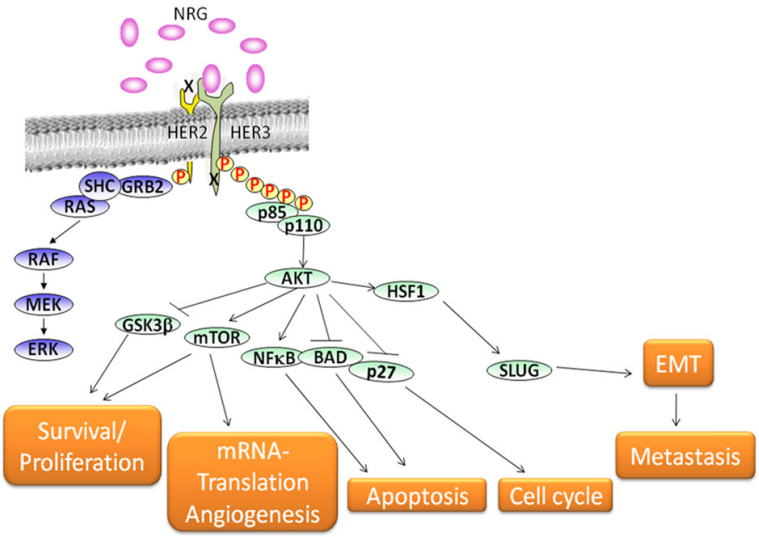

Figure 1. A schematic of HER3 engagedment in heterodimerization and signaling. General features of HER2:HER3 signaling. HER2 fails to bind any known HER-family ligands and HER3 has impaired catalytic activity. Following ligand engagement, HER3 engages and allosterically activates its kinase partner, in this case HER2. Phosphorylation of its C-terminal tail leads to recruitment of adapter proteins leading to activation of PI3K and RAS pathways. Activation of PI3K leads to phosphorylation of membrane phosphoinositides producing PIP3, which in turn docks the PH domain-containing proteins PDK1 and AKT. Membrane-bound AKT is phosphorylated and activated by PDK1 and mTOR. Activated AKT proceeds to phosphorylate a plethora of cellular substrates involved in diverse biological processes. HER3 ligand neuregulin (NRG) initiates HER2-HER3 dimerization which also leads to activate epithelial-mesenchymal transition (EMT) via phosphorylation of AKT-HSF1 (heat shock factor1)-SLUG (known EMT-regulator) signaling pathway (please see the text for details).

apy for HER2+ breast cancer, cannot block ligand-induced HER2:HER3 heterodimers as a single agent, suggesting it cannot effectively inhibit HER2 signaling [9]. As a result, the inhibition of HER2:HER3 heterodimerization may improve clinical outcome in co-overexpressed HER2/HER3+ subgroup.

It is not completely clear yet how HER3 contributes to various events in the HER2-mediated transformation of the breast carcinoma, although HER3 is expressed in HER2+ tumors in mice and humans [3-5]. One of the best known events by which the HER3 imparts the signal downstream is via the formation of HER2:HER3 heterodimers. Following ligand binding, receptors form homo- or heterodimers, leading to trans-phosphorylation and activation of downstream signaling pathways. HER2:HERB3 heterodimers are powerful oncogenic units, in part, because phospho-HER3 augments signaling through the PI3K-AKT-mTOR pathway [1, 10-13] (**Figure 1**). The p85 regulatory subunit of PI3K interacts directly with phospho-HER3 at 6 consensus PY p85- binding motifs (YXXM) within HER3 [14]. In contrast, HER2 cannot directly engage p85. It has been established that spontaneous formation of HER2:HER3 heterodimer can occur in tumors where HER2 expression on the cell surface is dramatically increased as a consequence of gene amplification [15]. Therefore, it is contemplated that HER3 functions primarily to drive HER2-mediated PI3K signaling [16, 17] which is important for tumor cells survival, proliferation, invasion, migration, angiogenesis and cellular metabolism.

Importance of HER3 ligands in HER2+ BC

In HER2-overexpressing cells following ligand receptor interaction, the signal is transduced via the PI3K-AKT-mTOR pathway (**Figure 2**). Several laboratory-based results implicate that upregulation of HER3 expression by inhibition of the RTKs (receptor tyrosine kinases)-PI3K-AKT pathway is prerequisite for neuregulin (NRG) ligand-mediated activation of HER3 that might be important in the context of tumor microenvironment and autocrine/paracrine resistance to receptor tyrosine kinase inhibitors. Breast cancer cells often express high levels of HER family receptor-activating ligands, and this plays an important role in developing anti-HER2 therapy resistance including trastuzumab [16, 18-20]. The overexpression of heregulin/neuregulin has also been shown to be a driver of breast cancer progression. It is known that growth factor ligands EGF, betacellulin, and heregulin reduce the growth inhibitory effect of trastuzumab in a preclinical model by 57-90 % [21]. Autocrine or paracrine-derived HER3 ligand neuregulin β1 (heregulin β1) can trigger the formation of HER2:HER3 heterodimers, which are blocked by pertuzumab, but not by trastuzumab [22]. In the December 15th, 2013 issue of Clinical Cancer

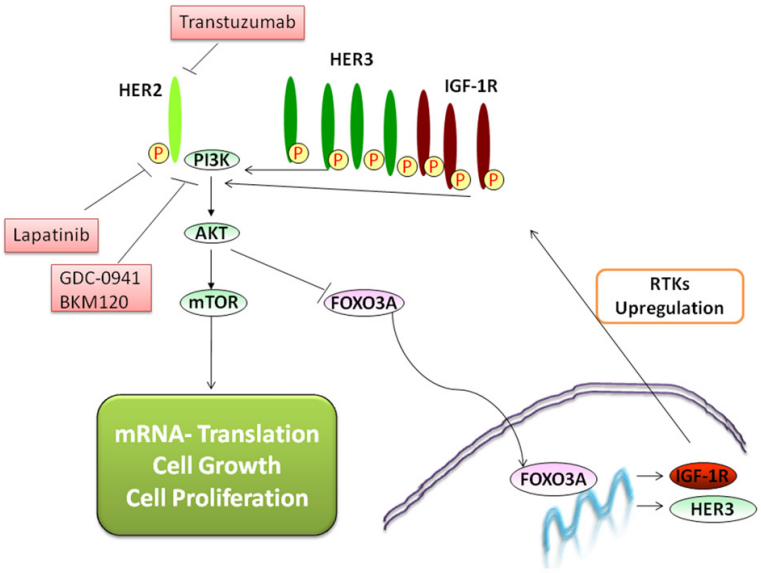

Figure 2. HER2 and PI3K-mediated therapies result in FOXO3A-associated feedback upregulation of RTKs. In the presence of PI3K inhibition through upstream receptor tyrosine kinase (RTK) inhibition or small molecule PI3K inhibitor (like pan-PI3K inhibitor, GDC-0941 or BKM120), inhibited AKT phosphorylation, allows FOXO3A (forkhead box O3A) to translocate to the nucleus and effect transcription of FOXO3A target genes, e.g. *HER3* and *IGF-1R*. The increased RTKs expression-associates resistance to PI3K inhibition by enhancing input into the PI3K pathway and alternate cell survival pathway, e.g. MAPK pathway.

Research, Lewis Phillips et al described (the first reported article) the mechanism of therapeutic resistance to T-DM1. In this article, the authors demonstrated that the HER3 ligand neuregulin β1 can abrogate the antitumor efficacy of T-DM1. Most importantly, neuregulin β1-mediated T-DM1 resistance was reversed when cells were treated with T-DM1 in combination with pertuzumab [23]. Although, it is not very surprising since the backbone of the T-DM1 is trastuzumab and one of the causes of trastuzumab resistance is the high level expression of HER-family receptor activating ligands. Lewis Phillips and group's study also ruled out the ADDC property of pertuzumab for enhanced anti-tumor efficacy of the combination therapy (T-DM1 plus pertuzumab). Using an engineered pertuzumab molecule that lacks ADCC property, authors elegantly demonstrate that the synergistic activity of adding pertuzumab to T-DM1 was not due to enhanced ADCC. This suggests that pertuzumab blocks the most important cell survival/proliferating HER2-HER3-PI3K-AKT-mTOR signaling pathway that may cause T-DM1 resistance, especially in the presence of HER-family receptor ligand. They also showed that HER2 non-overexpressing breast cancer cells (MDA-MB-175VII), which produce autocrine heregulin, were resistant to T-DM1. Interestingly, these cells were sensitive to the combination of T-DM1 plus pertuzumab [23]. Recently, Baselga and group also demonstrated that growth factor receptor ligands including neuregulin1 were upregulated following the treatment of p110α-specific inhibitor (BYL719). More interestingly, in the above mentioned condition, BYL719 failed to inhibit tumor cell growth *in vitro* [24]. Recently, Lin and group demonstrated that heregulin promoted anchorage-independent breast cancer cell growth more potently than EGF and that the heregulin-dependent activation of the PI3K-mTOR pathway is a necessary event for cell transformation [25]. Heregulin/neuregulin1 can confer resistance to lapatinib mediated growth inhibition in *HER2* amplified breast and gastric cancer cells through HER3 and its downstream AKT activation. Additionally, downregulation of HER3 with SiRNA in the presence of heregulin re-sensitized *HER2* amplified cancer cells to lapatinib [19, 26]. Yonesaka et al reported that the activation of HER2 signaling by increased heregulin production causes acquired resistance to cetuximab in colorectal cancer cells by leading to persistent activation of downstream signaling [27]. In the same line, a couple of years before Wang et al reported that hepatocyte growth factor (HGF) released by stromal fibroblasts induced resistance to EGFR inhibitors in non-small cell lung cancer due to MET activation [28, 29]. Recently in Targeting the PI3K-mTOR net work in Cancer meeting (Philadelphia, Sept.14-17th 2014) Dr. Levi A Garraway reported that upregualtion of neuregulin followed by HER2:HER3 signaling for ALK inhibitor (crizotinib)-resistant in ALK-driven NSCLC. These findings have tempted us to speculate that a subset of *HER2*-nonamplified tumors may respond to a pertuzumab-containing regimen (we will discuss more in the following section).

HER2-targeted drugs and HER3 mediated drug resistance in HER2+ BC

Trastuzumab, a humanized HER2 monoclonal antibody that binds domain IV of the extracellular domain of the HER2 receptor is used in combination with chemotherapy for treatment of HER2+ breast cancer. However, many metastatic *HER2*-amplified breast cancers do not respond to, or eventually escape, trastuzumab, often with recovery of phospho-HER3/PI3K-AKT-mTOR signaling [30-32]. In addition to trastuzumab, pertuzumab is another HER2-targeted humanized monoclonal antibody with efficacy confirmed in mouse xenograft models of breast, lung, prostrate and ovarian tumors [17, 33-35]. Pertuzumab binds domain II of the extracellular domain of HER2, preventing ligand-mediated association of HER2 with other HER-family members including HER3 [36, 37] and showed clinical activity as a single agent and with the combination with chemotherapy in ovarian, non-small cell lung and prostrate cancers [38-41]. Pertuzumab combined with trastuzumab has antitumor efficacy in both preclinical [36, 42, 43] and in patients with HER2+ metastatic breast cancer [44, 45]. The combination of trastuzumab plus pertuzumab also demonstrated antitumor activity in models of trastuzumab resistance, suggesting that trastuzumab and pertuzumab have complementary mechanisms of action [35]. Combining pertuzumab, trastuzumab plus docetaxel significantly improves the pathologic complete response (pCR) rate (45.8%) in the neoadjuvent setting (NeoSphere trial) compared with treatment without pertuzumab (29%) [45]. Pertuzumab was approved by FDA in 2012 on the basis of finding of CLEOPATRA, a phase III trial that demonstrated significantly improved progression free survival (PFS) (18.6 months versus 12.4 months) and overall survival [median overall survival was 37.6 months (95% CI 34.3-NE [not estimable] in the placebo group but had not been reached (95% CI 42.4-NE) in the pertuzumab group (hazard ratio 0.66, 95% CI 0.52-0.84; p = 0.0008] with addition of pertuzumab to trastuzumab plus docetaxel for treatment of patients with HER2+ metastatic breast cancer who have not received prior anti-HER2 therapy or chemotherapy [46, 47]. The FDA on Sept. 30 2013 also granted accelerated approval to pertuzumab injection for use in combination with trastuzumab and docetaxel for the neoadjuvant treatment of patients with HER2+, locally advanced, inflammatory, or early stage breast cancer (either greater than 2 cm in diameter or node positive) as part of a complete treatment regimen for early breast cancer. Genentech has also developed a FDA approved cytotoxic drug-conjugate of trastuzumab (T-DM1) through a stable linker for treatment of patients with HER2+ metastatic breast cancer who have received prior treatment with trastuzumab (Herceptin) and taxane chemotherapy. T-DM1 approval was based mainly on data (from EMILIA trial) showing significantly prolonged progression-free and overall survival and less toxicity compared with standard-of-care treatment, lapatinib (Tykerb) plus capecitabine (Xeloda) [48]. Lewis Phillips and colleagues [23] and our group [36] also showed that dual targeting HER2 with the combination of T-DM1 plus pertuzumab in HER2+ cell culture (in both trastuzumab-sensitive and trastuzumab-resistant models) and mouse xenograft models resulted in enhanced antitumor activity. Lewis Phillips and colleagues also showed that this combination was effective in terms of safety and tolerability in patients with preliminary evidence of efficacy [23]. A phase III clinical trial (MARIANNE trial, *NCT 01120184*) in HER2+ advanced metastatic breast cancer with T-DM1 plus pertuzumab is completed, data analysis is ongoing and should be available soon.

Interestingly, the HER3 receptor is emerging as a critical element not only in HER2-mediated transformation and tumor progression but also in drug resistance. Recently, Berghoff and group showed that co-expression of HER2/HER3 exist in 11% of their breast tumor specimens and HER3 overexpression observed a statistically significant association with HER2 overexpression (p = 0.02) [5]. The success of HER2-directed therapies relies partly on their ability to inhibit downstream PI3K-AKT-mTOR signaling. In fact, inhibition of HER2 phosphorylation by tyrosine kinase inhibitor (lapatinib) targeting EGFR and HER2 in HER2+ breast cancer cells is followed by feedback upregulation of activated HER3 [49] and the feedback HER3 upregulation seems to be clinically relevant. This evidence highlights the importance of HER3 in HER2 addicted breast cancers. We have undertaken a study to determine the importance of HER3 receptors in the HER2+ BC

using data from cBioPortal. **Figure 3A** shows that the HER3 gene is altered in 15.5% of all cases (58 samples) of PAM50 HER2 enriched subtype Breast Invasive Carcinoma (TCGA, Nature 2012; Case Set: PAM50 HER2 enriched). The alteration involves mutation, amplification, downregulation of mRNA and RPPA upregulation. **Figure 3B** shows the plot for HER3 gene where the putative copy number alteration (Y-axis) has been plotted against mRNA expression z-scores (X-axis) in HER2 enriched PAM50 samples from cBioPortal. The plot demonstrates that most of the variation in the expression of HER3 mRNA (z-scores) was observed in diploid conditions. **Figure 3C** shows the overall survival plot by Kaplan-Meier estimate for cases with alterations in the HER3 genes and cases with no alterations in the HER3 genes in HER2 enriched PAM50 samples as obtained from cBioPortal. The plot demonstrates that the median months of survival in cases with alterations in HER3 gene was 30.26 as compared to the 100.60 months in the cases without alterations in the HER3 gene. These data clearly indicate that there can be a subpopulation identifiable within HER2 enriched BC where the expression level/gene status is associated with the tumorigenic signaling in the cells and hence the status can have bearings on the treatment/patient outcome.

In HER2-overexpressing cells, inhibitors of the PI3K-AKT-mTOR pathway induce a compensatory upregulation of the expression and phosphorylation of HER3 [50, 51] (**Figure 2**). Furthermore, knocking down of HER3 results in sensitization to PI3K inhibitors [52]. Tao et al reported that EGFR or PI3K inhibitors have not led to durable responses in triple negative breast cancer, possibly due to compensatory activation of other receptor tyrosine kinases. They found that the pan-PI3K inhibitor GDC-0941 and the AKT inhibitor GDC-0068 each increased HER3 abundance and induced HER3 and EGFR phosphorylation in EGFR-positive, *PTEN*-null TNBC cell lines. Combining MEHD-7945A, an antibody that targets both EGFR and HER3 (see later, **Table 1**) with either GDC-0941 or GDC-0068 impeded ligand-induced EGFR and HER3 activation and significantly impaired triple negative cell proliferation when compared with PI3K-AKT inhibition alone. Furthermore, combined MEHD7945A and either GDC-0941 or GDC-0068 treatment markedly blocked tumor growth of triple negative breast cancer patient-derived xenografts (PDX) compared with monotherapy and also prevented HER3 and EGFR activation in tumor xenograft samples. Interestingly, pharmacologic or genetic inhibition of HER3 was significantly more effective than the anti-EGFR antibody cetuximab in sensitizing triple negative breast cancer cells to PI3K-AKT inhibitors [53]. Garner et al also demonstrated from their preclinical study that an antibody which has the capacity to lock HER3 in its inactive confirmation led to inhibit tumor growth driven by HER2 or neuregulin [54]. Very recently Robert Torka and group elegantly demonstrated that inhibition of AXL (a receptor tyrosine kinase) by short interfering RNA or tyrosine kinase inhibitor BMS777607 induces the expression of HER3 and neuregulin-mediated phosphorylation of HER3 in MDA-MB231 and Ovcar8 cells. Cell lines treated with MPCD84111 simultaneously blocked AXL and HER2/3 signaling and thereby prohibited HER3 feedback activation. Moreover, dual inhibition of AXL and HER2/3 using BMS777607 and lapatinib led to a significant inhibition of the cell viability [55]. Together, these findings implicate HER3 as a compensatory mechanism to PI3K-AKT inhibition and/or RTK inhibition and provide a rationale for clinical evaluation of combined HER3, other RTK and PI3K-AKT inhibition in cancer patients. Interestingly, enhanced neuregulin (NRG)-HER3 signaling is also observed in RAF (V600E) inhibited (using vemurafenib, FDA approved drug) melanoma cells and the combination of RAF inhibition with HER3-antibody was more efficacious than either treatment alone at inhibiting tumor growth and promoting durable response *in vivo* [56].

Oncogenic signaling of HER3 in HER2+ BC

The unique feature that separates HER3 from the other HER- family members is its evolutionary divergence at critical residues within the kinase domain, locking it in the inactive conformation, thus devoid of catalytic kinase activity [57-59]. In the HER family, dimerization of the kinase domain occurs in an asymmetric configuration leading to the allosteric activation of one kinase domain by the other [60]. In contrast to the other HER proteins, HER3 is not transforming when overexpressed or constitutively triggered by continuous ligand stimula-

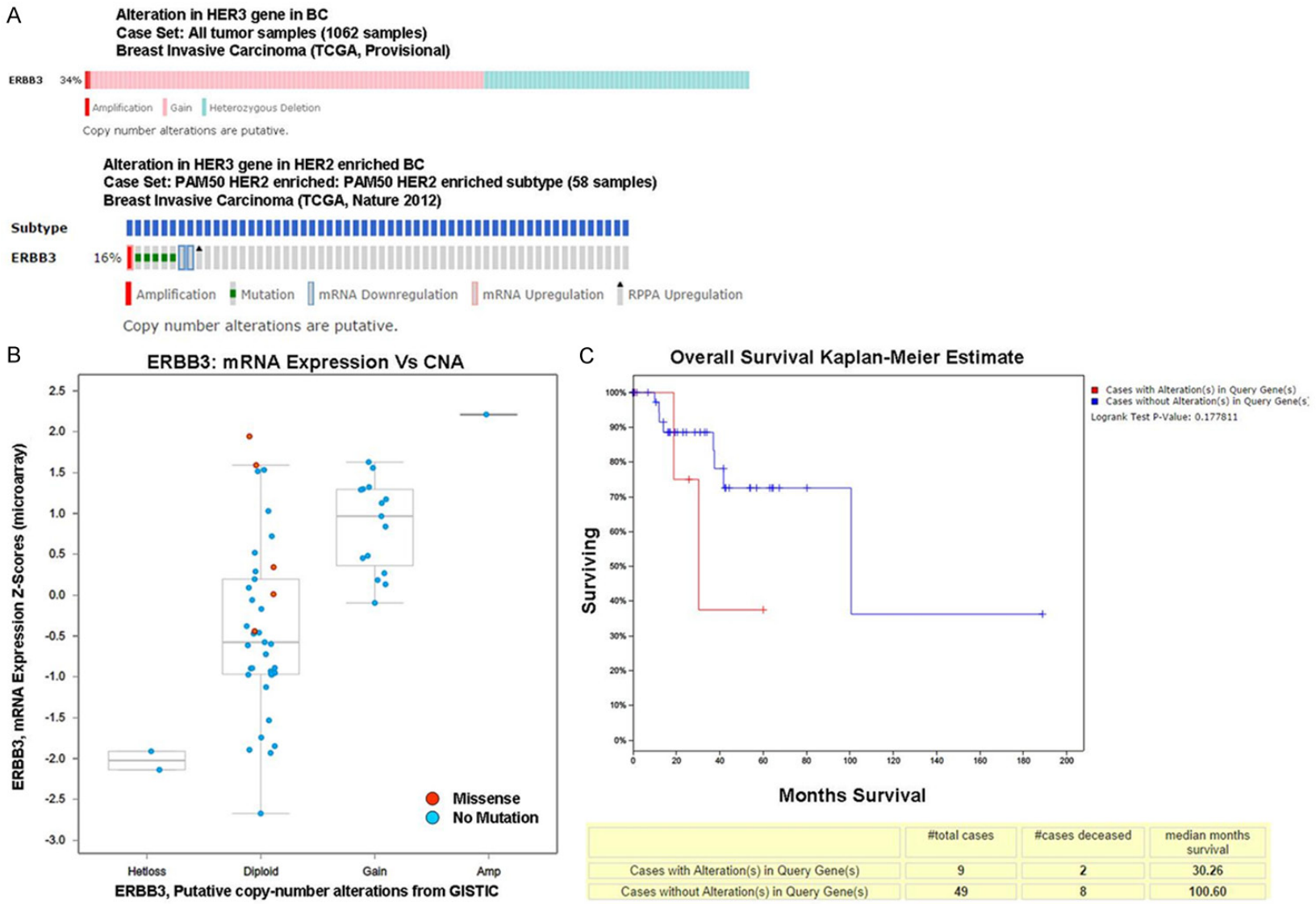

Figure 3. Alterations of HER3 gene in Breast cancer (A-C). HER3 gene alteration in invasive breast carcinoma, TCGA, Provisional (A: upper panel; Unaltered cases are removed) and PAM50 HER2 enriched cases, TCGA, Nature 2012 (A: lower Panel) are presented in (A). The putative copy number alteration (Y-axis) has been plotted against mRNA expression z-scores (X-axis) in HER2 enriched PAM50 samples (B). mRNA z-scores (Agilent microarray) compared to the expression distribution of each gene tumors that are diploid for this gene. Putative copy-number from GISTIC 2.0. Values: -2 = homozygous deletion; -1 = hemizygous deletion; 0 = neutral/ no change; 1 = gain; 2 = high level amplification. The overall survival plot by Kaplan-Meier estimate for cases with alterations in the HER3 genes and cases with no

alterations in the HER3 genes in HER2 enriched PAM50 samples as obtained from cBioPortal (C). Data was obtained using cBioPortal. We acknowledge the cBioPortal for Cancer Genomics site (http://cbioportal.org) which provides a Web resource for exploring, visualizing, and analyzing multi-dimensional cancer genomics data. The portal reduces molecular profiling data from cancer tissues and cell lines into readily understandable genetic, epigenetic, gene expression and proteomic events (Gao et al., 2013, Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal, Sci. Signal., 2 April, Vol. 6, Issue 269, p. pl1 [DOI: 10.1126/scisignal.2004088]) . We acknowledge works of Cerami et al. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multi-dimensional Cancer Genomics Data [99]. and Gao et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal [100]. We acknowledge the TCGA Research Network for generating TCGA datasets. Data show that the gene is altered in 15.5% of all cases.

Table 1. List of anti-HER3 antibodies under development

Antibody name	Type of antibody	Company name	Stage of development
AV-203	Mono-specific	Aveo Pharmaceuticals	Phase 1 (NCT01603979)
GE-huMab-HER3	Mono-specific	Roche Diagnostics	Phase 1
AMG 888	Mono-specific	Daiichi-Sankyo	Phase 1 (NCT00730470)
			Phase 1b/2 (NCT01211483)
MM-121	Mono-specific	Merrimack pharmaceuticals sanofi-aventis	Phase 1 and 2 (NCT01151046, NCT01421472, NCT00994123, NCT01451632, NCT01209195)
LMJ716	Mono-specific	Novartis pharmaceuticals sanofi-aventis	Phase 1 (NCT01598077)
REGN1400	Mono-specific	Regeneron pharmaceuticals	Phase 1 (NCT01727869)
H4B-121Ab	Mono-specific	Institut recherche en cancerologie de Montpellier, INSER Unit 869 Universite Montpellier, France	Preclinical
MM-111	Bi-specific	Merrimack pharmaceuticals	Phase 1 (NCT00911898, NCT01097460, NCT01774851, NCT01304784)
MEHD7945A	Bi-specific	Genentech Inc	Phase 2 (NCT01577173)
MM-141	Bi-specific	Merrimack pharmaceuticals	Phase 1 (NCT01733004)
GSK2849330	Mono-specific	GlaxoSmithKline pharmaceuticals	Phase 1 (NCT01966445)
KTN3379	Mono-specific	Kolltan	Phase 1 (NCT02014909)

tion [61], and there are currently no mutational alterations known to confer oncogenic activities to HER3. HER3 functions not only as a specialized allosteric activator of other HER proteins, but also as their signaling substrate. The 14 tyrosines in the C-terminal signaling tail of HER3, when phosphorylated, can potentially dock various SH2 or PTB binding proteins involved in a number of different intracellular signaling pathways. Whether all of these tyrosines are phosphorylated in cells, and whether all of the described interactions are physiologically relevant remain to be defined. But, the one critically important and well-established signaling activity of HER3 is its unique and potent ability to activate downstream PI3K and AKT pathway signaling by virtue of six consensus phosphotyrosine sites, not present on EGFR or HER2, [62-64]. Activated PI3K phosphorylates membrane phosphoinositides, leading to recruitment and activation of PDK1 and AKT. AKT lies at the hub of a plethora of downstream pathways, in particular in an intricate upstream and downstream relationship with two mTOR containing complexes, and is in a position to control many biological processes critical for tumorigenesis, including translation, survival, apoptosis, nutrient sensing, metabolic regulation, and cell cycle control [65, 66]. The link between HER3 and the AKT pathway not only confers oncogenic abilities to its kinase-active HER family partners especially with HER2 in HER2-overexpressing breast cancer, but provides a signaling node that can potentially be exploited by other signaling pathways to engage the activities of AKT. Most tumors require PI3K-AKT-mTOR signaling for their survival, and this is often achieved by upstream receptor tyrosine kinase activation, by mutational activation of PI3K (*PIK3CA*), or inactivation of tumor-suppressor gene *PTEN [66]*. Through its activating interface following ligand engagement, HER3 allosterically activates its kinase partners including HER2 and leads to recruit of adapter proteins (e.g. GRB2, SHC) leading to activation of the oncogenic RAS-RAF-MEK signaling pathway. In most of these scenarios, it is assumed that HER3 phosphorylation is driven by one of it's HER family kinase partners. A more promiscuous role for HER3 as a substrate of other kinases is possible, and at least suggested by the c-MET-induced activation of HER3 signaling [67], however, at this point additional evidence is needed before implementing this research finding. It has been recently reported by Carpenter et al that HER3-ligand heregulin initiates HER2:HER3 dimerization which leads to activate epithelial-mesenchymal transition (EMT) via phosphorylation of AKT-HSF1 (heat shock factor1)-SLUG (known EMT-regulator), and potentially contributes to progression of HER2+ breast cancer [68]. It has been reported by others that expression of HER3 has been associated with the epithelial phenotypes in cell lines, as well as sensitivity to EGFR inhibition [69-73]. Recently, McCormick and group showed that *HER3* mRNA expression was highly co-expressed with epithelial genes (e.g. *CLDN4*, *CLDN7*, *CDH1*, *MUC1* etc) and was strongly anti-correlated with tumors in the mesenchymal state (e.g. *FN1, VIM, TWIST1* etc) [73].

HER3 signaling in cancer stem cells in HER2+ BC

Breast cancers are heterogeneous and contain a subpopulation of cells called tumor initiating cells [TIC, also called cancer stem cells (CSC)] that have the ability to give rise to new tumors that recapitulate the fullest heterogeneity of the parental tumors [74]. HER2 overexpression has also been linked to CSCs, as exogenous overexpression of HER2 appears to increase numbers of CSCs and facilitates the mammary tumorigenesis, invasion and inhibition of HER2 can target CSC-like cells [75-77]. Recently, Lee and group showed that HER2:HER3 signaling in breast CSCs promotes self renewal and survival. They also demonstrated by using tissue microarray that neuregulin produced by CSCs and helps to initiate HER2:HER3-mediated signaling and enhances their proliferation/self renewal even in HER2-low tumors, including triple negative breast tumors [74]. It has been recently reported by other that HER3 plays a positive role in HER2 negative breast cancers [78]. Moreover, it was recently shown by other that exogenous neuregulin promotes mammosphere formation in established cell lines and cultured cells from primary breast tumor tissues [79]. Although, clinical data regarding the efficacy of anti-HER2 therapies in HER2-low breast cancers are mixed/controversial. While multiple trials have shown no benefit of targeting HER2 in metastatic HER2-low tumors, recent evidence specifically NSABP trial B-31 and N9831 trial suggest that anti-HER2 thera-

py may be benefited in the adjuvant settings of patients with HER2-low to no display of *HER2* amplification [80, 81]. Taken together, we can speculate why the combination of pertuzumab plus trastuzumab or pertuzumab plus T-DM1 is much more efficacious in the clinical settings.

HER3-targeted antibodies

From the mechanistic standpoint the intracellular region of this receptor is rich of tyrosine residues that, upon phosphorylation, become high affinity binding sites for PI3K and other proteins involved in signal transduction. Numerous attempts are therefore being put in the development of antibodies that target this receptor either singly or in combination with other synergizing receptors. Some of these compounds have already entered clinical development, although, clinical proof-of-concept has not yet been achieved.

A wealth of antibodies targeting human HER3 has been generated in the past years. In this section we will review their biochemical/biological properties and current status of development (Please see **Table 1**). Interestingly while the majority of these agents recognize only HER3, a subset of them has been engineered thanks to new technologies, to be able to bind not only HER3 but also an additional co-receptor.

AV-203

AV-203 is a humanized immunoglobulin IgG1 kappa antibody that is being developed by Aveo Pharmaceuticals. AV-203 is a clinical stage HER3-inhibiting antibody designed to inhibit both ligand-dependent and ligand-independent HER3 signaling. AV-203 has showed preclinical activity in a number of different tumor models including breast, head and neck, lung, ovarian and pancreatic cancers (according to company website). AV-203 is currently being investigated in a phase 1 clinical trial to evaluate safety and preliminary efficacy, as well as exploratory biomarkers in patients with advanced solid tumors (*NCT 01603979*).

GE-huMab-HER3

GE-huMab-HER3 is a novel humanized and glycoengineered IgG1 antibody (developed by Roche Diagnostics GmbH, Penzberg, Germany) that binds to HER3 with high affinity. This antibody prevents ligand binding and receptor heterodimerization thereby blocking receptor phosphorylation. In various tumor xenograft models treatment with this antibody leads to substantial tumor growth inhibition. A unique feature of GE-huMab-HER3 that differentiates it from other anti-HER3 antibodies is its ability to bind to human FcγRIIIa on immune effector cells with a 50-fold higher affinity than standard IgG1 antibodies. Consequently, GE-huMab-HER3 exhibits superior potency and efficacy in ADCC, as shown *in vitro* using recombinant A549 cells and *in vivo* by its Fc mediated greater anti-tumor effect in A549 orthotopic mouse models compared to a non-glyco-engineered variant of the antibody, WT-huMab-HER3. The combination of strong signaling inhibition and enhanced ADCC capability renders GE-huMabHER3 a highly potent HER3-targeting agent. Phase 1 clinical testing of this promising novel compound is ongoing [82].

AMG 888

AMG 888 (also known as U3-1287/patritumab) is a fully human anti HER3 monoclonal antibody isolated from U3 Pharma using Amgen's Xenomouse® technology. It is currently being developed by Amgen in partnership with Daiichi-Sankyo. AMG 888 was reported to block HER3-induced AKT and ERK signaling and to inhibit *in vitro* and *in vivo* growth of multiple tumor cell lines as single agent or, even better in combination with other HER-family inhibitors, such as cetuximab. However, the exact biochemical properties of the antibody have not been disclosed yet [83]. AMG 888 is currently undergoing clinical trials. A phase 1 clinical study (*NCT00730470*) to investigate the safety, tolerability, pharmacokinetics and pharmacodynamics of U3-1287 in patients with advanced solid tumors has been completed. Other phase 1b/2 clinical studies (*NCT01211483*) of U3-1287 with or without erlotinib in patients with advanced non-small cell lung cancer are ongoing.

MM-121

MM-121/SAR256212 is a fully human IgG2 monocolonal antibody that targets the HER3 receptor. MM-121 is being developed by Merrimack in partnership with Sanofi- Aventis. MM-121 functions by inhibiting ligand-induced signaling through HER3, and activation of asso-

ciated survival pathways. MM-121 is currently being tested clinically in combination with exemestane in postmenopausal women with hormone receptor-positive breast cancer (*NCT01151046*) and also with paclitaxel in estrogen receptor-positive/HER2- preoperative breast cancer patients (*NCT01421472*). Phase 1 or phase 2 studies of MM-121 in combination with multiple anticancer therapies in patients with advanced solid tumors (other than breast tumors) are ongoing (*NCT00994123; NCT01451632; NCT01209195*).

LMJ716

LJM716 is a fully humanized monoclonal antibody selected from a Human Combinatorial Antibody Library (HuCAL) and being developed by Novartis and Sanofi Aventis. Recently, Garner et al found that LJM716 binds to an epitope, within domains II and IV that traps HER3 in an inactive conformation [54]. LJM716 is a potent inhibitor of HER3/AKT phosphorylation and proliferation in a range of *HER2* amplified and nuregulin expressing cell lines *in vitro*. LJM716 induced tumor regression in Fadu (nuregulin expressing, HNSCC) tumor xenografts and significant tumor growth inhibition (> 80%) in a variety of xenograft models including BT474 (*HER2* amplified breast). Furthermore, the combination of LJM716 with trastuzumab, cetuximab or PI3K- targeted agents was synergistic in a panel of *in vitro* cell lines while the *in vivo* combination of LJM716 with trastuzumab or erlotinib was efficacious in BT474 and L3.3 (pancreatic) tumor xenografts respectively [84]. It has been reported by other that treatment with LJM716 reduced HER2:HER3 and HER3-p85 dimers, phospho-HER3 and phospho-AKT, both *in vitro* and *in vivo*. LJM716 and BYL719 (p110α-specific inhibitor, developed by Novartis Pharmaceuticals) synergistically inhibited growth in a panel of HER2+ and *PIK3CA* mutant cell lines. The combination also inhibited phospho-AKT in HER2+ breast cancer cells and growth of HER2+ NCI-N87 gastric cancer xenografts. More importantly, trastuzumab-resistant HER2+/*PIK3CA* mutant MDA453 xenografts regressed completely after 3 weeks of therapy with LJM716 and BYL719 [85]. A phase 1 study of LJM716 in squamous cell carcinoma of head and neck, or HER2+ breast cancer or gastric cancer is ongoing (*NCT01598077*).

REGN1400

REGN1400 is a fully-human anti-HER3 monoclonal antibody under development by Regeneron Pharmaceuticals Inc. REGN1400 inhibited phospho-HER3, phospho-AKT and the growth in multiple human tumor cell lines *in vitro*, including A431 (epidermoid carcinoma), MDA-MB-175-VII (breast cancer) and FaDu (head and neck cancer). Consistent with its potent effects on tumor cell growth *in vitro*, REGN1400 strongly inhibited the growth of A431 and FaDu tumor xenografts in a dose-dependent manner. Furthermore, combination treatment with REGN1400 plus trastuzumab inhibited the growth of BT474 breast tumor xenografts more potently than either of the agent alone [86]. A phase 1 study of REGN1400 in combination with erlotinib or cetuximab in patients with advanced non-small cell carcinoma, colorectal cancer or head and neck cancer is continuing (*NCT01727869*).

H4B-121 Ab

Yassamine Lazrek and team recently generated specific antibodies (Abs) against domain 1 (D1) and domain 3 (D3) of HER3 that recognize epitopes that do not overlap with the neuregulin-binding site. The fully human H4B-121 Ab inhibited tumor growth in nude mice xenografted with epidermoid, pancreatic, or triple-negative breast cancer cells. The combination of one anti-HER3 Ab and trastuzumab improved tumor growth inhibition in mice xenografted with HER2-low cancer cell lines, for which trastuzumab alone shows no or moderate efficiency [87].

MM-111

MM-111 is a first-in-class bi-specific antibody that has been shown in preclinical studies to bind with both specificity and avidity to HER2 and HER3 expressing tumor cells, and developed by Merrimack Pharmaceuticals. The HER2 arm is responsible for initial tumor cell targeting and docking, while the therapeutic HER3 arm is designed to block HER3-ligand (heregulin)-induced cell signaling. MM-111 is designed to allow the specific inhibition of HER3 signaling in cancer cells having elevated HER2 expression, a subtype representing a large population of breast and also gastric cancers. Preclinical studies showed that MM-111

as a single agent is more potent than lapatinib at inhibiting heregulin-mediated phospho-HER3 and phospho-AKT signaling in *in vitro* assays. A combination of MM-111 and lapatinib more effectively inhibits the growth of HER2-overexpressing breast tumors [88]. It has been also reported by others that MM-111 synergizes with various treatment regimens in both the first- and second-line treatment setting in HER2+ gastric cancer xenografts. Exogenous and endogenous heregulin can lead to resistance to paclitaxel *in vitro* and *in vivo,* and MM-111 is able to restore the sensitivity to paclitaxel [89]. These findings suggest that HER2+ gastric and gastroesophageal junction tumors that stop responding to trastuzumab-based therapies may benefit from MM-111 -based regimens. MM-111 is being tested clinically in combination with multiple anticancer therapies in patients with advanced solid tumors including heregulin positive breast cancer are currently ongoing (*NCT-00911898; NCT01097460; NCT01774851; NCT01304784*).

MEHD7945A

MEHD7945A is a fully human dual specificity anti-HER3 and anti-EGFR (HER1) monoclonal antibody under development by Genentech Inc. The objective to develop dual-specificity antibody is to obtain a wider spectrum monoclonal antibody capable of affecting ligand dependent proliferation of cancers which require signaling by EGFR or HER3 or both receptors simultaneously. MEHD7945A is a novel dual-action human IgG1 antibody. Each antigen-binding fragment blocks ligand binding to both EGFR and HER3, which is meant to inhibit the activity of the major ligand-dependent HER dimers in cancer. MEHD7945A also elicits antibody-dependent cell-mediated cytotoxicity, and has single-agent activity in a broad panel of tumor models, including those resistant to anti-EGFR or anti-HER3 treatment alone. Gabriele Schaefer and team demonstrated the advantage of dual blockade (by using MEHD7945A) to inhibit diverse intracellular signals in a number of *in vitro* systems and also showed significant tumor growth inhibition in 12 xenograft models that represent six different types of solid tumors [90]. A Phase 1, multicenter, open-label study in patients with refractory or relapsed epithelial tumors showed MEHD7945A is well-tolerated with a favorable safety profile treated at doses up to 30 mg/kg q2w [91]. Dual EGFR and HER3 antibody may be more effective for EGFR driven cancer than existing antibody therapies and worth trying as a single agent or in combination with the PI3K-AKT-mTOR pathway-specific inhibitor in *EGFR*-amplified triple negative breast cancer. Genentech sponsored a phase 2 study of MEHD7945A versus cetuximab in patients with recurrent/metastatic squamous cell carcinoma of head and neck is ongoing (*NCT01577173*).

MM-141

The IGF-IR and HER3 signaling pathways have been implicated as potential escape routes in cancers exhibiting resistance to targeted therapies and chemotherapies [92-95]. The evidence that a subset of cancer cell lines show simultaneous production of nuregulin and IGF1 and this gives rise to simultaneous activation of both HER3 and IGF1R receptors led Merrimack to generate a bi-specific antibody directed against those receptors which was called MM-141. MM-141 blocks both nuregulin binding to HER3 and IGF-1/IGF-2 binding to IGF1R and inhibits common downstream of PI3K-AKT-mTOR signaling pathway activation. MM-141 inhibits phosphorylation of IGF1R and HER3 as well as downstream activation of the PI3K pathway signaling. Inhibition of growth by MM-141 has been observed *in vitro* as well as *in vivo* in multiple xenograft models including human pancreatic cancer (BxPC-3) and human prostate cancer (DU145) [96]. A phase 1 study of MM-141 in patients with advanced solid tumors is ongoing and currently recruiting patients (*NCT01733004*).

GSK2849330

GSK2849330 is an IgG1/Ig3 chimeric, glyco-engineered humanized monoclonal antibody directed against HER3 with enhanced potency to moderate ADCC and CDC (complement-dependent cytotoxicity), resulting in different potential models of antitumor activity. It has been recently reported by Clarke et al that GSK2849330 inhibits HER3 signal transduction *in vitro* and *in vivo* and is up to two orders of magnitude more potent in mediating ADCC and CDC than WT antibody. This is the first AccretaMab antibody to initiate phase 1 clinical studies (*NCT01966445*) [97].

KTN3379

KTN3379 is a human monoclonal antibody designed to block the activity of HER3. KTN3379 inhibited both ligand dependent and HER2 dependent (ligand independent) HER3 activation in different tumor types. The crystallographic structure of the KTN3379 antigen-binding fragment (Fab) was determined in complex with the complete HER3 extracellular domain. The structure of the complex revealed that the antibody binds with very high affinity to a novel epitope in the boundary between domains 2 and 3 and locks HER3 in its inactive state. KTN3379 is now in phase 1 clinical trial in adult subjects with advanced tumors (*NCT02014909*) and presented in 26th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics in Barcelona, Spain.

HER3 driven therapy in HER2+ BC: A clinical perspective

It is clear that there is exciting evidence to support the importance of HER3 signaling in the treatment of cancer. As described previously, preclinical studies implicate HER3 as a major cause of treatment failure in cancer therapy, mainly through the activation of the PI3K-AKT, MEK-MAPK, and JAK-STAT signaling pathways [98]. In the clinic, the data on the single agent activity of HER3 selective drugs may be viewed as modest at best at this point despite several reagents that have been investigated in phase I and II trials. However, concomitant inhibition is most certainly required to overcome resistance and effectively treat cancer patients. As professor P. Lorusso recently mentioned (EORTC-NCI-AACR meeting in Barcelona, Spain 18th-21st November 2014) that NRG1 overexpression across cancer types. CLEOPATRA success provide the confidence both in clinical and in research communities that anti-ERBB3 agent will be successful with targeted agent in clinical setting e.g. with cetuximab in colorectal/head and neck cancers or with earlotinib in NSCLC or with trastuzumab in HER2+ breast cancer.

The complex mechanism of activation of HER family members presents multiple opportunities that can be pharmacologically targeted including inhibition of ligand binding to the extracellular domain, inhibition of receptor dimerization, and inhibition of the partner tyrosine kinase activity. The significance of HER3 in HER2-driven tumors has been proven by multiple studies implicating the upregulation of HER3 in resistance to HER2-targeted therapy. Prospective genomic sequencing-guided therapeutic selection in clinical trials will also likely help us elucidate additional targets and possible combinations.

Conclusion

The cellular signals that regulate HER3's functions in HER+ BC are only beginning to be acknowledged. Ongoing preclinical and clinical studies in conjunction with the arrival of many HER3-targeting agents will provide more insights into the clinical relevance and functions of this receptor in HER+ BC. However, it has to be recognized that the success of HER3-targeting therapies is essentially dependent on the validity of HER3-specific biomarker(s) including but not limited to the phospho-HER3 or its ligands expression. Thus the validity as well as the reliability of those assays will be critical to get an answer. Future clinical trials with prospective evaluation of tumor signaling and genomic changes are likely to identify novel resistance mechanisms as well as categorize the subset of HER2+ patients who may derive maximal benefit from HER3 signaling inhibitor(s).

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Disclosure of conflict of interest

The author(s) confirm that this article content has no conflicts of interest.

Address correspondence to: Dr. Pradip De, Department of Molecular and Experimental Medicine, Avera Cancer Institute, Sioux Falls. E-mail: pradip.de@avera.org

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