Activated d16HER2/SRC axis predicts Trastuzumab benefit

**Activated d16HER2 homodimers and Src kinase mediate optimal efficacy for trastuzumab**

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ABSTRACT

A splice isoform of the HER2 receptor which lacks exon 16 (d16HER2) is expressed in many HER2-positive breast tumors, where it has been linked to resistance to the HER2-targeting antibody trastuzumab, but the impact of d16HER2 on tumor pathobiology and therapeutic response remains uncertain. Here, we provide genetic evidence in transgenic mice that expression of d16HER2 is sufficient to accelerate mammary tumorigenesis and improve the response to trastuzumab. A comparative analysis of effector signaling pathways activated by d16HER2 and wild-type HER2 revealed that d16HER2 was optimally functional through a link to SRC activation (pSRC). Clinically, HER2-positive breast cancers from patients who received trastuzumab exhibited a positive correlation in d16HER2 and pSRC abundance, consistent with the mouse genetic results. Moreover, patients expressing high pSRC or an activated "d16HER2 metagene" were found to derive the greatest benefit from trastuzumab treatment. Overall, our results establish the d16HER2 signaling axis as a signature for decreased risk of relapse after trastuzumab treatment.
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PRECIS

Analyses of mice transgenically expressing human d16HER2 and WHER2 and of Trastuzumab-treated HER2-positive breast cancer patients show that the correlation between high expression of activated d16HER2 and of SRC kinase is a marker of Trastuzumab susceptibility.
INTRODUCTION

HER2 is a 185-kDa transmembrane receptor that belongs to the HER family of receptor tyrosine kinases (RTK), including HER1 (EGFR), HER3 and HER4. Binding of specific ligands to the extracellular domain (ECD) of HER1, HER3 and HER4 induces the formation of homo- and heterodimers, with activated HER2 as a preferred partner (1). Overexpression or amplification of HER2 occurs in 15-20% of invasive breast cancers (BCs) and is associated with more aggressive disease and, until the advent of HER2-targeted agents, a worse outcome (2). In the metastatic setting, the addition of HER2-targeted agents to chemotherapy improved disease-free survival (~37%), overall survival (~22%) and overall response rate (~67%) (3). Trastuzumab, a recombinant humanized anti-HER2 monoclonal antibody, combined with chemotherapy is a foundation of care for patients with HER2-positive BCs (2, 3). However, most HER2-positive BC patients who initially respond to Trastuzumab subsequently become refractory and disease progresses. Several intrinsic mechanisms whereby tumors escape HER2 inhibition by Trastuzumab have been suggested (4), including altered forms of HER2 itself (5, 6) and activating HER2 mutations identified in HER2 gene amplification-negative BC (7). We and others (8, 9) reported that the splice variant of human HER2 lacking exon 16, here named d16HER2 and characterized by an imbalance in the number of cysteines in the ECD portion and by the constitutive generation of stable HER2 homodimers, is a highly penetrant HER2 oncogenic alteration. d16HER2, identified in most human HER2-positive primary BCs, effects a
decrease in Trastuzumab binding in vitro (9) and promotes resistance to Trastuzumab in multiple cell lines (10).

While transgenic (tg) mouse models of the rodent form of HER2 have been instrumental in the study of basic oncogene activity (11-14), the inherent limitations of the rodent neu tg models have led to the development of tg mouse models for the human wild-type, full-length HER2 (WTHER2) (15-17) to study the mechanisms regulating HER2-driven cancer recurrence, Trastuzumab sensitivity and resistance. However, both rodent and human WTHER2 transgenes require activating mutations to become oncogenic, implying that genetic changes in addition to HER2 overexpression are required for mammary tumorigenesis (17, 18). In that context, we generated a FVB mouse line that transgenically expresses the human d16HER2 isoform and stochastically develops metastatic multifocal mammary tumors expressing heterogeneous levels of constitutively activated stable HER2 homodimers (pd16HER2D); these homodimers couple to multiple oncogenic downstream signal transduction pathways, including SRC kinase (19).

The oncogenic activity and Trastuzumab susceptibility of d16HER2-positive mammary tumors (9, 10, 20), as well as the relationship of d16HER2 with WTHER2-driven pathobiological and clinical features in human HER2-overexpressing BCs, await clarification.

Here, we provide evidence in both mice and humans that d16HER2-positive tumors respond significantly to Trastuzumab and that this response depends on the functional relationship and co-expression of activated d16HER2 stable homodimers and SRC kinase.
MATERIALS AND METHODS

Tumor cell lines
The d16HER2- and WHER2-positive mammary tumor cell lines MI6 and WHER2 were established from spontaneous primary mammary carcinomas of an 18-week-old virgin FVB-d16HER2 and a 34-week-old virgin FVB-huHER2 tg female mouse, respectively. Briefly, primary mammary tumors excised from sacrificed mice were finely minced, incubated in erythrocyte lysis buffer, enzymatically digested (Collagenase/Hyaluronidase, StemCell Technologies, Vancouver, Canada) and extensively washed before examination in four high-power fields based on trypan blue staining (see Supplementary Fig. 1A and B for cell membrane expression of d16HER2 and WHER2, respectively). In the case of d16HER2-positive tumors, whole mammary tumor cell suspensions were selectively separated under sterile conditions by AutoMACS™ separator (Miltenyi Biotec, Bergisch Gladbach, Germany) to obtain homogenous EpCAM- and d16HER2-positive neoplastic cell cultures (manuscript in preparation). The MI6 and WHER2 cell lines were maintained in complete culture medium (MammoCult, StemCell Technologies) supplemented with 1% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and penicillin-streptomycin (SIGMA-ALDRICH) and cultured at 37°C in a 5% CO₂ atmosphere. MI6 and WHER2 tumor cell lines were routinely tested by flow cytometry and qRT-PCR.

Tg mice and in vivo therapy
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A breeding colony of FVB d16HER2 tg mice was generated as described (19) and bred in the Animal Facility of Fondazione IRCCS Istituto Nazionale dei Tumori. Animal care and experimental procedures were approved by the Ethics Committee for Animal Experimentation of the Institute according to Italian law. DNA extracted from tail biopsies was used for routine genotyping by PCR analysis (primers: F: 5'-GGCTCAGTGACCTGTTTTGG-3’ and R: 5’-TGATGAGGATCCCAAAGACC-3), with an expected amplicon length of 231 bp. Mice were inspected twice weekly by palpation.

FVB-huHER2 (WTHER2) transgenic mouse line MMTV.f.hu.HER2#5 (Fo5) carries the full-length normal huHER2 gene under the control of the MMTV promoter on an FVB background (17) and was obtained from Genentech, Inc. (South San Francisco, CA). FVB-huHER2 mice were bred in animal facilities of the DIMES Department of the University of Bologna and genetically screened by PCR using a primer set specific to human growth hormone exons 4 and 5 included in the transgene backbone (17). Mice were inspected weekly by palpation. In vivo experiments were performed in compliance with the Italian and European guidelines and were approved by the institutional review board of the University of Bologna. Progressively growing masses ≥50 mm³ were scored as tumors in both tg models. Susceptibility of d16HER2 to Trastuzumab treatments was assessed in d16HER2-positive tg spontaneous and in orthotopic d16HER2 and WHER2-positive models. In the first set of in vivo experiments, d16HER2 tg mice were injected i.p. with Trastuzumab (Roche, Basel, Switzerland) or diluent NaCl solution (0.9%) in a short (n=8/group) and prolonged (n=7-8/group) administration protocol. In the short treatment, tg mice were treated with Trastuzumab (8 mg/kg) once per week for 5 weeks starting from 8 weeks, when only microscopic
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tumor lesions are present (19), until 12 weeks of age. The study was terminated at 29 weeks of age, when all d16HER2 mice developed the first spontaneous tumor. In the prolonged protocol, d16HER2 tg mice received Trastuzumab (4 mg/kg) twice weekly from 8 until 42 weeks of age. In each experiment, tumors were calibrated twice weekly and tumor volume was calculated as 0.5 X d1^2 X d2, where d1 and d2 are the smaller and larger diameters, respectively. FVB female mice (6-8 weeks old, body weight 20-25 g) were purchased from Charles River (Calco, Italy). Mice (n=10/group) were injected into the mammary fat pad (m.f.p.) with 1x10^6 MI6 or WHER2 tumor cells. When tumors reached 50 mm^3, mice were randomized into two groups to receive biweekly i.p. injections of 4 mg/kg Trastuzumab or diluent NaCl solution (0.9%). The use of the two different dosing schedules of Trastuzumab administration is based on the reliable results we previously obtained (21, 22). Tumors were calibrated twice weekly and tumor volume was calculated as above. Mice were sacrificed when tumor volumes reached ~2000 mm^3. Each tumor specimen was placed into liquid nitrogen for biochemical analyses. For histopathological analyses, tumors and lungs were fixed overnight in 10% neutral-buffered formalin and transferred into 70% ethanol before processing and paraffin-embedding. Paraffin sections (5-µm thick) were stained with H&E. Lung metastases were induced with 10^5 and 10^6 viable MI6 and WHER2 tumor cells, respectively, injected i.v. in 0.4 ml of PBS in FVB female mice. Mice were randomized into two groups (n=8/group) to receive biweekly i.p. injections of 4 mg/kg Trastuzumab or diluent NaCl solution 0.9%, respectively. Treatment started 7 days after cell injection. Mice were sacrificed and necropsied 11 weeks after d16HER2 and WHER2 cell
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Injection. Lungs were perfused with black India ink to outline metastases and fixed in Fekete’s solution. Lung metastases were counted using a dissection microscope.

**Quantitative real-time PCR (qRT-PCR)**

Of 84 HER2-positive human BC specimens, 43 frozen primary BC were available for analysis by qRT-PCR to determine the amount of d16HER2 transcript, as normalized to the amount of Wther2 mRNA. Total RNA from human primary BC frozen specimens was extracted with Trizol® (Invitrogen) according to the manufacturer’s instructions. cDNAs were reverse-transcribed from 1 µg of total RNA in a 20-µl volume with SuperScript III (Invitrogen) using random-hexamer primers and examined by qRT-PCR using Applied Biosystems SYBR® Green dye-based PCR assay on the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). d16HER2 and WHER2 isoforms were amplified using 200 nM primers (10). Data were normalized to GAPDH (23). Relative abundance of d16HER2 mRNA compared with that of WHER2 was calculated by the comparative Ct method (24), with d16HER2 transcript levels indicated as the ratio $2^{-\Delta Ct}d16HER2/2^{-\Delta Ct}WHER2$. To correlate d16HER2 transcript and pSRC expression levels in human BCs, gene expression data were split in two groups according to tertiles: low, containing values under the 1st tertile, and high, containing values greater than the 1st tertile.

**Statistical analyses**

Differences in tumor multiplicity curves in both d16HER2 and WHER2 tg models and differences in Trastuzumab antitumor activity in orthotopic MI6 and WHER2 models...
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were calculated, by two-tailed unpaired t-test. Differences were considered significant at p<0.05.

Linear regression and Pearson’s correlation coefficient $r$ were calculated to estimate the correlation of: 1) pd16HER2M and pd16HER2D with pSRC and of pWTHER2 with pSRC levels both under non-reducing and reducing conditions in protein extracts from both d16HER2 and WTHE R2 tg models; 2) d16HER2 with WTHE R2 gene expression levels; and 3) pSRC (%) with d16HER2 transcript levels in human primary BCs.

Survival was assessed using the Kaplan-Meier estimator, while log-rank test was used to compare survival distributions. Survival analysis was carried out using Cox proportional hazards regression models, and the effects of explanatory variables on event hazard were quantified by hazard ratios (HR) (25).

Data for the “activated-d16HER2 metagene”, constructed based on the Illumina Whole-Genome DASL® gene expression profiling of 21 HER2-positive BCs characterized for pSRC and d16HER2 expression (GSE55348, see Supplementary Table 4), were quantile-normalized using BeadStudio software and filtered with a data matrix containing 22121 probes, corresponding to 15715 Entrez Ids. Pathways differentially enriched in activated d16HER2 tumors were evaluated by Gene Set Enrichment Analysis using GSEA v2.0.13 (26) on a 193-cancer-related gene set (24). Permutation type was applied 1000 times. Core members of each significantly (p<0.05) enriched gene set were extracted and their mean expression levels were considered as the “activated-d16HER2 metagene” value. The metagene was calculated in HER2-positive BC biopsies of two publically available datasets, GSE22358 (27) and GSE41656 (28), for which pathological complete response information was available. Differences in
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“activated-d16HER2 metagene” values between responders and non-responders were evaluated by unpaired t-test. Area under the ROC curve was calculated by nonparametric ROC analysis (29).
RESULTS

Pathobiological characteristics of mouse lines transgenically expressing human d16HER2 and WTHE R2

We first investigated the oncogenicity driven by d16HER2 and WTHE R2 in tg models. Kaplan-Meier disease-free survival analysis (Fig. 1A) clearly revealed the significant survival advantage (p<0.001) of WTHE R2 compared to the d16HER2 variant. Indeed, mammary tumors in tg WTHE R2 virgin females (n=40) arose after 8 months of age and progressively thereafter only in 85% of mice, whereas all d16HER2 tg virgin females (n=87) developed multiple asynchronous mammary tumors between 8 and 32 weeks of age. Tumor multiplicity (Fig. 1B) was also significantly higher in d16HER2 tg mice (p<0.001), with a mean number of 5 lesions at 30 weeks of age (n=45) versus a mean of 2 in WTHE R2 females at 60 weeks (n=39).

FISH analysis to evaluate the genetic status of HER2 in ex vivo d16HER2 and WTHE R2-positive tumor cells derived from the spontaneous tg corresponding lesions (Fig. 1C) revealed a single FISH signal on two chromosomes both in metaphase spreads and in interphase nuclei (arrows) from d16HER2-positive tumor cells (left panel), whereas in WTHE R2-positive cells (right panel), amplified signals were identified within 2-3 chromosomes (arrows). Cytogenetic analysis revealed a near-tetraploid karyotype (76-88 chromosomes) of WTHE R2-overexpressing cells compared to a diploid karyotype observed in d16HER2-positive cells. Since our tg models are heterozygous for d16HER2 and WTHE R2, these results suggest selective duplication of
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the chromosome carrying the human transgene in mammary tumor cells derived from tg mice.

Histopathological analysis of tumors showed that both strains develop mammary ductal adenocarcinomas; however, while all d16HER2 tumors (Fig. 1D) and the vast majority of WHER2 tumors (Fig. 1E) grew with a solid pattern, some WHER2 tumors showed papillary differentiation (data not shown). Moreover, while WHER2 tumors were composed of uniform cells growing with a homogeneous solid appearance (Fig. 1E), different zones were detected in d16HER2 tumors: an outer zone composed of cells with an epithelial appearance and pale cytoplasm (*)&; an intermediate zone formed of fusiform cells with darker nuclei (**); and an inner zone of cells with an epithelial appearance and pinkish cytoplasm (****) (Fig. 1D). Immunohistochemical analysis classified both tumors in the same intrinsic subtype, i.e., ErbB2-overexpressing (30), since they are E-Cadherin-positive, confirming their ductal type, and express only low levels of estrogen receptor (ER), undetectable levels of progesterone receptor (PR), high levels of the proliferation marker PCNA (>14%) and a positivity for HER2 on most tumor cells (Fig. 1D, E). Interestingly, the intensity and distribution of HER2 expression differed considerably in the two strains. WHER2 tumors showed strong and uniform membrane staining for HER2-tg protein on most tumor cells, with only a slight increase at the edges of the tumors (Fig. 1E), while in d16HER2 tumors, membrane staining for d16HER2 tg protein was especially strong on the outer zone (*), faded in the intermediate zone (***) and again well detectable in the inner zone (****) (Fig. 1D).
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Trastuzumab-mediated antitumor activity in d16HER2 and WTEGR2 pre-clinical models

To address the critical controversy regarding Trastuzumab susceptibility, we performed a series of in vivo therapeutic bioassays using Trastuzumab in d16HER2 tg mice and in FVB mice orthotopically transplanted with MI6 d16HER2-positive and WTEGR2-positive tumor cell lines (Fig. 2). Trastuzumab treatment of tg mice for either a short (Fig. 2A, B) or prolonged (Fig. 2C, D) time starting at 8 weeks of age, when only microscopic tumor lesions are present (19), led in both cases to a significant reduction in mammary tumor incidence (p=0.0038, short; p=0.0065, prolonged) and tumor multiplicity (***p=0.0004, short; ***p=0.0002, prolonged) as compared with the control groups, suggesting a clear survival advantage upon Trastuzumab treatment. In the prolonged Trastuzumab treatment, 1 out of 7 treated tg mice was completely protected until the 42th week of observation (Fig. 2C). The validity of d16HER2- (MI6) and WTEGR2-positive cancer cells grown in the m.f.p. of parental FVB females as appropriate therapeutic models, especially useful for WTEGR2-positive tumors which typically have a long latency, was confirmed by histological examination and HER2 staining of MI6 and WTEGR2 orthotopic transplants and their spontaneous tg primary tumor of origin; both the d16HER2 and WTEGR2 m.f.p. models (Fig. 2E, left) strictly recapitulated histological and immunohistochemical features of spontaneous primary mammary tumors (Fig. 2E, right), reproducing both the morphological differentiation and differences in HER2 expression. Moreover, flow cytometry to assess expression levels of d16HER2 and WTEGR2 forms in the corresponding tumor cell lines showed a lower MFI of d16HER2-
positive cells than that of WHER2-positive cells (Supplementary Fig. S1), consistent with the HER2 staining pattern (Fig. 1E).

We then tested the therapeutic activity of Trastuzumab in parental FVB females (n=10/group) orthotopically implanted with MI6 (Fig. 2F) and WHER2 (Fig. 2G) cells. Trastuzumab treatment was started when mammary tumors became palpable (~50 mm³) and continued until tumor volume reached 2000 mm³. As compared to controls (n=10/group), Trastuzumab effectively suppressed d16HER2-driven tumor growth (p<0.001) (Fig. 2F), whereas the benefits of Trastuzumab in mice with WHER2 tumors were evident but not statistically significant (Fig. 2G). Assessment of the effect of Trastuzumab treatment on metastases apart from that on the primary tumors using mice injected i.v. with 10⁵ d16HER2 or 10⁶ WHER2 tumor cells showed that Trastuzumab significantly reduced the metastatic ability of MI6 (89% inhibition) and, to a lesser extent, that of WHER2 tumor cells (75% inhibition) (Table 1). Together, these results indicate that Trastuzumab can inhibit the oncogenic properties of d16HER2-expressing mammary tumor cells.

**Signal transduction axes downstream of d16HER2 and WHER2 isoforms**

Activation of the intrinsic tyrosine kinase activity of d16HER2 was analyzed by Western blotting both under non-reducing and reducing conditions in 8 primary tumor protein extracts (Fig. 3A, B). The signaling activity downstream of WHER2 was analyzed only under reducing conditions (n=9) (Fig. 3E), since HER2 stable homodimers were never detected in the WHER2 model. Analysis consistently revealed basal d16HER2 homodimers (d16HER2D) migrating above 225 kDa, whose phosphorylation levels...
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(pd16HER2D) were particularly marked in four samples (lanes 3, 6, 7 and 8), less intense in three (lanes 1, 4 and 5) and absent in one (lane 2) of all tested tumor lysates (Fig. 3A, upper and lower panels). Constitutive basal d16HER2D expression was less abundant and more difficult to resolve than its d16HER2 monomeric counterpart (d16HER2M), while both d16HER2M and D were always significantly activated within the same tumor sample (Fig. 3A, lanes 3, 6, 7 and 8). This scenario confirms our previous findings (19) and demonstrates that stable d16HER2D is constitutively and heterogeneously activated in d16HER2-positive lesions. Analysis of cell signaling downstream of d16HER2 and WHER2 receptors, evaluated under reducing conditions (Fig. 3B and E), revealed that phosphotyrosines of pd16HER2M and activated WHER2 (pWHER2) act as docking sites for proteins initiating signals that are transduced to the nucleus through different circuitries, including the mitogen-activated protein kinases (MAPK), AKT, SRC and STAT3. However, the activation levels of d16HER2 and WHER2 signal transduction pathways differed, with higher levels of pd16HER2D and pd16HER2M always significantly coupled to elevated pSRC levels (Fig. 3A-D). This finding strongly suggests the existence of a pd16HER2D-pSRC signaling axis that amplifies d16HER2-driven oncogenic signals, consistent with a significant direct correlation between pd16HER2D and pSRC ($r=0.8787$, $p=0.0041$) (Fig. 3C) and between pd16HER2M and pSRC ($r=0.8199$, $p=0.0127$) (Fig. 3D). Note that despite very high-level expression of native SRC kinase only in WHER2-positive tumors in all samples, SRC was activated in only 6 out of 9 cases independent of pWHER2 status (Fig. 3E), such that no direct significant correlation between pWHER2 and pSRC was apparent in the WT model (Fig. 3F).
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To test whether the signal in the d16HER2 model is orchestrated mainly by downstream pSRC, we examined MI6 cells treated or not at different times with anti-HER2 ECD MAbs MGR2 and 4D5 and solubilized under non-reducing and reducing conditions; Western blotting showed that activation levels of d16HER2D, d16HER2M and SRC in treated cells decreased in parallel in the same time frame compared to untreated cells, whereas the phosphorylation status of MAPK and AKT was kinetically less dependent on the downmodulation of pd16HER2D, pd16HER2M and pSRC levels (Fig. 3G, H). Consistent with our biochemical analyses indicating a significant functional direct interaction only between d16HER2 and pSRC (Fig. 3) and with in vitro data demonstrating a physical interaction between d16HER2 and pSRC (10), the tumor cell membrane in both d16HER2-positive primary lesions and lung metastases co-expressed d16HER2 and pSRC, as indicated by IHC, immunofluorescence and confocal microscopy analyses (Fig. 4).

**Correlation of d16HER2 and SRC activity with Trastuzumab-mediated clinical efficacy**

To evaluate the potential association of d16HER2 and pSRC in the human setting and to test whether patient outcome after Trastuzumab treatment might be influenced by d16HER2 signaling through pSRC activity, we examined a retrospective series of 84 primary human HER2-positive cases treated adjuvantly with Trastuzumab (see Supplementary Table 1 for BC patient pathobiological and clinical characteristics). Evaluation of pSRC expression in formalin-fixed, paraffin-embedded BC sections by confocal microscopy (Fig. 5A, B) revealed high pSRC positivity (>20%) in 34 of 84
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tumors (Fig. 5B), while the remaining 50 BCs expressed pSRC levels ranging from 0 to <20% (Fig. 5A, left and right panel, respectively). qRT-PCR analysis of 43 of the 84 available frozen BC samples for d16HER2 transcript levels, scored as low or high, revealed a significant association between pSRC (%) and d16HER2 (p=0.0482) expression (Fig. 5C). Moreover, tumors with pSRC (%) >0 showed a significant direct correlation between d16HER2 transcript and pSRC expression (r=0.6880, p=0.0016) (Fig. 5D), strongly suggesting that the presence of active d16HER2D in primary HER2-positive BCs is reflected by high SRC activation. Finally, BC patients with tumors expressing high d16HER2 and pSRC levels exhibited a lower relapse rate after Trastuzumab treatment than did d16HER2-high/pSRC-low or d16HER2-/pSRC-low patient subgroups (1/12 vs 9/31). In light of these results, we revisited the entire 84 case series, in which 34 showed high pSRC positivity (>20%); while no differences in clinical-pathobiological parameters were found between high- and low-pSRC-expressing tumors (Supplementary Table 2), the relapse-free survival of patients with a high pSRC score in their primary tumors showed a significantly lower progressive disease rate after Trastuzumab treatment than those with a low pSRC score (HR=0.28, 95% CI=0.09-0.83, p=0.022) (Fig. 5E), suggesting that high pSRC levels in early tumors predicts benefit from Trastuzumab-containing treatment.

To further investigate whether patients with high d16HER2 transcript/signaling are those more sensitive to Trastuzumab-mediated HER2 blocking, we generated an “activated-d16HER2 signature” by comparing gene expression profiles of 21 of the 43 qRT-PCR-tested BC cases according to d16HER2 and pSRC expression. Tumors expressing d16HER2 and pSRC-high were significantly enriched in hypoxia, tumor metastasis and
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cell motility pathways in GSEA analysis (Supplementary Fig. S2). Moreover, a metagene consisting of 73 leading genes (Supplementary Table 3) in the enrichment of these pathways discriminated, with good performance, cases with active d16HER2 (“activated-d16HER2 metagene”) (AUC=0.94, 95%CI=0.83-1.04, p=0.0039) (Fig. 6A). *In silico* analysis of “activated-d16HER2 metagene” expression in two datasets, GSE22358 (27) and GSE41656 (28), of HER2-positive BC patients treated or not with Trastuzumab-based neoadjuvant therapy showed significantly higher expression of this metagene (p=0.0305) in patients who achieved a complete or near-complete response to Trastuzumab than in partial responders (Fig. 6B), with a good performance prediction (Fig. 6C). By contrast, responders and non-responders to neoadjuvant therapy consisting of chemotherapy alone revealed no difference in the “activated-d16HER2 metagene” expression level (Fig. 6D), strongly suggesting that human BCs with high d16HER2 signaling benefit significantly from the addition of Trastuzumab to chemotherapy treatment.
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DISCUSSION

In this study, we provide evidence that d16HER2 variant constitutes a more aggressive HER2 isoform susceptible to Trastuzumab treatment. The significantly shorter latency and the consistently higher tumor multiplicity in the d16HER2 tg line as compared to the WThER2 tg line imply that genetic changes in addition to WT gene amplification are required for mammary tumorigenesis. Moreover, cytogenetic and FISH analyses of ex vivo d16HER2 tg tumor cells revealed a diploid karyotype and a single signal in two chromosomes, while ex vivo WThER2 tg cancer cells showed a marked aneuploidy and amplified signals on 2-3 chromosomes, supporting the notion of a “firestorm” genomic pattern (31) needed to drive WThER2-associated mammary tumorigenesis.

About 90% of women with HER2-positive BC and locally disseminated disease have been reported to co-express the oncogenic d16HER2 isoform (9, 10). The co-existence of d16HER2 with the other two naturally occurring HER2 splice variants, herstatin and p100, with contrasting roles in tumor cell biology (32), with truncated HER2 isoforms (33) and with HER2 somatic mutations (7) greatly contributes in complicating the HER2-derived proteome and increasing the heterogeneity of HER2-positive disease. In this context, it is important to note that if all the described HER2 forms are driver events, then HER2-positive BCs patients might benefit clinically from existing HER2-targeted drugs, although this seems unlikely (34).

It has remained unclear whether d16HER2 is sensitive to Trastuzumab treatment (9, 10, 20, 35) and whether d16HER2 represents a mechanism of resistance to Trastuzumab in patients with HER2-overexpressing BC (36, 37). In keeping with a pilot study of
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immunodeficient mice injected in the mammary gland with a d16HER2-positive transfectant (20), we found that spontaneous tumor development in d16HER2 tg mice was significantly impaired by Trastuzumab administered as monotherapy and that prolonged treatment was even curative in one d16HER2-positive female observed until 42 weeks of age. Moreover, tumors formed after m.f.p. injections of MI6 and WHER2 cells showed marked benefit of Trastuzumab treatment only in d16HER2-positive tumors, whereas WT tumors benefited only moderately. Also, the anti-metastatic effects of Trastuzumab on experimental lung metastases induced by MI6 and WHER2-positive cells were more consistent in the d16HER2 model, indicating that only d16HER2-driven tumor growth and aggressiveness remain highly dependent on oncogenic signaling pathways directed by and downstream of pd16HER2D. These in vivo data are consistent with implications of an in vitro study reporting that Trastuzumab is preferentially active against tumors driven predominantly by HER2 homodimer-induced signaling (38).

The d16HER2 variant, which appears to stabilize HER2 homodimer expression and activation, activates multi-signaling cascades (10, 19, 20), including consistent phosphorylation of SRC kinase (10, 19). SRC is the prototypic member of a non-receptor tyrosine kinase family with broadly pleiotropic effects on mammalian cells, including effects on cell morphology, adhesion, angiogenesis, migration, invasiveness, proliferation, differentiation and survival (39). Aberrant expression and activation of SRC occurs in several tumor types and has been correlated with poor outcome; SRC is also a potent mediator of many downstream effects of both HER1 and HER2 (39). Additionally, SRC is a reportedly common node downstream of multiple resistance
Activated d16HER2/SRC axis predicts Trastuzumab benefit pathways and a driver of Trastuzumab resistance, since it is hyperactivated in various Trastuzumab-resistance cell models (40). While Mitra and coauthors (10) speculated that in d16HER2-positive transfectants, SRC kinase might act as a “master regulator” of the spliced isoform, stabilizing its expression and coupling to mitogenic and cell motility pathways and contributing to Trastuzumab resistance, we found that high levels of pd16HER2D and M, and not pWHER2, were directly linked to marked SRC activity and that \textit{in vivo} d16HER2-driven tumorigenicity was significantly halted by Trastuzumab treatment. We also found a consistent decrease in SRC activity upon knockdown of activated d16HER2D and M with the anti-HER2 MAb MGR2 and with MAb 4D5, the murine precursor of Trastuzumab, strongly suggesting that the pd16HER2D/pSRC signaling axis is particularly sensitive to Trastuzumab administration. Additional evidence for a functional cross-talk between pd16HER2D and pSRC came from both IHC and confocal microscopy analyses indicating that such molecules are co-expressed at high levels at the cell surface by the same tumor cells in either primary mammary lesions or lung metastases of the d16HER2 tg line. Overall, our preclinical findings suggest that intense co-expression of the d16HER2 variant and pSRC at the tumor cell membrane reflects pd16HER2D-driven signaling.

In light of our previous speculation that the proportion and relevance of d16HER2 in HER2-positive BCs might redefine its role in sensitivity/resistance to Trastuzumab and can have an impact on current therapeutic strategies (32), we sought clinical verification of our pre-clinical data by examining tissue from 84 HER2-positive BCs treated with adjuvant Trastuzumab (41). In 43 out of 84 BC specimens for which frozen samples were available for d16HER2 qRT-PCR analysis, 12 out of 13 high-pSRC-expressing
primary tumors expressed elevated levels of d16HER2 transcript, strongly suggesting that pSRC reflects activated d16HER2 homodimers in human HER2-positive BCs. Indeed, such tumors are enriched in “tumor metastasis”, “hypoxia” and “cell motility” pathways, all features of aggressiveness revealed in the d16HER2 tg model. Thus, the better prognosis observed in the Trastuzumab-treated HER2-positive BC patients with elevated pSRC could be a direct consequence of the expression on their tumors of an activated d16HER2/SRC signaling axis, as observed in the Trastuzumab-sensitive d16HER2-driven mouse model. Indeed, in silico analyses to better define the high-pd16HER2/pSRC tumor profile indicated an “activated-d16HER2 metagene” that was expressed at significantly higher levels in tumors completely responsive to neoadjuvant Trastuzumab-based therapy as compared to those only partially responsive, whereas “activated-d16HER2 metagene” expression levels did not differ between complete and partial responders to neoadjuvant chemotherapy alone.

Our findings appear to contrast directly with those of Zhang et al. (38), who reported a lower clinical response rate and a higher progressive disease rate after Trastuzumab treatment in HER2-positive BC patients with high pSRC expression; however, it should be noted that their series consisted of 57 BC patients who received first-line Trastuzumab-based therapy in a metastatic setting, whereas our series includes BC patients treated with Trastuzumab-based regimens in an adjuvant setting (41). We speculate that while HER2-positive primary BCs expressing high levels of pSRC are initially dependent on HER2 and all its potential driver isoforms and are thus responsive to Trastuzumab, the progression of such BCs due to a high HER2-dependent growth...
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rate might lead to accumulation of genetic alterations that result in less HER2 dependency and, in turn, significantly less or even no responsiveness to Trastuzumab. Such a hypothesis would reconcile the contrasting findings of Zhang, et al. (40) with our own, since HER2 signaling-dependent tumors benefitting from Trastuzumab at an early stage may just be those that, if not treated early, gain additional dependencies in a metastatic setting to allow escape from Trastuzumab therapeutic effects. Together, our findings indicate the need for further evaluation of the role of pSRC in primary and advanced HER2-positive disease before clinical decision-making. While the relatively small size of our HER2 patient samples precluded analysis of whether BC patients with high d16HER2/low pSRC transcript levels might express inactive d16HER2 homodimers due to a failure to couple with pSRC, and despite a lack of a specific anti-pd16HER2D reagent, our present pre-clinical, clinical and in silico data support the notion that activated HER2 signaling is indicative of benefits from the addition of Trastuzumab to chemotherapy and that d16HER2 expression is not a reliable indicator of Trastuzumab resistance but instead a mirror of pSRC activity, reflecting d16HER2 homodimer-mediated driver activity leading to high responsiveness to Trastuzumab. These data might shed light on the very complex “HER2 world” and help clinicians identifying the “real” HER2 drivers for targeting by appropriate pharmacological strategies.
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References


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Table 1. Effect of Trastuzumab treatment on experimental lung metastasis after i.v. injection of MI6 and WHER2-positive tumor cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MI6</th>
<th>WHER2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incidence</td>
<td>Median</td>
</tr>
<tr>
<td>Control</td>
<td>8/8</td>
<td>211</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>7/8</td>
<td>^a24***</td>
</tr>
</tbody>
</table>

^a *p<0.05 and ***p<0.001 by Wilcoxon-Mann-Whitney test in Trastuzumab vs control mouse groups.
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FIGURE LEGENDS

Figure 1. Pathobiological characteristics of mouse lines transgenically expressing human d16HER2 and Wther2 receptors. A, Tumor-free survival of d16HER2 and Wther2 tg mice. P-value by log rank test. B, Mean number of palpable mammary carcinomas developed in d16HER2 (▼) and Wther2 (●) tg lines. Data are mean ± SEM. ***p<0.001 by unpaired t-test. C, FISH analysis of a metaphase spread and an interphase nucleus obtained from ex vivo d16HER2 (left panel) and on a metaphase spread of WT (right panel) HER2-positive mammary tumor cells derived from the corresponding spontaneous tumor. D, E, Histological and immunohistochemical analysis of primary tumors from d16HER2 and Wther2 tg mice, respectively. Hematoxylin and eosin (H&E) staining showed three zones in d16HER2 tumors: an outer zone (pale cells, ★), an intermediate zone (darker fusiform cells, ★★), and an inner zone (pinkish cytoplasm, ★★★), as compared to a homogeneously solid and uniform appearance of Wther2 tumors. Magnification x200. Consistent with the histological appearance, E-cadherin (E-cadh) and HER2 positivity were more marked in the outer and inner zones in d16HER2 tumors and uniformly and strongly positive in Wther2 tumors. Proliferative activity, indicated by PCNA positivity, was similar in the two tumors. Estrogen and progesterone receptor staining was negative in both tumors.

Figure 2. Trastuzumab-mediated antitumor activity in d16HER2 pre-clinical models. A, Tumor-free survival and B, tumor multiplicity of tg d16HER2 mice treated with a short Trastuzumab protocol (▲, 8 mg/kg i.p. once weekly for 5 weeks) and diluent saline solution (●, 150 μl, i.p once weekly for 5 weeks). C, Tumor-free survival and D, tumor
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multiplicity of tg d16HER2 mice treated with a prolonged Trastuzumab protocol (▲, 4 mg/kg i.p twice weekly until sacrifice) and diluent saline solution (●, 150 μl, i.p twice weekly until sacrifice). Short and prolonged treatments started when mice were 8 weeks of age. Data are mean ± SEM. Differences were assessed by log-rank test (A and C) and by unpaired t-test (B, ***p=0.0004 and D, ***p=0.0002). E, H&E and HER2 staining of MI6 (upper panel) and WT (lower panel) HER2 tumor cells injected in the m.f.p. of parental FVB females (left panel) and of their spontaneous tg primary mammary tumor of origin (right panel). F, G, Trastuzumab-mediated antitumor activity in parental FVB mice following orthotopic injection of MI6 and WT HER2-positive tumor cells, respectively. Tumor-bearing mice were treated i.p. with Trastuzumab (▲, 4 mg/kg twice weekly until sacrifice) and diluent saline solution (●, 150 μl, twice weekly until sacrifice) in the presence of evident disease. Data are mean ± SEM. ***p<0.001 by unpaired t-test.

Figure 3. Western blotting analyses of the signal transduction axis downstream of d16HER2 and WHER2 forms. A, Protein extracts from d16HER2 specimens (n=8) were separated by 3-8% gradient SDS-PAGE under non-reducing conditions and probed with anti-HER2 (d16HER2M and D, upper panel) and anti-phosphoHER2 (pd16HER2M and D, lower panel) antibodies. B, The same protein extracts were separated by 4-12% gradient SDS-PAGE under reducing conditions to evaluate the basal and activation status (p) of d16HER2M, SRC, STAT3, AKT and MAPK. Actin was used to normalize protein loading. C, Linear regression analysis of pd16HER2D vs pSRC in the d16HER2 protein extracts (see Methods) analyzed in panels A and B. D, Linear regression analysis of pd16HER2M vs pSRC in the d16HER2 protein extracts.
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(see Methods) analyzed in panel B. E, Protein extracts from WHER2 specimens (n=9) were separated by 4-12% gradient SDS-PAGE under reducing conditions to evaluate the basal and activation status (p) of WHER2, SRC, STAT3, AKT and MAPK. Vinculin was used to normalize protein loading. Autoradiographs of panels B and E were acquired at different exposure times to obtain optimal image resolution. F, Linear regression analysis of pWHER2 vs pSRC in WHER2 protein extracts (see Methods) analyzed in panel E. G, MI6 protein extracts from cells treated with the anti-HER2/ECD MAbs MGR2 and 4D5 for different times (5 min, 30 min, 4 h and 24 h) were separated by 3-8% gradient SDS-PAGE under non-reducing conditions and probed with anti-HER2 (d16HER2M and D) and anti-phosphoHER2 (pd16HER2M and D) antibodies. H, The same protein extracts as in G were separated by 4-12% gradient SDS-PAGE under reducing conditions to evaluate the basal and activation status (p) of d16HER2M, SRC, AKT and MAPK. Actin was used to normalize protein loading.

**Figure 4.** Immunohistochemical and immunofluorescence analyses of pSRC and HER2 expression in primary tumor and lung metastasis from a tg d16HER2 mouse. Immunohistochemistry showed pSRC and HER2 expression in the same zones. Confocal microscopy revealed colocalization of the two proteins on mammary tumor cell membranes.

**Figure 5.** Expression and coexpression of HER2 and pSRC markers and association between d16HER2 transcript and pSRC expression and risk of relapse in human HER2-overexpressing BCs patients treated with Trastuzumab. A, B, Representative immunofluorescence images of human BC tissues were evaluated by confocal microscopy and classified according to low (A) and high (B) pSRC scores. pSRC
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(green) and HER2 (red) staining indicate BC cells. Nuclei were counterstained with DRAQ5 (blue). C, Association between d16HER2 transcript levels measured by qRT-PCR and pSRC (%) expression in 43 human HER2-overexpressing BCs. *p=0.0482 by unpaired t-test. D, Pearson correlation between pSRC (%) and d16HER2 transcript expression levels in 18 qRT-PCR-tested cases positive for pSRC (>0). E, Association between pSRC levels (low <20%; high ≥20%) with relapse-free survival in 84 HER2-positive BC patients treated with Trastuzumab.

**Figure 6.** A, Receiver-operator characteristics (ROC) curve for expression of “activated-d16HER2 metagene” in d16HER2-high, pSRC-high tumors. B, Association between “activated-d16HER2 metagene” expression and response to Trastuzumab-based neoadjuvant therapy in the GSE22358 dataset. *p=0.0305 by unpaired t-test. C, ROC curve of Trastuzumab response prediction using “activated-d16HER2 metagene” in the GSE22358 dataset. D, Association between “activated-d16HER2 metagene” expression and response to neoadjuvant chemotherapy in the GSE41656 dataset. CR: complete response; PR: partial response.
Figure 4

pSRC HER2 pSRC-HER2 Colocalization

d16HER2 primary tumor

d16HER2 lung metastasis
Figure 5

A. pSRC < 20%
B. pSRC > 20%

C. pSRC (%)

D. pSRC (%)

E. Relapse-free survival (%)

No. at risk
- pSRC high: 34, 32, 24, 10, 4
- pSRC low: 50, 42, 25, 15, 4
Figure 6

A

B

Trastuzumab-based neoadjuvant therapy

C

D

Neoadjuvant chemotherapy

Activated-dHER2 metageno

CR

PR

Activated-dHER2 metageno

CR

PR

AUC=0.94
p=0.0039

AUC=0.77
p=0.0174
Activated d16HER2 homodimers and Src kinase mediate optimal efficacy for trastuzumab.

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