Characterisation of HER Heterodimers in Breast Cancer Using in situ Proximity Ligation Assay

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Abstract

Introduction: HER2 plays an important role in breast cancer progression and provides predictive and prognostic information. HER2 receptor family members function through dimerisation, which can lead to impact on cell function, growth and differentiation; however their value in breast cancer development remains to be defined. This study aims to examine the relationships of HER2 heterodimers to breast cancer characteristics in trastuzumab naïve and treated cases.

Methods: HER2 protein (IHC), HER2 gene (chromogenic ISH) and HER2 heterodimerisation status (chromogenic in situ proximity ligation assay; PLA) was assessed in two breast cancer series prepared in tissue microarray (TMA) format.

Results:

A range of signals/cell for each HER2 heterodimer was detected (0 – 34.6 signals/ cell). The vast majority of cases with HER2 heterodimers showed HER2 gene amplification and/or protein expression. There was an association between HER2 dimerisation with HER3 and HER4 and their protein expression level but no such association was found in with HER1 (EGFR). Of the HER2+ cases, 74%, 66% and 58% showed heterodimers with EGFR, HER3 and HER4 respectively. 51% of HER2+ tumours expressed all three heterodimers whereas 23% of the cases did not show expression of any of the three heterodimers. There was an inverse association between the presence and levels of HER2 heterodimers and hormone receptor expression in HER2+ tumours. Tumours exhibiting high levels of HER2 heterodimers demonstrated aggressive clinicopathological features and poor outcome. In the HER2+ cases, dimerisation with EGFR and HER3 but not with HER4 showed an association with aggressive features. There was no association between HER2 heterodimers with patient breast cancer specific survival or recurrence in HER2+ breast cancer in those patients receiving trastuzumab or not.

Conclusions: Our results demonstrate that HER2 dimerisation is a complex process that may underlie the biological heterogeneity of HER2 positive tumours and may identify patients suitable for a specific targeted therapy but does not predict patient outcome for those receiving trastuzumab. PLA proved to be a useful tool for detecting, visualising and quantifying the frequency of protein-protein interactions in archival formalin fixed paraffin embedded tissue samples.

Keywords: HER2, PLA, Heterodimer, Breast Cancer, Trastuzumab

Introduction

Breast carcinomas are heterogeneous in terms of different histological characteristics and genetic and molecular variations, which control patient prognosis and tumour performance [1,2]. Human Epidermal Growth Factor Receptor (HER)2 (neu or c-erbB-2) overexpression is related with higher level of metastasis development and are amongst those with the worst prognosis [3] representing 10%-23% of breast tumours [4-6]. The HER family is composed of four different types: HER1 (EGFR or c-erbB-1), HER2, HER3 (c-erbB-3) and HER4 (c-erbB-4) [5]. These compile the type I group of the Receptor Tyrosine Kinases (RTKs) and regulate several cellular metabolic reactions [7,8]. RTKs can function as controllers of cellular progress, however they may influence the development and improvement of different types of carcinoma [9].

All members of the HER family, apart from HER2, are activated by a group of transmembrane precursor protein molecules, which possess a conserved epidermal growth factor (EGF)-like domain [10]. Each HER member has specific ligands that they interact with and this ligand-ectodomain interaction encourages either homo- or hetero-dimerisation [11]. HER2 does not interact with any ligand and therefore its conformation arm is constantly predisposed to exist in a competent form and ready to connect with any of the other monomers from the same family [12], HER2 the being preferred receptor for dimerisation incidence [13,14].

HER2 positive breast cancer patients are submitted to targeted therapies that include trastuzumab (Herceptin™; Genentech), which disrupts its signalling mechanisms. Only 35% of patients with HER2 metastatic breast cancer demonstrate a response when submitted to trastuzumab [15]. Nonetheless, when the therapeutic antibody is

applied with first-line chemotherapy, the levels of success can increase up to 84% [16,17]. Therefore alternative mechanisms that are not disturbed by trastuzumab might be implicated in carcinoma expansion [18]. Trastuzumab might be effective at initial treatments, however after a period of time acquired resistance increases substantially. Our understanding of these resistance mechanisms remain unclear although several have been proposed (reviewed in ref [19]). The semi-quantitative methods for determination of HER2, such as immunohistochemistry (IHC), might not be adequate and/or sufficient to predict high levels of therapy success.

Moreover, the majority of preceding studies on the HER family in breast cancer have paid attention on either individual expression of the receptor monomers or their co-expression, describing the association with the different clinicopathological parameters [20]. In breast cancer, both EGFR and HER2 expression are highly associated with poor outcome characteristics [21-24] and with negative oestrogen receptor (ER) status [21]. The co-expression of two or more receptors indicate an even more unfavourable outcome [23-26]. Accurate quantification of protein expression and other biological characteristics, such as HER heterodimerisation, could be important to elucidate and predict patient outcome, particularly with HER targeted therapies. The action of these molecules is controlled by a complex system that includes structures modifications or connections with other molecules such as ligands; the consequence heterodimer arrangements will trigger different pathways and consequently different outcomes [27,28]. Thus, quantification of HER heterodimerisation status might be important to elucidate their roles for detailed biological outcome. Therefore this study was aimed at investigating the co-expression and dimerisation of the HER family in HER2+ breast cancer in order to understand their role in the disease.

Material and Methods

Patient samples

Primary breast cancer series (Unselected Series)

The patient series comprised 1,858 unselected primary operable invasive breast carcinoma cases, presenting between 1986-1998, from the well-characterised Nottingham-Tenovus Primary Breast Carcinoma Series. [29-31]. Amongst all patients, 1,256 (67.6%) were 50 years or over at presentation. A total of 354 (19.1%) tumours were grade I, 616 (33.3%) grade II and 880 (47.6%) grade III. A total of 1,367 (73.9%) of patients had tumours of 1.5cm or larger in size. Additionally 1,187 (64.1%) cases revealed tumour stage N0, 505 (27.3%) stage N1 and 159 (8.6%) stage N2.

Biological characterisation, including immunoreactivity, marking and categorising of ER, progesterone receptor (PgR), and Triple Negative Phenotype (TN) were delineated in this series as previously described [25,26,32,33].

HER2+ adjuvant trastuzumab series

The HER2+ adjuvant trastuzumab series consisted of 143 primary operable breast tumours from patients presenting between 2003 and 2010 who received adjuvant trastuzumab. A total of 79 (56.4%) patients were aged over or equal to 50 years, and 61 (43.6%) were less than 50 years old. The age of patients at presentation ranged between 31-79 years (median and mean of 52 years). At primary diagnosis, 3

(2.1%) tumours were grade 1, 37 (25.9%) grade 2, and 103 (72.0%) cases were grade 3. A total of 120 (83.9%) patients had tumours 1.5cm or larger. Relapse occurred in 23 (16.1%) cases, distant metastasis in 17 (12.1%) cases, and 10 (7.0%) patients died from breast cancer. Local recurrence occurred in 7 (4.9%) of cases and regional recurrence in 1 (0.8%) case.

Immunohistochemistry and Chromogenic in situ Hybridisation

TMAs were prepared as previously described [25]. Immunohistochemistry was performed using Novolink Polymer Detection Systems (Leica). The primary antibodies were used to detect HER2 (Dako, 1:400), EGFR (clone 31G7, Invitrogen, 1:30), HER3 (clone RTJ1, Leica, 1:30), HER4 (Rabbit polyclonal, Thermo Scientific, 1:100), ER (clone SP1, Dako, 1:50) and PgR (clone PgR 636, Dako, 1:125) for 30 minutes incubation. TMA sections were counterstained with Mayer's haematoxylin. Immunoreactivity of HER2 in TMA cores was scored using standard HercepTest guidelines (Dako). Chromogenic in situ Hybridisation (CISH) was used to quantify HER2 gene amplification using the HER2 FISH pharmDx™ plus HER2 CISH pharmDx™ kit (Dako) as previously described [26]. HER2 classification was assessed by using American Society of Clinical Oncology guidelines as previously described [26].

The expression of ER, PgR, HER3, HER4 and EGFR were evaluated by assessing percentage staining [34]. Only invasive cancer cells localised within tissue cores were considered and only cores exhibiting at least 15% of tumour cells were scored. TMAs were scored using high-resolution digital images (NanoZoomer; Hamamatsu Photonics), at x20 magnification, using a web-based interface (Distiller; Slidepath Ltd).

in situ Proximity Ligation Assay (PLA)

Quantification of HER heterodimers was measured using in situ PLA for brightfield microscope as per the manufacturer's instructions (Duolink kit, Olink). 4 µm TMA sections were mounted on X-tra™ adhesive micro slide (Surgipath, Leica). Deparaffinisation was performed and heat induced antigen retrieval was executed for 20 min in citrate buffer (pH 6.0). Endogenous peroxidase was quenched using 0.3% hydrogen peroxide for 5 min and followed by a blocking solution for 30 min at 37˚C. To detect heterodimers, target antibodies from two different species were applied at previously determined optimal conditions. The anti-HER2 rabbit antibody (Dako, 1:200) was used for all PLA detections. For HER2/HER3 heterodimer detection, anti-HER3 mouse antibody (clone 2F12, Neomarkers, 1:40) was used, and incubated with the HER2 antibody for 30 min at room temperature (RT). For HER2/EGFR heterodimer detection, the anti-EGFR mouse antibody (clone EGFR-R2, Santa Cruz, 1:10) and for HER2/HER4 interaction the anti-HER4 antibody mouse (clone HFR1, Abcam Ltd, 1:50) were used respectively. Both the HER2/EGFR and HER2/HER4 antibodies were incubated for 60 min at RT. This was followed by incubation with the PLA-probe in a pre-heated humidity chamber for 90 min at 37˚C. Hybridisation/ ligation incubation took place for 30 min and amplification for 120 min at 37˚C. To detect hybridisation, Horse Radish Peroxidase was used and incubated for 30 min at RT followed by appliance of substrate solution for 10 min at RT. Counterstaining was performed using Duolink® nuclear staining for 2 min at RT followed by washing the slides under running tap water for 10 min. Slides were mounted with a coverslip after dehydration of the sections.

Image Analysis

To quantify the HER heterodimerisation, image analysis was employed using Duolink® ImageTool (Olink, Sweden). High-resolution images of TMA sections were acquired at x40 magnification (NanoZoomer).

One observer (FFTB) scored all IHC and PLA results, which were rescored arbitrarily revealing a high concordance between both occasions. Furthermore the PLA procedure was performed three times on HER2+ cases (n=143) revealing a good correlation amongst the three experiments (kappa value, 0.735).

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, Illinois). Pearson's χ^2 association analysis was employed in support of inter-relationships between dimerisation occurrence status with clinicopathological parameters, and biomarkers including HER2 gene amplification. For any relationship to be considered significant a p-value of <0.05 was used.

Ethics

Nottingham Research Ethics Committee 2 approved this research project under the title of "Development of a molecular genetics classification of breast cancer".

RESULTS

HER2 status in the Unselected series

Regarding HER2 protein expression, 1,604 (86.3%) of patients in the Unselected series were negative, 74 (4.0%) were 2+ equivocal faction and 180 (9.7%) were 3+ positive. HER2 gene amplification was observed in 160 (14.4%) patients resulting in a total of 224 (12.1%) HER2+ cases.

HER heterodimer frequency in the Unselected series

The unselected primary breast cancer cases revealed a range of signals/cell for each heterodimer which were predominantly detected on the membrane of tumour cells: HER2/EGFR (0–20.2 signals/cell), HER2/HER3 (0–34.6 signals/cell) and HER2/HER4

(0–17.2 signals/cell). The cut-off point to define the heterodimer status was determined using X-tile [35] which dichotomised data into low and high levels of heterodimerisation. It was therefore considered that low levels of dimers were in those cases showing less than 1.0 HER2/EGFR signals per cell, less than 4.5 HER2/HER3 signals per cell and less than 3.4 HER2/HER4 signals per cell (Figure 1A, B and C, respectively). Any incidence above these limits were accepted as positive levels of heterodimerisation (Figure 1D, E and F respectively).

The incidence of HER2/EGFR heterodimers in the Unselected series revealed positive levels of HER2/EGFR heterodimers in 104/812 (12.8%) cases (Table 1). Similar levels of the HER2/HER3 (97/743, 13.1%) and HER2/HER4 (96/891, 10.8%) heterodimers were observed. There were significant positive associations between the incidences of all HER heterodimers (Tables 2A-B, all $p<0.001$).

Association of HER heterodimers and biomarkers expression in the Unselected series

As predicted, there were strong positive correlations between all three HER heterodimers and HER2 status (all $p<0.001$, Table 3A). As a consequence, the heterodimers were associated with poor clinicopathological parameters such as high lymph node stage, tumour grade, NPI and a significantly higher risk of recurrence (Supplementary Table 1). A total of 81/96 (84.4%) breast cases showing HER2/EGFR heterodimers were HER2+. Of the 15 cases that showed HER2/EGFR dimers but not HER2 over-expression, the majority (11/15, 73%) had very low levels of heterodimers (<2.4 signals/cell). Two cases had HER2 protein expression, regarded as 2+ determined by IHC but did not show gene amplification in CISH. The remaining two cases had high levels of HER2/EGFR dimers but no over-expression of HER2. There were a proportion of HER2+ cases (27/108, 25%) that did not express

HER2/EGFR heterodimers. In terms of the presence of HER2/HER3 heterodimers, a significant proportion of cases showing HER2/HER3 heterodimers were HER2+ (79/89, 89%). Of those remaining, 7/10 cases had relatively low levels of heterodimers as detected by PLA (<8.9 signals/cell) and 2/10 cases had increased HER2 protein expression (2+) but showed no gene amplification by CISH. There were a number of HER2+ cases, 33/112 (30%) that did not show dimerisation of HER2/HER3. A similar observation was made with the HER2/HER4 heterodimers where 76/86 (88%) of cases with these heterodimers were HER2+. The majority of remaining cases had low levels of heterodimers (6/10 cases; <4.7 signals/cell) or showed overexpression of HER2 protein but not amplification of the gene. A total of 62/138 (45%) HER2+ cases did not show HER2/HER4 heterodimers.
Interestingly, 59/89 (66%) cases with HER2/EGFR heterodimers did not express EGFR protein (p=0.002). In contrast, the majority of tumours (71/78, 91%) showing HER2/HER3 heterodimers expressed HER3 protein. Likewise, all but 5 cases (6.3%) that were positive for HER2/HER4 heterodimers were positive for the HER4 protein (p=0.004). All HER heterodimers were significantly correlated with ER and PgR negativity and Triple Negative tumours (Table 3A, all p<0.001).

Association of HER heterodimers and HER2 gene amplification in the Unselected series

There was significant correlation between HER2 gene amplification with the number of detected signals of A) HER2/EGFR (r^2=0.381, p<0.001), B) HER2/HER3 (r^2=0.429, p<0.001) and C) HER2/HER4 (r^2=0.377, p<0.001) as shown in Figures 2 A, B and C However some cases harbouring HER2 gene amplification did not reveal heterodimerisation of HERs.

HER heterodimer frequency in HER2+ breast cancer

In HER2+ breast cancer, 168/229 (73.4%) of cases showed HER2/EGFR heterodimers, 149/226 (65.9%) had high levels of HER2/HER3 heterodimers and approximately half of the cases (131/243, 53.9%) had HER2/HER4 heterodimers (Table 1). Similar to the Unselected series, the presence of all three heterodimers were significantly associated with each other (Table 3B, $p<0.001$). A total of 73/142 (51.4%) HER2+ tumours expressed all three heterodimers (Table 4) whereas only 32/142 (22.5%) cases did not show expression of any of the heterodimers investigated. Expression of only one heterodimer or two heterodimers were less commonly observed.

Association of HER heterodimers and clinicopathological parameters in HER2+ breast cancer

Correlations between the different HER2 heterodimers and clinicopathological parameters are summarised in Table 5. Both HER2/EGFR and HER2/HER3 heterodimers were associated with high tumour grade ($p=0.006$ and $p=0.017$, respectively). The presence of HER2/EGFR heterodimers was also significantly associated with a poor NPI score ($p=0.048$). The HER2/HER3 interaction was differentiated from the other HER family interactions by an association with development of distant metastases ($p=0.039$). Finally, HER2/HER4 dimerisation incidence was not associated with any of the clinicopathological parameters investigated.

Association of HER heterodimers and biomarker expression in HER2+ breast cancer

Table 3B summarises the association of the different HER family heterodimers against the protein expression of the HER family (EGFR, HER2, HER3 and HER4), as detected by IHC, and the hormone receptors ER and PgR. Similar to that observed in

the Unselected series, the expression of the HER2/EGFR heterodimer in the HER2+ series was independent of EGFR expression where approximately half of the HER2/ EGFR heterodimer positive cases were negative for EGFR protein. In contrast, only 6/121 (5.0%) and 2/108 (1.9%) cases positive for the HER2/HER3 and HER2/HER4 heterodimers showed negative expression of HER3 and HER4 respectively.

There was a significant association of HER2/EGFR, HER2/HER3 and HER2/HER4 heterodimers with negative ER ($p=0.002$, $P<0.001$ and $p<0.001$, respectively) and PgR status ($p=0.016$, $P=0.001$ and $p<0.001$, respectively). HER2/HER3 heterodimers were not associated with either ER or PgR.

HER2/HER3 was the only heterodimerisation not highly correlated with Ki67 expression, the nuclear protein severely correlated with ribosomal RNA transcription and is expressed mostly in proliferative cells [36,37].

HER heterodimers and their association with patient outcome

High levels of all three HER2 dimers showed a significantly worse outcome for both BCSS (Figures 3A,C,E) and DFI (Figures 3B,D,F) in the Unselected series. Multivariate Cox regression including tumour size, stage and grade, in the Unselected series demonstrated high levels of HER2 heterodimers were independent predictors for worse BCSS: HER2/EGFR (HR=0.64, 95% CI=0.45-0.89, p=0.009), HER2/HER3 (HR=0.62, 95% CI=0.45-0.62, p=0.006), HER2/HER4 (HR=0.66, 95% CI=0.48-0.92, p=0.014), and worse DFI: HER2/EGFR (HR=0.64, 95% CI=0.47-0.88, p=0.005,) HER2/HER3 (HR=0.72, 95% CI=0.53-0.98, p=0.037), HER2/HER4 (HR=0.69, 95% C =0.51-0.94, p=0.017). However, the association between HER2 heterodimers with patient outcome was not significant in patients with HER2+ breast cancer only (trastuzumab naïve or trastuzumab treated, data not shown).

Discussion

This study has utilised chromogenic PLA as an innovative procedure for in situ exposure of HER2 heterodimers such as EGFR/HER2, HER2/HER3 and HER2/HER4, in a large series of paraffin embedded TMA breast tumours. This technique has demonstrated high levels of specificity, sensitivity and consistency with low levels of background. The quantification analysis demonstrated to be robust and with reproducible results amongst the different dimers here considered.

Previously, IHC has been used as a semi-quantitative approach to detect the existence of distinct proteins being limited to single protein or co-expression recognition. Consequently it is not possible to observe protein-protein interactions using this limited technique. Moreover, co-expression of two proteins, such as the HER family, does not necessarily mean that heterodimerisation will occur, as the process is dependent on interaction with ligands to trigger this reaction. So methods, such as PLA, are a suitable solution to quantify protein interaction and visualise and quantify in situ heterodimerisation of the HER family.

For all three HER heterodimers investigated in breast cancer, it is apparent that whilst HER2 over-expression is the dependent factor in dimerising with the other members of the HER family, the protein expression of EGFR, HER3 and HER4 in HER2+ breast cancer does not necessarily result in heterodimerisation. HER2 has previously been suggested as the dominant monomer for heterodimerisation comparing with any others from the same family [13]. HER2 does not include an ectodomain where a ligand can attach, however it is competent to perform as a co-receptor with an extraordinary affinity to interact with the same family members forming heterodimers [9]. Within RTKs Type I group, HER2 is the most favourable

molecule to interact with the others due to extend ligand/heterodimer linkage and therefore prolonging MAPK pathway [5,38,39] relating these cases with high biological activity. Additionally HER2 does not interact with any ligand and therefore its conformation arm is constantly predisposed to exist in a competent form and ready to connect with any of the other monomers from the same family [12].
Even though HER2/HER3 dimer has been reported as the most frequent dimer in breast cancer [20], it was found that all three HER heterodimers were expressed at a similar frequency. This might be explained by critical factors of the PLA such as the use of different antibodies, different cut-off points and/or the designation of the overexpression status. The results of brightfield versus fluorescent could also contribute to the evident discrepancies. However both these two techniques produce similar results in both cell culture or tissue samples where chromogenic PLA is as specific and sensitive on quantification analysis as fluorescent PLA [40]. Despite these variances, we similarly showed an association between HER2/HER3 heterodimers and poor prognostic factors including high tumour grade and stage, ER negative tumours and HER2 over-expression/amplification.

Although both EGFR and HER2 protein overexpression are correlated with worse outcome [22,21], HER2/EGFR dimerisation has not been well documented in breast cancer. We show for the first time, that HER2/EGFR heterodimers are highly prevalent in HER2+ breast cancer and are correlated with poor outcome. This is in contrast to a previous study which showed EGFR and HER2 co-expression had no significant association with survival [26].

The number of HER heterodimer signals in tumours showing HER2 over-expression/ amplification revealed a significant association and therefore a directly relation between these two factors, suggesting and confirming the dependency of high levels

of heterodimerisation and HER2. However some cases did not reveal the presence of any heterodimers even though HER2 gene amplification or protein over-expression was apparent. Some occurrences, such as alternative splicing, modification of the transcription mechanism or even the lack of ligands might be the cause for the absence of the heterodimerisation signals detection in these cases [41].

Within HER2+ breast cancer, it was difficult to discriminate between each of the interactions since such a high association between the three different HER2 heterodimers occurred. However, HER2/HER3 is distinguished from the other heterodimers by being the only interaction with a significant association with high distant metastasis ($p = 0.039$). This is supported by the idea that HER3 is highly associated with the development of metastases [42]. Also HER2/HER3 was the only heterodimerisation not associated with ki67, suggesting that HER2/HER3 promotes high rates of cell proliferation, though not related with Ki67 pathway. EGFR/HER2 heterodimer was correlated with high tumour grade and poor NPI. On the other hand HER2/HER4 was not highly associated with any of the clinicopathological parameters, revealing the least influence in the poor outcome features. Other studies have already revealed HER4 is associated with a better outcome compared with other members of the HER family [24], which is in concordance with our results. However in general, i.e. primary series, heterodimerisation HER2/HER4 was highly associated with all clinicopathological parameters excluding vascular invasion and regional recurrence.

Despite levels of the three HER heterodimers were revealed to be associated with worse DFI and BCSS in breast cancer, we were not able to discriminate divergent outcomes based on HER2 dimerisation levels within the HER2+ population only whether treated by trastuzumab or not. However, the results for the trastuzumab adjuvant series have a limited number of cases and relatively short follow-up. Further

studies are therefore required to determine the role of HER2 heterodimers in patients receiving trastuzumab.

Conclusions

We have observed that HER2 heterodimers are significantly correlated with poor outcome features in breast cancer and note that HER2 overexpression encourages heterodimerisation, in order to stimulate growth, malignant development [43] and migration [44], despite of being impossible to interact with any ligand. Furthermore, despite the interaction levels undoubtedly hold beneficial knowledge, it will be fundamental to discriminate the different ligand/receptor interactions and reveal their value on patient outcome. Protein or protein-protein interaction quantification, using PLA, could be used to investigate sub-cellular developments helping to divulge both upstream and downstream pathways in HER2+ breast cancer in order to identify possible targets for therapeutic interventions and avoid development of resistance which occurs in current treatment strategies. However, our results strongly suggest there is no evidence to suggest that the identification of HER2 heterodimers will currently enhance the management of HER2+ patients receiving trastuzmab.

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Abbreviations

HER: Human Epidermal Growth Factor Receptor; RTKs: Receptor Tyrosine Kinases; EGF: Epidermal Growth Factor; STATs: Signal Transducer and Activation of Transcription; PI3K: Phosphatidylinositol (PtdIns) 3-Kinase; IHC: Immunohistochemistry; ER: Oestrogen Receptor; NPI: Nottingham Prognostic Index; PgR: Progesterone Receptor; TN: Triple Negative Phenotype; ASCO: American

Society of Clinical Oncology; CISH: Chromogenic in situ Hybridisation; TMA: Tissue Microarray; PLA: in situ Proximity Ligation Assay; HRP: Horse Radish Peroxidase.

Author's Contributions

FFTB, CCN and ACD generated, assembled data on protein expression, gene amplification, and heterodimerisation quantification. TAF and PM acquired clinical data. FFTB, ACD, TAF, PM, ARG analysed and interpreted the data. IOE, ARG, EAR and SC conceived the study, participated in its design and subsequent coordination. All the authors participated in the interpretation of data and drafting the manuscript. All authours have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. *in situ* Proximity Ligation Assay dimerisation detection in breast carcinomas showing: negative/low levels of (A) HER2/EGFR, (B) HER2/HER3, (C) HER2/HER4 and positive levels of (D) HER2/EGFR, (E) HER2/HER3 and (F) HER2/HER4

Figure 2. Linear regression analysis between HER family heterodimerisations A) HER2/EGFR, B) HER2/HER3 and C) HER2/HER4 and *HER2* gene amplification in the Unselected series. There was a positive correlation between *HER2* gene amplification and the number of heterodimers signals

Figure 3. Patient outcome according to HER2 dimer status in the Unselected series of breast cancer. (A) HER2/EGFR – BCSS, (B) HER2/EGFR - DFI, (C) HER2/HER3 – BCSS, (D) HER2/HER3, (E) HER2/HER4 – BCSS, (F) HER2/HER4 DFI

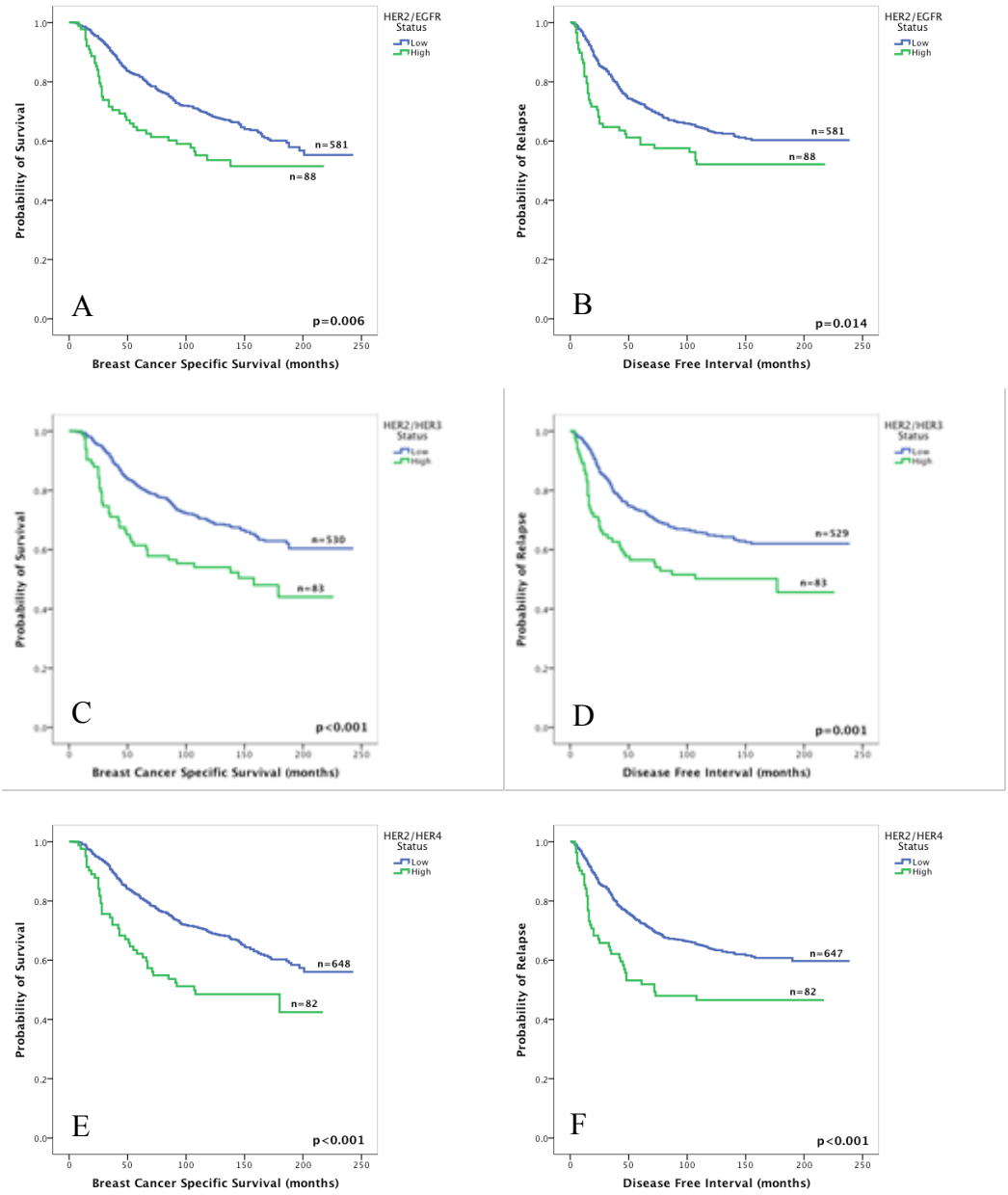
HER2/EGFR Status
Low
High
Probability of Survival
n=581
n=88
A
p=0.006
Breast Cancer Specific Survival (months)
Probability of Relapse
n=581
n=88
B
p=0.014
Disease Free Interval (months)
HER2/HER3 Status
Low
High
Probability of Survival
n=530
n=83
C
p<0.001
Breast Cancer Specific Survival (months)
Probability of Relapse
n=529
n=83
D
p=0.001
Disease Free Interval (months)
HER2/HER4 Status
Low
High
Probability of Survival
n=648
n=82
E
p<0.001
Breast Cancer Specific Survival (months)
Probability of Relapse
n=647
n=82
F
p<0.001
Disease Free Interval (months)

Figure 3

Table 1. Frequency of HER heterodimers in unselected and HER2+ breast tumours

Heterodimer	Unselected			HER2+		
	Low (%)	High (%)	Total	Low (%)	High (%)	Total
HER2/EGFR	708 (87.2)	104 (12.8)	812 (100)	61 (26.6)	168 (73.4)	229 (100)
HER2/HER3	646 (86.9)	97 (13.1)	743 (100)	77 (34.1)	149 (65.9)	226 (100)
HER2/HER4	795 (89.2)	96 (10.8)	891 (100)	112 (46.1)	131 (53.9)	243 (100)

Table 2A. Association between HER heterodimers in Unselected breast cancer series

	HER2/EGFR			HER2/HER3		
Variable	Low (%)	High (%)	p Value (Chi- Square)	Low (%)	High (%)	p Value (Chi- Square)
HER2/						
Low	387 (97.5)	11 (16.7)	<0.001 (306.265)			
High	10 (2.5)	55 (83.3)				

HER2/HER4						
Low	432 (98.0)	24 (31.6)	<0.001 (274.489)	36 (63.2)	45 (38.8)	<0.001 (187.288)
High	9 (2.0)	101 (68.4)		21 (36.8)	71 (61.2)	

Table 2B. Association between HER heterodimers in HER2+ breast cancer

	HER2/EGFR			HER2/HER3		
Variable	Low (%)	High (%)	p Value (Chi- Square)	Low (%)	High (%)	p Value (Chi- Square)
HER2/HER3						
Low	38 (84.4)	18 (13.7)	<0.001 (77.182)			
High	7 (15.6)	113 (86.3)				
HER2/HER4						
Low	41 (95.3)	36 (26.3)	< 0.001 (63.787)	46 (74.2)	38 (32.2)	< 0.001 (28.793)
High	2 (4.7)	101 (73.7)		16 (25.8)	80 (67.8)	

Table 3A. Relation of HER heterodimers with biomarkers in unselected breast cancer series

Variable	EGFR/HER2			HER2/HER3			HER2/HER4		
	Low (%)	High (%)	p Value (Chi- Square)	Low (%)	High (%)	p Value (Chi- Square)	Low (%)	High (%)	p Value (Chi- Square)
ER									
Negative	197 (29.4)	64 (66.0)	<0.001 (50.658)	169 (28.3)	56 (60.2)	<0.001 (37.424)	234 (30.9)	57 (64.8)	<0.001 (40.035)
Positive	474 (70.6)	33 (34.0)		429 (71.7)	37 (39.8)		523 (69.1)	31 (35.2)	
PgR									
Negative	296 (44.6)	72 (75.8)	<0.001 (32.266)	264 (44.2)	65 (70.7)	<0.001 (22.321)	335 (44.5)	66 (75.9)	<0.001 (30.769)
Positive	367 (55.4)	23 (24.2)		333 (55.8)	27 (29.3)		418 (55.5)	21 (24.1)	
EGFR									
Negative	476 (80.7)	59 (66.3)	0.002 (9.578)	430 (79.2)	60 (74.1)	0.296 (1.094)	527 (78.5)	57 (69.5)	0.064 (3.421)
Positive	114 (19.3)	30 (33.7)		113 (20.8)	21 (25.9)		144 (21.5)	25 (30.5)	
HER2									
Negative	655 (96.0)	15 (15.6)	<0.001 (455.221)	599 (94.8)	10 (11.2)	<0.001 (414.975)	709 (92.0)	10 (11.6)	<0.001 (369.562)
Positive	27 (4.0)	81 (84.4)		33 (5.2)	79 (88.8)		62 (8.0)	76 (88.4)	
HER3									
Negative	48 (8.5)	3 (3.6)	<0.119 (2.427)	47 (9.0)	7 (9.0)	0.999 (0.0)	74 (11.7)	1 (1.3)	0.006 (7.704)
Positive	519 (91.5)	81 (96.4)		477 (91.0)	71 (91.0)		560 (88.3)	75 (98.7)	
HER4									
Negative	97 (17.1)	6 (7.0)	0.017 (5.743)	82 (15.4)	5 (6.8)	0.048 (3.919)	125 (19.5)	5 (6.3)	0.004 (8.219)
Positive	471 (82.9)	80 (93.0)		452 (84.6)	69 (93.2)		517 (80.5)	74 (93.7)	
TN									
No	20 (10.6)	20 (80)	<0.001 (69.600)	14 (8.6)	22 (100)	< 0.001 (102.727)	30 (14.0)	18 (94.7)	< 0.001 (69.871)
Yes	168 (89.4)	5 (20)		148 (91.4)	0 (0)		185 (86.0)	1 (5.3)	
Ki67									
Low	173 (30.6)	12 (14.1)		172 (33.8)	21 (25.3)		197 (31.7)	12 (15.4)	
Moderate	209 (37.0)	34 (40.0)	0.004 (11.186)	176 (34.6)	25 (30.1)	0.062 (5.573)	211 (34.0)	27 (34.6)	0.004 (10.933
High	183 (32.4)	39 (45.9)		161 (31.6)	37 (44.6)		213 (34.3)	39 (50.0)	

Table 3B. Relation of HER heterodimers with biomarkers in HER2+ breast cancer

Variable	EGFR/HER2			HER2/HER3			HER2/HER4		
	Low (%)	High (%)	p Value (Chi- Square)	Low (%)	High (%)	p Value (Chi- Square)	Low (%)	High (%)	p Value (Chi- Square)
ER									
Negative	23 (39.7)	102 (63.4)	0.002 (9.775)	23 (31.1)	91 (62.8)	< 0.001 (19.699)	47 (42.7)	81 (65.9)	< 0.001 (12.545)
Positive	35 (60.3)	59 (36.6)		51 (68.9)	54 (37.2)		63 (57.3)	42 (34.1)	
PgR									
Negative	32 (54.2)	111 (71.6)	0.016 (5.819)	36 (48.6)	100 (71.4)	0.001 (10.845)	62 (56.9)	92 (76.7)	0.001 (10.153)
Positive	27 (45.8)	44 (28.4)		38 (51.4)	40 (28.6)		47 (43.1)	28 (23.3)	
EGFR									
Negative	31 (59.6)	81 (53.6)	0.455 (0.558)	45 (66.2)	73 (55.3)	0.139 (2.194)	64 (66.0)	64 (54.7)	0.094 (2.807)
Positive	21 (40.4)	70 (46.4)		23 (33.8)	59 (44.7)		33 (34.0)	53 (45.3)	
HER3									
Negative	4 (8.3)	5 (3.6)	0.190 (1.716)	5 (7.6)	6 (4.9)	0.450 (0.570)	6 (6.8)	2 (1.9)	0.080 (3.055)
Positive	44 (91.7)	133 (96.4)		61 (92.4)	117 (95.1)		82 (93.2)	106 (98.1)	
HER4									
Negative	0 (0.0)	5 (3.6)	0.184 (1.763)	1 (1.8)	3 (2.1)	0.618 (0.249)	3 (3.5)	4 (3.7)	0.936 (0.006)
Positive	47 (100.0)	132 (96.4)		68 (98.2)	118 (97.5)		83 (96.5)	104 (96.3)	
Ki67									
Low	2 (11.1)	7 (10.6)	0.216 (3.062)	4 (19.0)	14 (21.2)	0.400 (1.831)	7 (15.9)	9 (14.3)	0.404 (1.811)
Moderate	11 (61.1)	26 (39.4)		10 (47.6)	21 (31.8)		21 (47.7)	23 (36.5)	
High	5 (27.8)	33 (50.0)		7 (33.4)	31 (47.0)		16 (36.4)	31 (49.2)	

Table 4. **Frequency of HER heterodimers in HER2+ breast cancer**

HER2/EGFR	HER2/HER3	HER2/HER4	Frequency
+	-	-	4
-	+	-	4
-	-	+	0
+	+	-	24
+	-	+	11
-	+	+	1
+	+	+	73
-	-	-	32

Table 5. Relation of HER heterodimers with clinicopathological features in the HER2+ breast cancer series

	EGFR/HER2			HER2/HER3			HER2/HER4		
Variable	Low (%)	High (%)	p Value	Low (%)	High (%)	p Value	Low (%)	High (%)	p Value
Lymph Node Stage									
1	23 (39.0)	70 (41.7)		35 (46.7)	66 (44.3)		52 (47.3)	60 (45.8)	
2	23 (39.0)	64 (38.1)	0.926 (0.154)	29 (38.7)	49 (32.9)	0.334 (2.191)	36 (32.7)	47 (35.9)	0.866 (0.289)
3	13 (22.0)	34 (20.2)		11 (14.7)	34 (22.8)		22 (20.0)	24 (18.3)	
Tumour Size									
<1.5 cm	10 (16.4)	26 (15.5)	0.866 (0.028)	10 (13.0)	24 (16.1)	0.534 (0.387)	17 (15.2)	21 (16.0)	0.855 (0.033)
≥1.5 cm	51 (83.6)	142 (84.5)		67 (87.0)	125 (83.9)		95 (84.8)	110 (84.0)	
Tumour Grade									
I	1 (1.6)	6 (3.6)		1 (1.3)	6 (4.0)		0 (0.0)	4 (3.1)	
II	20 (32.8)	25 (14.9)	0.006 (9.3267)	21 (27.3)	19 (12.8)	0.017 (8.159)	22 (19.6)	17 (13.0)	0.075 (5.187)
III	40 (65.6)	137 (81.5)		55 (71.4)	124 (83.2)		90 (80.4)	110 (84.0)	
Tubule									
1	0 (0.0)	2 (1.2)		0 (0.0)	1 (0.7)		0 (0.0)	2 (1.6)	
2	14 (23.3)	26 (15.9)	0.314 (2.230)	16 (21.3)	23 (15.6)	0.455 (1.576)	21 (19.1)	17 (13.2)	0.207 (3.147)
3	46 (76.7)	136 (82.9)		59 (78.7)	123 (83.7)		89 (80.9)	111 (85.3)	
Pleomorphism									
1	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
2	10 (16.7)	15 (9.1)	0.113 (2.506)	11 (14.7)	12 (8.2)	0.137 (2.209)	12 (12.0)	9 (7.0)	0.265 (1.244)
3	50 (83.3)	149 (90.1)		64 (85.3)	134 (91.8)		96 (88.9)	120 (93.0)	
Distant Metastasis									
No	47 (78.3)	118 (70.7)	0.252 (1.310)	61 (79.2)	97 (66.0)	0.039 (4.259)	81 (72.3)	86 (66.7)	0.343 (0.901)
Yes	13 (21.7)	49 (29.3)		16 (20.8)	50 (34.0)		31 (27.7)	43 (33.3)	
Mitotic Frequency									
1	11 (28.3)	23 (14.5)		13 (17.3)	21 (14.3)		10 (9.1)	18 (14.0)	
2	18 (23.3)	42 (26.1)	0.490 (1.426)	22 (29.3)	33 (22.4)	0.355 (2.069)	31 (28.2)	27 (20.9)	0.281 (2.538)

3	31 (48.3)	99 (59.4)		40 (53.3)	93 (63.3)		69 (62.7)	84 (65.1)	
Vascular Invasion									
No	34 (56.7)	90 (54.2)	0.744 (0.1076)	36 (47.4)	81 (54.7)	0.296 (1.091)	58 (54.5)	73 (56.3)	0.501 (0.453)
Yes	26 (43.3)	76 (45.8)		40 (52.6)	67 (45.3)		53 (45.5)	56 (43.4)	
NPI									
Good	15 (24.6)	21 (12.5)	0.048 (11.152)	12 (15.6)	17 (11.5)	0.200 (7.295)	14 (12.5)	21 (16.1)	0.259 (6.516)
Moderate	37 (60.7)	95 (56.2)		51 (66.3)	84 (56.4)		73 (65.2)	67 (51.1)	
Poor	9 (14.7)	52 (30.9)		14 (18.2)	48 (32.2)		25 (22.3)	43 (32.9)	
Death									
No	47 (79.7)	114 (72.2)	0.261 (1.265)	58 (79.5)	97 (69.8)	0.131 (2.276)	78 (73.6)	81 (67.5)	0.318 (0.999)
Yes	12 (20.3)	44 (27.8)		15 (20.5)	42 (30.2)		28 (26.4)	39 (32.5)	
Local Recurrence									
No	49 (80.3)	117 (70.1)	0.123 (2.379)	59 (76.6)	100 (67.6)	0.157 (2.004)	83 (74.8)	85 (65.4)	0.114 (2.500)
Yes	12 (19.7)	50 (29.9)		18 (23.4)	48 (32.4)		28 (25.2)	45 (34.6)	
Regional Recurrence									
No	55 (93.2)	140 (90.9)	0.587 (0.295)	71 (95.9)	127 (91.4)	0.214 (1.547)	103 (94.5)	111 (91.7)	0.411 (0.675)
Yes	4 (6.8)	14 (9.1)		3 (4.1)	12 (8.6)		4 (5.5)	10 (8.3)	