Frequency, impact and a preclinical study of novel ERBB gene family mutations in HER2-positive breast cancer

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Abstract

Background: Somatic mutations in the *ERBB* genes (epidermal growth factor receptor: *EGFR, ERBB2, ERBB3, ERBB4*) promote oncogenesis and lapatinib resistance in metastatic HER2+ (human epidermal growth factor-like receptor 2) breast cancer *in vitro*. Our study aimed to determine the frequency of mutations in four genes: *EGFR, ERBB2, ERBB3* and *ERBB4* and to investigate whether these mutations affect cellular behaviour and therapy response *in vitro* and outcomes after adjuvant trastuzumab-based therapy in clinical samples.

Methods: We performed Agena MassArray analysis of 227 HER2+ breast cancer samples to identify the type and frequency of *ERBB* family mutations. Of these, two mutations, the somatic mutations *ERBB4*-V721I and *ERBB4*-S303F, were stably transfected into HCC1954 (PIK3CA mutant), HCC1569 (PIK3CA wildtype) and BT474 (PIK3CA mutant, ER positive) HER2+ breast cancer cell lines for functional *in vitro* experiments.

Results: A total of 12 somatic, likely deleterious mutations in the kinase and furin-like domains of the *ERBB* genes (3 *EGFR*, 1 *ERBB2*, 3 *ERBB3*, 5 *ERBB4*) were identified in 7% of HER2+ breast cancers, with *ERBB4* the most frequently mutated gene. The *ERBB4*-V721I kinase domain mutation significantly increased 3D-colony formation in 3/3 cell lines, whereas *ERBB4*-S303F did not increase growth rate or 3D colony formation *in vitro*. ERBB4-V721I sensitized HCC1569 cells (PIK3CA wildtype) to the pan class I PI3K inhibitor copanlisib but increased resistance to the pan-HER family inhibitor afatinib. The combinations of copanlisib with trastuzumab, lapatinib, or afatinib remained synergistic regardless of *ERBB4*-V721I or *ERBB4*-S303F mutation status.

Conclusions: *ERBB* gene family mutations, which are present in 7% of our HER2+ breast cancer cohort, may have the potential to alter cellular behaviour and the efficacy of HER- and PI3K-inhibition.

Keywords: breast cancer, HER2+ BC, PI3K, PI3K inhibition, somatic mutations, trastuzumab resistance

Background

Human epidermal growth factor-like receptor 2 (HER2) is overexpressed by *ERBB2* gene amplification in approximately 20% of human breast cancers (HER2-positive breast cancers). HER2 and its fellow HER family receptors epidermal growth factor receptor (*EGFR*), HER3 and HER4 together drive oncogenesis, signalling predominantly through the phosphoinositide 3-kinase (PI3K) and mitogen-activated kinase (MAPK)

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pathways.[1] HER2 overexpression is associated with a worse prognosis[2] in breast cancer, and increased risk of metastasis.[3]

The HER2-targeted therapy, trastuzumab, has established efficacy in the treatment of women with HER2+ breast cancer in the metastatic and adjuvant settings.[4,5] Other HER2-targeted therapies such as lapatinib, ado-trastuzumab-emtansine (T-DM1), pertuzumab and afatinib are also currently in use or being trialled for HER2+ breast cancer. However, resistance to HER2-targeted therapies remains a problem.[1] Potential mechanisms of trastuzumab resistance include reduced receptor-antibody binding due to HER2 masking, altered signalling through alternative HER family receptor tyrosine kinases (RTKs) or non-HER family receptors, as well as altered intracellular signalling involving loss of phosphatase and tensin homolog (PTEN), reduced p27kip1, or increased PI3K/AKT activity.[1] However, it is generally accepted that not all mechanisms that mediate trastuzumab resistance are fully known.[1]

Emerging evidence suggests that *ERBB* family gene mutations may play a role in the pathogenesis of HER2+ breast cancer and in response to HER2-targeted therapy. Somatic mutations in *ERBB3* are found in 11% of gastric and colon cancers and have demonstrated oncogenic activity *in vitro* and *in vivo.*[6] Somatic *ERBB4* mutations have been seen in breast, gastric, colorectal and non-small cell lung cancers and affect signal transduction *in vitro.*[7] Activating mutations in *ERBB2* may increase phosphorylation of *EGFR* and HER3 in breast cancers which were classed as HER2-negative.[8] A previous study identified 12 kinase domain mutants across *EGFR* (6 mutations), *ERBB2* (3 mutations), or *ERBB4* (3 mutations) (*n* = 76) in HER2+ breast cancers.[9] Patients whose tumours carried these mutations did not respond to HER2-targeted therapy in the metastatic setting.[9] These *ERBB2* mutations also conferred a more aggressive phenotype *in vitro.*[9] A T798M somatic mutation in HER2 was identified in an *in vitro* screen using a randomly mutagenized HER2 library[10] and HER2-T798M was shown to confer resistance to lapatinib.[11]

Our study aimed to determine the frequency of mutations in *EGFR, ERBB2, ERBB3,* and *ERBB4* and to investigate whether these mutations affect cellular behaviour and therapy response *in vitro* and outcomes after adjuvant trastuzumab-based therapy in clinical samples.

In contrast with other known HER family members, increased HER4 protein expression has been associated with a better prognosis[12] and increased sensitivity to trastuzumab in metastatic breast cancer.[12] However, some studies argue that knockdown of *ERBB4* reverses resistance to trastuzumab, and high HER4 expression is associated with a poor outcome in HER2+ breast cancer.[13] Given this ambiguous role, the presence of a hotspot mutation, and as it was the most frequently mutated gene in our set, we selected two *ERBB4* mutations to functionally interrogate *in vitro*: *ERBB4*-S303F and *ERBB4*-V721I. *ERBB4*-S303F occurred in three of our samples and as a furin-like domain mutation, offered the potential to affect dimerization; a crucial first step in HER family signalling. *ERBB4*-V721I was a kinase domain mutation and thus offered the potential to alter HER4 signalling and tyrosine kinase inhibitor (TKI) efficacy.

Methods

Patients
Formalin-fixed, paraffin-embedded (FFPE) patient samples were confirmed to have at least 50% tumour content by the Royal College of Surgeons in Ireland (RCSI) pathology department using haematoxylin and eosin (H&E) staining. Patients were selected for this study if they had received trastuzumab as part of their neoadjuvant or adjuvant treatment for HER2-positive breast cancer. A total of 227 patients were used for Agena analysis. Samples included primary HER2+ breast cancer surgical specimens, initial staging biopsies, and pretreatment biopsies from patients who had received neoadjuvant trastuzumab. Patient characteristics are summarized in Supplementary Table 3. Some patients had to be removed from our survival analysis owing to their receiving trastuzumab for the treatment of metastatic disease. The 133 patients included in our survival analysis were treated between 1994 and 2012 in either St Vincent's University Hospital or Beaumont Hospital, Ireland. Ethical approval was granted by both institutions for this study. For the relapse free survival analysis 29 events occurred and follow up ranged from 3 to 167 months. For the overall survival analysis 21 events occurred and follow up ranged from 11 to 259 months. In our patient cohort only 11 *ERBB* family mutations were detected.

This research was performed in accordance with the Declaration of Helsinki. All clinical samples used in these studies were obtained from Beaumont Hospital and St Vincent's University Hospital, Ireland with the full approval of each hospital's ethics committee, who are, respectively, the Beaumont Hospital Ethics Committee (Beaumont Hospital, Beaumont Road, Dublin 9) and the St Vincent's Healthcare Group Ethics and Medical Research Committee (Education and Research Centre, Elm Park, Dublin 4). Written, informed consent was granted by the patients whose samples were used in this study.

DNA extraction from FFPE HER2+ breast cancer clinical samples

DNA extraction was performed using a QiaAMP DNA FFPE Kit from Qiagen (Hilden, Germany) as per manufacturer's protocol and quantified using QuBit. We designed an Agena MassARRAY panel to assay for 67 novel *ERBB* gene family somatic mutations in 227 HER2+ breast cancer patients (Supplementary Table 1). Typically, 10 ng per assay was used for mass spectrometry-based genotyping (Agena MassARRAY, San Diego, CA, USA), which was applied as previously described.[14] Reactions where >15% of the resultant mass ran in the mutant site were scored as positive.

Protein extraction and reverse phase protein array analysis of FFPE HER2+ breast cancers

Protein was extracted from 85 FFPE breast cancer samples and reverse phase protein array (RPPA) analysis was carried out as previously described[15] (Table 1).

Site-directed mutagenesis

A plasmid encoding wildtype (WT) *ERBB4* was obtained from Addgene (29536) and *ERBB4*-WT DNA isolated using a Qiagen Maxiprep kit and QIAfilter as per the manufacturer's instructions. This *ERBB4* WT DNA was used as a template to generate *ERBB4*-S303F and *ERBB4*-V721I DNA with the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent®, Agilent, Santa Clara, California) as per manufacturer's instructions. Primers (Supplementary Table 2) were designed using Agilent's QuikChange Primer Design programme at www.agilent.com/genomics/qcpd. MassArray analysis was used to confirm each mutation had been generated.

Transduction of exogenous DNA into HER2+ breast cancer cells

We selected 2 *ERBB4* mutations for functional analysis, the potential hotspot mutation S303F (furin-like domain) and V721I (kinase domain). *ERBB4*-WT, *ERBB4*-S303F and *ERBB4*-V721I plasmids, along with the pCDF1-MCS2-EF1-puro empty vector, were stably expressed in HCC1569, BT474 and HCC1954 HER2+ breast cancer cell lines. These cell lines were chosen as transfection hosts as all are WT for all four known *ERBB* family members. Lentiviral expression constructs were prepared using 20 μl of the pPACKF1 Lentivector Packaging Kit (Systems Biosciences, Palo Alto, California). After 48 h post-transfection, the viral-enriched supernatant was collected from HEK293T cells and filtered through a 0.45 μM syringe filter. Then, 3.5 ml of supernatant was then added to T75 flasks containing host cells. Successfully transfected cells were selected in 2 μg/ml puromycin, beginning 48 h post-transfection, for a minimum of 10 days prior to experiments, and were maintained in this concentration of puromycin thereafter. Cells were removed from puromycin prior to experiments.

Although transfection with *ERBB4* WT, *ERBB4* V721I and empty vector controls were successfully established across our three cell lines, *ERBB4*-S303F transfection could be established only in the HCC1569 cell line. We note that, unlike the other two cell lines used in our study, HCC1569 is WT for *PIK3CA*, and that in our clinical study, *ERBB4*-S303F did not co-occur with a PIK3CA mutation.

Cell culture assays

Human HER2+ breast cancer cell lines were gifted by the National Institute for Cellular Biotechnology, Dublin City University, Ireland. All cell lines were grown in RPMI-1640 media (Sigma Aldrich, St Louis, Missouri) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin and maintained at 37°C with 5% CO_2. Cell line identity was confirmed by DNA fingerprinting, performed by Source Biosciences (Nottingham, England). Cell lines were *Mycoplasma* tested before and after *in vitro* experiments. Trastuzumab (21 mg/ml), prepared in bacteriostatic water, was obtained from St James University Hospital. Lapatinib (10.8 mM) and Afatinib (20.6 mM) were purchased from Sequoia Chemicals (Pangbourne, United Kingdom) and prepared in dimethylsulfoxide

Table 1. Primary antibodies used in our RPPA experiments.

Antibody	Manufacturer	Catalogue number	Species	Dilution
AKT	Cell Signaling	9272	Rabbit	1:3000
AKT (S473)	Cell Signaling	9271	Rabbit	1:250
AKT (T308)	Cell Signaling	9275	Rabbit	1:500
AKT2 (5B5)	Cell Signaling	2964	Rabbit	1:50
C-Raf	Millipore	04-739	Rabbit	1:250
C-Raf (S338) 56A6	Cell Signaling	9427	Rabbit	1:200
EGFR	Santa Cruz	SC-03	Rabbit	1:100
EGFR (Y1173)	Epitomics	1124	Rabbit	1:50
EGFR (Y992)	Cell Signaling	2235	Rabbit	1:100
EGFY (Y1068)	Cell Signaling	2234	Rabbit	1:100
MAPK – ERK 1/2	Cell Signaling	9102	Rabbit	1:200
HER2	Lab Vision	MS-325-P1	Mouse	1:1000
HER2 (Y1248)	Upstate	06-229	Rabbit	1:750
HER3	Santa Cruz	285	Rabbit	1:500
HER3 (Y1289)	Cell Signaling	4791	Rabbit	1:50
HER4	Cell Signaling	4795S	Rabbit	1:50
MAPK (T202/Y204) - ERK 1/2	Cell Signaling	4377	Rabbit	1:1200
MEK1	Epitomics	1235-1	Rabbit	1:1200
MEK1/2 (S217/S221)	Cell Signaling	9121	Rabbit	1:1000
mTOR	Cell Signaling	2972	Rabbit	1:400
mTOR (S2448)	Cell Signaling	2971	Rabbit	1:100
mTOR (S2481)	Cell Signaling	2974	Rabbit	1:100
NF-kB-p65 (S536)	Cell Signaling	3033	Rabbit	1:100
p38_MAPK	Cell Signaling	9212	Rabbit	1:300
p38 MAP Kinase (T180/Y182)	Cell Signaling	9211	Rabbit	1:250
p70 S6 Kinase	Epitomics	1494-1	Rabbit	1:250
p70 S6 Kinase (T389)	Cell Signaling	9205	Rabbit	1:250
PDK1 (S241)	Cell Signaling	3061	Rabbit	1:100
PI3K-p110-alpha	Cell Signaling	4255	Rabbit	1:100
PTEN	Cell Signaling	9552	Rabbit	1:1000
S6 Ribosomal Protein (S235/S236) (2F9)	Cell Signaling	2211	Rabbit	1:200
S6 Ribosomal Protein (S240/S244)	Cell Signaling	2215	Rabbit	1:3000
SRC	Upstate	05-184	Mouse	1:200
SRC (Y527)	Cell Signaling	2105	Rabbit	1:400

HER, human epidermal growth factor-like receptor; PTEN, phosphatase and tensin homolog; RPPA, reverse phase protein array.

(DMSO). Copanlisib (10 mM) was obtained under materials transfer agreement (MTA) from Bayer Pharmaceuticals (Berlin, Germany) and prepared in DMSO and 5% trifluoracetic acid. The 3D soft agar colony-forming assays were carried out as described previously.[16] Proliferation assays over 5 days were used to determine the half maximal inhibitory concentration (IC_{50}) values of copanlisib, afatinib and lapatinib, the growth inhibition at maximum effective dose for trastuzumab, and the combination index (CI) values of the combination of HER2-targeted therapies and copanlisib, as previously described.[17] To investigate the effect of our *ERBB4* mutations on protein signalling, 4.5×10^5 cells were seeded into each well of six-well plates and left to adhere overnight. Cells were then either treated with 100 ng/µl epidermal growth factor for 5 min or left as untreated controls prior to protein lysis, dilution and RPPA analysis as previously described.[17]

ERBB4 *protein immunoprecipitation and kinase assay*

ERBB4 immunoprecipitation was carried out using Dynabeads Protein A (Thermo Fisher Scientific, Waltham, Massachusetts) as per the manufacturer's instructions, with anti-rabbit *ERBB4* antibody (cell signalling, 4795S) at 1:50 dilution. Standard curves were prepared with fixed ratios of adenosine triphosphate (ATP): adenosine diphosphate (ADP). Then, 50 µM of ADP was added to 10 µl sample, 10 µl *ERBB4* enzyme and 5 µl DMSO in a 96-well white opaque plate. The plate was covered with tin foil and incubated in the dark for 1 h at room temperature. A standard ATP-to-ADP conversion curve was generated by combining appropriate volumes (µl) of 1 µM ADP and 1 µM ATP stock solutions as follows: ADP:ATP 100:0, 80:20, 60:40, 40:40, 20:80, 10:90, 5:95, 4:96, 3:97, 2:98, 1:99, 0:100. Then, 25 µl ADP-Glo reagent was added to the plate, which was then left at room temperature for 40 min. 50 µl kinase detection reagent was added and the plate incubated in the dark at room temperature for 30 min. Luminescence was read on a PerkinElmer plate reader and the kinase activity calculated by the level of unconverted ATP remaining in the sample.

Statistical analysis

The Kaplan–Meier method was used to estimate the survival curve from the observed survival times. The log rank test was used to compare the survival curves from the input data dichotomized based on the presence or absence of the *ERBB* mutations. Fisher's exact test (two variables) or Chi-square test ($\geqslant$ three variables) were used to search for significant correlations between mutations and clinical/pathological features. IC_{50} and CI values at effective dose 50 (ED_{50}) were calculated using CalcuSyn software (BioSoft, Cambridge, UK). A CI value of < 0.9 is considered synergistic, 0.9–1.1 is considered additive and > 1.1 is considered antagonistic. A Student's *t* test was used to compare the protein signalling activity of *ERBB* mutated and WT tumour and cell lines, and to compare the 3D colony formation of the *ERBB4*-S303F and V721I expressing cells relative to *ERBB4*-WT cells. Statistical testing was carried out when $n \geqslant 3$. $p < 0.05$ was considered statistically significant.

Results

Frequency and function of ERBB *mutations in HER2-positive breast cancer*

MassArray analysis confirmed the presence of 12 somatic, likely deleterious mutations in the *ERBB* family genes (Table 2). To our knowledge, these mutations have not been previously reported in HER2+ breast cancer, although the three *ERBB3* mutations were reported and functionally tested in gastric and colon cancer.[6] Our mutations, which together were present in 7% (16/227) of our samples (Table 2), were predicted as likely deleterious by two independent bioinformatics tools, AVSIFT[18] and Mutation Assessor.[19] All mutations were confirmed as somatic by sequencing matched normal DNA.

As *ERBB4* was the most commonly mutated *ERBB* family member in our dataset, contained the potential hotspot S303F and due to the ambiguous role of *ERBB4* mutations in HER2+ breast cancer, we chose to transfect the *ERBB4* V721I (kinase domain) and S303F (furin-like domain) mutations into cell line models of HER2-positive breast cancer, which are either PIK3CA mutant (HCC1954, BT474) or WT (HCC1569).

ERBB4 *mutations affect signalling and kinase activity as well as 3D colony formation* in vitro

Transfection of the *ERBB4*-V721I mutation into HCC1569 cells (Figure 1, *p*-values in Supplementary Figure 4) resulted in a significant increase in HER4 expression, and HER3 signalling was increased in both HCC1569 *ERBB4* mutants. Kinase activity was significantly lower in

Table 2. The identity and properties of somatic *ERBB* family mutations confirmed in our Agena study in 227 HER2-positive breast cancers. A low AVSIFT$_{18}$ value predicts that a mutation is likely to be deleterious. Mutation Assessor$_{19}$ is a bioinformatics tool which predicts the likely effect of a mutation. No mutation reported herein was reported in the TCGA HER2-positive breast cancer study as of 1 April 2017. All *ERBB* family mutations listed here were confirmed somatic by sequencing matched normal DNA.

Gene/ mutation	Domain	AVSIFT value	Predicted effect (Mutation Assessor)	COSMIC ID	Accession number
EGFR V769L	Kinase	0.24	T, PD	6242	P00533
EGFR A839T	Kinase	0	D, PD	13430	P00533
EGFR K846R	Kinase	0.03	Borderline D/B	13431	P00533
HER2 H878Y	Kinase	0.03	D, PD	21985	P04626.1
HER3 P262H	1st furin-like	N/A	N/A	N/A	P21860
HER3 Q809R	Kinase	N/A	N/A	N/A	P21860
HER3 E928G	Kinase	0	D, PD	94228, 1363010	P21860
HER4 S303F	1st furin-like	0	D, PD	N/A	Q15303
HER4 V721I	Kinase	N/A	N/A	N/A	Q15303
HER4 G599W	2nd furin-like	0	D, PD	N/A	Q15303
HER4 D595G	2nd furin-like	0.27	T, PD	1405173	Q15303
HER4 K935R	Kinase	0.1	T, B	1405163	Q15303

B, benign; D, deleterious; EGFR, epidermal growth factor receptor; N/A, not available; PD, probably damaging; T, tolerated.
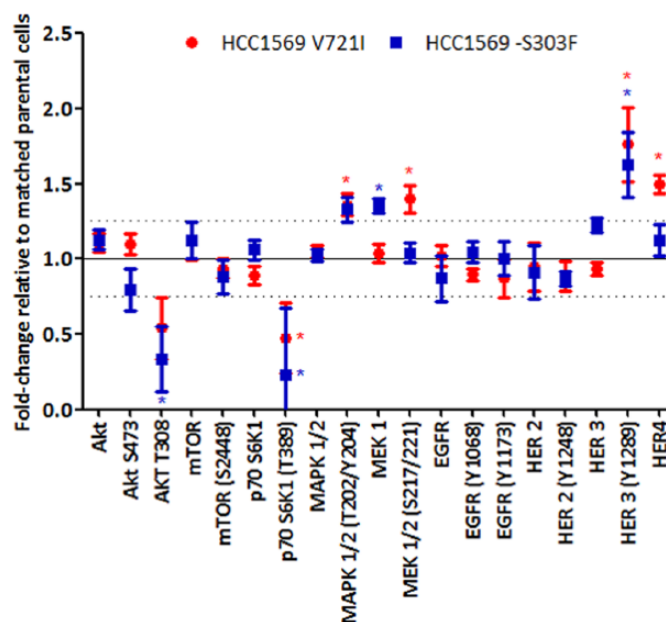

Figure 1. Signalling differences as determined by RPPA in HCC1569 *ERBB4* V721I- or *ERBB4* S303F- (transfected) HER2-positive cell lines relative to matched HCC1569 cells transfected with WT *ERBB4* normalized to 100.
* = $p < 0.05$ relative to matched *ERBB4*-WT cells. p-values were calculated using the Student's *t* test (two-tailed with equal variance).
HER, human epidermal growth factor-like receptor; RPPA, reverse phase protein array; WT, wildtype.
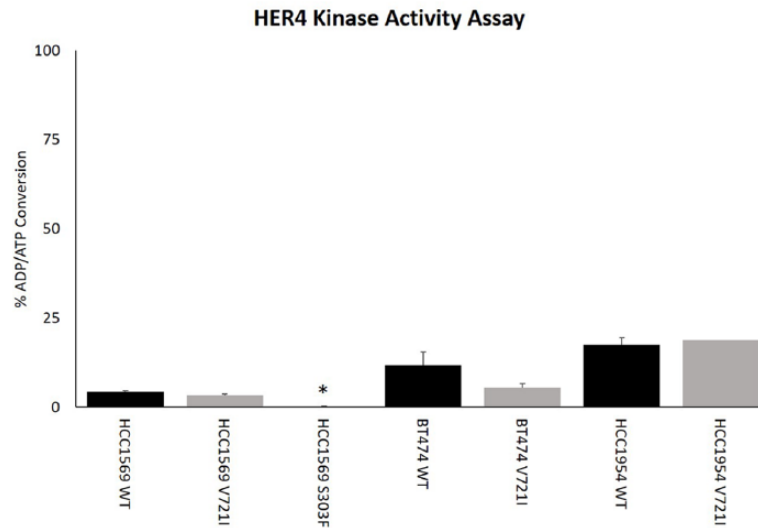
HER4 Kinase Activity Assay

Figure 2. The effect of the *ERBB4*-V721I and *ERBB4*-S303F mutations on the kinase activity if the HER4 protein in the HER2-positive breast cancer cell lines HCC1569, BT474 and HCC1954. Error bars are representative of independent duplicate experiments.
* = $p < 0.05$ as calculated by the Student's *t* test (two-tailed with equal variance).
HER, human epidermal growth factor-like receptor.

ERBB4-S303F than *ERBB*4-WT cells (Figure 2). In HCC1954 cells transfection of the *ERBB4*-V721I mutation significantly increased HER4 and HER2 expression, and decreased HER3 phosphorylation (Y1289) (Figure 3, *p*-values in Supplementary Figure 4). BT474 V721I cells showed an increase in phosphorylation of *EGFR* (Y1173) (Figure 3, *p*-values in Supplementary Figure 4).

Transfection of both the S303F and V721I *ERBB4* mutations altered signalling through the *ERBB* family protein receptors, the PI3K and MAPK pathways (Figure 3, *p*-values in Supplementary Figure 4).

These results demonstrate that the *ERBB4*-V721I mutation impacts the expression and phosphorylation of key drivers in both the PI3K/AKT and MAPK/ERK signalling pathways. These impacts are cell line specific and likely dependent on the mutational drivers already present in the cell line models. This however should not diminish the fact that these mutations are activating and will likely have significant impact on cellular function.

To validate these results, we extracted protein from HER2-positive FFPE breast cancer patient tumour samples and analyzed them on the RPPA platform (Figure 4). A limitation of this study is that the *ERBB* family mutated patients selected for our analysis had mutations in either the *EGFR, ERBB2, ERBB3* or *ERBB4* genes making it difficult to decipher specific functional impacts of specific ERBB family members. However, we found that ribosomal protein S6 (RPS6), which is activated by the PI3K pathway and involved in translation, has significantly increased phosphorylation (S240/244) ($p = 0.0313$) in *ERBB* family mutated *versus ERBB* family WT breast cancers. SRC kinase phosphorylation was significantly increased at (Y416) and (Y527) in *ERBB* mutated tumours relative to *ERBB* family WT tumours (Y416, $p < 0.01$, Y527, $p = 0.03$) (Figure 4). Overall, our analysis identifies that *ERBB* family mutated tumours have elevated signalling in pathways which are PI3K/AKT dependent, a pathway commonly activated in HER2-positive breast cancer; likely indicating that *ERBB* family mutations will impact response to agents which target the PI3K/AKT pathway.

When grown in 3D soft agar assays, V721I-expressing cells formed significantly more colonies than the corresponding WT *ERBB4* transfected cell lines (HCC1569, 0.74-fold increase, $p = 0.02$; BT474, 0.68-fold increase, $p = 0.02$; HCC1954, 1.10-fold increase, $p = 0.04$) (Figure 5), demonstrating a more aggressive phenotype. In contrast, S303F transfected HCC1569 cells did not show increased colony formation relative to *ERBB4* WT-transfected HCC1569 cells ($p = 0.17$) (Figure 5).
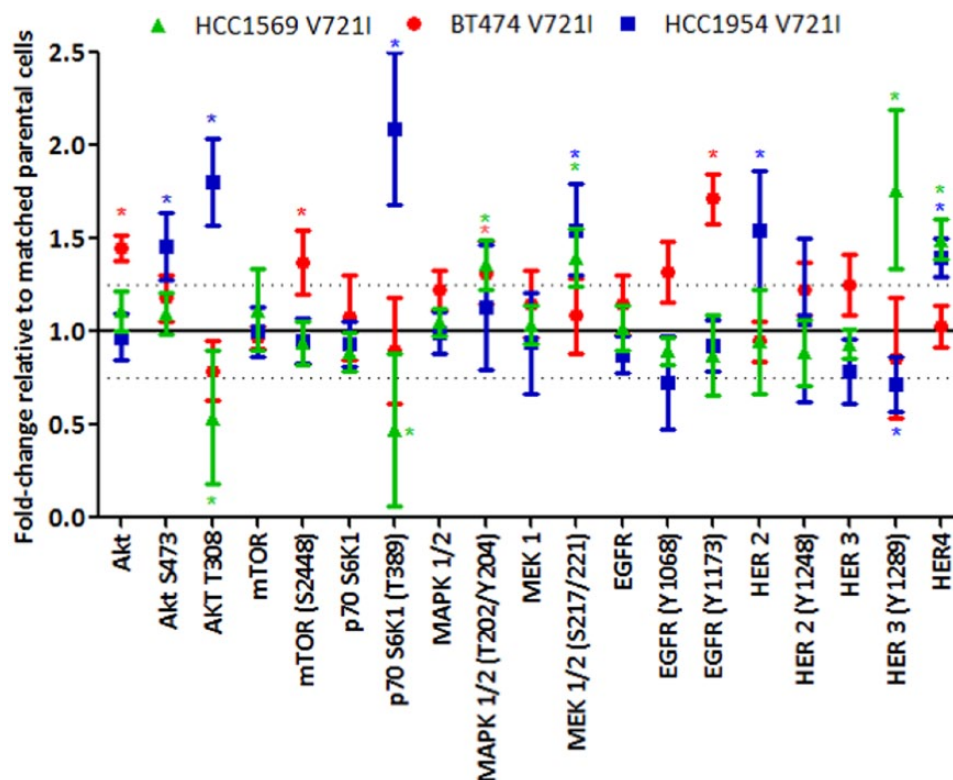
Figure 3. Signalling differences as determined by RPPA in *ERBB4* V721I- (transfected) HCC1569, BT474, and HCC1954 HER2-positive cell lines relative to matched cells transfected with WT *ERBB4* normalized to 100.
* = $p < 0.05$ relative to matched *ERBB4*-WT cells. *p*-values were calculated using the Student's *t* test (two-tailed with equal variance).
HER, human epidermal growth factor-like receptor; RPPA, reverse phase protein array; WT, wildtype.

The *ERBB4* mutations tested herein did not appear to affect levels of migration or invasion (Supplementary Figure 1). However, all models were poorly invasive and the BT474 and HCC1569 cells were poorly migratory, making it difficult to confidently draw conclusions from these experiments (Supplementary Figure 1). We noted no change in the morphology of the cells carrying ERBB4-V721I or *ERBB4*-S303F mutations (Supplementary Figure 2).

ERBB *family mutations affect sensitivity to HER2- and PI3K-targeted therapies in vitro*
Transfection of *ERBB4*-V721I resulted in a small but significant increase in resistance to afatinib, relative to cells transfected with *ERBB4*-WT in 2/3 cell lines, although this (HCC1569 $p = 0.02$, HCC1954 $p = 0.005$, Table 3) was not enough to classify the cell line as clinically resistant to afatinib. Again, transfection of *ERBB4*-S303F resulted in a small but significant increase in the sensitivity of

HCC1569 cells to afatinib ($p = 0.0006$ *versus ERBB4*-WT, Table 3).

ERBB4-V721I transfection did not significantly influence lapatinib sensitivity in any of the cell lines tested (Table 3). Interestingly though, the *ERBB4*-S303F transfection was associated with resistance to lapatinib in the HCCC1569 model ($p = 0.03$, Table 3).

Transfection of both V721I and S303F mutations significantly increased sensitivity to the PI3K inhibitor copanlisib in the *PIK3CA* WT cell line HCC1569 (V721I −0.42-fold change, $p = 0.01$, S303F −0.37-fold change, $p = 0.001$). Transfection of V721I increased resistance to copanlisib in the *PIK3CA* mutated cell line HCC1954 in comparison with the same cell line transfected with WT *ERBB4* ($p = 0.001$), although copanlisib retains low nM IC$_{50}$s in both cell lines (Table 3).

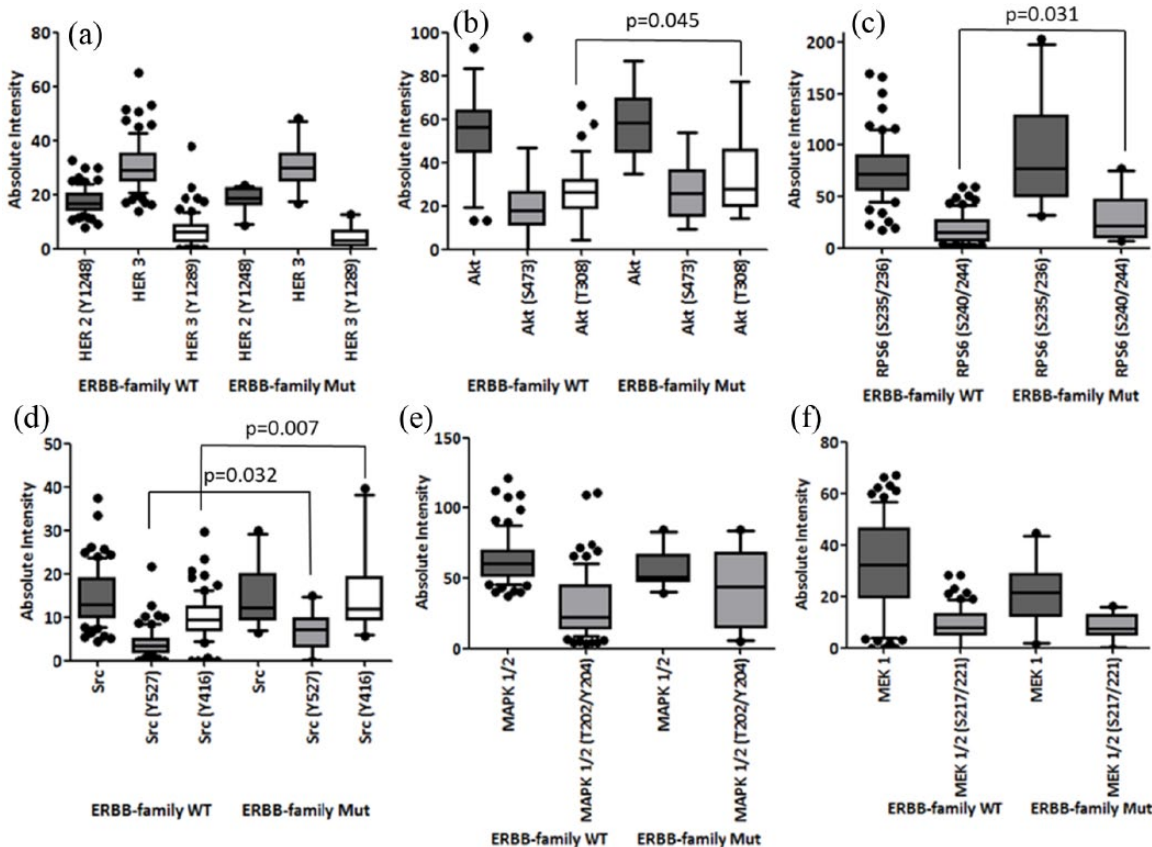We have previously shown that co-targeting PI3K and HER2 has the potential to be an improved

Figure 4. RPPA analysis of expression and phosphorylation, levels of (a) HER2 (Y1248), HER3, HER3 (Y1289), (b) AKT, AKT (S473), and AKT (T308), (c) S6 Ribosomal Protein (RPS6) (s235/236), RPS6 (S240/244) and (d) SRC kinase, SRC (Y527), SRC (Y418), (e) MAPK 1/2, MAPK 1/2 (T202/Y204) and (f) MEK 1, MEK 1 (S217/221) in *ERBB* family WT (n = 75)) *versus ERBB* family mutant (n = 10) patient tumours. Significant p-values which were calculated using a two-tailed Student's t test with equal variance are indicated on the figure.
HER, human epidermal growth factor-like receptor; RPPA, reverse phase protein array; WT, wildtype.

treatment strategy for patients with HER2+ breast cancer.[17] Here, we report that the combination of copanlisib with trastuzumab, lapatinib or afatinib is effective in all cell lines tested, regardless of their *ERBB4*-S303F or V721I mutation status.

In general, additivity or synergy was noted between afatinib and copanlisib in the HCC1569 and HCC1954 cell models used herein (Figure 6).

Encouragingly, the minor afatinib resistance induced by the V721I mutation in both HCC1569 and HCC1954 cells was overcome by combining afatinib with copanlisib. No additivity or synergy between afatinib and copanlisib occurred in the BT474 models. However, both drugs were highly potent as single agents in this cell line (Figure 6).

The combination of lapatinib and copanlisib remained synergistic in all of our cell lines regardless

of *ERBB4* mutational status (Supplementary Figure 3). CIs indicate that the combination of copanlisib and lapatinib is less synergistic in HCC1954 V721I transfected cells relative to *ERBB4* WT-transfected HCC1954 cells. However, this could be due to the slightly enhanced sensitivity to lapatinib in the HCC1954-V721I model.

Neither of the *ERBB4* mutations we tested affected trastuzumab sensitivity *in vitro* (Table 3). A log rank test was used to compare the survival curves for both progression-free survival and overall survival (OS) and assign significance (or lack thereof) (Figure 7). While the assumption of the log rank test is that of proportional hazards, small departures from this assumption do not invalidate the test (potentially in the mutant subgroup). Furthermore, the p-values are not even close to significance (relapse-free survival, p = 0.477; OS p = 0.666).
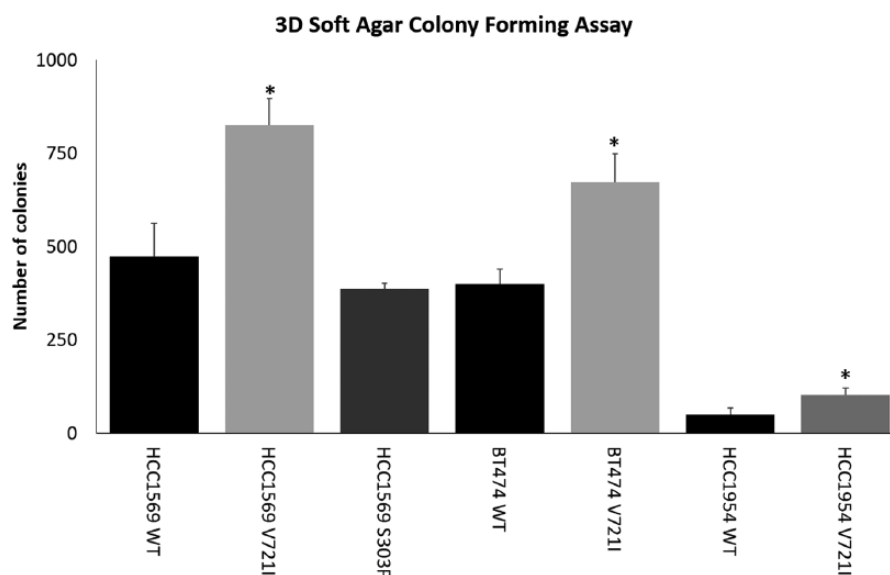
Figure 5. The effect of the *ERBB4-V721I* and S303F mutations on 3D colony-forming ability of the HER2-positive breast cancer cell lines HCC1569, BT474 and HCC1954 in 3D soft agar. Error bars are representative of independent triplicate experiments.
* = $p < 0.05$ as calculated by the Student's *t* test (two-tailed with equal variance).
HER, human epidermal growth factor-like receptor.

We also found no significant correlation between the presence of *ERBB* family mutations and clinicopathological features (Supplementary Table 4), although the low numbers in our test groups preclude confidently drawing conclusions.

The combination of trastuzumab and copanlisib inhibited the growth of all cell line models, with no significant difference observed between the transfected models and the WT models. (Supplementary Figure 5).

Discussion

To date, little work has been undertaken to determine the frequency and implications of *ERBB* family mutations in HER2+breast cancer. Herein we report somatic *ERBB* family mutations in 7% of our HER2+breast cancer cohort. To the best of our knowledge, these have not previously been reported in HER2-positive breast cancer. Within the full the cancer genome atlas (TCGA) breast cancer study (but not the TCGA HER2-positive study), *ERBB3*-E928G and *ERBB4*-S303F were reported at a similar frequency as to our dataset. The frequencies we report are in line with previous reports of mutations in 5% (4/78) of HER2-positive breast tumours[9] (*ERBB2*), but lower than the 11% reported in gastric and colon cancers[6] (*ERBB3*). We argue that a 7% frequency,

while low, may be clinically relevant, as low frequency mutations can be targeted in cancer. For example, *ALK* mutations, although occurring in approximately 5% of lung cancers, guide therapy in that disease.[20] Low frequency mutations in 8% or less were found to be an independent predictor of poor treatment-free survival in chronic lymphocytic leukemia (CLL) and monoclonal B-cell lymphocytosis,[21] and low frequency *KRAS* mutations (<10%) have been shown to predict a worse response to anti-*EGFR* therapies in metastatic colorectal cancers.[22]

The three *ERBB3* mutations reported herein have been previously reported in colon and gastric cancer by Jaiswal and colleagues who found that *ERBB3* mutations could transform cells in culture and *in vivo*, promote anchorage-independent growth of breast and colonic epithelial cells, and promote cell survival.[6] Intriguingly they found that *ERBB3* mutants' oncogenic effects were dependent on the expression of kinase-active *ERBB2*.[6]

Our *ERBB* mutations clustered in either the furin-like domain or the kinase domain. Kinase domain mutations can affect both cell signalling and drug sensitivity, with *EGFR* kinase domain mutations known to sensitize lung cancers to therapies such as gefitinib and erlotinib. The furin-like domain mediates the formation of homo- and heterodimers,

Table 3. IC$_{50}$ values of copanlisib, lapatinib and afatinib, and the effect of trastuzumab on percent growth inhibition in a panel of HER2-positive breast cancer cell lines stably expressing *ERBB4*-WT, ERBB4-V721I, or *ERBB4*-S303F. Standard deviations are representative of triplicate independent experiments. * = $p < 0.05$ compared with the matched cell line transfected with *ERBB4*-WT by Student's *t* test. Student's *t* tests were two-tailed with equal variance.

Mutation	Copanlisib IC$_{50}$ (nM)	Afatinib IC$_{50}$ (nM)	Lapatinib IC$_{50}$ (nM)	Trastuzumab % inhibition at 10 µg/ml
HCC1569 (PIK3CA WT)				
ERBB4-WT	74.74 ± 9.69	6.93 ± 0.51	514.02 ± 30.50	3.59 ± 2.21
ERBB4-V721I	43.20 ± 9.22*	12.34 ± 2.38*	503.67 ± 33.42	2.98 ± 2.03
ERBB4-S303F	47.09 ± 5.09*	2.98 ± 0.46*	736.41 ± 51.55*	0.26 ± 2.89
BT474 (PIK3CA mutated)				
ERBB4-WT	4.72 ± 0.65	1.49 ± 0.13	42.69 ± 4.97	43.02 ± 5.63
ERBB4-V721I	4.27 ± 1.04	1.13 ± 0.25	44.78 ± 4.12	42.05 ± 5.34
HCC1954 (PIK3CA mutated)				
ERBB4-WT	5.96 ± 0.29	6.54 ± 2.15	894.24 ± 42.16	3.58 ± 2.76
ERBB4-V721I	9.25 ± 0.63*	13.74 ± 0.27*	789.48 ± 89.44	1.41 ± 0.73

EV, empty vector; IC50, half maximal inhibitory concentration; WT, wildtype.

which is a crucial first step in *ERBB* family signalling.[1] Further, crystallography studies suggest these mutations may lie at the base of the dimerization arm of *ERBB4* and be involved in the dimerization contacts by the receptor.[23] An analysis of data from over 5000 tumours across 22 cancer types from TCGA found that mutations cluster in domains involved in tyrosine kinase receptor signalling, including the kinase and furin-like domain, and identified *ERBB4*-S303F as a mutation warranting further study.[24]

ERBB4-S303F, which shows an effect only in some of our functional studies, is analogous to *ERBB2*-S310F, which has been observed at low frequencies in lung, breast and ovarian cancers, and in a bladder cancer cell line.[25] This mutation-induced anchorage-independent growth, and cells bearing it were more sensitive to anti-*ERBB2* small molecule inhibitors than corresponding *ERBB4*-WT cells.[25] Although not directly analogous, *ERBB4*-V721I is just four residues away from the *ERBB4* residue analogous to *EGFR*-G719S. *EGFR*-G719S has been shown to activate the kinase by activating the p-loop, and to alter nucleotide binding.[26]

HER4 overexpression may be associated with a better outcome clinically[12,27] and an antiproliferative response *in vitro*.[28] Conversely, *ERBB4* has been shown *in vitro* to promote the proliferation of breast cancer cells,[29] while there is evidence to suggest HER4 has a role in resistance to therapy in breast and other cancers.[30] In fact, a recent study demonstrated that in both HER2-positive and triple negative breast cancer, HER4 expression was significantly associated with a favourable prognosis in a univariate analysis, however the results were not significant after multivariate analysis.[31] In part, due to the conflicting studies described above, and because it was the most frequently mutated gene in our study, and contained the unpublished hotspot S303F, we selected the *ERBB4* gene as a candidate for functional analysis *in vitro*.

Neither *ERBB4*-V721I nor *ERBB4*-S303F co-occurred with PIK3CA mutations in our clinical samples. *In vitro* we could only establish the
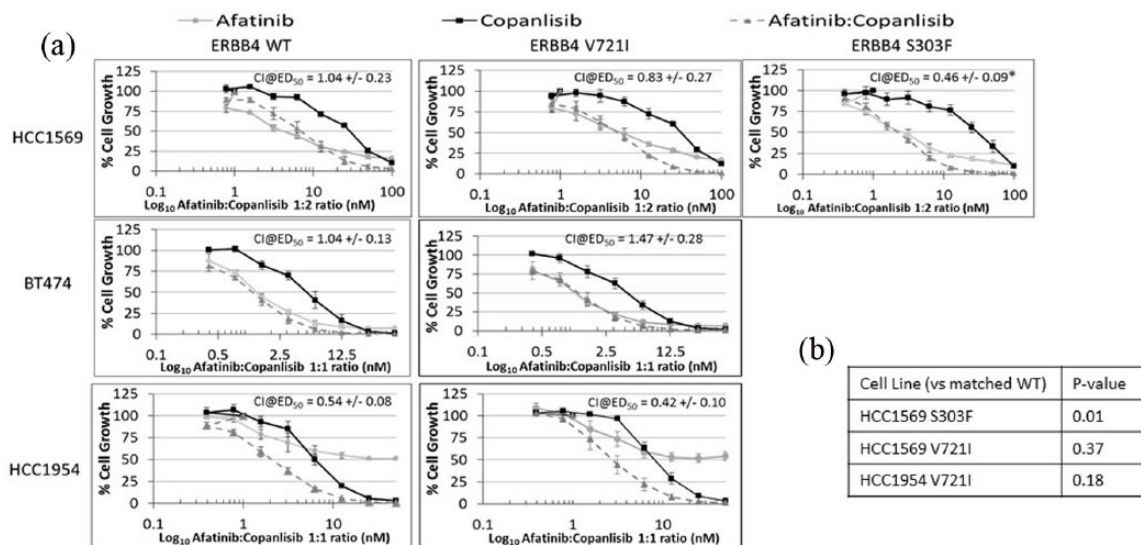
Figure 6. (a) Efficacy of afatinib (-◊-), copanlisib (-□-) and a combination of afatinib and copanlisib (–Δ–) in the HER2-positive cell lines HCC1569, BT474, and HCC1954 stably expressing *ERBB4*-WT, V721I or S303F and (b) *p*-values for the difference in efficacy of the combination. Acid phosphatase toxicity assays were used to investigate the effect of a serial dilution of the drugs on these HER2-positive breast cancer cell lines over a 5-day period and the resulting dose-response curves were analyzed with CalcuSyn (Biosoft). Error bars are representative of standard deviations across triplicate experiments.
* = $p < 0.05$ relative to WT as calculated by the Student's *t* test (two-tailed with equal variance). Afatinib:Copanlisib was tested at a 1:1 ratio (top conc 100nM:100nM).
HER, human epidermal growth factor-like receptor; WT, wildtype.
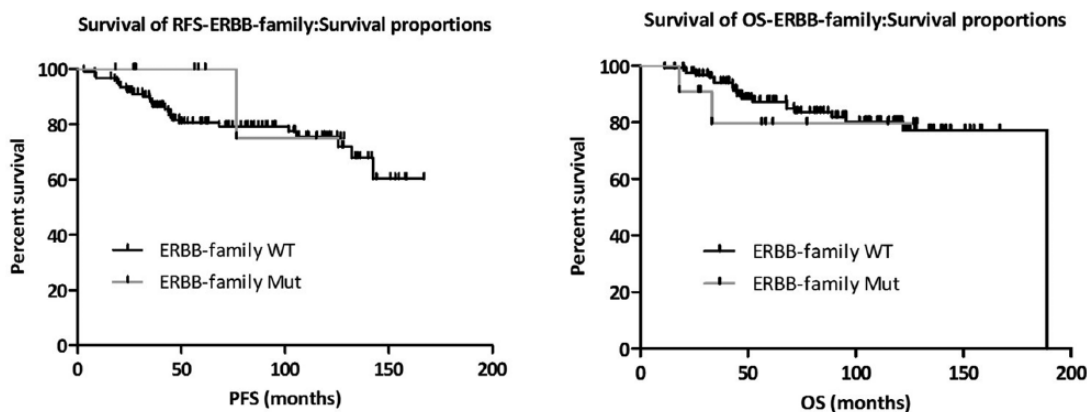
Figure 7. Impact of somatic *ERBB* family mutations on RFS (left, *n* = 133) and OS (right, *n* = 134) after adjuvant trastuzumab-based therapy in our sample set. *p*-values were calculated using the log rank (Mantel–Cox) test with Graphpad Prism. *ERBB* family mutations were identified using Agena's MassARRAY mass spectrometry-based genotyping platform.
OS, overall survival; RFS, relapse-free survival.

ERBB4-S303F mutation in the HCC1569 cell line (PIK3CA-WT), possibly indicating that the combination of *ERBB4*-S303F with a PIK3CA mutation is lethal. 3D colony formation is a surrogate for anchorage-independent growth, one of the hallmarks of cancer.[32] All *ERBB4*-V721I mutated cells formed significantly more 3D colonies than *ERBB4*-WT cells. This finding was in contrast with the HCC1569-S303F cell line which did not form greater numbers of colonies than the HCC1569 *ERBB4*-WT cells, thus suggesting *ERBB4*-V721I mutations may be oncogenic, whereas the S303F mutation may be less so. Transfection of an *ERBB4*-mutation (both

V721I and S303F) resulted in altered activation and expression of *EGFR, ERBB2* and *ERBB3*; however, these effects were cell line dependent.

Interestingly we found that in the HCC1569 cells, p-HER3 (Y1289) signalling was increased while p70S6 kinase phosphorylation (T389) was decreased in response to transfection with either *ERBB4*-V721I or *ERBB4*-S303F. We also observed an increase in either expression or phosphorylation of MAPK (Y202/T204) and MEK (S221/222) in both cell lines. These findings support the idea that in the HCC1569 cells (PIK3CA WT), transfection of either *ERBB4*-V721I or *ERBB4*-S303F increases MAPK/ERK signalling.

However, in the BT474 and HCC1954 cell lines which are PIK3CA mutated, transfection of *ERBB4*-V721I resulted in an increase in AKT phosphorylation AKT (S473 and T308) in HCC1954 cells and an increase in mTOR (S2448) in BT474 cells. These results indicate that in PIK3CA-Mutant cells, transfection of these *ERBB4* mutations will likely promote an increase in PI3K/AKT signalling.

ERBB4 can signal through ectodomain cleavage and translocation of the remaining intracellular half of the protein into the nucleus, a process which requires ADAM 17 protease.[33] We used the cleavable isoform of *ERBB4* in our *in vitro* studies,[34] and although a full study of ERBB4 cleavage was beyond the scope of our work, it may present an interesting future study (although the unclear physiological relevance of *ERBB4* cleavage in breast cancer and the difficulties in detecting the cleaved intracellular domain will present difficulties).[33]

As mentioned previously, our FFPE experiments required the pooling together of all *ERBB* family mutations, due to the limited number of mutations in our dataset. However, our RPPA analysis of FFPE-preserved HER2+breast cancer tumours demonstrates that *ERBB* family mutant tumours show increased phosphorylation of AKT (T308), and ribosomal protein S6 (RPS6) (S240/244) relative to the *ERBB*-WT tumours. Phosphorylation of AKT (T308) is essential for activation of mTORC1, which activates protein synthesis and can further activate S6 Kinase, which subsequently activates RPS6. Inhibition of RPS6 has been shown to correlate with trastuzumab-mediated growth inhibition *in vitro*[35] indicating that *ERBB*-mutant cells may proliferate at a faster rate.

We also found that phosphorylation of SRC kinase (Y527 and Y416) was significantly increased in *ERBB*-mutant cell lines. This finding suggests that future studies should examine the targeting of *ERBB*-mutant cancers with SRC kinase inhibitors such as dasatinib, which have recently been reported to overcome trastuzumab resistance in HER2-amplified gastric and biliary tract cancer cell lines.[36]

Pan-HER family inhibitors such as neratinib have and afatinib have recently entered clinical trials for the treatment of cancer patients who have a mutated or altered HER family gene or protein. We chose to use trastuzumab, lapatinib and afatinib in this study as trastuzumab and lapatinib are currently the standard of care for HER2+ advanced breast cancer, and afatinib it is currently being tested clinically in patients with altered HER family members.

ERBB4-V721I HCC1569 and HCC1954 cells had significantly higher levels of HER4 expression than the corresponding *ERBB*-WT cells. However, it is important to note that both HCC1569 and HCC1954 *ERBB4*-V721I mutant cell lines retain their sensitivity to afatinib. HCC1569-S303F cells demonstrated increased resistance to lapatinib, but not afatinib. Although lapatinib and afatinib are both anti-HER2 TKIs, their mechanism differs in that lapatinib is a reversible inhibitor of HER2 and *EGFR*, whereas afatinib is an irreversible, pan-HER inhibitor, which has been shown to bind strongly to *ERBB4*.[12] These different drug mechanisms may explain the difference in sensitivity.

Both HCC1569 S303F and HCC1569 V721I have increased sensitivity to the small molecule PI3K inhibitor copanlisib, previously found by us to have single-agent efficacy and, when used in combination with the HER2-targeted therapies trastuzumab, lapatinib and afatinib, to restore sensitivity to these HER2-targeted therapies in cell lines with acquired resistance.[17] Therefore we tested combinations of copanlisib and HER2-targeted therapies in our WT and S303F/V721I-expressing cell lines. We found these combinations largely remain synergistic regardless of *ERBB4* mutation status. In the case of the S303F hotspot mutation, synergy between afatinib and copanlisib may be enhanced.

We did not find any significant association between *ERBB* family mutations and clinical/histological features at surgery or diagnosis

(Supplementary table 4), or survival after adjuvant trastuzumab therapy (Figure 7). This analysis though is limited by the low frequency of *ERBB* family mutations in our dataset which required us to group all mutations. This grouping also limits the opportunity to evaluate individual *ERBB* gene mutations and their impact on clinical response.

Conclusions

We found *ERBB* family members to be mutated in 7% of our sample set ($n = 227$). *ERBB4* was the most frequently mutated gene of all four *ERBB* family members in our sample set, carrying five mutations, including the *ERBB4* S303F hotspot. *ERBB4* V721I and S303F affect the biology and a preclinical study suggests they may affect therapy responsiveness of HER2-positive breast cancer. This work suggests that *ERBB* family mutations have potential to alter the efficacy of PI3K-inhibition and may be potential biomarkers, if not to trastuzumab, then to emerging anti-HER TKIs such as afatinib.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Supplementary material

Supplementary material for this article is available online.

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