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Genomic analysis of the *HER2/TOP2A* amplicon in breast cancer and breast cancer cell lines

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HER2 and *TOP2A* are targets for the therapeutic agents trastuzumab and anthracyclines and are frequently amplified in breast cancers. The aims of this study were to provide a detailed molecular genetic analysis of the 17q12–q21 amplicon in breast cancers harbouring *HER2/TOP2A* co-amplification and to investigate additional recurrent co-amplifications in *HER2/TOP2A*-co-amplified cancers. In total, 15 breast cancers with *HER2* amplification, 10 of which also harboured *TOP2A* amplification, as defined by chromogenic *in situ* hybridisation, and 6 breast cancer cell lines known to be amplified for *HER2* were subjected to high-resolution microarray-based comparative genomic hybridisation analysis. This revealed that the genomes of 12 cases were characterised by at least one localised region of clustered, relatively narrow peaks of amplification, with each cluster confined to a single chromosome arm (ie 'firestorm' pattern) and 3 cases displayed many narrow segments of duplication and deletion affecting the vast majority of chromosomes (ie 'sawtooth' pattern). The smallest region of amplification (SRA) on 17q12 in the whole series extended from 34.73 to 35.48 Mb, and encompassed *HER2* but not *TOP2A*. In *HER2/TOP2A*-co-amplified samples, the SRA extended from 34.73 to 36.54 Mb, spanning a region of ~1.8 Mb. Apart from *HER2* and *TOP2A*, this region encompassed four additional genes whose expression levels as defined by quantitative real-time PCR are significantly higher in *HER2/TOP2A*-co-amplified *vs HER2*-amplified breast cancers: *CASC3*, *CDC6*, *RARA* and *SMARCE1*. Of the cell lines studied, SKBR3 and UACC812 showed *HER2/TOP2A* co-amplification. In conclusion, this is the first detailed genome-wide characterisation of *HER2/TOP2A*-amplified breast cancers; cell lines were identified that can be used to model these cancers *in vitro*. The 17q12 amplicon is complex and harbours multiple genes that may be associated with breast cancer development and progression, and potentially exploitable as therapeutic targets.

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Oncogene addiction refers to the phenomenon in which a tight dependence on the continued activity of a specific oncogene drives tumour development and progression, even in the presence of additional tumorigenic lesions.[1] Consequently, identifying and inhibiting the activity or expression of the proteins encoded by these 'addictive oncogenes' are highly attractive therapeutic strategies in human cancer. This concept has been well illustrated in the case of HER2-positive (HER2+) breast cancer, where amplification and overexpression of the *HER2 (ERBB2)* oncogene in approximately 20% of breast cancers on chromosome 17q12 have been shown to predict clinical response and improved outcome following treatment with the humanised HER2 monoclonal antibody, trastuzumab.[2–4]

It has recently been proposed that the association of breast cancers harbouring *HER2* amplification with response to anthracyclines[5–7] may be a direct result of the frequent co-amplification of *TOP2A*,[8–10] which maps close to the *HER2* gene. *TOP2A* is co-amplified with *HER2* in approximately 40–50% of breast cancers,[11,12] and codes for topoisomerase IIα, a key protein involved in resolving topological problems such as DNA supercoiling, encountered during DNA

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transcription and replication. This enzyme is a known direct molecular target of anthracyclines. We and others demonstrated that *TOP2A* amplification may predict sensitivity to anthracyclines in the adjuvant setting,[8–10,13] suggesting that, at least in HER2+ breast cancer, more than one amplified gene may be predictive of response to therapy.

Previous studies of *HER2*-amplified tumours have demonstrated that the smallest region of amplification (SRA) involving *HER2* spans 280 kb and contains a number of genes in addition to *HER2* that have elevated levels of expression, including *GRB7*, *MLN64* (*STARD3*) and *PNMT*.[14,15] Despite the genomic proximity of *HER2* and *TOP2A*, and the fact that *TOP2A* amplification seems to be restricted to tumours harbouring *HER2* amplification, this SRA does not include *TOP2A*, and there is evidence to suggest that these two genes pertain to separate amplicons.[16] In fact, tumours harbouring *HER2* gene amplification have been shown to display remarkably complex aberrations on the long arm of chromosome 17.[17] Clearly, while *HER2* has been established as the key amplicon driver on chromosome 17q12 in *HER2*-amplified breast cancers, and both *HER2* and *TOP2A* are consistently overexpressed in *HER2/TOP2A*-co-amplified breast cancers, it is yet to be determined if other genes may also have a role as amplicon drivers in this region. More intriguingly, the specific patterns of gene co-amplification and overexpression, and thus the presence of other putative therapeutic targets, in both *HER2*-amplified and *HER2/TOP2A*-co-amplified tumours remain unknown.

In a recent study by Neve *et al*[18] the recurrent genomic and gene expression changes of 51 breast cancer cell lines were characterised using a 1-Mb resolution BAC microarray-based comparative genomic hybridisation platform and Affymetrix high-density oligonucleotide array human HG-U133A chips, respectively, and were found to mirror those of 145 primary breast tumours. Here, we characterise the molecular genetic profile of *HER2/TOP2A*-co-amplified breast cancers and define the SRA and the boundaries of the 17q12 amplicon harbouring *HER2* and *HER2/TOP2A* co-amplification (as identified by chromogenic *in situ* hybridisation (CISH)). In addition, we provide a detailed characterisation of the molecular genetic profiles of tumours in both groups, including other regions of co-amplification in these tumours. The genes within the SRAs on the amplicon 17q12 were correlated with gene expression data to identify additional putative oncogene candidates. Furthermore, given that the accuracy of CISH in defining *HER2* and *TOP2A* amplifications remains contentious,[8,11] we tested whether CISH can reliably identify cases with *TOP2A* and *HER2* co-amplification.

MATERIALS AND METHODS

Breast Cancer Samples

A series of 295 primary breast cancer cases were assessed for *HER2* and *TOP2A* copy numbers by means of CISH on two tissue microarrays (TMAs). The clinicopathological details of the TMA containing 245 cases have been reported elsewhere.[8] In brief, this TMA comprises replicate 0.6 mm cores of 245 invasive breast carcinomas (186 invasive ductal carcinomas, 27 invasive lobular carcinomas, 24 invasive mixed carcinomas and 8 invasive breast carcinomas of other special types) obtained from patients primarily treated with curative surgery (69 mastectomies and 155 wide local excisions) at the Royal Marsden Hospital, London, UK. Patients in this series had been treated with standard anthracycline-based adjuvant chemotherapy. The clinicopathological and immunohistochemical findings of the second TMA containing 50 cases will be the focus of a separate publication. Briefly, this series comprises 50 invasive micropapillary breast carcinomas retrieved from the archives of The Royal Marsden Hospital, London, UK and Department of Biomedical Sciences and Human Oncology, University of Turin, Torino, Italy. All cases were reviewed by CM and JSR-F. Paraffin-embedded blocks of the cases with either *HER2* amplification or *HER2/TOP2A* co-amplification were retrieved from the archive. This study has been approved by the Research Ethics Committees of The Royal Marsden Hospital and University of Turin, Italy.

Breast Cancer Cell Lines

The cell lines BT474, MDA-MB-361, MDA-MB-453, SKBR3, UACC812 and ZR-75.30, known to harbour *HER2* amplification, were grown as previously described.[18]

CISH

CISH for *HER2* and *TOP2A* was performed as previously described,[8] using the ready-to-use digoxigenin-labelled SpotLight *HER2* and *TOP2A* amplification probe (Zymed®) and the biotin-labelled chromosome 17 centromeric probe (CEP17) (Zymed®). Heat pretreatment of deparaffinised sections consisted of incubation for 15 min at 98°C in CISH pretreatment buffer (SpotLight tissue pretreatment kit; Zymed®) and digested with pepsin for 5 min at room temperature according to the manufacturer's instructions. In each case, at least 60 non-overlapping nuclei were analysed by two of the authors on a multi-headed microscope. Amplification was defined as the presence of large gene clusters and/or >5 gene signals in the nuclei of at least 50% of neoplastic cells.[8]

DNA Extraction

Paraffin sections of *HER2*- and *HER2/TOP2A*-amplified tumours were stained with nuclear fast red and microdissected with a sterile needle (Terumo Corporation®, Japan) under a stereomicroscope (Olympus SZ61®, Japan) to obtain a percentage of tumour cells in the remaining tissue greater than 75%, as previously described.[19]

Genomic DNA was extracted using a standard proteinase K digestion followed by phenol–chloroform extraction and resuspended in TE buffer pH 7.5, as previously described.[19] The concentration of the DNA was measured with Picogreen® according to the manufacturer's instructions (Invitrogen, Paisley, UK). Multiplex PCR was performed to

assess the quality of the DNA.[20] DNA was extracted from cell lines following standard protocols.[21]

Microarray CGH

The aCGH platform used for this study was constructed in the Breakthrough Breast Cancer Research Centre and comprises ~16 000 clones, spaced at approximately 100 kb throughout the genome and spotted onto Corning GAPSII-coated glass slides (Corning, NY, USA).[22] This platform contains 418 BACs mapping to chromosome 17, with tiling path coverage of 61.15% (ie, overlapping BACs). For the remaining 38.85%, the median interval between BACs was 106.90 kb (range = 0.216–897.82 kb). Labelling, hybridisation and washes were carried out as previously described.[22] Briefly, 400 ng of test and reference genomic DNA was labelled with Cy3-dCTP or Cy5-dCTP (Amersham BioScience, Amersham, UK) using a Bioprime® labelling kit (Invitrogen), according to the manufacturer's protocol modified to incorporate 0.6 mM dCTP, and 1.2 mM dATP, dGTP and dTTP. The unincorporated nucleotides were removed with MinElute® purification columns (Qiagen Ltd, UK). All experiments were performed in duplicate ('dye swaps') to minimise dye biases.

Image Acquisition and Data Analysis

The aCGH slides were scanned using an Axon 4000B scanner (Axon Instruments, Burlingame, CA, USA) and images were processed using Genepix Pro 4.0 image analysis software (Axon Instruments). The $\log_2$ ratios were normalised for spatial- and intensity-dependent biases using a two-dimensional loess regression and then averaged across the 'dye swaps'. This left a final data set of 14 277 clones with unambiguous mapping information according to the March 2006 build (hg18) of the human genome (http://www.ensembl.org). Data were smoothed using a local polynomial adaptive weights smoothing (aws) procedure for regression problems with additive errors. Thresholds for defining genomic gains and losses were obtained using data from unamplified female *vs* female and female *vs* male genomic DNA, as previously described.[22] A categorical analysis was applied to each clone on the array after classification as gain, loss or no change according to their smoothed $\log_2$ ratio values. Smoothed $\log_2$ ratio values < -0.12 were categorised as losses, those > 0.12 as gains, and those in between as unchanged. Amplifications were defined as smoothed $\log_2$ ratio values > 0.4. Data processing and analysis were carried out in R 2.0.1 (http://www.r-project.org/) and BioConductor 1.5 (http://www.bioconductor.org/), making extensive use of modified versions of the packages aCGH, marray and aws in particular.

Statistical Analysis for Differential Gene Expression

Statistical analysis was carried out using SPSS statistical package. Affymetrix mRNA gene expression data from 54 breast cancer cell lines were retrieved from the Supplementary Material of Neve *et al*.[18] All cell lines that were not classified as luminal or basal like, that have proven not to be breast cancer cell lines (ie MDA-MB-435, which is a melanoma cell line) and that were reported to be positive for HER2 and could not be profiled in this study (ie HCC1954, HCC202, SUM190PT and SUM225CWN) were excluded. Expression of genes pertaining to the SRA of *HER2*-amplified cases and *HER2/TOP2A*-co-amplified samples were collated in a SPSS spreadsheet. Analysis of the correlation between *HER2* amplification and expression of genes pertaining to the SRA of *HER2* amplicon was performed with Mann–Whitney *U*-test. Analysis of the correlation between *HER2/TOP2A* co-amplification and expression of genes pertaining to the SRA defined in cases harbouring *HER2/TOP2A* amplification was performed with Mann–Whitney *U*-test. We also compared the expression of genes pertaining to the SRA of *HER2/TOP2A*-co-amplified cases in cell lines with *HER2* amplification *vs* those with *HER2/TOP2A* co-amplification; owing to the limited number of cell lines ($n = 2$ for *HER2/TOP2A* co-amplified and $n = 4$ for *HER2* amplified), the two-tailed Student's *t*-test was employed.

Quantitative Real-Time (qRT)-PCR

We then investigated the expression of genes shown to be significantly overexpressed in cell lines with *HER2/TOP2A* co-amplification when compared to those with only *HER2* amplification in a series of *HER2*-amplified and *HER2/TOP2A*-co-amplified breast cancers by means of quantitative real-time (qRT)-PCR.

The 5 *HER2*- and the 10 *HER2/TOP2A*-co-amplified cancers analysed in this study and additional 10 *HER2*-amplified and 10 *HER2/TOP2A*-co-amplified breast cancers were subjected to RNA extraction. RNA was extracted from FFPE tissue using the RNeasy FFPE RNA Isolation Kit (Qiagen Ltd) as per manufacturer's instructions, with the modification of one 30 min xylene treatment at 37°C. An additional DNase treatment step was performed; following RNA extraction, 6 U of DNase (Ambion) was incubated with RNA at 37°C for 1 h, to enable efficient DNA digest. All RNA quantification and qualification were performed using the Agilent 2100 Bioanalyzer with RNA Nano LabChip Kits (Agilent Biosystems). Criterion for use in reverse transcription was a concentration of > 75 ng/μl. Twelve *HER2*-amplified cancers (5 subjected to array CGH analysis + 7 additional cases) and 15 *HER2/TOP2A*-co-amplified cases (10 subjected to array CGH analysis + 5 additional cases) rendered optimal results. Reverse transcription was performed with Superscript III (Invitrogen) using 400 ng of RNA per reaction. Triplicate reactions were performed for each sample (RT+), in addition to an RT− reaction to check for the absence of detectable DNA contamination.

qRT-PCR was performed using TaqMan® chemistry on the ABI Prism 7900HT (Applied Biosystems), using the standard curve method.[23] Assays were purchased from Applied Biosystems, with the exception of *TBP* where primers

and probes (Operon; forward: GCCCGAAACGCCGAATAT, reverse: CGTGGCTCTCTTATCCTCATGA) were designed using Primer Express software (Applied Biosystems) and sequences were checked for specificity using BLAST. All selected amplicons were less than 85 bp to accommodate the degraded nature of the RNA. In addition, three reference genes (*TBP*, *TFRC* and *MRPL19*) were used, having been previously selected as effectively normalising for degradation of RNA. Each gene of interest was normalised to the geometric mean of the three references. The Mann–Whitney *U*-test was applied to determine significance.

RESULTS

Molecular Genetic Profiles of *HER2* and *HER2/TOP2A*-Amplified Cases

Genome-wide aCGH profiling of *HER2*-amplified and *HER2/TOP2A*-co-amplified breast cancers revealed several genomic regions that were consistently gained or lost in association with the *HER2* or *HER2/TOP2A* amplification. These regions are illustrated in Figure 1. Although some of these changes were common to several tumours, the profiles of the tumours were heterogeneous as shown in Figure 1. In fact, 12 out of 15 (80%) cases showed a 'firestorm' pattern as described by Hicks *et al*,[24] presenting clustering of multiple amplicons on single chromosome arms;[24] three cases showed a 'sawtooth' pattern (many narrow segments of duplication and deletion affecting all chromosomes) and none of the cases demonstrated a 'simplex' pattern (broad segments of duplication and deletion comprising entire chromosomes or chromosome arms). If classified according to the patterns identified by Chin *et al*,[25] 12, 3 and 0 would be categorised as 'amplifiers' (low-level gains and losses and recurrent amplifications), 'complex' (many low level copy number alterations) or '1q/16q/simple' (very few copy number changes), respectively. The three cases with a 'sawtooth'/complex pattern belonged to the *HER2/TOP2A*-co-amplified group (Figure 1). Interestingly, only 2 out of the 15 (13.3%) cases analysed displayed losses of the whole long arm of chromosome 16 (Figure 1).

The 17q12–q21 Amplicon in *HER2* and *HER2/TOP2A* Breast Cancers

Following DNA extraction and quality evaluation, 15 cases with *HER2* gene amplification as defined by CISH were subjected to aCGH, of which 10 also harboured *TOP2A* amplification (Table 1). The minimal region of amplification in the 15 *HER2*-amplified cases on 17q12–q21 comprised four BACs with a total length of 746.48 kb, from 34 730.32 to 35 476.80 kb (Figure 2a). This region encompasses >20 genes, including *HER2*, *PERLD1*, *PNMT*, *PPP1R1B*, *GSDML*, *PSMD3*, *STARD3*, *THRAP4*, *TCAP*, *GRB7*, *THRA* and *PPARBP*, reported to be overexpressed when amplified.[12,15–18,21]

In the 10 cases with *HER2/TOP2A* co-amplification, the smallest region of overlap extended from 34 730.32 to 36 547.20 kb, comprising a region of 1816.88 kb and encompassing >50 genes (Figure 2b). Apart from the genes pertaining to the *HER2* amplicon and *TOP2A*, this region included additional genes that are reported to be overexpressed when amplified: cancer susceptibility candidate 3 (*CASC3*), retinoic acid receptor alpha (*RARA*), cell division cycle homologue 6 (*CDC6*), SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily e, member 1 (*SMARCE1*) and keratin 10 (*KRT10*).[12,16–18,22,25]

Owing to the selection of criteria of samples harbouring *HER2/TOP2A* co-amplifications, the prevalence of *TOP2A* gene deletions could not be analysed.

The 17q12–q21 Amplicon in Breast Cancer Cell Lines with *HER2* Amplification

The cell lines BT474, MDA-MB-361, MDA-MB-453, SKBR3, UACC812 and ZR-75.30 have been shown to harbour *HER2* gene amplification.[18] In our study, the *HER2* amplicon in these cell lines is remarkably similar to that seen in the 15 breast cancers encompassing the region between 34 730.32 and 35 476.80 kb and the BACs RP11-62N23, RP11-94L15, RP11-610O22 and RP11-32H6 (Supplementary Table 1). *TOP2A* amplification was only observed in the cell lines SKBR3 and UACC812; MDA-MB-361, MDA-MB-453 and ZR-75.30 harboured *TOP2A* gene deletions and BT474 showed normal *TOP2A* copy number (Figure 2c). Complex rearrangements on 17q, similar to those observed in our series of *HER2* and *HER2/TOP2A*-co-amplified cases, were also found in the cell lines: all cell lines showed rearrangements distal to *HER2* amplicon, whereas rearrangements proximal to *HER2* amplicon were only seen in MDA-MB-361 and ZR-75.30. Genome-wide analysis of the cell lines revealed complex 'firestorm' patterns in all but MDA-MB-453 cells, which displayed a complex 'sawtooth' pattern (Supplementary Figure 1).

Differential Expression of Genes Mapping to the SRA of the *HER2* and *TOP2A* Amplicons in Breast Cancer Cell Lines and Human Tumours

Our analysis, including 46 breast cancer cell lines,[18] demonstrated that the expression of 9 genes pertaining to the SRA of *HER2* amplicon was strongly correlated with amplification of this genomic region in breast cancer cell lines: *PPARB*, *IKZF3*, GRB7, *PERLD1*, *STARD3*, *PNMT*, *ZNFN1A3* and *ERBB2* (all $P<0.05$, Mann–Whitney *U*-test) (Supplementary Table 2). When we compared the expression of each gene within the *HER2* SRA in each cell line with its median expression in all cell lines, we observed that only *GRB7*, *PERLD1*, *STARD3*, *PNMT* and *ERBB2* were consistently overexpressed in all *HER2*-amplified cell lines.

When we compared the expression of each gene within the *HER2/TOP2A* SRA in each cell line with its median expression in all cell lines, we observed that *CCR7*, *CDC6*, *CRKRS*, *CSF3*, *TNS4*, *GRB7*, *PERLD1*, *CASC3*, *STARD3*, *NEUROD2*, *NR1D1*, *PNMT*, *PPARBP*, *GSDML*, *PSMD3*, *RARA*, *SMARCE1*, *TCAP*, *THRA*, *TOP2A*, *THRAP4* and *HER2* were

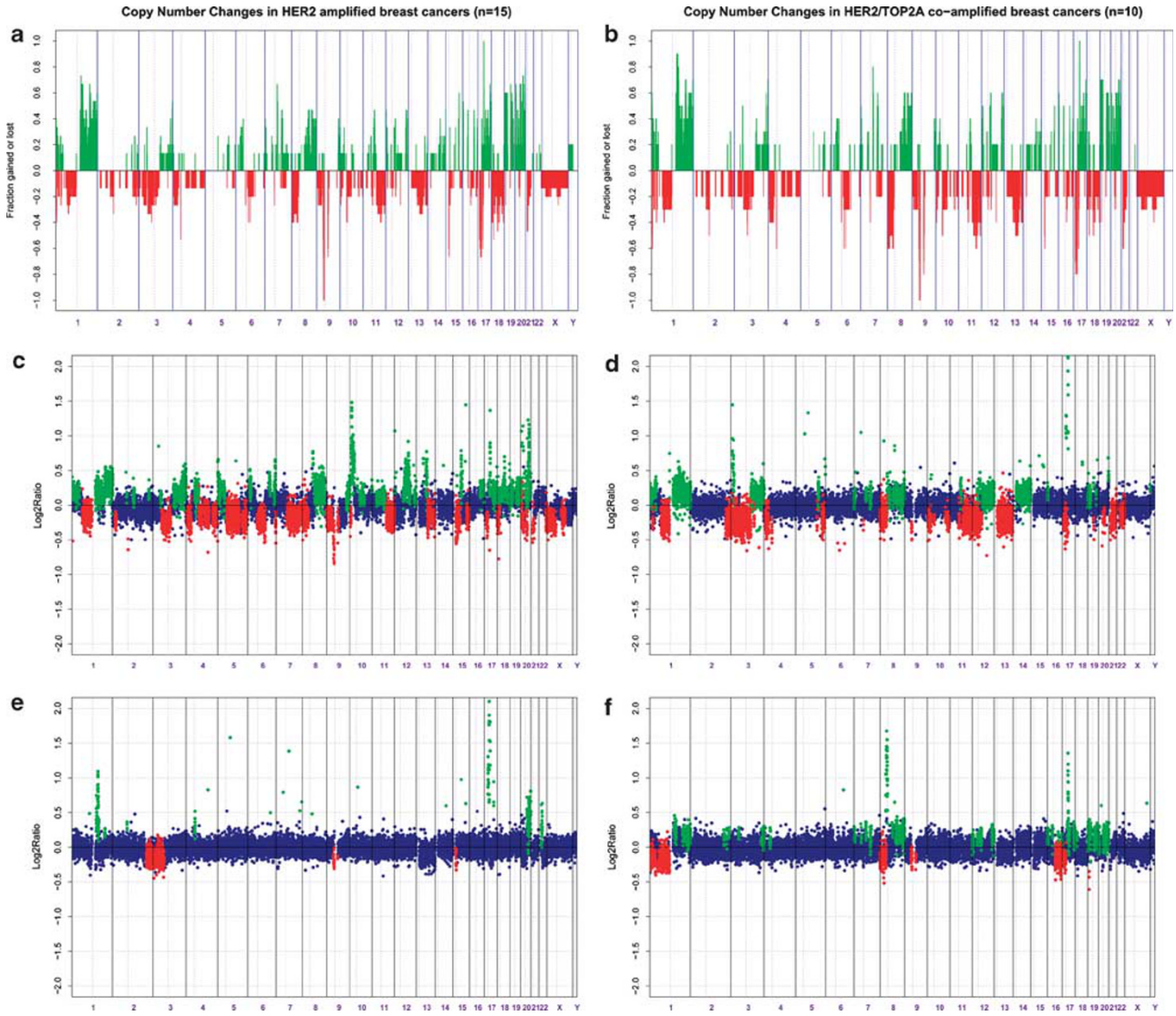

Figure 1 Array CGH analysis of *HER2*- and *HER2/TOP2A*-amplified breast cancers. (**a**) Frequency plot of chromosomal gains and losses in 15 *HER2*-amplified breast cancers. (**b**) Frequency plot of chromosomal gains and losses in the subset of *HER2/TOP2A*-co-amplified breast cancers. The proportion of tumours in which each clone is gained (green bars) or lost (red bars) is plotted (*Y* axis) for each BAC clone according to genomic location (*X* axis). Vertical dotted lines represent chromosome centromeres. (**c**–**f**) Representative genome plots of *HER2/TOP2A*-co-amplified breast carcinomas showing either a complex amplifier/firestorm pattern (**c**, **d**) or a complex/sawtooth pattern (**e**, **f**). Log_2 ratios are plotted on the *Y* axis against each clone according to genomic location on the *X* axis. The centromere is represented by a vertical dotted line. BACs categorised as displaying genomic gains as defined by aws ratios >0.12 are highlighted in green and those categorised as genomic losses as defined by aws ratios <-0.12 are highlighted in red. BACs with aws ratios between -0.12 and 0.12 were considered unchanged and highlighted in blue.

consistently overexpressed in the two *HER2/TOP2A*-co-amplified cell lines (ie SKBR3 and UACC812). Of these genes, the following showed a statistically significant correlation with *HER2/TOP2A* co-amplification: *CCR7*, *CDC6*, *CRKRS*, *CSF3*, *GRB7*, *CASC3*, *STARD3*, *PNMT*, *PPARB*, *GSDML*, *RARA*, *SMARCE1*, *TOP2A*, *THRAP4* and *HER2* (all $P<0.05$, Mann–Whitney *U*-test) (Supplementary Table 2).

When we compared the expression of each gene pertaining to the *HER2/TOP2A* SRA in each cell line with its median expression in the six *HER2*-amplified cell lines, we observed that *CCR7*, *CDC6*, *CRKRS*, *CSF3*, *TNS4*, *KRT10*, *CASC3*, *PNMT*, *PPARBP*, *PRO2521*, *PSMD3*, *RARA*, *SMARCE1*, *TCAP*, *THRA*, *TOP2A* and *THRAP4* were consistently overexpressed in the two *HER2/TOP2A*-co-amplified cell lines. Out of these genes, only *CDC6*, *CASC3*, *SMARCE1*, *RARA* and *TOP2A* were statistically significantly expressed at high levels in *HER2/TOP2A*-co-amplified cases ($P<0.05$, Student's *t*-test). We next compared the expression of these genes and *HER2* and *KRT10* (two genes not differentially expressed in the two groups) in a series of 12 *HER2*-amplified and 15 *HER2/TOP2A*-co-amplified invasive breast cancers by means of qRT-PCR (Table 2). No significant differences between the

Table 1 Summary of cases subjected to chromogenic *in situ* hybridisation and microarray-based comparative genomic hybridisation

Case	Type	Grade[a]	ER/PR[b]	*HER2* CISH	*HER2* aCGH	*TOP2A* CISH	*TOP2A* aCGH	qRT-PCR
A11735P	MIMP	3	−ve/−ve	Amp	Amp	Not Amp	Not Amp	Yes
M13	IDC	3	+ve/−ve	Amp	Amp	Not Amp[c]	Not Amp	Yes
M324	IDC	3	+ve/+ve	Amp	Amp	Not Amp	Not Amp	Yes
M345	IDC	3	+ve/+ve	Amp	Amp	Not Amp	Not Amp	Yes
M348	IDC	3	−ve/−ve	Amp	Amp	Not Amp	Not Amp	Yes
M37	IDC	3	+ve/−ve	Amp	Amp	Amp	Amp	Yes
M61	P-ILC	3	+ve/−ve	Amp	Amp	Amp	Amp	Yes
M166	IDC	3	+ve/−ve	Amp	Amp	Amp	Amp	Yes
M168	IDC	3	+ve/+ve	Amp	Amp	Amp	Amp	Yes
M180	IDC	3	−ve/−ve	Amp	Amp	Amp	Amp	Yes
M275	IDC	3	+ve/+ve	Amp	Amp	Amp	Amp	Yes
M300	IDC	2	+ve/+ve	Amp	Amp	Amp	Amp	Yes
M309	IDC	3	−ve/−ve	Amp	Amp	Amp	Amp	Yes
M312	Mixed	3	+ve/+ve	Amp	Amp	Amp	Amp	Yes
X644	MIMP	3	−ve/−ve	Amp	Amp	Amp	Amp	Yes
M346	Mixed	2	+ve/+ve	Amp	NA	Not Amp	NA	Yes
M144	IDC	3	−ve/+ve	Amp	NA	Not Amp	NA	Yes
M347	IDC	3	+ve/+ve	Amp	NA	Not Amp	NA	Yes
M327	IDC	3	−ve/+ve	Amp	NA	Not Amp	NA	Yes
M19	IDC	3	+ve/+ve	Amp	NA	Not Amp	NA	Yes
M230	IDC	3	+ve/+ve	Amp	NA	Not Amp	NA	Yes
M259	IDC	3	+ve/+ve	Amp	NA	Not Amp	NA	Yes
M257	IDC	3	−ve/+ve	Amp	NA	Amp	NA	Yes
M316	IDC	3	+ve/+ve	Amp	NA	Amp	NA	Yes
M337	IDC	2	+ve/+ve	Amp	NA	Amp	NA	Yes
M245	IDC	3	+ve/+ve	Amp	NA	Amp	NA	Yes
M270	IDC	2	−ve/−ve	Amp	NA	Amp	NA	Yes

[a]Bloom–Richardson–Scarff histological grade.

[b]As defined by an Allred score ≥ 3.

[c]This case showed low-level copy 'amplification' using only the *TOP2A* probe. When ratios *TOP2A*:CEP17 were devised, the case lacked *TOP2A* gene amplification.

−ve, negative; +ve, positive.

aCGH, microarray-based comparative genomic hybridisation; Amp, amplified; CISH, chromogenic *in situ* hybridisation; IDC, invasive ductal carcinoma of no special type; MIMP, mixed invasive micropapillary carcinoma; mixed, mixed invasive ductal and lobular carcinoma; not Amp, not amplified; NA, not assessable due to failed quality control PCR; P-ILC, pleomorphic lobular carcinoma.

mitotic counts of these *HER2* and *HER2/TOP2A*-amplified cancers were observed. Our results demonstrate that *CASC3* ($P<0.0001$), *CDC6* ($P<0.0001$), *RARA* ($P<0.001$), *SMARCE1* ($P<0.005$) and *TOP2A* ($P<0.01$) were differentially expressed in both subgroups.

CISH and aCGH Analysis of *HER2* and *TOP2A* Gene Status

From a total of 295 samples analysed by CISH on two TMAs, 276 cases yielded optimal results for both *HER2* and *TOP2A* probes. Applying the guidelines[26] for *HER2* and *TOP2A* CISH amplification testing, 43 (15.6%) displayed *HER2* amplification of which 24 (8.7%) also displayed *TOP2A* amplification (Figure 3). There were no cases with *TOP2A* amplification in the absence of *HER2* amplification. In one case of *HER2/TOP2A* co-amplification, while *HER2* was amplified throughout the tumour, *TOP2A* amplification was heterogenous, with areas containing >10 copies of *TOP2A* signals per nucleus (70% tumour) and others with cells harbouring <5 gene copies per nucleus (30% tumour). One case harboured high-level *HER2* amplification (ie >10 gene

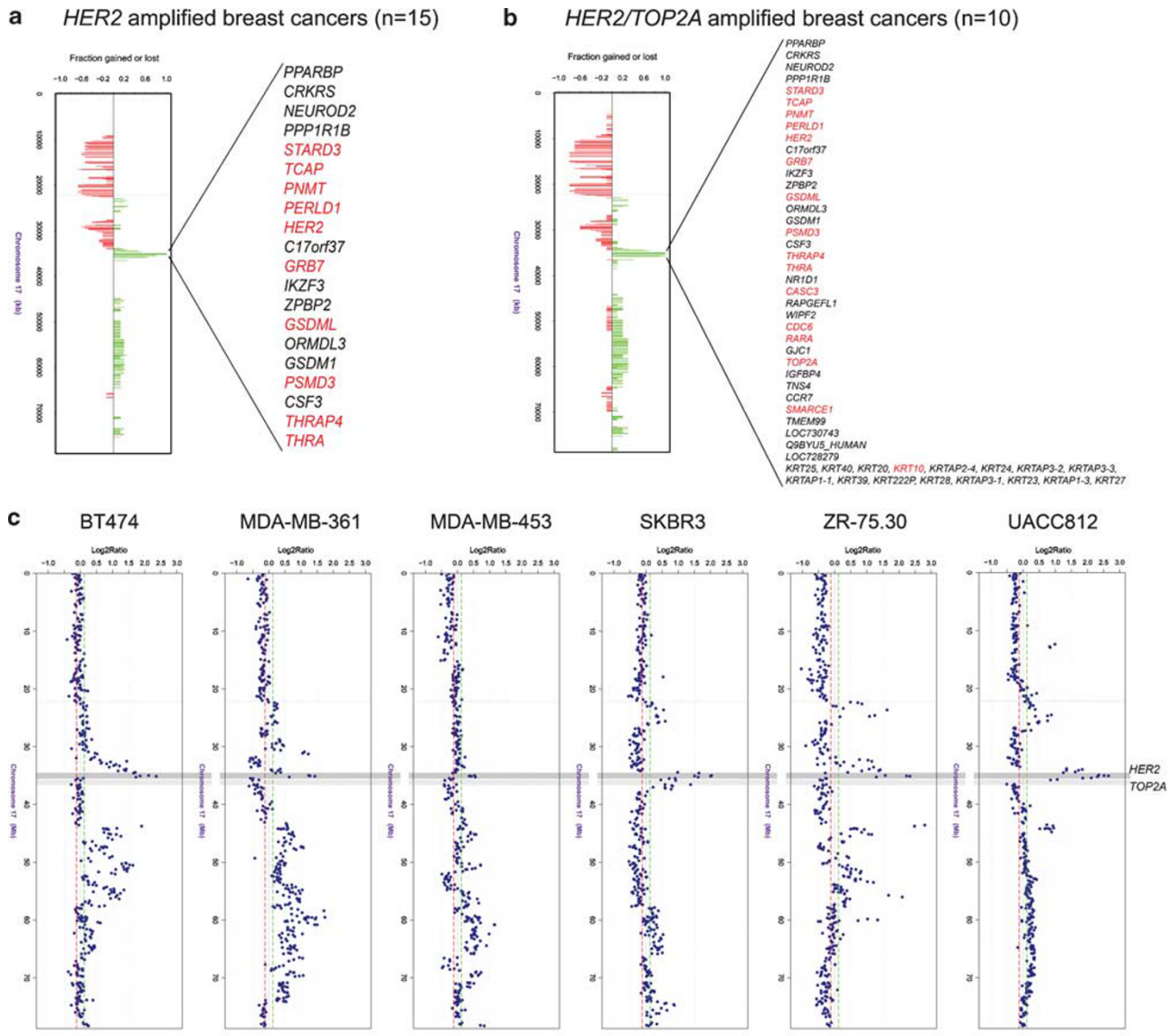

Figure 2 Frequency of amplifications and deletions on chromosome 17 in 15 *HER2*-amplified breast cancers (**a**) and in the subset ($n = 10$) of *HER2/TOP2A*-co-amplified breast cancers (**b**). The proportion of tumours in which each clone is amplified (green bars) or lost (red bars) is plotted (*X* axis) for each BAC clone according to genomic location (*Y* axis). Horizontal dotted lines represent chromosome centromeres. The smallest regions of overlap and the genes mapping to these regions in *HER2*-amplified cancers and *HER2/TOP2A*-co-amplified breast cancers are highlighted on the right. In red, genes whose expression is reported to correlate with gene amplification.[14–18,21] (**c**) Representative chromosome 17 plots of five *HER2*-amplified breast cancer cell lines. Note that out of these cell lines, only SKBR3 and UACC812 harboured *HER2/TOP2A* co-amplification. MDA-MB-361, MDA-MB-453 and ZR-75.30 displayed deletions of the *TOP2A* gene. Log_2 ratios are plotted on the *X* axis against each clone according to genomic location on the *Y* axis. The centromere is represented by a horizontal dotted line. *HER2* and *TOP2A* amplicons are highlighted by dark and light grey boxes, respectively.

copies per nucleus), but low-level *TOP2A* amplification (average of 5.2 *TOP2A* copies per nucleus). As recommended by the current guidelines for CISH assessment, we derived the *TOP2A*/CEP17 ratios for the case with *TOP2A* low-level amplification, which revealed no *TOP2A* amplification.

In four cases, BACs mapping to the pericentromeric region of 17q also showed high-level copy number gains. However, no difference in CEP17 copy numbers by CISH was seen when cases with and without pericentromeric 17q gains were analysed with chromosome centromere 17 CISH probes: CEP17 copy numbers ranged from 1.3 to 2.6 in cases with these pericentromeric gains, whereas in the remaining cases the number of CEP17 signals ranged from 1.4 to 2.3 (*t*-test, not significant).

Other Genomic Aberrations on Chromosome 17

Given the reported complexity of genomic rearrangements on chromosome 17 in cases with *HER2* amplification,[17] we performed a detailed analysis of the molecular genetic aberrations affecting this chromosome. Two discrete regions of

Table 2 Quantitative real-time PCR analysis of genes mapping to *HER2/TOP2A* amplicon in invasive breast cancers

Gene	*TOP2A* not amplified ($n = 12$)	*TOP2A* amplified ($n = 15$)	Fold change	*P*-value[a]
CASC3	0.38 ± 0.07	1.50 ± 0.12	3.93	< 0.0001
CDC6	0.21 ± 0.09	3.03 ± 0.63	14.12	< 0.0001
RARA	5.85 ± 1.2	6.55 ± 0.96	4.74	< 0.001
SMARCE1	1.26 ± 0.41	5.99 ± 1.33	2.82	< 0.005
TOP2A	0.37 ± 0.05	1.04 ± 0.19	5.27	< 0.01
ERBB2	0.05 ± 0.02	0.26 ± 0.05	1.12	NS
KRT10[b]	NA	0.25 ± 0.21	NA	NS

[a]Mann–Whitney *U*-test.

[b]Detectable levels of *KRT10* were seen in only one *HER2*-amplified cancer and in three *HER2/TOP2A*-co-amplified cancers.

NA, not assessable; NS, not significant.

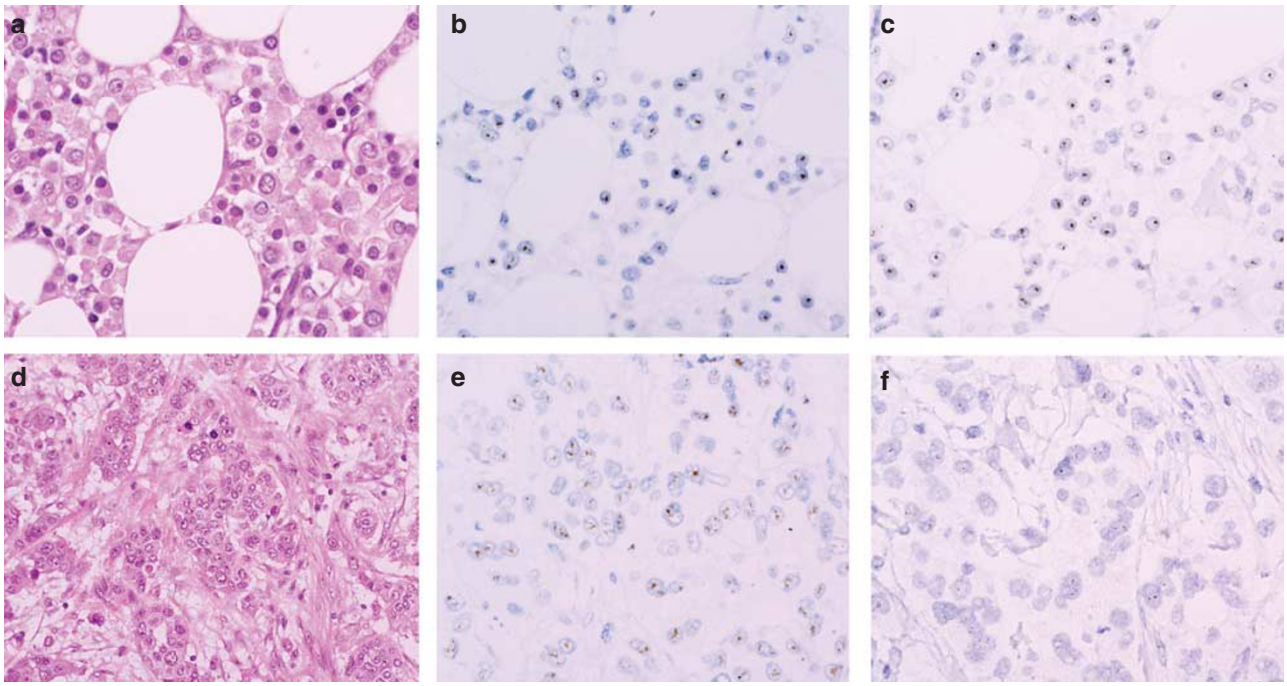

Figure 3 *HER2*-amplified invasive breast carcinomas. Grade III pleomorphic lobular carcinoma (**a**) with *HER2* (**b**) and *TOP2A* (**c**) co-amplification. Grade III invasive ductal carcinoma (**d**) harbouring *HER2* gene amplification (**e**) and lacking *TOP2A* gene amplification (**f**). (**a**, **d**) H&E; (**b**, **e**) chromogenic *in situ* hybridisation with probes for the *HER2* gene; (**c**, **f**) chromogenic *in situ* hybridisation with probes for the *TOP2A* gene; original magnification × 200 (**d**); × 400 (**a**–**c**, **e**, **f**).

deletion but no amplifications were observed on 17p (Supplementary Table 3). Only one case harboured a genomic deletion mapping to *TP53* locus (case X644). Recurrent losses on 17p13.1–p11.2 were seen in nine cases, with two smallest regions of overlap being identified: 17p12 (12 930–13 775 kb) and 17p11.2 (16 396–16 800 kb), both of which are known copy number polymorphisms (http://projects.tcag.ca/variation/) and frequently deleted in normal genomic DNA.[27,28] The second region of deletion affected 10 cases and mapped to 17p11.2-pcen (18 287–22 767 kb), with two smallest regions of overlap, one mapping to 18 287–18 697 kb and the other to 19 859–21 843 kb. The latter has not been reported to map to a copy number polymorphism and encompasses the following genes: mitogen-activated protein kinase kinase 3 (*MAP2K3*), potassium channel inwardly rectifying subfamily j member 12 (*KCNJ12*), spectrin and coiled-coil domains protein 1 (*SPECC1*), dehydrogenase/reductase (SDR family) member 7B (*DHRS7B*), transmembrane protein 11 (*TMEM11*), C17orf51 and family with sequence similarity 27-like (*FAM27L*).

Unlike 17p, 17q frequently displayed gains rather than losses of genomic material (Supplementary Table 3). The only recurrent region of deletion found on 17q mapped to 17q11.2–q12 (27 614–34 174 Mb); its smallest region of overlap mapped to 17q12 (29 014–29 555 Mb), which was seen in eight cases. This region encodes the neuronal amiloride-sensitive cation channel 1 (*ACCN1*), whose expression does not seem to be regulated by deletions or gains of genomic material.[18,21] In addition to the *HER2* and *TOP2A* amplicons, six other regions of recurrent amplification were observed on the long arm of chromosome 17 (Table 3).

Recurrent Co-Amplifications in *HER2*-Amplified Cases

We studied regions of amplification associated with amplification of *HER2* and/or *TOP2A*. We found 21 additional regions of recurrent amplification throughout the genome. We next analysed the SRA within each amplicon (Table 3). After excluding all regions included in the SRAs with reported copy number polymorphisms (http://projects.tcag.ca/variation/), 10 regions of recurrent amplifications in *HER2*-amplified cases were identified, three of which mapped to chromosome 20q (Table 3).

DISCUSSION

Genome-wide analysis of *HER2*- and *HER2*/*TOP2A*-amplified cases revealed that the large majority of these breast cancers and breast cancer cell lines display a 'firestorm'/'amplifier' genomic pattern, which is often seen in a subgroup of luminal and HER2 cancers,[25] and less commonly seen in grade I and basal-like breast cancers.[18,21,24,25] These findings suggest that different molecular subgroups of breast cancers are associated with distinct types of genetic instability and/or evolve through distinct genetic pathways. Unlike grade I breast cancers that harbour deletions of the whole of 16q in 65–85% of cases,[29,30] only 15% of *HER2*-amplified breast cancers display this genetic aberration. These findings suggest that progression from grade I to grade III breast cancer is an uncommon biological phenomenon and that *HER2*-amplified breast cancers are unlikely to originate from luminal A cancers, which are characterised by concurrent gains of 1q and deletions of 16q.[21]

The *HER2* SRA identified in this study encompasses that described by Kauraniemi *et al*[14,15] and Orsetti *et al*.[17] Interestingly, in all breast cancers and cell lines analysed here, four BACs (RP11-62N23, RP11-94L15, RP11-610O22 and RP11-32H6) were consistently amplified. The reason why the *HER2* SRA defined in this study is apparently larger than that reported by Kauraniemi *et al*[15] ($\sim$280 kb) stems from the updated mapping positions of BAC clones in more recent genome builds. In that study,[15] the boundaries of the 'core region' of *HER2* amplicon were defined by BAC clones RP11-390P24 and RP11-387H17, which according to the current genome build (hg18, http://www.ensembl.org), map to 34.80–34.98 Mb and 35.23–35.44 Mb, respectively. Hence, the actual 'core region' of *HER2* amplicon defined by Kauraniemi *et al*[15] spans approximately 633 kb.

In *HER2*-amplified tumours, the SRA encompassed *HER2* and *PPARBP*, *PPP1R1B*, *STARD3*, *TCAP*, *PNMT*, *PERLD1*, *GRB7*, *GSDML*, *PSMD3* and *THRAP4*, of which all but *PPARBP* and *PPP1R1B* have been reported to correlate with gene expression.[14,15,17,18,21] Our analysis of publicly available data on *HER2*-amplified and non-amplified breast cancer cell lines[18] revealed that *PPARB*, *GRB7*, *PERLD1*, *STARD3* and *PNMT* are also overexpressed when co-amplified with *HER2*. siRNA-mediated downregulation of *GRB7* and *STARD3* in *HER2*-amplified breast cancer cell lines is reported to lead to decreased cell proliferation and cell cycle progression,[31] suggesting that amplification and overexpression of other genes mapping to the SRA may also contribute to tumour growth. This is further corroborated by preclinical models, which have shown that a Grb7 peptide inhibitor in combination with Herceptin treatment enhances the inhibitory effect on the proliferation of SKBR3 cells and inhibit the proliferation of MDA-MB-361 cells.[32] Taken together, these findings suggest that *HER2* may not be the sole oncogene in this amplicon.

This study provides the first fine mapping of the 17q12–q21 amplicon in breast cancers harbouring *HER2*/*TOP2A* co-amplification (SRA: 34 730.322–36 547.206 kb). Apart from the genes known to be amplified and overexpressed in cases harbouring *HER2* amplification, *TOP2A*, *CASC3*, *CDC6*, *RARA* and *SMARCE1* are overexpressed in *HER2*/*TOP2A*-co-amplified breast cancers and breast cancer cell lines. *CASC3* or *MLN51* is overexpressed in breast cancers and encodes a core component of the exon junction protein complex, which is deposited on spliced mRNAs at exon–exon junctions and functions in nonsense-mediated mRNA decay.[33] *RARA* is frequently rearranged in promyelocytic leukaemias and has recently been reported to be a separate amplicon in breast cancers with complex patterns of 17q rearrangements.[34] Our findings demonstrate that *RARA* does not form a separate amplicon and is actually part of *TOP2A* amplicon.[34] The *SMARCE1* or *BAF57*, is part of the large ATP-dependent chromatin remodelling complex SWI/SNF, which is required for transcriptional activation of genes normally repressed by chromatin remodelling. This gene is reported to mediate ERα signalling in breast cancer cells.[35] The *CDC6*, which is regulated by E2F proteins, encodes a protein essential for the initiation of DNA replication. Amplification and overexpression of *CDC6* result in *INK4*/*ARF*-dependent transcriptional repression, recruitment of histone deacetylases and heterochromatinisation of the *INK4*/*ARF* locus, and a concurrent decrease in p16 expression.[36] These amplified and overexpressed genes may be additional amplicon drivers with important biological roles in *HER2*/*TOP2A* co-amplified breast cancers. Further studies to test whether these genes could also be explored as additional therapeutic targets are warranted.

Table 3 Summary of smallest regions of recurrent amplification in 15 *HER2*-amplified invasive breast cancers

Cytoband	Start (kb)	End (kb)	CNP	Amplified cases (*TOP2A* amplified)	Genes	MicroRNAs
1q23.1	155 533.39	155 942.01	No	2 (1)	*FCRL3*, *FCRH3*, *FCRL4*, *FCRL5*	
1q23.2–23.3	158 012.91	158 762.75	No	2	*NHLH1*, *ATP1A2*, *CR625159*, *VANGL2*, *KIAA1355*, *VSIG8*, *SLAMF8*, *IGSF9*, ***PEA15***, *TAGLN2*, *KCNJ10*, *PIGM*, *SLAMF9*, *SLAMF6*, *SUMO1*, *IGSF8*, *KCNJ9*, *ATP1A4*, *CCDC19*, *CASQ1*, ***WDR42A***, *NESG1*, ***PEX19***, *UNQ1938*, *DUSP23*, ***COPA***, ***NCSTN***, *FCRL6*	
1q32.1–32.2	204 706.01	208 215.77	Yes	2 (1)	*DYRK3*, *G0S2*, *C1orf74*, *CD34*, *C4BPB*, *ZCYTO10*, ***LGTN***, *AK123177*, *C4BPA*, *PFKFB2*, *LAMB3*, *AY882314*, *IL10*, *IL19*, *C1orf107*, *CR1*, *IL20*, ***IRF6***, *FCAMR*, *TRAF3IP3*, *SYT14*, *PLXNA2*, *IKBKE*, *C1orf116*, *CD55*, *CD46*, *C1orf147*, *RED*, *IL24*, *CAMK1G*, *FAIM3*, *FKSG87*, *CR2*, *BC063704*, *PIGR*, *YOD1*, *RASSF5*, *CR1L*, ***MAPKAPK2***, *DAF*, *HSD11B1*	hsa-mir-29c, hsa-mir-29b-2, hsa-mir-205
1q41–q42.12	219 587.07	223 218.85	Yes	2 (1)	*TP53BP2*, *CNIH4*, *C1orf58*, *DISP1*, *RP11-367A10.1-001*, *TAF1A*, *TLR5*, *MIA3*, *FBXO28*, *CNIH3*, *WDR26*, *KIAA1822L*, *NVL*, *C1orf65*, *DUSP10*, *C1orf80*, *CAPN2*, *C1orf67*, *DKFZp666C185*, *SUSD4*, *FLJ43505*, *CR625980*, *DKFZp586E1222*, ***DEGS1***	
1q43	234 816.89	236 546.39	Yes	2 (1)	*HEATR1*, *MTR*, *ZP4*, *RYR2*, *ACTN2*, *MT1P2*	
1q44	243 448.79	244 960.36	Yes	2 (1)	*SCCPDH*, *KIF26B*, *TFB2M*, *C1orf71*, *DQ593601*, *SMYD3*	
6p21.1	44 057.11	44 543.40	No	2 (1)	*HSP90AB1*, *ENT1*, *SPATS1*, *SLC29A1*, *SLC35B2*, *MGC33600*, *MRPL14*, *NFKBIE*, *CDC5L*, *TMEM63B*, *AARS2*, *MGC45491*, *CAPN11*	
6q36.3	154 985.71	155 707.99	Yes	2 (1)	*SHH*, *RBM33*, *PRR8*, *CNPY1*	
6q36.3	157 543.30	157 954.14	No	2 (1)	*DQ583756*, *PTPRN2*	
6q36.3	158 595.97	158 660.28	No	2 (1)	*VIPR2*	
8p12	37 307.82	38 168.97	Yes	2 (2)	***LSM1***, *AX747135*, *EIF4EBP1*, *ADRB3*, *GOT1L1*, *STAR*, ***BAG4***, *ZNF703*, ***ASH2L***, ***PROSC***, *RAB11FIP1*, *GPR124*, *KIAA1531*, ***BRF2***, ***SPFH2***, *ERLIN2*	
8q21.2	86 717.11	86 899.50	Yes	2 (2)	*REXO1L1*	
11q13.3	68 461.88	68 945.85	No	3 (2)	*OCIM*, *MRGPRD*, *MYEOV*, *IGHMBP2*, *TPCN2*, *MRGPRF*	
17q11.2	22 766.90	25 931.27	Yes	4 (3)	*SUPT6H*, *POLDIP2*, *FLOT2*, *PIGS*, *SARM1*, *PCFT*, *TIAF1*, *ERAL1*, *SEZ6*, *FLJ00234*, *TMEM97*, *TP53I13*, *CRYBA1*, *DHRS13*, *LOC201229*, ***GIT1***, *NUFIP2*, *PIPOX*, *PHF12*, *ALDOC*, *RAB34*, *IFT20*, *UNC119*, *TRAF4*, *NLK*, *C17orf63*, *NEK8*, *SLC13A2*, *SDF2*, *FLJ00295*, *PROCA1*, *DKFZp547N203*, *FOXN1*, *TLCD1*, *VTN*, *RPL23A*, *SPAG5*, *FLJ40504*, *LOC116236*, *MYO18A*, *UNQ419*, *BCOX*, *TNFAIP1*, *TAOK1*, *SEBOX*, *C17orf32*	hsa-mir-195, hsa-mir-497, hsa-mir-451, hsa-mir-144, hsa-mir-423, hsa-mir-324, hsa-mir-33b
17q11.2–q12	28 369.71	29 170.12	Yes	2 (1)	*ACCN1*	
17q12	29 347.57	29 842.55	Yes	2 (0)	*CCL8*, *CCL1*, *CCL2*, *CCL11*, *CCL7*, *ACCN1*, *CCL13*	
17q21.33	45 192.43	45 766.56	Yes	3 (2)	***ITGA3***, *PPP1R9B*, ***PDK2***, ***MYST2***, *COL1A1*, *FAM117A*, *TAC4*, *DLX3*, *TMEM92*, ***DLX4***, *SAMD14*, *SGCA*, *HILS1*	
17q21.33	46 753.68	47 115.08	Yes	3 (2)	*FLJ42842*, *CA10*	
17q23.2–q23.3	57 237.42	57 657.12	Yes	4 (3)	***THRAP1***, ***BRIP1***, *INTS2*	
17q25.1	69 945.06	71 453.61	Yes	2 (2)		

Table 3 Continued

Cytoband	Start (kb)	End (kb)	CNP	Amplified cases (*TOP2A* amplified)	Genes	MicroRNAs
					C17orf77, ***ICT1***, ***SLC16A5***, ***LLGL2***, *OTOP2*, ***MRPS7***, *ACOX1*, *CD300C*, *NHERF*, *CDR2L*, *HN1*, ***CASKIN2***, *GALK1*, *TMEM104*, ***KCTD2***, *UNC13D*, *MRPL38*, *C17orf28*, *FBF1*, ***H3F3B***, *UNK*, ***ATP5H***, ***WBP2***, *NUP85*, *GRIN2C*, *HTRG*, ***SUMO2***, *USH1G*, *NT5C*, *DKFZp564D012*, *FADS6*, *TRIM65*, *TSEN54*, *SLC25A19*, *OTOP3*, *FDXR*, ***ARMC7***, *SLC9A3R1*, *DKFZp667C0210*, ***GPRC5C***, *ITGB4*, *NAT9*, ***RECQL5***, *TRIM47*, ***GGA3***, *MIF4GD*, *SAP30BP*, *RAB37*, *ARM2*, ***GRB2***	
17q25.3	73 330.75	75 566.34	Yes	3(3)	***SYNGR2***, *CBX8*, *TK1*, *EPR-1*, *ENPP7*, *FLJ21865*, *TMC6*, *LOC651900*, *SOCS3*, *LOC146713*, *TMC8*, ***PGS1***, *LGALS3BP*, *CTRP1*, *CBX4*, *DNAHL1*, *CBX2*, ***PSCD1***, ***BIRC5***, *DNEL2*, *DNAH17*, *C1QTNF1*, *TNRC6C*, *TIMP2*, *CANT1*, *AFMID*, *DKFZp434P174*, *FLJ45079*, *DKFZp762M186*, *TBC1D16*	
18q21.1	42 616.60	42 806.27	Yes	2 (2)	*TCEB3C*, ***PIAS2***, *KATNAL2*	
18q23	74 479.69	74 556.23	No	3 (2)		
20q13.13	48 297.35	48 599.68	No	2 (2)	***PTPN1***, *LOC284751*	
20q13.2	49 661.58	49 726.34	No	3 (2)	*KIAA0611*, ***ATP9A***	
20q13.2	51 672.43	52 291.75	Yes	3 (2)	*BCAS1*, *CYP24A1*, ***PFDN4***	
20q13.31	53 780.45	55 362.29	Yes	2 (1)	*AURKA*, *SPO11*, *C20orf43*, ***RAE1***, *C20orf32*, *C20orf108*, *C20orf107*, *MC3R*, *C20orf106*, ***TFAP2C***, *CBLN4*, *BMP7*, ***CSTF1***	
20q13.32	56 267.79	56 673.02	No	2 (1)	***STX16***, *VAP-B*, *AK123388*, *AK091704*, ***VAPB***, ***RAB22A***, *APCDD1L*, *PPP4R1L*	
20q13.33	61 234.05	61 880.00	Yes	3 (2)	*ARFGAP1*, *KIAA1269*, *ZBTB46*, *PRIC285*, *SRMS*, *STMN3*, *GMEB2*, *CHRNA4*, *TNFRSF6B*, *LIME1*, *PTK6*, *RTEL1*, *C20orf58*, *COL20A1*, ***ARFRP1***, *YTHDF1*, ***SLC2A4RG***, *KCNQ2*, *ZGPAT*, *EEF1A2*, *BIRC7*, *C20orf195*, *C20orf149*	hsa-mir-124a-3

CNP, copy number polymorphism.

In bold, genes that are reported to be overexpressed when amplified.[13,15–18,21,23]

Analysis of the six *HER2*-amplified cell lines revealed that only SKBR3 and UACC812, which harbour *HER2*/*TOP2A* co-amplification, are appropriate models for *HER2*/*TOP2A*-amplified breast cancers. Furthermore, MDA-MB-361, MDA-MB-453 and ZR-75.30 do not appear to be ideal *in vitro* models for *HER2*-amplified breast cancers in general, given that these cell lines have a luminal expression profile,[18] are positive for ER and harbour *TOP2A* deletions. *TOP2A* gene deletions are likely to have a significant impact on the biology of *HER2*-amplified breast cancers and are rather infrequent in human tumours.[37–39]

We have also identified complex patterns of copy number changes on chromosome 17 in *HER2/TOP2A*-co-amplified breast cancers. Owing to the high resolution of our aCGH platform, we could define the smallest regions of recurrent deletions on 17p and recurrent high-level gains/amplifications either proximal or distal to but not in continuity with the *HER2* amplicon. These additional amplicons on 17q may harbour potential oncogenes and therapeutic targets (Table 3) as some of these genes are consistently overexpressed when amplified.[18,21,24] For example, *BIRC5* (17q25.3) encodes the apoptosis inhibitor survivin, which has been shown to correlate with lack of ER, high histological grade and a poor prognosis in breast cancer when overexpressed.[40] In addition, there also appears to be a link between HER2 overexpression and aberrant regulation of survivin in breast cancer.[41] There are burgeoning data to suggest that survivin may be an useful therapeutic target given that (i) survivin small-molecule inhibitors are reported to induce regression of human hormone-refractory prostate tumour xenografts[42] and

(ii) histone deacetylase inhibitors have also shown to decrease the expression of survivin and may have a role alone or in combination with the standard treatments for HER2+ tumours.[43] *GRB2* (17q25.1) encodes the growth factor receptor-binding protein 2, which is an adaptor protein that binds to an autophosphorylated site within the cytoplasmic tail of the HER2 receptor. The HER2–Grb2 coupled signalling pathway has been shown to induce the development of mammary tumours with unique morphological characteristics and a high metastatic potential in transgenic mouse models.[44] Inhibition of Grb2 expression using siRNA has been shown to inhibit proliferation of bcr-abl-positive leukaemia cell lines and prolong survival in mice bearing bcr-abl-positive leukaemia xenografts.[45] Our results suggest that further studies designed to test survivin and *GRB2* as additional therapeutic targets for *HER2/TOP2A*-amplified breast cancers are warranted.

Additional regions of recurrent amplification are apparent in our analysis (Table 3). Although amplification of *HER2* may be the best characterised genetic event in the development of these tumours, there are several genes included in these co-amplified regions that may also play a critical role in tumour development and response to therapy. For instance, the amplicon on chromosome 1q23 contains *PEA15*, which has a role in the development of other tumour types and interacts with the ERK/MAPK pathway,[46] whereas *RAB22A* on 20q13.2 belongs to the well-defined RAS oncogene family, which plays a crucial role in many types of cancer.[47] Although trastuzumab therapy is already changing the natural history of HER2+ breast cancer, these tumours harbour multiple additional amplifications of genes that may either explain the primary resistance of specific subgroups of HER2+ tumours to this therapy or could be tested as possible additional therapeutic targets for these tumours.

Despite the limited sample size of our study, combined CISH and aCGH analysis has revealed that CISH provides a reliable assessment of *HER2* and *TOP2A* amplification. The accuracy of this technique, when compared to the results of aCGH analysis, was 100% for highly amplified cases. However, cases with low-level amplification may require correction with CEP17 probes.

In conclusion, our study provides the first detailed molecular characterisation of *HER2/TOP2A*-amplified breast cancer, defines the SRA on 17q12–q21 in cases harbouring *HER2/TOP2A* co-amplification and identifies the SKBR3 and UACC812 cell lines as *in vitro* models for this breast cancer subtype. Unlike basal-like cancers, these tumours less frequently display multiple low-level gains and deletions and more frequently harbour recurrent amplifications. Further studies on the genetic mechanisms behind these multiple amplifications and the recurrently amplified and overexpressed genes identified in our analysis may lead to a better understanding of this molecular subgroup of breast cancers. Most importantly, some of the genes identified in this study can be targeted by drugs in early phases of development with promising antitumoural activity. Our results pave the way for further studies testing these genes as therapeutic targets in HER2+ breast carcinomas.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org).

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DISCLOSURE/DUALITY OF INTEREST
None.

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