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Molecular profiling of invasive breast cancer by multiplex ligation-dependent probe amplification-based copy number analysis of tumor suppressor and oncogenes

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Several oncogenes and tumor-suppressor genes have been shown to be implicated in the development, progression and response to therapy of invasive breast cancer. The phenotypic uniqueness (and thus the heterogeneity of clinical behavior) among patients' tumors may be traceable to the underlying variation in gene copy number of these genes. To obtain a more complete view of gene copy number changes and their relation to phenotype, we analyzed 20 breast cancer-related genes in 104 invasive breast cancers with the use of multiplex ligation-dependent probe amplification (MLPA). We identified *MYC* gene amplification in 48% of patients, *PRDM14* in 34%, *topoisomerase IIα* (*TOP2A*) in 32%, *ADAM9* in 32%, *HER2* in 28%, *cyclin D1* (*CCND1*) in 26%, *EMSY* in 25%, *IKBKB* in 21%, *AURKA* in 17%, *FGFR1* in 17%, *estrogen receptor alpha* (*ESR1*) in 16%, *CCNE1* in 12% and *EGFR* in 9% of patients. There was a significant correlation between the number of amplified genes and the histological grade and mitotic index of the tumor. Gene amplifications of *EGFR*, *CCNE1* and *HER2* were negatively associated with estrogen receptor status whereas *FGFR1*, *ADAM9*, *IKBKB* and *TOP2A* revealed a positive association. Amplifications of *ESR1*, *PRDM14*, *MYC* and *HER2* were associated with a high mitotic index, and *PRDM14* and *HER2* amplifications with high histological grade. *MYC* amplification was detected more frequently in ductal tumors and high-level *MYC* amplifications were significantly associated with large tumor size. *HER2*/*MYC*, *HER2*/*CCNE1* and *EGFR*/*MYC* co-amplified tumors were significantly larger than tumors with either of these amplifications. Gene loss occurred most frequently in *E-cadherin* (*CDH1*) (20%) and *FGFR1* (10%). In conclusion, MLPA analysis with this 'breast cancer kit' allowed to simultaneously assess copy numbers of 20 important breast cancer genes, providing an overview of the most frequent (co)amplifications as well as interesting phenotypic correlations, and thereby data on the potential importance of these genes in breast cancer.
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Several genes have been shown to be involved in the development, progression and response to therapy of invasive breast cancer. Among these, HER-2/*neu* is likely the most important proto-oncogene. Amplification of the *HER2* gene is present in approximately 15–30% of breast carcinomas and leads to protein overexpression, which correlates with a poor outcome[1–3] and is associated with a good response to treatment with trastuzumab, a recombinant humanized monoclonal anti-*HER2* antibody.[4,5] Furthermore, amplification of *HER2* has also been shown to correlate with resistance to conventional adjuvant chemotherapy and tamoxifen.[6–10] *Topoisomerase IIα* (*TOP2A*) gene amplification seems to be predictive of response to a class of cytostatic agents called TopoII inhibitors, which include the anthracyclines.[11–16] Recently, *estrogen receptor alpha* (*ESR1*) gene amplification has been implicated in response to tamoxifen therapy,[17] but its significance was doubted by others.[18] Amplification of *MYC* has been associated with poor prognosis and

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resistance to anti-estrogen therapy.[19] Therapeutic or prognostic significance of other frequently amplified genes such as *cyclin D1* (*CCND1*)[20] or frequent loss of genes such as *E-cadherin* (*CDH1*) is less clear, and comparative genomic hybridization (CGH) studies have pointed to many more genes and chromosomal loci with potentially important copy number changes.[21–23]

Nevertheless, no single gene copy number seems to completely explain prognosis or response to therapy of individual breast cancer patients. A simultaneous analysis of copy number changes of a variety of genes involved in prognosis and therapy response may thus be very useful for molecular profiling of individual breast cancer patients. This can be achieved by array CGH but this is still a costly and labor-intensive technique that requires a relatively large amount of sample DNA and specialized personnel to deal with the complexity of the data. In this study, we used an easier and faster high-throughput PCR-based technique, called multiplex ligation-dependent probe amplification (MLPA).[24] This assay determines relative gene copy numbers in a quantitative way and requires only minute quantities of small DNA fragments, which makes it very suitable for DNA isolated from paraffin-embedded material. In previous studies, we obtained promising results with *HER2* MLPA in comparison with immunohistochemistry[25] and *in situ* hybridization[26] and evaluated this technique to simultaneously determine copy number changes of *HER2*[27] and *TOP2A*.[16] The goal of this study was to apply MLPA as a technique to simultaneously detect amplifications and/or losses of a large set of breast cancer-related genes. These genes (including *HER2*, *EGFR*, *TOP2A*, *MYC*, *CCND1*, *CCNB1*, *ESR1*, *AURKA*, *EMSY*, *CDH1*, *FGFR1*, *PRDM14*, *ADAM9* and *IKBKB*) were selected based on their prognostic and/or therapeutic implications in breast cancer, or their proven frequent copy number change by CGH. We sought to obtain a more complete view of the clinical significance of MLPA-detected gene copy number alterations and therefore analyzed their mutual interactions, as well as their associations with common prognostic factors such as age, histological type and grade, HER2 immunohistochemistry, estrogen and progesterone receptor (ER, PR) status, mitotic index[28] and tumor size.

Materials and methods

Patient Material

Tissue samples of invasive breast cancer patients were collected between November 2004 and December 2008 at the Department of Pathology of the University Medical Center in Utrecht (UMCU), The Netherlands. This study randomly selected 104 tissue samples from this consecutive series. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in the UMCU.[29] All tissue samples were analyzed with immunohistochemistry to assess HER2, ER and PR protein expression and MLPA to determine gene copy number alterations. In addition, age at diagnosis, histological type, tumor size, histological grade and mitotic activity index (MAI)[30] were determined for all patients.

Immunohistochemistry

Immunohistochemistry for HER2 was performed using the Hercep test (Dako, Glostrup, Denmark) according to the manufacturers' instructions on 4 μm thick sections from the neutral buffered formaldehyde fixed tissue blocks. Immunohistochemistry membrane staining was semiquantitatively scored as negative (0), weakly positive (1+), equivocal (2+) and strongly positive (3+) according to the DAKO FDA-approved scoring system. Interpretation of staining was conducted by two experienced breast pathologists. As control, a small tissue array containing a 0, 1+, 2+ and 3+ breast tumor samples was taken along on the same slide as the tumor to be analyzed. Immunohistochemical staining for ER (1D5, 1:80, Dako) and PR (PGR636, 1:200, Dako) was performed using a Bond-Max automated staining machine (Vision Biosystems, Newcastle, UK) with the Bond polymer refine detection kit (Vision BioSystems, cat. no DS9800). Negative controls were used throughout.

Multiplex Ligation-Dependent Probe Amplification

Invasive tumor areas as identified on serial H&E sections were harvested from one or two whole 4 μm thick paraffin sections (corresponding to approximately 1 square cm tumor tissue) with a scalpel. We have estimated the tumor percentages of all samples before MLPA and used samples with tumor percentages of at least 70%. In a previous study,[26] we showed that tumor percentages $>30\%$ are already sufficient for reliable MLPA performance and that more than half of the tumors show a tumor percentage $>60\%$. DNA was isolated from these tissue fragments by 1-h incubation in proteinase K (10 mg/ml; Roche, Almere, The Netherlands) at 56 °C followed by boiling for 10 min. This DNA solution (50–100 μl) was, after centrifugation, used in the MLPA analysis according the manufacturers' instructions, using the P078-A1 kit (MRC Holland, Amsterdam, The Netherlands). The contents of this kit are depicted in Table 1. All tests were performed in duplicate in an ABI 9700 PCR machine (Applied Biosystems, Foster City, CA, USA). PCR products were analyzed on an ABI310 capillary sequencer (Applied Biosystems). Gene copy numbers were analyzed using Genescan (Applied Biosystems) and Coffalyser (version 7.0) software (MRC-Holland). For genes with more than one probe present in the kit, the mean of all the probe peaks of this gene in duplicate was calculated. If this mean value was below 0.7 the

Table 1 Contents of the P078-A1 MLPA kit (MRC Holland)

Gene	*Chr*	*Mapview position*	*No. of probes*	*Transcript description*	*Ref.*
ESR1	06q25	06–152.307247 06–152.423838 06–152.457215	3	Transcription factor	Holst *et al*[17]
EGFR	07p11	07–055.191055 07–055.196767 07–055.233957	3	Receptor tyrosine kinase involved in signal transduction	Park *et al*[50]
FGFR1	08p12	08–038.391533 08–038.434092	2	Receptor tyrosine kinase involved in signal transduction	Chin *et al*[37] and Kwek *et al*[40]
ADAM9	08p11	08–038.998319	1	Metalloproteinase associated with protein metabolism	Chin *et al*[37] and Kwek *et al*[40]
IKBKB	08p11	08–042.292902 08–042.302676	2	Serine/threonine kinase associated with signal transduction	Chin *et al*[37]
PRDM14	08q13	08–071.130073	1	Transcription regulatory protein	Nishikawa *et al*[43]
MYC	08q24	08–128.821796 08–128.822001 08–128.822151	3	Transcription factor involved in apoptosis and cell proliferation	Rodriguez-Pinilla *et al*[51]
CCND1	11q13	11–069.165399 11–069.167779 11–069.175089	3	Cell cycle control protein involved in signal transduction	Kirkegaard *et al*[47]
EMSY	11q13	11–075.902087 11–075.926543	2	Transcription regulatory protein	Kirkegaard *et al*[47]
CDH1	16q22	16–067.328716 16–067.404826 16–067.419579	3	Adhesion molecule associated with signal transduction	Cleton-Jansen[52]
NOS2A	17q11	17–023.114082	1	Enzyme with oxidoreductase activity involved in metabolism	—
TRAF4	17q11	17–024.098403	1	Adaptor molecule involved in signal transduction, cell proliferation and apoptosis	Camilleri-Broet *et al*[53]
CPD	17q11	17–025.795018	1	Carboxypeptidase involved in protein metabolism	—
LASP1	17q12	17–034–308187	1	Cytoskeletal associated protein involved in signal transduction	Grunewald and Butt[54]
PPARBP	17q12	17–034.840858	1	Transcription regulatory protein involved in signal transduction	Zhu *et al*[55]
HER2	17q12	17–035–118101 17–035–122165 17–035.127183	5	Receptor tyrosine kinase associated with signal transduction	Moelans *et al*[26]
CDC6	17q21	17–035.699283	1	Cell cycle control protein involved in signal transduction	Arriola *et al*[56]
TOP2A	17q21	17–035.812698 17–035.816651 17–035.818297	3	DNA topoisomerase protein involved in regulation of the topological status of DNA	O'Malley *et al*[14]
CCNE1	19q12	19–034.999920 19–035.000150 19–035.005214	3	Cell cycle control protein involved in signal transduction	Callagy *et al*[46] and Jensen *et al*[57]
AURKA	20q13	20–054.389980	1	Serine/threonine kinase involved in signal transduction	Ginestier *et al*[58] and Sen *et al*[59]

For each gene the chromosomal position, the number of probes present in the MLPA kit, a description of the transcript protein and if possible a relevant (breast cancer) reference is given.

respective gene was defined as lost, a value between 0.7 and 1.3 was defined as normal, a value between 1.3 and 2.0 as low-level amplification and values >2.0 as high-level amplified as previously established.[31,32]

Statistics

Statistics were performed using SPSS statistical software. Data were dichotomized as follows: amplified *vs* non-amplified, grade I *vs* grade II/III, age <50 *vs* ≥ 50, tumor size pT1 *vs* pT2/pT3, ER and PR positive *vs* negative, MAI <13 *vs* ≥ 13, ductal *vs* lobular, HER2 IHC 0/1+ *vs* 2+/3+. Associations were examined using χ^2 test and Fisher's exact tests, if applicable. Correlations were calculated with Spearman's rho. Unsupervised hierarchical cluster analysis (Euclidean distance, average linkage analysis) was performed using the open-source R statistical software (http://www.r-project.org).

Results

Amplifications and Losses

Frequencies of gains and losses for the 20 analyzed genes in 104 invasive breast cancers are depicted in Figure 1 and Table 2. All analyzed regions were involved in amplification with varying frequencies. A majority of the amplifications (low and high level) were found on chromosome 8 (particularly *MYC*, *PRDM14* and *ADAM9* in 48%, 34% and 32% of the patients, respectively) and on chromosome 17 (particularly *TRAF4*, *CDC6*, *TOP2A* and *HER2* in 36, 35, 32 and 28% of the patients, respectively). Although *MYC* showed amplification in almost half the patients, only 16% of these amplifications were high level (ratio > 2.0). For *HER2*, in contrast, most amplifications (72%) were high level. In a previous study, we already established a good correlation between *HER2* gene amplification by MLPA and *HER2* gene amplification by *in situ* hybridization.[26] Of 56 out of 104 (54%) patients, we had previously determined *HER2* chromogenic *in situ* hybridization data: of 11 out of 21 MLPA amplified patients there were CISH data available and all 11 patients showed CISH amplification. Of 41 out of 75 MLPA *HER2* normal patients, CISH data were available and all 41 tumors were normal by CISH. Of four out of eight *HER2* MLPA low-level amplified patients, CISH data were available: three-fourths were normal and one-fourth was amplified by CISH. *CCND1* amplification was found in 26% of the patients, and 56% of these amplifications were high level. *ESR1* amplification was found in 16% of the patients although most were low level and only rarely high level (2% of all patients).

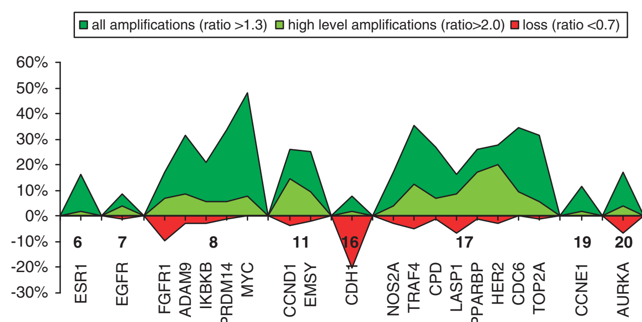

Figure 1 Amplifications (green) and losses (red) for 20 oncogenes and tumor-suppressor genes as found by analysis of 104 invasive breast cancer patients with the P078-A1 breast cancer dedicated MLPA kit. The chromosome numbers of the genes are shown on the horizontal axis.

Several regions showed loss by MLPA. The two regions with the most frequent loss were *CDH1* on chromosome 16 (20% of patients: 13 out of 21 of ductal and 6 out of 21 of lobular type) and *FGFR1* on chromosome 8 (10% of the patients).

On average, this study found five amplifications per patient (range 0–17 of the 20 analyzed genes) of which two were high-level amplifications (range 0–10). Only five patients (5%) did not show any amplification or loss for the analyzed regions. Five other patients showed no amplifications but did show loss of one or more genes. Of these five patients, there were three patients with only loss of *CDH1* (two-thirds were ductal carcinomas). One

Table 2 Frequencies of amplification (ratio > 1.3), high level (HL) amplification (ratio > 2.0) and loss (ratio < 0.7) for all 20 genes analyzed by multiplex ligation-dependent probe amplification in 104 invasive breast cancer patients

Gene	Chr	Amplifications (%)	High-level amplifications (%)	Loss (%)	Expected amps/ loss in % (range)	Ref.
ESR1	06q25	16	2	0	0–20.6	Holst *et al*[17] and Albertson[18]
EGFR	07p11	9	4	1	5–10 (7–65)	Lambros[19]
FGFR1	08p12	17	7	10	9	Letessier *et al*[35] and Elbauomy *et al*[60]
ADAM9	08p11	32	9	3	—	—
IKBKB	08p11	21	6	3	—	—
PRDM14	08q13	34	6	1	—	—
MYC	08q24	48	8	0	9–15 (1–94)	Lambros[19] and Jensen *et al*[57]
CCND1	11q13	26	14	4	15 (0–27)	Lambros[19] and Jensen *et al*[57]
EMSY	11q13	25	10	2	7–13	Kirkegaard *et al*[47] and Hughes-Davies *et al*[48]
CDH1	16q22	8	2	20	50 LOH 16q	Cleton-Jansen[52] and Chalmers *et al*[61]
NOS2A	17q11	17	4	3	—	—
TRAF4	17q11	36	13	5	—	—
CPD	17q11	27	7	1	—	—
LASP1	17q12	16	9	7	—	—
PPARBP	17q12	26	17	1	—	—
HER2	17q12	28	20	3	15–30	Owens *et al*[1] and Slamon *et al*[3]
CDC6	17q21	35	10	0	—	—
TOP2A	17q21	32	6	1	5–10	Knoop *et al*[62] and Di Leo *et al*[63]
CCNE1	19q12	12	2	0	3–6	Callagy *et al*[46] and Jensen *et al*[57]
AURKA	20q13	17	4	7	14 (5–20)	Letessier *et al*[35]

Abbreviations: amps, amplifications; Chr, chromosome position; Ref., reference.
The two last columns represent published amplification (or loss of *CDH1*) frequencies and corresponding references respectively.

other patient with a lobular carcinoma showed a loss of *CDH1* accompanied by *IKBKB*, *CCND1* and *LASP1* loss, and the fifth patient presented with a *PRDM14* and *FGFR1* loss. Ten patients showed amplifications of just one gene: *PRDM14* (3 out of 10), *MYC* (3 out of 10), *EMSY* (2 out of 10) and a high-level amplification of *AURKA* and *EGFR* in one patient each.

Co-amplified Regions, Loss of Regions and Their Association

A majority of the genes were never found amplified or lost alone. Nine of the 104 patients (9%) showed amplifications for all five analyzed chromosome 8 genes, possibly pointing to polysomy 8. In 13 patients (13%), both genes on chromosome 11q were amplified. Two patients (2%) were amplified for all eight chromosome 17q genes analyzed, possibly pointing to gain of 17q. Of these two patients, one was also amplified for all chromosome 8 and 11 genes, and the other patient was amplified for all chromosome 8 genes. None of the patients showed loss for all analyzed chromosome 8, 11 and/or 17 genes.

In all, 15% of all patients showed a co-amplification of *HER2* and *MYC*, 13% of *HER2* and *TOP2A*, 9% of *HER2* and *CCND1* and 7% of *HER2* and *CCNE1*. Eighteen percent of all patients showed a co-amplification of *MYC* and *TOP2A* and 16% of *MYC* and *CCND1*. Figure 2 shows the percentage of co-amplifications of *HER2*, *MYC*, *CCND1*, *CCNE1* and *TOP2A* amplified breast tumors. Of the 27 *HER2* amplified breast cancers, 52 and 45% were *MYC* and *TOP2A* co-amplified, respectively. Of the 12 *CCNE1* amplified patients, 10 were also *MYC* amplified (83%). When only high-level amplifications (MLPA ratio >2.0) were considered relevant, 5 out of 21 *HER2* high-level amplified patients were also *CCND1* (high level) amplified, 4 out of 21 *TOP2A* co-amplified, 1 out of 21 *MYC* co-amplified and 1 out of 21 *CCNE1* co-amplified. Of the 15 *CCND1* high-level amplified patients, 5 out of 15 were *HER2* co-amplified, 2 out of 15 *TOP2A* co-amplified and 1 out of 15 was *MYC* amplified. Patients with an amplification of *EGFR* had an increased likelihood to also have *CCNE1* amplifications ($P<0.001$) and tumors with a *TOP2A* amplification had an increased probability of *EMSY* amplification ($P=0.004$). Furthermore, patients with high-level *HER2* amplifications had an increased probability to have high-level *TOP2A* amplifications ($P=0.017$) and patients with high-level *EGFR* amplification a higher risk of having high-level *MYC* amplifications ($P=0.023$).

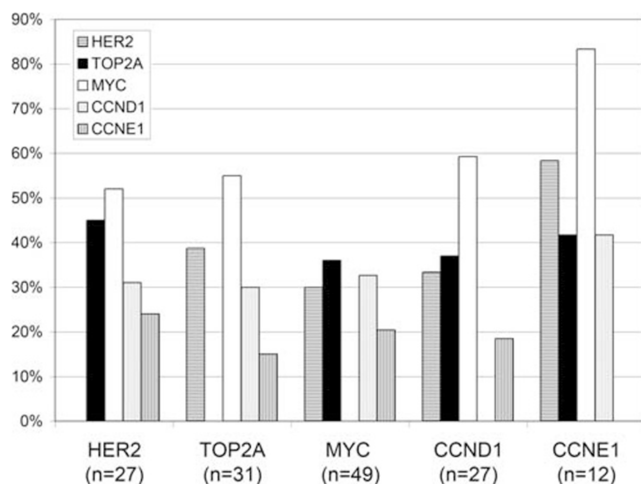

Figure 2 Co-amplifications of *HER2*, *TOP2A*, *MYC*, *CCND1* and *CCNE1* amplified breast tumors in a series of 104 invasive breast cancers analyzed by MLPA.

Cluster analysis, as illustrated in Figure 3, showed one apparent cluster of *HER2* (*ERBB2*) and *PPARBP*. Most other chromosome 17 genes (*TOP2A*, *CPD*, *CDC6*, *TRAF4* and *NOS2A*) were located in a different cluster. *ESR1*, *CCNE1* and all chromosome 8 genes except for *FGFR1* formed another cluster.

Supplementary Table 1 shows the co-amplifications (all and high level) for all 20 genes analyzed by MLPA.

Association between Amplified Regions and Clinical Characteristics

There was a significant correlation between the number of amplifications per tumor and grade ($P=0.030$) and even more between the number of high-level amplifications per tumor and grade ($P<0.001$). There was a significant association between the number of amplifications and *HER2* immunohistochemistry status. In addition, there was a significant correlation between the number of amplifications and high-level amplifications per tumor and mitotic index ($P=0.015$ and $P=0.004$, respectively) but there was no association with tumor size, with hormone receptor status nor with the patient's age. We also found significantly more high-level amplifications per tumor for tumors of the ductal subtype than for tumors of the lobular subtype ($P<0.001$, $P=0.083$) but not for all amplifications. Although not significant, we did observe more *CDH1* loss in lobular tumors (36%) than in ductal tumors (17%).

Table 3 shows the association of several amplified regions with clinicopathological characteristics. There was, as expected, a significant association between HER2 immunohistochemistry and *HER2* gene amplification ($P<0.001$), but there was also a significant association with *PRDM14* gene amplification ($P=0.027$). *ESR1* amplification was significantly associated with higher MAI ($P=0.007$) and showed a trend toward association with higher grade ($P=0.054$). *EGFR* amplification was significantly associated with negative ER status ($P=0.005$) and showed a trend toward association with

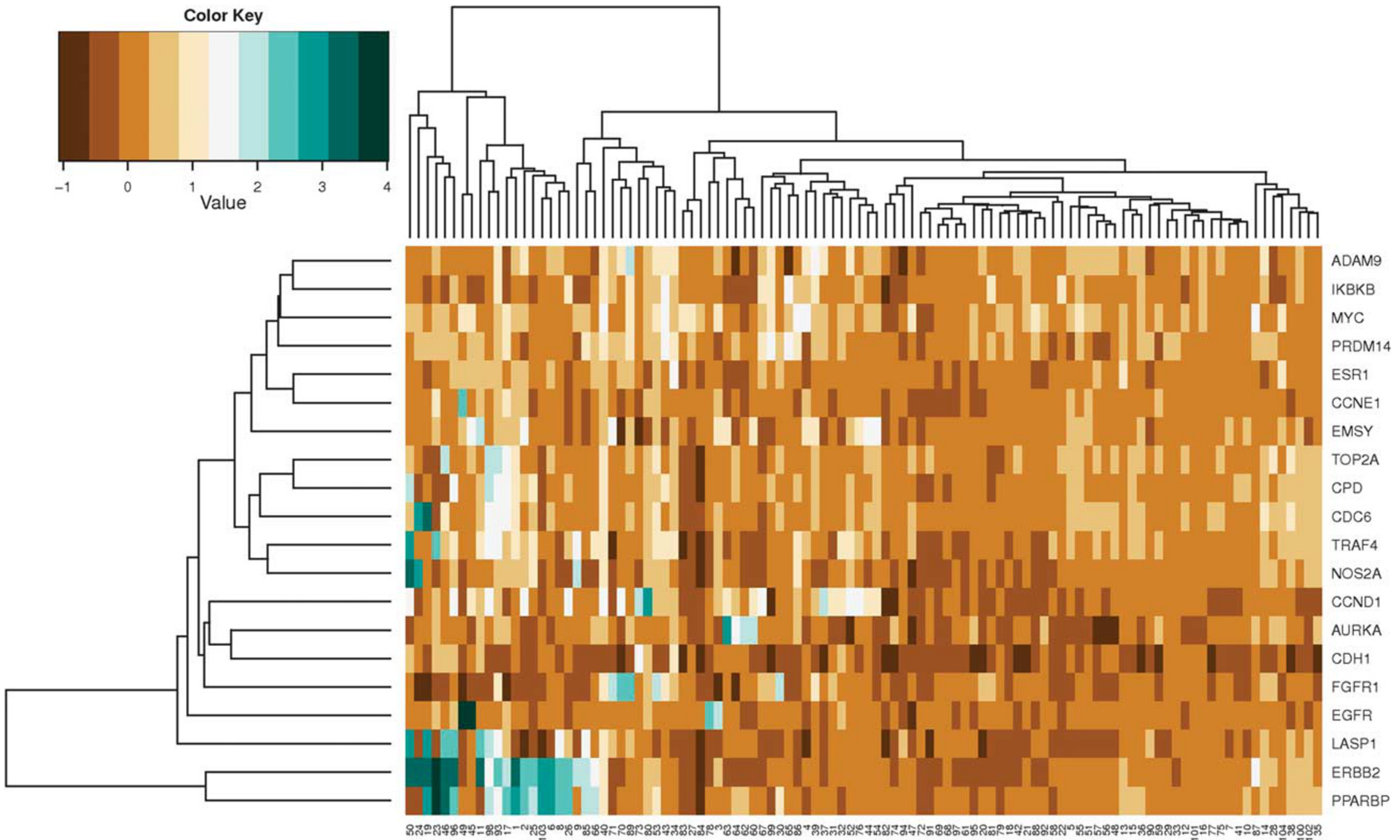

Figure 3 Hierarchical cluster analysis of 104 invasive breast cancer patients (horizontal axis) analyzed by MLPA for 20 breast cancer-related genes (vertical axis).

Table 3 Association of amplified regions with clinicopathological characteristics

		ER *0/1*	*PR* *0/1*	*Grade* *1/2–3*	*Age* *<50/⩾50*	*MAI* *<13/⩾13*	*Tumor size* *pT1/pT2–3*	*HER2 IHC* *0–1+/2+−3+*	*Type* *Ductal/lobular*
	n	*31/70*	*48/53*	*19/72*	*23/81*	*50/43*	*35/60*	*82/21*	*76/11*
ESR1	17			($P=0.054$)		$P=0.007$			
EGFR	9	$P=0.005$	($P=0.052$)						
FGFR1	18	$P=0.032$							
ADAM9	33	$P=0.019$							
IKBKB	22	$P=0.026$	$P=0.015$						
PRDM14	35			$P=0.049$		$P=0.010$		$P=0.027$	
MYC	50					$P=0.040$			$P=0.011$
CCND1	27								
EMSY	26								
HER2	29	($P=0.060$)		$P=0.040$		$P=0.036$		$P<0.001$	
TOP2A	33	$P=0.045$							
CCNE1	12	$P=0.004$							
AURKA	18								

For significant associations, the corresponding *P*-values following χ^2 statistics are depicted in the table. Trends are mentioned between brackets.

negative PR status ($P=0.052$). *FGFR1* and *ADAM9* amplifications were significantly associated with positive ER status ($P=0.032$ and $P=0.019$, respectively). *IKBKB* was significantly associated with positive ER and PR status ($P=0.026$ and 0.015, respectively). *PRDM14* amplification was correlated with higher grade ($P=0.049$) and MAI ($P=0.010$). *MYC* amplification was significantly associated with higher MAI ($P=0.040$) and with the ductal subtype ($P=0.011$). High-level *MYC* amplifications were significantly associated with a larger tumor size ($P=0.045$). *HER2* amplification was associated with higher grade ($P=0.040$) and MAI ($P=0.036$) and showed a trend toward association with ER status ($P=0.060$, for high-level amplifications $P=0.004$). *TOP2A* amplification was significantly associated with positive ER status of the tumor ($P=0.045$), in contrary to *CCNE1*, which was significantly associated with ER negativity ($P=0.004$). *CCND1*, *EMSY* and *AURKA* amplification did not show any significant associations with clinical–pathological features. We also did not observe any association between amplified regions and age.

Tumors with *HER2* and *MYC* co-amplification were significantly larger in size ($P=0.030$) than tumors with amplification of only one or neither of these genes, as were *HER2–CCNE1* co-amplified tumors ($P=0.017$). Tumors with *HER2–MYC* co-amplification were also significantly associated with higher HER2 immunohistochemistry status ($P<0.001$). There was also a trend toward an association between tumor size and *HER2–TOP2A* co-amplification ($P=0.061$). Tumors with *EGFR–MYC* co-amplification were significantly associated with ER negativity ($P=0.023$), were significantly larger ($P=0.017$) and showed a trend toward higher MAI ($P=0.059$) than tumors with either or neither of these amplifications.

Discussion

Several chromosomal regions are frequently amplified in breast cancer. Gene amplifications are essential features of advanced cancers and have prognostic as well as therapeutic significance in clinical cancer treatment. The aim of this study was therefore to simultaneously examine the copy number status of important or promising breast cancer genes (located on different chromosomal regions) by MLPA, to study the frequency of their co-amplifications, and to couple the obtained data to clinical–pathological characteristics currently used to determine treatment and/or prognosis.

It has long been known that the more advanced a cancer is, the more rearranged the genome is. We were therefore interested in verifying whether there was an association between the number of genetic alterations observed in a tumor and worse clinical–pathological tumor characteristics. On average, this study found 5 amplifications of the 20 analyzed genes per patient of which 2 were high-level amplifications. Ten patients (10%) showed single amplifications (of which 60% involved *MYC* or *PRDM14* amplifications), and interestingly these tumors were all grade 2 or 3 and were often highly proliferative with MAI>13. Only five patients (5%) did not show any amplification or loss in the analyzed regions. In these five tumors, the MAI was smaller than five, and three of them were grade 1 while two were grade 2. Although grade 1 tumors generally showed fewer genomic events than grade 2/3 tumors, they also rarely showed more complex genomic patterns associated with more advanced tumors indicating that there is not a strict relation between genomic state and histological grade. Nevertheless, this study found a significant correlation between the number of (high level) amplifications and the histological grade and MAI. Presence of gene amplifications may not only be important because of the resulting

overexpression of the oncogenes, it may also serve as a surrogate parameter for increased genetic instability of a cancer and, as such, represent an indicator of poor patient prognosis. Indeed, an association between patient survival and the number of amplifications was described by some studies.[33,34]

Amplifications and Losses

Amplifications involving chromosomes 8p (*FGFR1*, *ADAM9*, *IKBKB*), 11q (*CCND1*, *EMSY*) and 17q (*NOS2A*, *TRAF4*, *CPD*, *LASP1*, *PPARBP*, *HER2*, *CDC6*, *TOP2A*) are among the most common high-level copy number aberrations in breast tumors, occurring, for example, in one study, in 22.8, 19.6 and 9.9% of tumors, respectively.[35] Table 2 shows that the frequencies of amplification observed by MLPA for all analyzed genes in this study are in line with other studies. A majority of the *MYC* amplifications observed in this study were low level (84%), which is consistent with published results.[36] High-level amplifications of *ESR1*, a gene that is possibly involved in tamoxifen response,[17] were rare although we did observe 16% of patients with increased *ESR1* copy numbers. In a study by Chin *et al*,[37] low-level copy number aberrations by array CGH were not associated with reduced survival and they hypothesized that these aberrations are presumably selected during tumor development because they increase basal cell metabolism.

We found *CDH1* loss in 20% of all patients (36% in lobular carcinomas and 17% in ductal carcinomas), which is less than the reported frequency of LOH on 16q (78% in lobular carcinomas and 28% in ductal carcinomas).[38] *FGFR1* loss, which was found in 10% of cases in this study has previously been described and has been associated with poor outcome.[37]

Cluster analysis of all 20 breast cancer-related genes showed one apparent cluster of *HER2* (*ERBB2*) and *PPARBP*. Both genes are located near each other on chromosome 17 and have previously been shown to be often co-amplified.[39] Most other chromosome 17 genes (*TOP2A*, *CPD*, *CDC6*, *TRAF4* and *NOS2A*) were located in a separate cluster, indicating that these amplifications are probably independent of *HER2* amplification and represent a different advantage for tumor growth or survival. Another cluster was composed of *ESR1*, *CCNE1* and all chromosome 8 genes except for *FGFR1*, which was located in yet another small cluster with *AURKA*, *CDH1* and *CCND1*. Co-amplification of *FGFR1* on 8p12 and *CCND1* on 11q13 is one of the most common co-amplifications in breast cancer.[34,40]

Association of Genomic Regions with Clinical–Pathological Parameters

Amplification of 8p and 11q are most often observed in ER positive tumors whereas amplification of 17q occurs in both ER-positive and ER-negative tumors.[41,42] In our study, *EGFR* (7p), *CCNE1* (19q) and *HER2* (17q) were associated with a negative ER status, whereas *FGFR1* (8p), *ADAM9* (8p), *IKBKB* (8p) and *TOP2A* (17q) were associated with a positive ER status of the tumor. Contrary to the study of Holst *et al*,[17] we did not observe a significant association between *ESR1* amplification and ER protein overexpression (73% of tumors with *ESR1* amplification were ER-positive compared with 69% of tumors without *ESR1* amplification).

HER2 and *PRDM14* amplifications were associated with positive HER2 immunohistochemistry. *ESR1*, *PRDM14*, *MYC* and *HER2* amplifications were associated with a higher MAI and *PRDM14* and *HER2* amplifications were also correlated with higher grade. For *ESR1*, we found a strong trend toward association with higher grade, which was in contrast to a previous study,[17] but this study did not examine the association with MAI. For *PRDM14*, one study found no correlation between its expression levels and clinicopathological characteristics, which was assumed to reflect the small number of samples analyzed.[43] *MYC* amplification was more likely to be present in tumors of the ductal subtype compared with lobular ones, and high-level *MYC* amplifications were significantly associated with a larger tumor size.

Co-amplified Regions

Patients with more amplified loci had a significantly higher grade and MAI. As not only the number of amplified loci but also the function of the genes involved determine tumor characteristics, we selected pairs of frequently co-amplified genes and studied their relation to clinicopathological features. *HER2–MYC* co-amplification, for example, was present in 15% of the tumors, which could indicate the existence of a selective advantage associated with their co-amplification. This hypothesis is supported by our findings showing that concomitant amplification of *HER2* and *MYC* is associated with a significant larger tumor size and higher HER2 IHC status, and by other studies that found a relationship between *HER2* and *MYC* co-amplification and reduced survival.[33,34] From preliminary analyses from the NSABP B-31 trial, it was suggested that tumors that are *HER2–MYC* co-amplified have a remarkably favorable prognosis with adjuvant trastuzumab treatment.[44] Although not as frequent (7%) as *HER2–MYC* co-amplification, this study found that *HER2–CCNE1* co-amplified tumors were significantly larger than tumors with either of these amplifications. *CCNE1* protein overexpression has previously been associated with positive *HER2* status and poor prognosis,[45] but *CCNE1* amplification on itself was shown to have no prognostic role in breast cancer so far.[46] Tumors with *EGFR–MYC* co-amplification (7%) were larger and showed a trend toward higher MAI than tumors

with either or neither of these amplifications. Several other frequent co-amplifications in this study (eg, 17% *MYC–TOP2A*, 15% *MYC–CCND1*, 12% *TOP2A–EMSY* and 9% *HER2–CCND1*) did not show any association with clinicopathological characteristics. These data imply that there is no relationship between the frequency of the co-amplification and the association with current prognostic markers and that the type of genes involved in the co-amplifications determines the association with prognostic factors.

Co-amplification of 8p12 (*FGFR1*) has been reported in 30–40% of tumors with *CCND1* (11q13) amplification. In our study, 33% (9 out of 27) of *CCND1* amplifications were concomitant with *FGFR1* amplifications. Co-amplification of these genes is associated with significantly reduced survival,[34] but in our study this co-amplification was not associated with any clinical–pathological characteristics.

In this study 50% of *EMSY* amplifications were also *CCND1* amplified, which is less than the 70% described by another study.[47] *CCND1* and *EMSY* amplifications have both been associated with poor overall survival,[47,48] but there is no a straightforward association between *CCND1* amplification and expression, and *CCND1* expression has been associated with ER and good survival.[49] The mechanism for the frequent co-amplification of genes spread over different chromosomes is yet unclear.

In conclusion, this study introduces a dedicated breast cancer MLPA kit that provides data on the copy number of 20 tumor suppressor and oncogenes in a single PCR reaction on paraffin-derived DNA. MLPA is an easy and high-throughput PCR-based technique that provided potentially important information on associations with essential clinicopathological features and on the frequency of co-amplifications of different genes in breast cancer. Such detailed knowledge of potential driver oncogenes and their gene–gene interactions may help to refine patient-tailored treatment of breast cancer patients in the future.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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