EDITORIAL

Absence of chromosome 17 polysomy in breast cancer: analysis by CEP17 chromogenic in situ hybridization and multiplex ligation-dependent probe amplification

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Abstract Amplification of the HER2 gene, present in 15–30% of breast carcinomas, correlates with poor outcome and is an indication for treatment with trastuzumab. Standard testing methods for HER2 amplification are fluorescence (FISH) or chromogenic in situ hybridization (CISH). In FISH/CISH scoring, correction for chromosome 17 polysomy is believed to be critical for determination of true HER2 amplification as opposed to increased chromosome 17 copy number. The term "polysomy 17" is widely used and defined as ≥ 3 copies of the chromosome 17 centromere (probe CEP17, D17Z1). Thus, the centromere is assumed to be representative for the entire chromosome. This study aimed to investigate the frequency of polysomy 17 and its association with HER2 amplification in 111 invasive breast cancer patients by CEP17 CISH and by copy number analysis of a set of 17 genes along chromosome 17 using multiplex ligation-dependent probe amplification (MLPA).

Chromosome 17 usually showed a complex pattern of gains and losses by MLPA, unrelated to the copy number status of the centromere. Increase in centromere 17 copy number (denoted "polysomy 17"), as assessed by CEP17 CISH, was found in 19% of the patients. Of these patients, 60% also showed amplification of HER2 measured by MLPA. However, none of the 111 patients showed a true polysomy of chromosome 17 by MLPA. Only two patients (1.8%) had a possible gain of 17q. Amplification of 17p was not found in any of the patients, although a possible loss of 17p was found in one patient. In conclusion, this extensive analysis of amplicons along chromosome 17 shows that true polysomy of chromosome 17, either of the whole chromosome or of the short or the long arm, is very rare in invasive breast cancer. Abnormal CEP17 copy numbers may therefore actually stem from high level gains or amplification of CEP17 regardless of copy number gains of the short and long arms of chromosome 17 and, at least in some cases, correction with CEP17 probes may provide misleading HER2 gene status assessment results.

Keywords Breast cancer · MLPA · CEP17 · Polysomy · HER2

Introduction

Several genes have been shown to be implicated in the development, progression and response to therapy of invasive breast cancer. Among these, HER-2/*neu* is likely the most important proto-oncogene. HER2 is located on chromosome 17q21 and encodes an epidermal growth factor receptor family protein involved in signal transduction [1, 2]. Amplification of the HER2 gene, which is present in about 15–30% of breast carcinomas and leads to protein overexpression, correlates with a poor outcome [3] and is an indication for treatment with the recombinant humanized monoclonal anti-HER2 antibody trastuzumab [4, 5]. Standard testing methods include, besides immunohistochemistry, analysis of HER2 gene copy number by fluorescence (FISH) or chromogenic in situ hybridization (CISH). In FISH (and to a lesser extent CISH) scoring, correction for chromosome 17 polysomy is believed to be

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critical for determination of true HER2 gene amplification as opposed to increased chromosome 17 copy number [6, 7]. The term "polysomy 17" is widely used and is defined as ≥3 copies of the chromosome 17 centromere (probe CEP17, D17Z1). Thus, the centromere is assumed to be representative for the entire chromosome. Reported incidence of polysomy 17 ranges from 10 to 49%, depending on the criteria used to define polysomy [8]. In a recent study by Bartlett et al. [9], it was stated that the presence of polysomy 17, as established by CEP17 FISH rather than HER2 and TOP2A amplification, was predictive for response to anthracyclins. This further underlines the importance of assessing chromosome 17 copy number increase. Overall, chromosome 17 is one of the smallest and the second most densely gene-loaded human chromosome. It is rearranged in at least 30% of breast cancers with short and long arms differing in the type of events they harbor [10, 11]. Chromosome 17p is mainly involved in losses, some of them possibly focal, whereas comparative genomic hybridization (CGH) on 17q shows complex combinations of overlapping gains and losses. Therefore, increase in the centromeric region of chromosome 17 as detected by FISH/CISH may not at all reflect "polysomy" 17 and thereby be unsuitable to correct for 17q status. Also, the long arm of chromosome 17 (17q) is frequently characterized by a "firestorm" pattern in CGH studies (many narrow peaks of amplification and/or loss), but 5–10% of the firestorms do not include amplification of HER2 giving weight to the notion that other loci in the region may contribute to oncogenesis [12]. There have been many CGH studies to characterize breast cancer in terms of gene and class discovery [12–16], and although array CGH will undoubtedly have a number of clinical applications in the future, it is still too early to be used routinely by clinicians because it is still a costly and labor intensive technique that requires a relatively large amount of sample DNA and trained personnel to deal with the complexity of the data.

In the present study, we used an easier and faster high-throughput technique, called multiplex ligation-dependent probe amplification (MLPA), to characterize chromosome 17 status in paraffin-embedded invasive breast cancer samples. We specifically investigated the frequency of true polysomy 17 and the presence of co-amplifications. MLPA can simultaneously determine copy number gains and/or losses of multiple genes [17] (the centromere region included) along chromosome 17. In previous studies, we obtained promising results with MLPA in comparison with ISH [18] and evaluated this technique to simultaneously determine copy number changes of HER2 and TOP2A, a gene that has shown involvement in the response to anthracyclins by some groups [19, 20]. To analyze the centromere status of chromosome 17, we performed CEP17 chromogenic in situ hybridization. Additionally, we evaluated WSB1, located very near the centromere region at 17q11.1 (22,645,233–22,664,772 bp from pter), as an alternative for CEP17 in our MLPA analysis.

Methods

Patient material

Tissue samples of 111 invasive breast cancer patients were randomly selected from a previous study at the Department of Pathology of the University Medical Center in Utrecht [18]. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital [21]. All tissue samples were analyzed with immunohistochemistry (IHC) to assess HER2 protein expression and MLPA to determine gene copy number alternations. Subsequently, CEP17 analysis by CISH was performed on 106 samples; the other 5 samples were excluded from analysis as there was too little material left.

Immunohistochemistry

IHC for HER2 was performed using the Hercept test (Dako, Glostrup, Denmark) according to the manufacturers' instructions on 4-µm-thick sections from neutral buffered formaldehyde fixed tissue blocks. IHC membrane staining was semiquantitatively scored as negative (0), weakly positive (1+), positive (2+) and strongly positive (3+) according to the DAKO FDA-approved scoring system. Areas with intraductal carcinoma were excluded from the evaluation and cytoplasmic staining was ignored. Interpretation of staining was done 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. Appropriate negative controls were used throughout.

Multiplex ligation-dependent probe amplification

Invasive tumor areas as identified on serial H&E sections were harvested from two to four whole 4-µm-thick paraffin sections (corresponding to ~1 cm^2 tumor tissue) with a scalpel. 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 P004-B1 kit (MRC Holland, Amsterdam, The Netherlands). The contents of this kit are depicted in Table 1. It contains probes for 17 chromosome

Table 1 Genes on chromosome 17 targeted by probes in the P004-B1 MLPA kit (MRC Holland)

Gene	Location	Distance to pter	Number of probes
PAFAH1B1	17p13.3	2.530	1
PMP22	17p12	15.083	1
TOM1L2	17p11.2	17.727	1
WSB1	17q11.1	22.654/663/663	3
NOS2A	17q11.1	23.133	1
TRAF4	17q11.2	24.098	1
CPD	17q11.2	25.795	1
RNF135	17q11.2	26.336	1
PEX12	17q21	30.928	1
NEUROD2	17q21	35.014	1
ERBB2/HER2	17q21	35.118/133/137	3
RARA	17q21	35.762	1
TOP2A	17q21	35.817/818/823	3
BRCA1	17q21.31	38.469/496	2
SGCA	17q21.33	45.603	1
GH1	17q24.1	59.350	1
METRNL	17q25	78.636	1
REFERENCE PROBES			15

17 genes: 3 on 17p, one very close to the centromeric region (WSB1), 13 on 17q, as well as 15 control probes. All tests were performed in duplicate in an ABI 9700 PCR machine. PCR products were analyzed on an ABI310 capillary sequencer (Applied Biosystems, Foster City, CA, USA). Gene copy numbers were analyzed using Genescan and Coffalyser (version 7.0) software. 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 respective gene was defined as lost, a value between 0.7 and 1.3 was defined as normal, 1.3–2.0 as low level amplification and values >2.0 as high level amplified, according to the definitions in the Coffalyser software [22].

Centromere 17 chromogenic in situ hybridization

CEP17 CISH was performed using SpoT-Light chromosome 17 centromeric probe (Zymed, San Francisco, 84-0500) and SpoT-Light CISH centromere detection kit (Zymed, 84-9248) according to the manufacturer's instructions. CISH was mainly performed on 4-μm-thick tissue microarray sections ($n = 57$), supplemented with whole sections ($n = 49$) for further cases not present on the tissue microarray. Polysomy was defined as three or more copies of CEP17, counted in at least 30 tumor cells.

Statistics

Associations between two categorical variables were examined using chi-square with continuity correction and Fisher's exact tests if necessary. Unsupervised hierarchical cluster analysis (Euclidean distance, average linkage analysis) was performed using the open-source R software (version 2.9.1, http://www.r-project.org).

Results

Copy numbers for genes on chromosome 17 by MLPA

Supplementary Table 1 shows the copy numbers for the 17 genes along chromosome 17 for all cases. Most individual cases showed a complex pattern of amplifications and losses over chromosome 17. Frequencies of amplification and losses along chromosome 17 are depicted in Fig. 1. There were four major regions of amplification: 17q11.1-11.2 with the WSB1, NOS2A, TRAF4 and CPD genes; 17q12 with the PEX12, NEUROD2 and HER2 genes; 17q21.2 with TOP2A; and 17q21.33-q25.3 with SGCA, GH1 and METRNL. High level amplifications (ratio >2.0) are mainly localized in the second region containing HER2 (73% of its amplifications were high level). We found two regions on chromosome 17 with frequent loss: 17p11.2-p12 containing TOM1L2 and PMP22 and 17q21.2 containing RARA.

Centromere analysis

CEP17 analysis in 106 patients showed copy number increase (ranging from three copies/nucleus to large clusters with >10 copies/nucleus) in 20/104 patients (19.2%); two samples were inconclusive. Copy number analysis of the centromere region by MLPA (three WSB1 probes) showed increased copy number in 16% (18/111) of all patients; 33% of these (6/18) showed a high level amplification. Overall, the concordance between CEP17 and WSB1 copy number status was 92.3% (96/104) (see Table 2).

Polysomy analysis

None of the 111 patients showed a true polysomy of whole chromosome 17, as reflected by a copy number increase of all probes along chromosome 17. Polysomy of whole 17p as defined by amplification of all three probes on 17p was not found in any of the patients either, although possible loss of whole 17p was found in one patient that showed loss of all three 17p probes. This patient had a normal

Fig. 1 Chromosome 17 copy number aberrations by MLPA in 111 breast cancer patients. *Top* percentage of patients showing amplifications (ratio > 1.3, *white*) and losses (ratio < 0.7, *black*) for all the chromosome 17 probes of the MLPA kit. *Bottom* percentage of patients showing amplifications (> 1.3, *white*) and high level amplifications (ratio > 2.0, *black*) for all the chromosome 17 probes of the MLPA kit
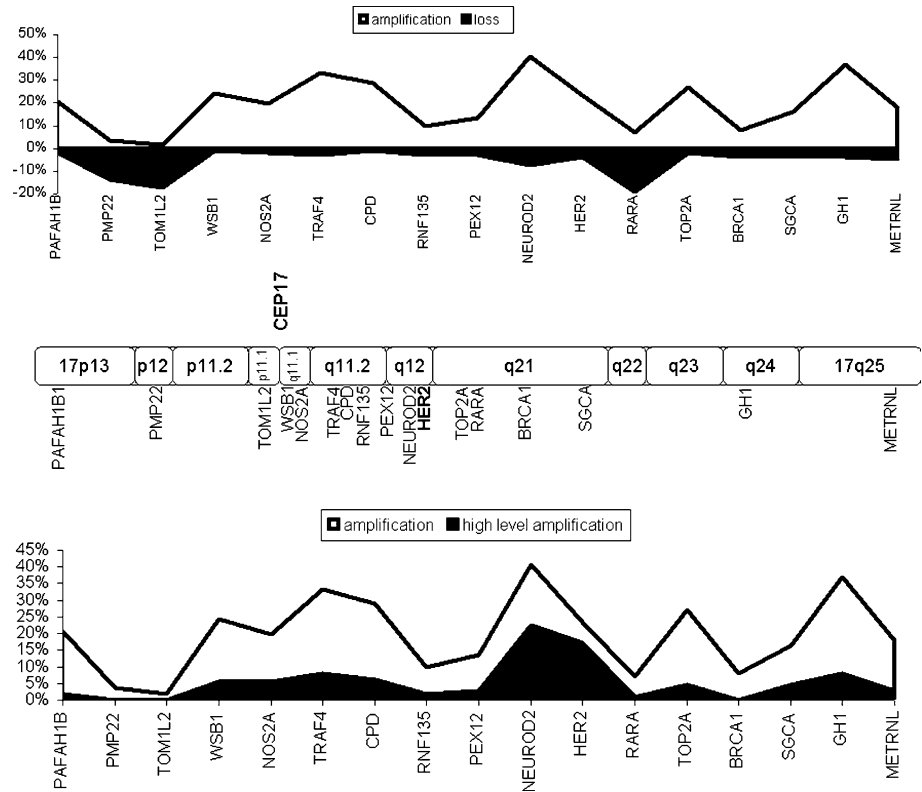
Table 2 Comparison between chromosome 17 centromere copy number by CEP17 CISH and WSB1 MLPA in 104 invasive breast cancer patients (concordance 92.3%)

	WSB1 MLPA	
	Normal	Increased
CEP17 CISH		
≤2 copies	82	2
>2 copies	6	14

Table 3 The association between centromere status by CEP17 CISH or WSB1 MLPA on the one hand and HER2 amplification by MLPA on the other

	CEP17 CISH		WSB1 MLPA	
	≤2 copies	>2 copies	Normal	Increased
HER2 status				
Normal	74	8	74	8
Amplified	10	12	14	8

centromere status by CEP17 (1–2 copies/nucleus) and MLPA (ratio 1.05). Only two patients (1.8%) showed amplification of all the tested genes on the 17q arm, pointing to a gain of whole 17q. These patients had increased centromere copy numbers by CEP17 (3–5 copies/nucleus and small clusters, respectively) and MLPA (ratio 1.86, 1.94, respectively).

Centromere status and HER2 amplification

Table 3 shows the association between centromere status by CISH and MLPA, and HER2 amplification. Eight of the 16 patients (50%) showing amplification of WSB1 and 12/20 patients (60%) showing amplification of CEP17 were also HER2 amplified. About 55% (12/22) and 36% (8/22) of HER2 amplified patients showed CEP17- and WSB1-based "polysomy 17," respectively. Only one patient presented with a loss of WSB1 and CEP17, but this was not a

true monosomy 17 since several other chromosome 17 genes had normal copy numbers.

Association between amplified regions

Most genes were never found amplified or lost alone. Amplification of HER2 (26/111 patients) was often associated with amplification of NEUROD2 (23/26, 88%), GH1 (17/26, 65%), TRAF4 (15/26, 58%), TOP2A (13/26, 50%) and WSB1 (12/26, 46%). Amplification of WSB1 (18 of 111 patients) was most frequently associated with amplification of TRAF4 (81%), CPD (74%), GH1 (70%), NEUROD2 (67%), NOS2A (63%) and TOP2A (59%).

Cluster analysis (Fig. 2) showed that only HER2 and NEUROD2 were frequently clustered together, which is consistent with other studies [23]. WSB1, NOS2A and TRAF4 seemed to form a second small gene cluster independent of the HER2/NEUROD2 cluster.

Fig. 2 Hierarchical cluster analysis of 111 invasive breast cancer patients analyzed for copy number status of 17 chromosome 17 genes by MLPA
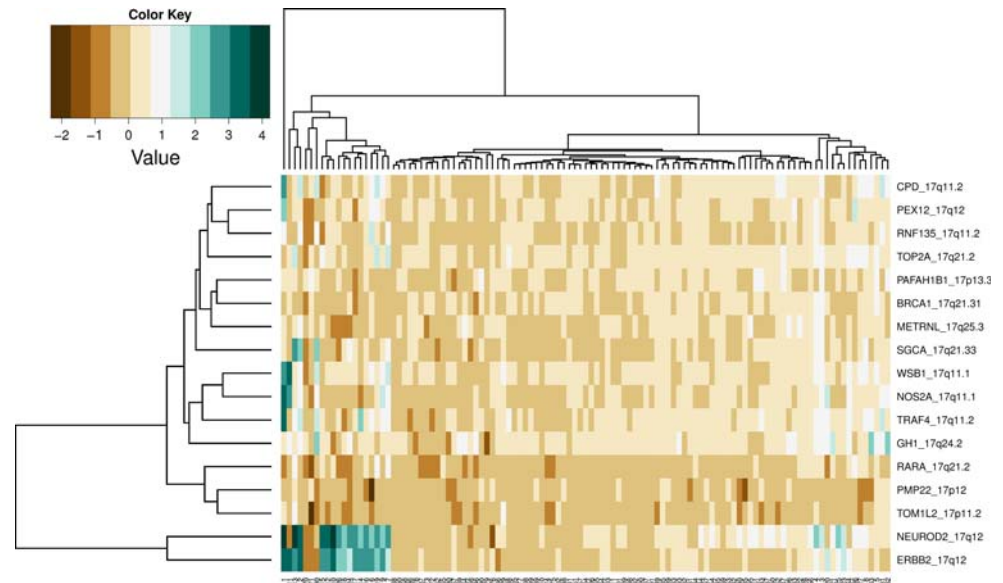
Discussion

This study aimed to investigate the frequency of polysomy 17 and its association with HER2 gene amplification in invasive breast cancer patients by CEP17 CISH and MLPA analysis. Also, we determined copy number aberrations of a set of genes along chromosome 17.

Chromosome 17 appears to undergo selective pressure to gain or lose specific regions as exemplified by the frequency plot of chromosome 17 shown in Fig. 1. A number of the events on chromosome 17 are bordered by sharp transitions, and these breakpoints tend to cluster in small intervals (0.2–2 Mb, [13]) that could represent fragile sites leading to the occurrence of copy number changes. Consistent with CGH studies, we found complex combinations of gains and losses on chromosome 17q and mainly losses on 17p [12, 13]. Some studies [12] showed that a fraction of the complex amplification patterns ("firestorms") on 17q (5–10%) did not include amplification of HER2, giving weight to the notion that other loci in the region may contribute to oncogenesis. In our study, cluster analysis of the analyzed chromosome 17 genes identified especially HER2 and NEUROD2 to be clustered together, which is consistent with other studies [23]. This implies that the HER2/NEUROD2 cluster is the most important amplicon on chromosome 17, which is also confirmed by the high number of high level amplifications in this amplicon when compared to the other chromosome 17 amplicons.

This study identified three other regions of amplification: 17q11.1-11.2 with the WSB1, NOS2A, TRAF4 and CPD genes; 17q21.2 with TOP2A; and 17q21.33-q25.3 with SGCA, GH1 and METRNL. These amplified regions are consistent with CGH studies [12, 13, 15], although the

last region can probably be subdivided into more regions if the gene probe density along 17qter is increased in the MLPA mix. Important to notice is the location of HER2 and TOP2A in different amplicons.

In the present study, none of the 111 analyzed patients showed a true polysomy 17 as reflected by simultaneous copy number increase of most genes and no losses along chromosome 17, although 16% of patients did show a higher WSB1 copy number and 19% of patients showed an increased CEP17 copy number. In our study, there was no perfect correlation between CEP17 and WSB1 (92%) indicating that WSB1 may not completely replace CEP17 for centromere analysis. But more importantly, our data imply that nor WSB1, nor CEP17, can be used to determine true polysomy of chromosome 17, since amplification of the centromere region by either techniques was not associated with amplification of other chromosome 17 loci. We believe that true polysomy 17 is extremely uncommon in breast cancer. There were no patients showing amplification of whole 17p and only 2/111 (1.8%) patients showed possible whole 17q amplification.

In our study, 50–60% of the patients showing WSB1- or CEP17-based polysomy 17 were HER2 amplified and up to 55% of HER2-amplified patients were polysomic based on WSB1 MLPA or CEP17 CISH analysis. The group of Hoffman et al. [24] showed that two "polysomic" patients that were FISH negative (but IHC3+) responded to trastuzumab indicating that FISH analysis can lead to false-negative results mainly based on CEP17 amplification. The HER2/CEP17 ratio may thus not be the best way to evaluate the HER2 status in all cases and the absolute HER2 gene copy number (whether increased through amplification or polysomy) may be the more important determinant for trastuzumab response for some patients. Except for this

study, it is presently unknown whether patients with amplicons spanning HER2 and CEP17 will respond to trastuzumab. Vanden Bempt et al. [6] showed that tumors displaying CEP17 amplification in the absence of HER2 amplification resemble more HER2-negative than HER2-positive tumors. These findings highlight the need for clinical trials to investigate whether patients with CEP17 amplification benefit from HER2-targeted therapy. At present, there is no clinical indication to determine CEP17 status on itself, although Bartlett et al. [9] did suggest a possible association between CEP17 amplification and response to anthracyclins.

In this study, we used MLPA as a technique to determine copy number changes of a set of genes along chromosome 17. In previous studies, we have already shown a good correlation between MLPA and FISH/CISH for HER2 and a good sensitivity and specificity of MLPA based on CISH as gold standard [18, 25]. Although interpretation of MLPA results is easier than FISH, one major disadvantage of MLPA is that it is a non-morphological technique that requires proper control of input material.

Since we randomly selected the breast tumor samples in this study, only 20/111 of the included patients were HER2 positive. Our results are consistent, however, with recent data from two studies using array CGH on breast tumors, which also suggest that polysomy of chromosome 17 is a rare event in breast cancer [26, 27]. Although extensive prognostic testing should be performed before drawing definite conclusions on the value of CEP17 in HER2 scoring, evidence from our study and other studies is mounting and at least raise questions concerning the proper selection of patients for trastuzumab/lapatinib therapy based on HER2 scoring with CEP17 correction.

In summary, this extensive analysis of amplicons along chromosome 17 shows that true polysomy of chromosome 17, either of the whole chromosome, or the short or the long arm, is likely very rare. Chromosome 17 usually shows a complex pattern of gains and losses, rather unrelated to the copy number status of the centromere and as a consequence, at least in some cases, correction with CEP17 probes may provide misleading HER2 gene status assessment results. Determining what CEP17 amplification means in terms of response to trastuzumab and anthracyclin treatments remains to be further studied. A more comprehensive analysis of amplicons along chromosome 17 rather than just HER2/CEP17 FISH or CISH may be indicated in IHC positive or doubtful breast cancers.

Conflict of interest statement   There are no conflicts of interest to be declared.

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