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Simultaneous detection of *TOP2A* and *HER2* gene amplification by multiplex ligation-dependent probe amplification in breast cancer

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***HER-2/neu* gene amplification, found in certain subtypes of (breast-) cancers, is an independent prognostic factor of poor outcome and determines eligibility for systemic treatment with trastuzumab. *TopoIIα* (*TOP2A*) gene amplification seems to be predictive of response to a class of cytostatic agents called *TopoII* inhibitors, which include the anthracyclines. The observed increased efficacy of anthracyclines in *HER2*-positive tumors is thought to arise from the close proximity of both genes on chromosome 17, where the *TopoII* amplification status will determine the anthracycline sensitivity. This study aimed to validate a new polymerase chain reaction-based test, called multiplex ligation-dependent probe amplification (MLPA), as a simple and quick method to simultaneously assess *HER-2/neu* and *TopoIIα* gene amplification status in paraffin-embedded breast cancer samples. To this end, MLPA results were compared with *TopoIIα*, *HER2* chromogenic *in situ* hybridization (CISH). We also assessed *TopoIIα* protein expression by immunohistochemistry. Of 353 patients, 9% showed *TopoIIα* amplification by MLPA and 13% of patients were *HER2* amplified. *TopoIIα* amplification was seen in 42% of *HER2*-amplified cases and showed no high level amplification without *HER2* amplification. Eleven patients displayed *TopoIIα* loss (3%). Concordance between MLPA and CISH was 91% for *TopoIIα* and 96% for *HER2*. Correlation between amplification and overexpression of *TopoIIα* was significant ($P = 0.035$), but amplification did not always predict protein overexpression. Loss of the *TopoIIα* gene was almost never associated with loss of its protein. In conclusion, MLPA is an easy and accurate method to simultaneously detect breast cancer *HER-2/neu* and *TopoIIα* copy number status in paraffin-embedded tissue, and thus an attractive supplement or alternative to CISH.**
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The topoisomerase IIα (*TopoIIα, TOP2A*) gene is located at chromosome 17q21.2 and encodes a 170 kDa protein that has a key role in cell division by controlling and modifying the topological status of DNA.[1] Furthermore, TopoIIα is the direct molecular target of TopoII inhibitors including anthracyclines, which are among the most powerful cytostatic agents in the treatment of invasive breast cancer. The binding of anthracyclines to TopoIIα is believed to stabilize the DNA double-strand breaks created by TopoIIα, leading to apoptosis. The TopoIIα gene is located next to the locus of the *HER2* (human epidermal growth factor receptor 2) gene, a proto-oncogene belonging to the *EGFR* family. The *HER2* gene encodes for a 185-kDa transmembrane glycoprotein, and overexpression of the protein is associated with poor prognostic as

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a consequence of increased cell proliferation, angiogenesis, invasive growth, and resistance to apoptosis. The *HER2* gene is amplified and overexpressed in 10–30% of breast cancers, in which it has an important role in oncogenesis.[2,3] The *HER2* protein is a direct target of trastuzumab (Herceptin^{R}), a humanized monoclonal antibody that has been approved for the systemic treatment of both primary and metastatic breast cancer.[4–6] With regard to the sensitivity of *HER2*-positive breast cancer, a number of studies have suggested an association with increased benefit of anthracycline-containing regimens. As a molecular basis for this association seems difficult to grasp, it has been suggested that the increased sensitivity of *HER2*/*neu*-positive breast cancer is a result from the proximity of the *TopoIIα* gene to the *HER2* gene.[7] Overall, *TopoIIα* amplification is considered to be an uncommon event in breast cancer, with a prevalence of approximately 5–10%.[8,9] Co-amplification of *HER2* and *TopoIIα* is seen in approximately 40% of *HER2*-amplified breast cancer patients[10,11] and results of—mainly retrospectively obtained—data seem to underline the hypothesis that *TopoIIα* and not *HER2* overexpression is the ultimate predictor of the response to anthracyclines.[10,12–14] Measurement of *TopoIIα* in the tumor could therefore potentially be useful in selecting the patients for treatment with *TopoII* inhibitors, including anthracyclines. Expression of *TopoIIα* protein has, however, not been shown to reliably predict response to anthracyclines, despite the fact that it is the direct target for these compounds.[11,15–17] In contrast, evaluation of *TopoIIα* gene copy number appears to be a good predictor of response to *TopoIIα* inhibitors.[18–20] Furthermore, contrary to *HER2*, *TopoIIα* amplification has shown an inconsistent correlation with *TopoIIα* protein expression,[21,22] mainly because *TopoIIα* protein is highly dependent on the stage of the cell cycle and proliferation rate.

Recently, we introduced *HER2* amplification detection in breast cancer by multiplex ligation-dependent probe amplification (MLPA). MLPA kits contain probes for up to 45 different targets allowing copy number assessment of different genes in the same PCR.[23] MLPA requires only small quantities of short DNA fragments, which makes it very suitable for analysis of paraffin-embedded material. In previous studies using MLPA, we obtained promising results for *HER2* in comparison with immunohistochemistry,[24] fluorescence *in situ* hybridization and chromogenic *in situ* hybridization (CISH).[25] As the applied *HER2* kit also contains a *TopoIIα* probe, we set out to test MLPA as a new method to simultaneously assess *HER2* and *TopoIIα* gene amplification status in a large group of breast cancer patients and to validate MLPA results with CISH in a subgroup of these patients. In addition, we investigated the correlation between *TopoIIα* protein expression levels and gene amplification status on tissue micro arrays, using immunohistochemistry and CISH, respectively.

Materials and methods

Patient Material

From a previously used study cohort ($n=518$), collected between November 2004 and June 2006 at the Department of Pathology of the University Medical Centre in Utrecht,[25] 353 consecutive tissue samples of invasive breast cancer patients were randomly selected. First, all tissue samples were analyzed by MLPA to determine *HER2* and *TopoIIα* gene amplification status. For *TopoIIα* CISH and immunohistochemistry, tissue microarrays were constructed from the original paraffin-embedded tumor blocks ($n=315$) using published guidelines.[26] In this study, the use of left over material was approved by the Tissue Science Committee of the UMC Utrecht.

Multiplex Ligation-Dependent Probe Amplification

Invasive tumor areas were harvested from 4 μm thick paraffin sections by dissection with a scalpel (using at least 1 cm^2 tumor tissue) and DNA was isolated 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-A1 *HER2* kit (MRC Holland, Amsterdam, The Netherlands). This kit contains three probes for the *HER2* gene, a probe for *TopoIIα*, 9 additional control probes for chromosome 17, and 25 control probes located on other chromosomes. Details of the probes in this kit can be found at http://www.mrc-holland.-com. All tests were performed in duplicate on an ABI 9700 PCR machine (Applied Biosystems, Foster City, CA, USA). PCR products were analyzed on an ABI310 capillary sequencer. *HER2* and *TopoIIα* gene copy numbers were normalized against the control probes in the kit, thereby excluding all chromosome 17 probes. The mean of all three *HER2* probe peaks in duplicate (6 values) and the *TopoIIα* peak in duplicate (2 values) was calculated. If this mean value was below 0.7, *TopoIIα* or *HER2* was considered lost, values between 0.7–1.5 were considered normal, values between 1.5 and 2.0 as low level amplified, and values >2.0 as *HER2* or *TopoIIα* amplified. The 2.0 threshold was used in accordance with previous *HER2* MLPA studies,[24,27] whereas the 1.5 threshold was empirically established during routine diagnostic application of MLPA kits for trisomy detection.

Chromogenic *In Situ* Hybridization

HER2 and *TopoIIα* CISH assays were performed on 4 μm thick paraffin serial tissue array sections using the SPoT-Light *HER2* or *TopoIIα* kits (Zymed, San Francisco, CA, USA) according to the manufac-

turers' instructions. First, sections were baked overnight at 56°C and deparaffinized in xylene and alcohol 100%. For *HER2* and *TopoIIα*, the slides were then boiled in pretreatment buffer for 15 min, followed by enzymatic digestion at room temperature (RT) for 10 min (Zymed). Then, slides were dehydrated with graded alcohols. After 20 min of air-drying, the digoxigenin-labeled *TopoIIα* or *HER2* probes were applied to the slides. Then, the sections were denatured on a hot plate (95°C) for 5 min and hybridization was carried out overnight at 37°C. After hybridization, appropriate stringency washes at 80°C were performed, followed by blocking with 3% hydrogen peroxide and CAS block (Zymed). Subsequently, the slides were incubated with mouse-anti-digoxigenin antibody (Zymed) for 30 min at RT and goat-anti-mouse antibody conjugated with horseradish peroxidase (HRP) for 30 min at RT. This was followed by diaminobenzidine (DAB) development for 30 min and counterstaining with hematoxylin. Finally, sections were dehydrated and mounted in Histomount (Zymed). A positive control was included in each CISH run and consisted of paraffin sections of a case known to be *TopoIIα*/*HER2* amplified by CISH. At least 30 preferably non-overlapping nuclei in every tumor sample were scored by two blinded observers to determine the number of *HER2* and *TopoIIα* signals. Amplification was defined to be present when large peroxidase-positive intra-nuclear clusters (or >10 individual small signals) were detected in at least 50% of tumor cells. The presence of small peroxidase-positive intra-nuclear clusters (or 6–10 individual small signals) was considered low level amplified. One to five individual small signals were scored as *HER2*/*TopoIIα* non-amplified.

Immunohistochemistry

Immunohistochemistry was performed using a mouse monoclonal antibody against the *TopoIIα* protein (clone Ki-S1, DAKO, Glostrup, Denmark) on 4 μm thick sections from neutral-buffered formaldehyde-fixed tissue array blocks. First, sections were baked overnight at 56°C, deparaffinized and rehydrated. The slides were then blocked in 3% hydrogen peroxide for 15 min and boiled in EDTA buffer (pH 9.0) for 20 min. After washing in 0.05% PBS Tween, the slides were incubated with the primary antibody at a dilution of 1/200 for 60 min at RT. Detection was performed with Envision (Dako, Glostrup, Denmark) using an HRP-conjugated secondary antibody followed by DAB development. The percentage of strongly positive nuclei was estimated (weakly positive nuclei were ignored). The median percentage of stained cells was 2%, we therefore defined >2% as overexpression. Immunohistochemistry expression was analyzed by one experienced (blinded) breast pathologist (PJvD) and at least 30 nuclei were scored.

Statistics

Results obtained with MLPA and CISH were compared by cross tables using SPSS for Windows and the concordance percentages were calculated. Correlations between continuous and categorical variables were performed with the non-parametric Mann–Whitney *U*-test. Correlations between categorical variables were performed using the χ^2-test. *P*-values below 0.05 were considered significant.

Results

Multiplex Ligation-Dependent Probe Amplification

Table 1 shows the frequencies of *TopoIIα* and *HER2* amplification. The *TopoIIα* gene was low level amplified in 7% of cases and highly amplified in 8 cases (2%), adding up to a total of 33/353 (9%) cases with amplification. *HER2* was low level amplified in 10/353 cases (3%) and highly amplified in 34/353 cases (10%), adding up to a total of 44/353 (13%) of amplified cases.

Co-amplification with *TopoIIα* was seen in 42% of *HER2*-amplified cases (including both low and high levels). There was no high level amplification of *TopoIIα* without *HER2* amplification. However, in some cases we found a low level amplification of *TopoIIα* without amplification of *HER2*. As to comparative copy numbers in co-amplified tumors, *HER2* was often amplified at a higher level than *TopoIIα* within the same tumor.

Eleven cases (3%) were deleted for *TopoIIα*, all having a normal *HER2* status.

Chromogenic *In Situ* Hybridization

TopoIIα and *HER2* CISH were performed on 284 patients who were analyzed by MLPA (see Table 2).

Table 1 Frequencies of *TopoIIα* and *HER2* amplification by multiplex ligation-dependent probe amplification analysis in 353 invasive breast cancer patients

Gene	*Low level amplification (target/control ratio 1.5–2.0)*	*High level amplification (target/control ratio >2.0)*	*Total amplification*
HER2	10/353 (3%)	34/353 (10%)	44/353 (13%)
TopoIIα	25/353 (7%)	8/353 (2%)	33/353 (9%)

For *TopoIIα* we found concordance in 259 out of 284 (91%) of these patients. Most discordance was found in cases scored as low level by MLPA. Only 5/25 of these cases were confirmed to be *TopoIIα* amplified by CISH, and the other 20 cases were scored normal by CISH. All MLPA highly amplified cases were confirmed by CISH, although two of these cases only showed a low level amplification by CISH. Of the non-amplified cases by MLPA, 249 (99%) were concordant with CISH, whereas two non-amplified cases were scored low level amplified by CISH.

For *HER2*, 273/284 (96%) cases were concordant between MLPA and CISH. Concordance was highest in MLPA amplified (27/28) and non-amplified (244/248) cases, whereas 4/7 MLPA low level cases were scored normal by CISH.

Sensitivity, specificity, positive-predictive value and negative-predictive value of MLPA for *HER2* and *TopoIIα* were calculated and depicted in Table 4 using CISH results as gold standard, and by taking low level and high level amplifications together. When the cut-off was set at 1.8, the number of low level amplified patients was reduced significantly, thereby increasing the concordance between MLPA and CISH (as gold standard) for *TopoIIα*. Nevertheless, increasing the cut-off value lead to a decrease in sensitivity of MLPA for both genes.

Immunohistochemistry

From tissue arrays containing cores of 315 patients, information for both immunohistochemistry and CISH was obtained for 265 patients. A strong positive nuclear staining for Topoisomerase IIα in 265 invasive breast tumors ranged from 0 to 90% of tumor cells. One hundred and seventeen cases (44%) showed overexpression. Topoisomerase IIα overexpression was significantly associated with *TopoIIα* amplification by MLPA ($P=0.035$), although 4/14 (29%) of amplified tumors did not overexpress the *TopoIIα* protein (Table 3, Figure 1). One patient showing amplification of *TopoIIα* by CISH was not analyzed by immunohistochemistry because there was not enough tissue left. Of the cases without *TopoIIα* amplification, 42% showed overexpression, in comparison with 71% for *TopoIIα*-amplified cases. There was also evidence of a difference ($P=0.01$) in the mean *TopoIIα* protein expression level for tumor samples with *TopoIIα* amplification by CISH ($n=14$, mean 28% immunohistochemistry positive) *vs* no *TopoIIα* gene amplification ($n=251$, mean 7% immunohistochemistry positive).

Loss of the *TopoIIα* gene ($n=15$) was rarely (2/15) accompanied by absence of its protein, but rather by overexpression (7/15) although not significantly ($P=0.421$).

Discussion

The aim of this study was to test MLPA as a new method to simultaneously assess *HER2* and *TopoIIα* gene amplification status in a large group of breast cancer patients, and to compare MLPA results with CISH data as gold standard in a selected group of patients. Of 353 patients analyzed by MLPA, 2% showed a high level amplification of the *TopoIIα* gene and 10% of patients manifested a high level amplification of the *HER2* gene. When including low amplification, the percentages of amplification rose to 9 and 13%, respectively. For *HER2* this is lower than the 20–30% positivity that has generally been described in the literature,[2,3,28,29] although several other studies have reported lower (10–18%) percentages[30–34] as well. It is likely that many of the series in which higher *HER2* overexpression/amplification frequencies were described have not been unselected, whereas frequencies below 20% have been reported before in unselected series. As our study group concerned consecutive patients, selection bias can be excluded. Furthermore, methodological variation is an

Table 2 Comparison between multiplex ligation-dependent probe amplification (MLPA) and chromogenic *in situ* hybridization (CISH) results for *TopoIIα* and *HER2* on 284 breast cancer patients when a cut-off value of 1.5 between normal and low level amplified was applied

	MLPA (cut-off = 1.5)			
	Not amplified	*Low level amplified*	*Amplified*	*Total*
TopoIIα CISH				
Not amplified	249	20	0	269
Low level amplified	2	4	2	8
Amplified	0	1	6	7
HER2 CISH				
Not amplified	244	3	1	248
Low level amplified	4	4	2	10
Amplified	0	1	25	26
				284

Table 3 Association between Topoisomerase IIα protein expression (by immunohistochemistry) and gene amplification status (by chromogenic *in situ* hybridization) in 265 invasive breast cancer patients ($P=0.035$)

	Topoisomerase IIα protein		*Total*
	Normal	*Overexpressed*	
TopoIIα not amplified	144	107	251
TopoIIα amplified	4	10	14

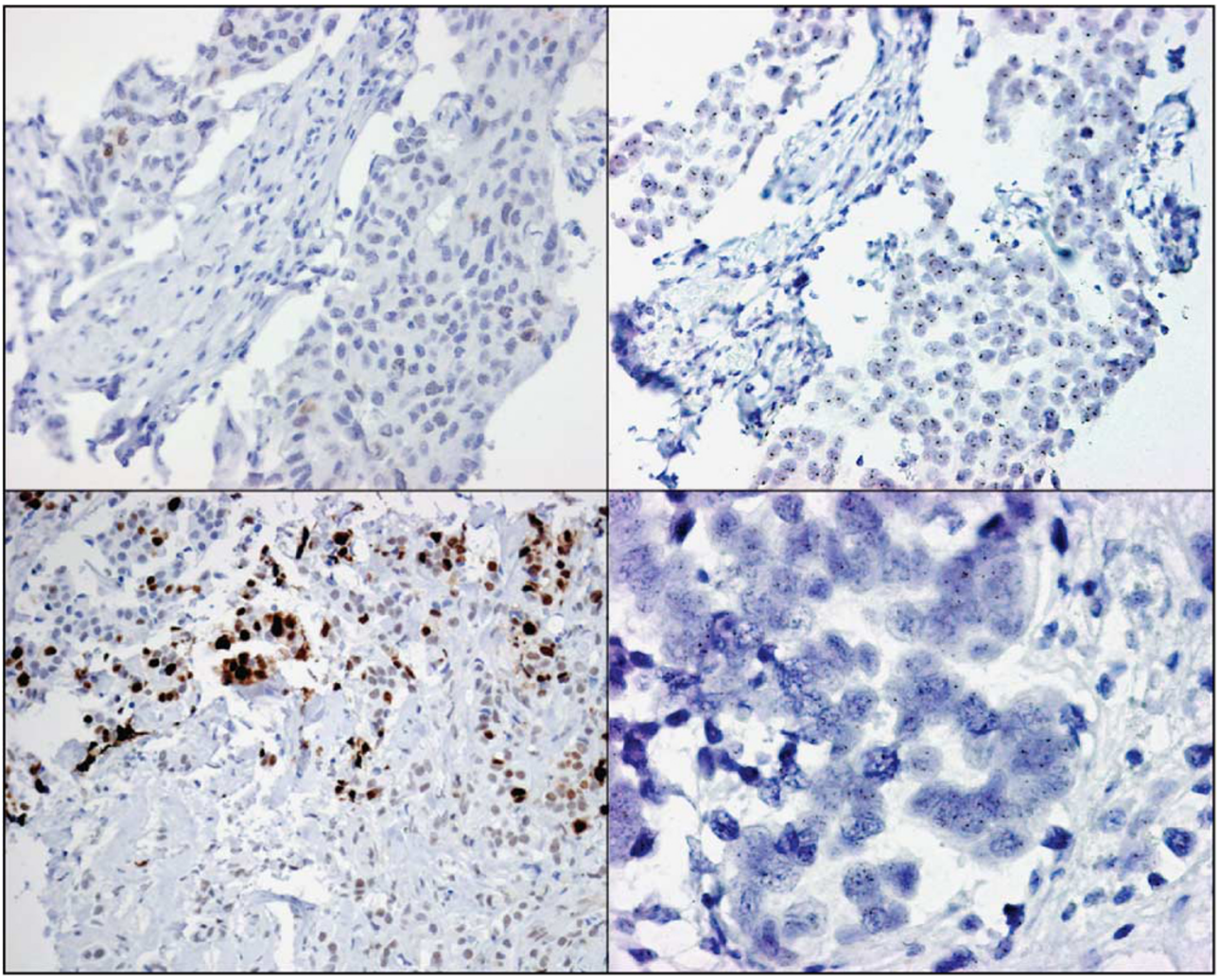

Figure 1 Correlation between gene amplification and protein expression in breast cancer as determined by chromogenic *in situ* hybridization and immunohistochemistry. Top left: Almost no *TopoIIα* protein expression is present. Top right: Large chromogenic *in situ* hybridization clusters indicate *TopoIIα* gene amplification in the same patient. Bottom left: strong *TopoIIα* protein expression is present in 5% of tumor cells. Bottom right: chromogenic *in situ* hybridization shows less than 5 signals per cell indicating no *TopoIIα* gene amplification.

unlikely explanation as the fraction of HER-2/*neu*-amplified cases by immunohistochemistry (10%, Moelans *et al*[25]) was similar. This implies that there may be geographic variations in HER-2/*neu*- and *TopoIIα*-amplification status. *TopoIIα* amplification has been described to be present in approximately 5–10% of the total population (about one-third of *HER2*-amplified tumors),[11] which is consistent with our data (9%).

Co-amplification of *HER2* and *TopoIIα* was seen in 42% of cases (low and high level) in line with previous studies that reported co-amplification rates of 32–57%.[10,11] We found no high level amplification of *TopoIIα* without *HER2* amplification, in contrast with some studies that did find *TopoIIα* amplification with normal *HER2* status.[8,35] However, in some cases we found a low level of *TopoIIα* gene amplification without any amplification of *HER2*, but this amplification could not be identified by CISH.

Copy numbers of *HER2* were higher than those of *TopoIIα*, which in addition to the different frequency of amplification of these loci supports the concept that the *HER2* gene is the hot spot for amplification on chromosome 17, with lower frequencies of amplification and lower level of amplification of the surrounding genes such as *TopoIIα*[36] and other chromosome 17q genes included in the kit (as depicted in Figure 2). Nevertheless, the mechanism of amplification of the *HER2* gene and surrounding loci is yet unknown. To which extent these co-amplified genes have an impact on response to the *HER2*-targeted treatment with trastuzumab is unknown.

Eleven patients showed a deletion for *TopoIIα* by MLPA (3%), which is consistent with literature, where overall prevalence of *TopoIIα* deletions in breast cancer has varied from 2 to 11% in different studies.[10,11,37] In our study, none of these deletions was accompanied by an amplification of *HER2*. The

significance of these deletions is still controversial, but contrary to what was previously thought,[18] one study claimed that it may also predict benefit from treatment with *TopoIIα* inhibitors.[8]

In our study, tumors with gene amplification of *Topoisomerase IIα* showed evidence of greater expression of topoisomerase IIα protein than did other tumors ($P=0.035$), but 4/14 (29%) amplified tumors did not overexpress the *TopoIIα* protein. All four cases displayed low level amplification by CISH, and two of these four cases were also amplified by MLPA. Previous studies have revealed that, contrary to *HER2*, where gene amplification is almost always correlated with protein overexpression, *TopoIIα* gene amplification apparently does not always lead to protein overexpression.[12,21,22] Other factors, specifically the tumor proliferation status, may interfere with the *TopoIIα* protein status as topoisomerase IIα is a marker of proliferation and topoisomerase IIα expression depends on the cell cycle status.

We found, similar to a large previous study,[25] a high concordance between amplification status by MLPA and CISH, which indicates that MLPA is a reliable test for detection of *HER-2/neu* and *TopoIIα* amplification. One can even wonder whether MLPA would be suitable as a pre-screening tool alternative to the Hercep test (*HER2* immunohistochemistry). Indeed, MLPA is not only easy but also cheaper than CISH. Consumables costs are €11 per reaction compared with €70 per reaction for *HER2* CISH and €56 per reaction for TopoIIα CISH. Furthermore, MLPA is more quantitative than immunohistochemistry allowing more straightforward interpretation, and in the same analysis several genes that are important in therapy selection and/or prognosis, such as *TopoIIα*, can be tested for amplification. Given the inherent molecular complexity of the malignant process, it seems unlikely that the assay of a single marker, regardless of methodology, will ever give us the complete answer as to the response to targeted therapeutics.

Concordance with CISH for *TopoIIα* was 91%, for *HER2* 96%. This difference could be due to a more accurate estimation of *HER2* status based on three probes instead of only one for *TopoIIα* in the current kit, indicating that the kit would benefit from more *TopoIIα* probes. More MLPA probes for *TopoIIα*

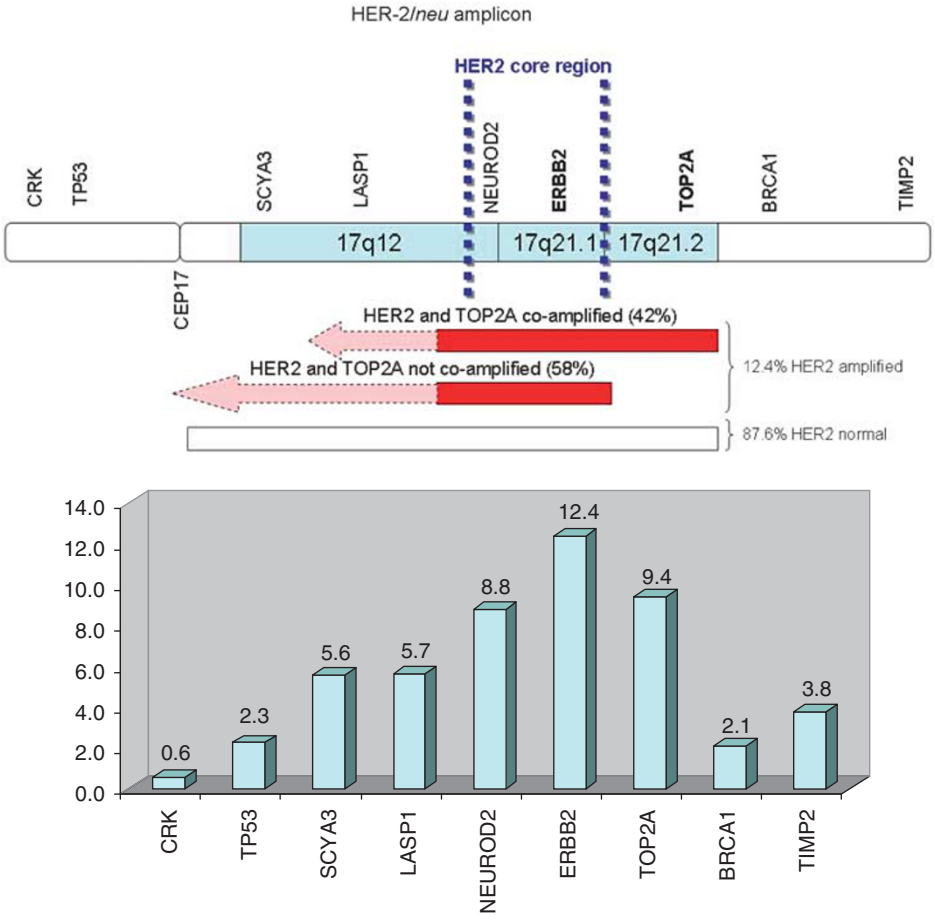

Figure 2 Schematic representation of the *HER-2/neu* amplicon. The *HER2* (*ERBB2*) core region as defined by Kauraniemi *et al*[46] is indicated by dashed lines. Genes corresponding to the chromosome 17 probes included in the MLPA P004 kit are depicted above the chromosome and their (low plus high level) amplification frequencies (in %) as found in this study are depicted in the chart below.

could make a more accurate estimate of whether a sample is low level or not amplified. Concordance between MLPA and CISH for *HER2* and *TopoIIα* was highest in MLPA amplified (96 and 100%, respectively) and non-amplified cases (98 and 99%, respectively). For MLPA low-level amplified cases, concordance was low (50 and 16%). However, low-level *HER2* amplification only occurs in 1–3% of the general population and in 4–25% of the critical group of immunohistochemistry 2+ carcinomas.[38] These low-level amplified cases probably do not respond as well to *HER2*-directed therapy as patients showing high level amplifications.[39] Preliminary data from the NSABP B-31 trial, however, suggest that there is a limited subset of patients with tumors that are fluorescence *in situ* hybridization negative and graded less than immunohistochemistry 3+ that do achieve significant benefit ($P = 0.03$) from adjuvant trastuzumab.[40] We therefore re-analyzed our MLPA results with higher cut-off values (than 1.5) between non-amplified and low-level amplified cases (see Table 4), which increased the positive predictive value and specificity but decreased the sensitivity of MLPA for both genes. Next to the number of probes and the choice of the cut-off value, another explanation of discrepancies could be the non-morphological aspect of MLPA. Small-amplified clones may be obscured by background non-amplified cells and thereby missed by MLPA. Careful manual microdissection is able to resolve some of the discrepancies, but is not necessary in routine practice and only advisable when tumor percentage is very low (<30%) or extensive ductal carcinoma *in situ* is present.[41] On the other hand, there were also cases with amplification by MLPA, while CISH was normal. This may be partly due to a lack of sensitivity by CISH for low level amplification.

These data show that MLPA is suited to detect amplification (as well as deletion) of *HER2* and *TopoIIα* in breast cancer patients in one test. Both *HER2* and *TopoIIα* gene alternations have independently been associated with an increased responsiveness to anthracycline-containing chemotherapy regimens relative to non-anthracyline regimens,[42,43] indicating that measurements of alternations of both genes can guide in the selection of anthracycline-containing regimens. Furthermore, this MLPA kit contains probes to several other chromosome 17 loci (see Figure 2) and can thereby easily determine chromosome 17 polysomy, likely better than using a single *in situ* hybridization centromere probe, and easier than additional *in situ* hybridization probes targeted to other chromosome 17 loci.[44] This is even more an advantage, as recently the definition of chromosome 17 polysomy based on CEP17 only is found most questionable.[45]

In conclusion, MLPA is an easy and cheaper method to simultaneously detect breast cancer polysomy 17, *HER-2/neu* and *TopoIIα* amplification in small quantities of short fragmented DNA extracted from paraffin blocks, and is thereby a good supplementary or even alternative technique to *in situ* hybridization.

Table 4 Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and concordance of MLPA with chromogenic *in situ* hybridization for *TopoIIα* and *HER2* using cut-off values of 1.5 and 1.8 to discriminate between no amplification and low level amplification status

	Sensitivity	*Specificity*	*PPV*	*NPV*	*Concordance*
TopoIIα 1.5	86.7	92.6	39.4	99.2	91.2
TopoIIα 1.8	73.3	99.6	91.7	98.5	97.5
HER2 1.5	88.9	98.4	88.9	98.4	96.1
HER2 1.8	75.0	99.6	96.4	96.5	95.8

Disclosure/conflict of interest

The authors declare no conflict of interest.

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