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HER-2/*neu* amplification testing in breast cancer by multiplex ligation-dependent probe amplification in comparison with immunohistochemistry and *in situ* hybridization

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Abstract. *Background*: Assessment of HER-2/*neu* status in invasive breast cancer is crucial to establish eligibility for trastuzumab and taxane based chemotherapy. Next to immunohistochemistry (IHC) to evaluate protein overexpression, a second line gene amplification test is required for cases with equivocal protein expression. This study aimed to validate a new PCR based test, called Multiplex Ligation-dependent Probe Amplification (MLPA), as a simple and quick method to assess HER-2/*neu* gene amplification status in invasive breast cancer.

Methods: MPLA results were compared with gene amplification status assessed by fluorescence *in situ* hybridization (FISH) and chromogenic *in situ* hybridization (CISH) as gold standard, and with protein overexpression by IHC in 518 breast carcinoma patients.

Results: About 10% of cases overexpressed HER-2/*neu* at the protein level (IHC), and 11% of cases showed gene-amplification by MLPA. A high concordance was found between FISH and CISH, MLPA and IHC, and MLPA and CISH. MLPA showed amplification in $7/36$ (19%) of the equivocal IHC 2+ cases. However, of the IHC 0/1+ cases, $6/434$ (1.4%) were also amplified by MLPA, and amplification was confirmed in all of these cases by FISH/CISH. On the other hand, one of the 48 (2%) IHC 3+ cases was normal by MLPA and lack of amplification was confirmed by FISH/CISH.

Conclusion: MLPA is a fast, accurate and cheap method to detect breast cancer HER-2/*neu* amplification in small quantities of DNA extracted from paraffin blocks, and thereby a reliable alternative to FISH and CISH.

Keywords: HER-2/*neu*, amplification, *in situ* hybridization, multiplex ligation dependent probe amplification, immunohistochemistry

1. Introduction

HER-2/*neu* is a proto-oncogene located on chromosome 17q21 that belongs to the human epidermal growth factor receptor (EGFR) family. It encodes a 185 kD transmembrane protein that is involved in signal transduction [1,31]. In about 20–30% of breast carcinomas HER-2 is amplified and the expression of its receptor protein is increased [27,35,39]. Such patients respond well to treatment with trastuzumab, a recombinant humanized monoclonal anti-HER-2 antibody [15, 40]. Since the costs for trastuzumab therapy are high and side effects are significant, accurate selection of eligible patients for this therapy is very important. Furthermore, amplification of HER-2 has also been shown to correlate with poor prognosis [19] and with resistance to conventional adjuvant chemotherapy and tamoxifen [4,5,37,38,44]. With the recognition of its prognostic, predictive and therapeutic implications, assessment of HER-2 status has now become of major

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importance in clinical practice for breast cancer patients.

At present, the most common method to assess HER-2 status is immunohistochemistry (IHC), which is a routine technique available in all pathology laboratories to detect protein levels. However, although staining and scoring methodology has been better standardized with the introduction of the Hercep®test than for most IHC assays, IHC is liable to poor fixation and there are still problems with reproducibility and interpretation of IHC assays [16,32,45], leading to both false negative and positive IHC results. In addition, there is some evidence that testing for HER-2 gene amplification provides better predictive information than IHC [2,23,29,51]. Originally, gene amplification was determined by Southern blotting, but this technique is not suited for daily practice since it is laborious and requires large quantities of DNA. Therefore, HER-2 gene amplification testing is usually done by fluorescence *in situ* hybridization (FISH). Comparative studies of FISH and IHC have generally shown a high level of concordance [18,21,29]. Discordant results were mainly observed for tumors that were scored 2+ by IHC. However, pathologists have been reluctant to embrace routine FISH testing, because it is a difficult, expensive and cumbersome technique that requires trained personnel which is not available in every pathology laboratory. Moreover, fluorescence fades upon storage, making it difficult to preserve the slides for future reference, and the fluorescent probes in the kits have a limited half life. Furthermore, detailed morphological features of the tumor are usually difficult to observe due to the required protein digestion and the fluorescent mode, and heterogeneity can be missed since spots are evaluated at $\times 100$ magnification using oil immersion. FISH is, therefore, usually limited to the 2+ IHC equivocal cases.

Chromogenic *in situ* hybridization (CISH) was introduced as an alternative for HER-2 FISH in 2000 by Tanner et al. [41], using an immunoperoxidase reaction to detect specific DNA probes, which makes visualization possible with a conventional bright field microscope. Furthermore, similar to IHC, a permanent staining record is retained and better morphologic examination is possible facilitating detection of heterogeneity. CISH is also easier to interpret for pathologists who are not trained in fluorescence microscopy and is less expensive than FISH. In several studies, HER-2 CISH was demonstrated to be well correlated with FISH and IHC [7,12,13,41,48,53]. However, CISH is still fairly difficult and amplification can only be assessed semi-quantitatively and therefore, detection of amplification by easier quantitative PCR techniques has been proposed as an alternative. One of the newly introduced techniques for detection of HER-2 amplification is multiplex ligation-dependent probe amplification (MLPA) [36]. This technique determines relative 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 a previous pilot study we obtained promising results with MLPA in comparison with IHC [33]. The aim of the present study was to compare MLPA as a new method to assess HER-2 gene amplification with FISH and CISH data as gold standard in a large group of breast cancer patients.

2. Materials and methods

2.1. Patient material

Tissue samples of 518 consecutive invasive breast cancer patients were collected between November 2004 and June 2006 at the Department of Pathology of the University Medical Center in Utrecht. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital [50]. All tissue samples were analyzed for IHC to assess HER-2 protein expression and MLPA to determine HER-2 gene amplification. In addition, ISH was performed, partly by FISH and CISH on full sections (including all 51 IHC/MLPA discrepant cases) and with CISH on a larger series for which we constructed tissue microarrays using published guidelines [28]. In total, 322 cases were thereby tested with FISH/CISH. Presence and amount of ductal carcinoma *in situ* (DCIS) was noted and the tumor content was estimated by a pathologist (PvD).

2.2. Immunohistochemistry (IHC)

IHC 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. 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 stain-

ing was done by 2 experienced breast pathologists. As control a small tissue array containing a 0, 1+, 2+ and 3+ breast tumor sample was taken along on the same slide as the tumor to be analyzed. Negative controls were obtained by omission of the primary antibody.

2.3. Multiplex ligation-dependent probe amplification (MLPA)

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 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 HER-2 kit (MRC Holland, Amsterdam, The Netherlands). This kit contains 3 probes for the HER-2 gene, 11 other chromosome 17 control probes, and 25 control probes located on other chromosomes. Details of the probes in this kit can be found at www.mrc-holland.com. 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). HER-2 gene copy number was determined by calculating the mean ratio of the HER-2 probe peaks with the two previous peaks and the two following peaks. The mean of all three HER-2 probe peaks in duplicate (6 values) was calculated. If this mean value was below 1.5 the test was scored HER-2 normal. A value 1.5–2.0 was scored as HER-2 low level amplification, and values >2 as HER-2 amplified. The 2.0 threshold was used in accordance with previous HER-2 MLPA studies [24,33], while the 1.5 threshold was empirically established during routine diagnostic application of MLPA kits for trisomy detection.

2.4. Fluorescence in situ hybridization (FISH)

All FISH assays were performed using the FDA approved PathVysion kit (Vysis, Abbott Laboratories, Abbott Park, IL, USA) which included probes for determining the copy number of both HER-2 (red) and the chromosome 17 (CEP17, green). FISH was performed according the manufacturers' instructions on 4 μm paraffin sections. Since it was not deemed cost-effective to perform FISH on many IHC normal cases, FISH was performed on some of the samples using the following selection criteria: all IHC-MLPA discrepant cases and at least 5 of each of the following concordant case groups: IHC 0/MLPA normal, IHC 1+/MLPA normal, IHC 2+/MLPA low level amplified and IHC 3+/MLPA amplified cases.

The slides were baked overnight at 56°C, deparaffinized, rehydrated in graded ethanol and immersed in a 0.2 N HCl solution for 20 min. After pressure cooking in phosphate buffered saline (PBS) for 25 min, slides were rinsed in 0.01 N HCl and digested in pepsin (0.04 g/80 ml) at 37°C for 10 min. Slides were then dehydrated in graded alcohols and air dried. Subsequently, 10 μl of Vysis PathVysion probe was applied and after denaturation at 73°C for 5 min, slides were hybridized overnight at 37°C in a humidified chamber. Post-hybridization washing was performed in a 2× SSC solution with 0.3% NP40 at 73°C in a water bath. Finally, slides were air dried and counterstained with 10 μl DAPI (4,6-diamindino-2-phenylinodole) at room temperature. A positive control was included in each run of FISH and consisted of paraffin sections of a case known to be HER-2 amplified by FISH. The FISH signals were visualized by using a fluorescence microscope. Enumeration was done following the manufacturers' guidelines. HER-2 and chromosome 17 signals were assessed simultaneously by two observers within areas of invasive carcinoma that were previously marked on the slides by serial H&E sections. A HER-2/CEP17 ratio > 2.2 was considered HER-2 gene amplified. All ratios <1.8 were scored as HER-2 non-amplified. Ratios between 1.8 and 2.2 were considered low-level amplified. Chromosome 17 polysomy was defined as 3 CEP17 signals or more. Borderline FISH/CISH was confirmed by counting additional cells. When FISH/CISH remained equivocal after recounting, FISH/CISH was repeated or the tumor sample was analyzed by another validated technique.

2.5. Chromogenic in situ hybridization (CISH)

All CISH assays were run using the Zymed SPoT-Light HER-2 CISH (Zymed, South San Francisco, CA) kit according the manufacturers' instructions. CISH was performed on 4 μm thick whole paraffin sections and/or on tissue microarray sections. First, sections were baked overnight at 56°C and deparaffinized in xylene and alcohol 100%. The slides were then boiled in pretreatment buffer for 15 min, followed by enzymatic digestion at room temperature for 10 min (Zymed). Then, slides were dehydrated with graded alcohols. Af-

ter 20 min of air drying, the digoxigenin-labeled Her2 probe (Zymed) was 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). Then, the slides were incubated with mouse-anti-digoxigenin antibody (Zymed) for 30 min at RT and goat-anti-mouse antibody conjugated with horseradish peroxidase 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 (Histomount, Zymed). CISH scoring was performed according the manufacturers' guidelines. Briefly, HER-2 was scored amplified when large peroxidase-positive intra-nuclear gene copy clusters or numerous individual small signals (>10 dots per nucleus in more then 50% of tumor cells) were present, or in case of a mixture of clusters and individual signals. Tumors were scored low-level amplified when small clusters were present or when tumor cells showed between 6–10 individual signals per nucleus, and were scored normal when tumor cells never showed more than 5 small dots per nucleus (thereby including polysomy). No CEP17 analysis was performed. A positive control was included in each CISH run and consisted of paraffin sections of a case known to be HER-2 amplified by CISH.

2.6. Statistics

Results obtained with the various techniques were compared by cross tables and the concordance percentages and correlations (Spearman's rho) were calculated using SPSS statistical software. For MLPA and IHC, sensitivity, specificity, positive (PPV) and negative predictive value (NPV) were calculated using CISH as gold standard.

3. Results

Table 1 shows the comparison between HER-2 IHC and HER-2 gene amplification by MLPA. About 53% of all patients tested negative for IHC, 30% was scored IHC 1+, 7% IHC 2+ (unequivocal) and 10% IHC 3+ (strongly positive). HER-2 amplification status by MLPA was normal in 86% of cases, low level amplified in 3% and amplified in 11% of cases. Of all IHC negative cases 99% was MLPA normal, and in the group of IHC 1+ cases 93% was MLPA normal. In these IHC 0 and IHC 1+ cases, 1% and 5%, respectively, was MLPA low level amplified, and 1% and 3% was, respectively, MLPA amplified.

In the IHC 3+ group 90% was MLPA amplified and 8% was MLPA low level amplified, whereas 2% was MLPA normal. In the IHC 2+ group discrepancy with MLPA was, as expected, most pronounced: 67% was not amplified, 14% was MLPA low level amplified and 19% was amplified. Overall, there was 90% agreement between both techniques (considering IHC 0 and IHC 1+ as equivalent to MLPA normal).

Table 2 compares HER-2 IHC and MLPA for biopsies (98/423, 23%) and resections (325/423, 77%), separately. There did not appear to be clear differences between biopsies and resections (85.7% agreement for biopsies and 88.7% agreement for resections), and the percentage of IHC 3+ or MLPA amplified cases was not significantly different between biopsies and resections.

Table 3 shows MLPA results for 423 cases divided into 9 groups according to the estimated tumor percentage. A tumor percentage below 50% was found in 31% of cases. Most cases had a tumor percentage between 60 and 70%. Amplification was detected by MLPA in similar frequencies in all groups, even when the tumor percentage was below 10%. To determine a tumor load cut-off from which MLPA results are reliable to detect amplification, we compared results from every

Table 1

Comparison of HER-2/*neu* protein overexpression by immunohistochemistry (IHC) with gene amplification by multiplex ligation-dependent probe amplification (MLPA) in 518 invasive breast cancer patients

	IHC				
	0	1+	2+	3+	Total
MLPA					
Normal	273	146	24	1*	444
Low level	2	7	5	4	18
Amplified	2**	4**	7	43	56
Total	277	157	36	48	518

*This case was not amplified by FISH/CISH. **These six cases were amplified by FISH/CISH.

Table 2
Comparison of the HER-2/*neu* protein overexpression by immunohistochemistry (IHC) with gene amplification by multiplex ligation-dependent probe amplification (MLPA) in biopsies and resections separately

Biopsy	IHC				
	0	1+	2+	3+	Total
MLPA					
Normal	34	39	9	2	84 (86%)
Low level	0	1	1	1	3 (3%)
Amplified	0	0	1	10	11 (11%)
Total	34 (35%)	40 (41%)	11 (11%)	13 (13%)	98
Resection					
MLPA					
Normal	166	92	17	0	275 (85%)
Low level	2	6	3	2	13 (4%)
Amplified	2	5	3	27	37 (11%)
Total	170 (52%)	103 (32%)	23 (7%)	29 (9%)	325

Table 3
MLPA HER-2 test results for 423 cases divided into groups according to tumor percentage

Tumor (%)	MLPA			Total
	Normal	Low level	Amplified	
0–10	25	2	2 (7%)	29 (7%)
10–20	19	0	3 (14%)	22 (5%)
20–30	13	0	2 (13%)	15 (3%)
30–40	7	0	2 (22%)	9 (2%)
40–50	50	2	8 (13%)	60 (14%)
50–60	65	1	5 (7%)	71 (17%)
60–70	88	3	13 (13%)	104 (25%)
70–80	68	7	12 (14%)	87 (21%)
80–90	24	1	1 (4%)	26 (6%)
Total	359 (85%)	16 (4%)	48 (11%)	423

tumor load group with IHC and FISH/CISH (data not shown). When the tumor percentage was higher than 30%, the correlation between IHC and MLPA was best with $32/36$ (89%) IHC 3+ cases showing amplification by MLPA. Three of the discrepant cases were low level amplified by MLPA and only one was MLPA normal (also by FISH/CISH). Two of the discrepant cases that were MLPA low level amplified, were amplified by FISH/CISH and one was not.

In Table 4, the results of the comparisons between IHC, MLPA and FISH/CISH are displayed. All cases negative by IHC lacked amplification by FISH whereas only one case showed a low-level amplification by CISH. Most of the IHC 3+ cases were amplified by FISH ($18/20$, 90%) and CISH ($29/31$, 94%). Of the IHC 1+ cases, $4/16$ (25%) were amplified by FISH and $4/85$ (5%) by CISH. The IHC 2+ cases showed amplification by FISH in $5/21$ cases (24%) and by CISH in $7/35$ cases (20%). HER-2 amplification by MLPA was not confirmed by FISH in $3/25$ cases (12%) and not by CISH in $4/40$ cases (10%). Of the MPLA normal cases, $1/29$ (3.5%) was amplified by FISH and $5/265$ (1.9%) were (low- or high-level) amplified by CISH. MLPA low level amplified cases were high level amplified by FISH/CISH in $4/16$ cases (25%) and low level amplified in $6/16$ cases (37.5%). In all IHC 0/1+ cases that were amplified by MLPA, amplification was confirmed by FISH/CISH. The IHC 3+ case that was normal by MLPA was also normal by FISH/CISH, and $6/7$ IHC 2+ cases that were amplified by MLPA were also amplified by CISH/FISH. Figure 1 shows examples of MLPA and CISH to detect HER-2 amplification in comparison with IHC in invasive breast cancer.

Table 5 shows the concordance percentages between the different techniques as well as Spearman's rho (all correlations were significant).

Table 6 shows sensitivity, specificity, PPV, NPV for MLPA and IHC using CISH as a gold standard.

4. Discussion

The aim of this study was to compare MLPA as a new method to assess HER-2 gene amplification in comparison with FISH and CISH data as gold standard in a large group of breast cancer patients. Gene amplification analysis by FISH or CISH was highly comparable, and MLPA correlated well with CISH. MLPA, FISH and CISH all detected amplification among cases

Table 4
Comparison of HER-2/*neu* gene amplification by fluorescence (FISH), chromogenic *in situ* hybridisation (CISH) and multiplex ligation-dependent probe amplification (MLPA) with immunohistochemistry (IHC) in a group of invasive breast cancer patients

	FISH ($n = 67$)			CISH ($n = 321$)			Total ISH
	Normal	Low level	Amplified	Normal	Low level	Amplified	
IHC							
0	10	0	0	169	1	0	170
1+	11	1	4	78	3	4	85
2+	15	1	5	21	7	7	35
3+	2	0	18	1	1	29	32
MLPA							
Normal	28	0	1	260	4	1	265
Low level	7	2	4	7	6	3	16
Amplified	3	0	22	2	2	36	41

without HER-2/*neu* overexpression, and all techniques could not confirm amplification in a fraction of HER-2/*neu* overexpressors. Results on biopsies were comparable to those on resection specimens.

In the present study, protein overexpression by IHC was detected in 10% of the 518 cases studied. This is lower than the 20–30% positivity that has generally been described in the literature [11,27,38,39] although several other studies have reported lower (10–18%) percentages [7,9,10,30,42] as well. It is likely that many of the series in which higher HER-2 overexpression/amplification frequencies were described may not have been unselected and frequencies below 20% are seen in unselected series. As our study group concerned consecutive patients, selection bias can be excluded. Further, methodological variation is an unlikely explanation as the fraction of HER-2/*neu* amplified cases by MPLA (10%) was similar. This implies that there may be geographic variations in HER-2/*neu* amplification status.

There was a high concordance between amplification by MLPA, FISH and CISH, which confirms results from a recent much smaller study [24]. This validates MLPA as a good alternative test for detection of HER-2/*neu* amplification in breast cancer. In only a few cases, MLPA failed to detect amplification that was found by FISH/CISH. Low tumor content may play a role here, since small amplified clones may be obscured by background non-amplified in such a non-morphological technique. On the other hand, there were also cases with amplification by MLPA while FISH/CISH were normal. This may be due to intra-tumor heterogeneity missed by FISH and lack of sensitivity by CISH for low level amplification. As the MLPA test contains controls for chromosome 17, polysomy can be excluded. For CISH, performing CEP17 analysis on a serial slide may be required to exclude polysomy, especially for borderline cases (4–6 copies of HER-2).

MLPA also correlated well with IHC as in a previous smaller study [33]. MLPA showed amplification in $12/36$ (33%) of the IHC 2+ cases that are generally regarded as equivocal and necessitating a second line amplification test, in line with previous studies [3,16,21,30,34,45]. This indicates that MLPA can aid therapeutic decision in these equivocal cases. However, of the IHC 0/1+ cases, $6/434$ (1.4%) were amplified by MLPA, which was confirmed in all of these cases by FISH/CISH. On the other hand, $1/48$ (2%) IHC 3+ cases was normal by MLPA and lack of amplification was confirmed by FISH/CISH. This shows that MLPA is able to detect amplification in a relevant fraction of IHC low cases as well as deny amplification in a fraction of IHC 3+ cases that are generally considered to be eligible for HER-2 directed therapy. MLPA therefore seems to be suited for detection of HER-2/*neu* amplification in perhaps all breast cancer cases, not just the IHC 2+ cases.

In view of these results, one can even wonder if amplification tests such as MLPA should be reserved as a second line test for the IHC 2+ cases. There are as yet only few data to indicate that amplified but not overexpressed cases respond to HER-2 directed therapy [22,23], but nevertheless one can wonder whether MLPA would be suitable as a pre-screening tool alternative to IHC. Indeed, MLPA is fast, easy and cheap, and more quantitative than IHC allowing more straightforward interpretation. Furthermore, in the same analysis several genes that are important in therapy selection and/or prognosis like TopoIIα can be tested for

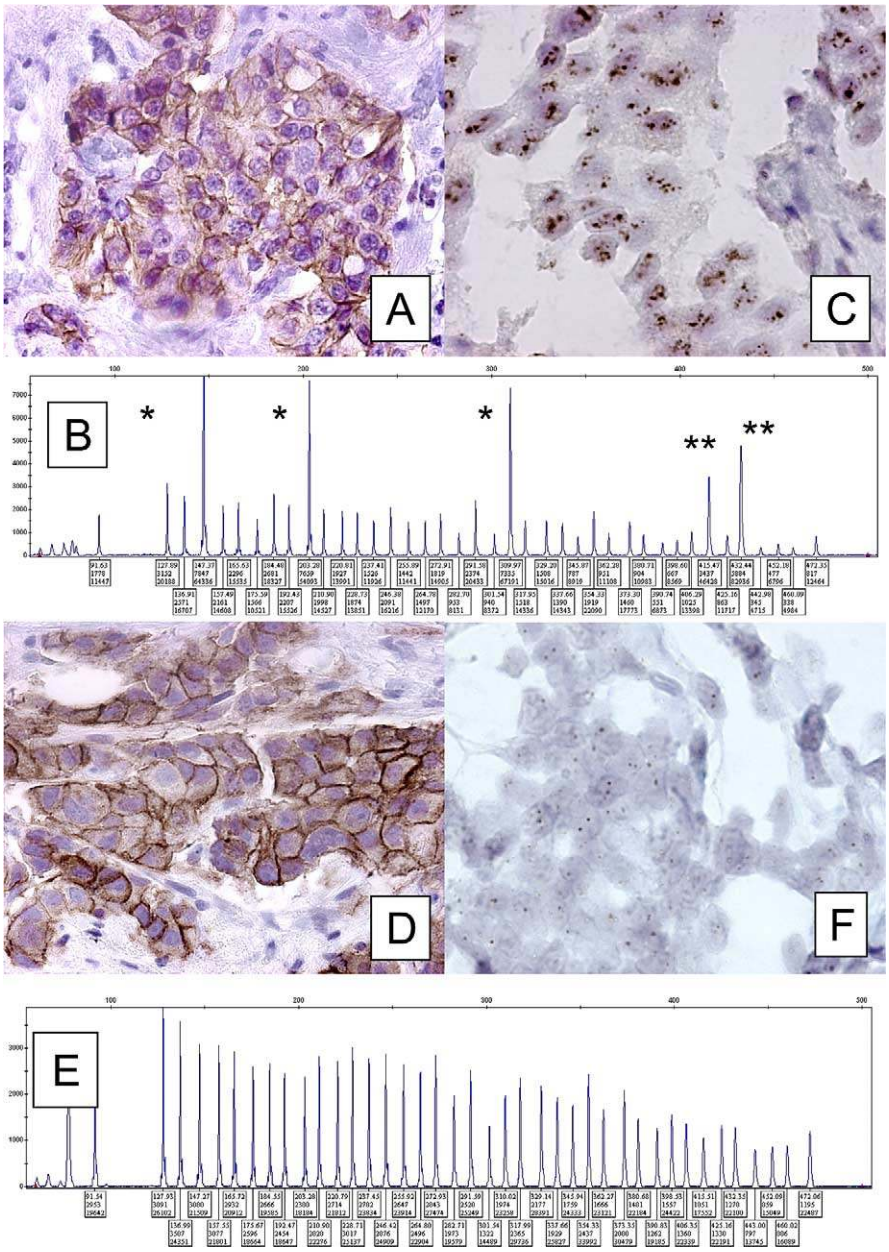

Fig. 1. MLPA, CISH and IHC to detect HER-2 amplification in invasive breast cancer. (A) HER-2 IHC 1+ case with in (B) amplification by MLPA (see 3 HER-2 probe peaks (*) way above the controls and two other chromosome 17 peaks (**) co-amplified) as confirmed by CISH in (C). (D) HER-2 IHC 3+ case with in (E) no amplification by MLPA (none of the 3 HER-2 probe peaks above the controls) or CISH in (F).

amplification. However, MLPA has the disadvantage of being a non-morphological technique that can result in the overlooking of heterogeneity and DCIS which could be partly resolved by H&E staining of a sequential slide.

Another disadvantage of MLPA is that results depend on the tumor percentage of the sample. The higher the tumor percentage, the more reliable the results will be, since also smaller or low level amplified clones, will then be picked up. Nevertheless, amplification was detected by MLPA even in cases with a tumor percentage below 10, indicating that this technique is quite sensitive. Since the best correlations between MLPA and IHC, FISH and CISH were obtained in cases with a tumor percentage higher than 30%, we advise to restrict the use of MLPA to these cases,

Table 5

Concordance percentages and correlation (Spearman's rho) between different techniques to detect HER-2 overexpression and gene amplification

	Agreement (%)	Correlation Spearman (rho)
FISH-CISH	91	0.93
IHC-FISH[#]	60	0.58
IHC-CISH	88	0.74
MLPA-FISH[#]	78	0.78
MLPA-CISH	94	0.87
IHC-MLPA	90	0.74

All correlations shown are significant.
[#]Smaller series of selected discrepant cases leading to lower agreement.

Table 6

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for immunohistochemistry (IHC) by Hercep test and multiplex ligation-dependent probe amplification (MLPA) as determined by analysis of 321 invasive breast cancer patients, considering CISH as gold standard

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
IHC	73	92	94	97
MLPA	90	97	90	98

and otherwise perform careful microdissection before MLPA or use CISH/FISH as an alternative.

An important issue is how to deal with MLPA low level amplification cases (3.5% of cases). We think that until MLPA has better been clinically validated, ISH should decide on amplification status for clinical decision making.

Since core needle biopsies are increasingly performed within the scope of primary diagnosis of breast cancer, they are also increasingly used for the assessment of prognostic and predictive markers such as HER-2. In cases that receive neoadjuvant therapy [14], or where the primary tumor will not be resected but ablated [49], marker studies completely rely on the core biopsies. Studies comparing the HER-2 status in needle biopsies and surgical resections have reported an overall concordance of 91–100% using IHC alone [6,8,17,25,43,46]. However some studies have also suggested that the validity of IHC score 3+ in core biopsies is limited [43], reporting high rates of false positives (19.3%). Therefore, we separately analyzed our biopsy and resections data. We found a slightly higher percentage of IHC 3+ positivity in biopsies compared to resections, but this did not reach statistical difference, and MLPA showed amplification in 11% of biopsies and resections.

Of the 65 tumors analyzed by both ISH techniques, only six samples showed a discordant result. This confirms previous papers showing a high concordance between these techniques [7,12,13,41,48,53]. Both methods are to some extent liable to observer subjectivity which could explain discrepancies, and by FISH intra-tumor heterogeneity may easily be missed when scanning under oil at a 100× magnification. Of the six cases with discrepancies between FISH and CISH, five were IHC 2+ and one case was IHC 1+ and MLPA low-level amplified, underlining the high discordance already reported in low-level amplified/overexpressed cases. One discordant case could be related to chromosome 17 polysomy. Although generally a high concordance has been reported between CISH and FISH, CISH is reported to be less sensitive for low-level amplification [47]. However, low-level amplification only occurs in 1–3% of the general population and in 4–25% of the critical group of IHC 2+ carcinomas [47], and these low level amplified cases probably do not respond well to HER-2 directed therapy [22].

Concordance between ISH and IHC was high as expected [3,20]. Only one case in the IHC 0 group ($n = 170$) showed a low level amplification by CISH. In the IHC 3+ group FISH was negative in $2/20$ cases (10%) and CISH in $1/31$ cases (3%). Absence of gene amplification in IHC 3+ cases has previously been observed [26,29] and was explained by upregulation or decreased degradation of the protein, although false positive IHC may also occur. It is therefore important to select a block with normal tissue present (that should not show membrane staining) for HER-2/*neu* IHC.

According to the ASCO guidelines [52], 90% respectively 95% of IHC 0 and IHC 1+ tumors should show no HER-2 gene amplification, while 90% of IHC 3+ scores should show amplification. For MLPA, these percentages were 99%, 93% and 90%, respectively, while for CISH these percentages were 99%, 92% and 94%. Thereby, MLPA results almost corresponded to the ASCO guidelines and were similarly good as CISH.

In conclusion, MLPA is a fast, accurate and cheap method to detect breast cancer HER-2/*neu* amplification in small quantities of DNA extracted from paraffin blocks, and thereby a good alternative or supplementary technique to FISH and CISH.

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