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Detection of HER2 amplification in breast carcinomas: Comparison of Multiplex Ligation-dependent Probe Amplification (MLPA) and Fluorescence In Situ Hybridization (FISH) combined with automated spot counting

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Abstract. In this study the detection of HER2 gene amplification was evaluated using Fluorescence In Situ Hybridization (FISH; PathVysion) in comparison with Multiplex Ligation-dependent Probe Amplification (MLPA), a PCR based technique. These two methods were evaluated on a series of 46 formalin fixed paraffin embedded breast carcinomas, previously tested for protein overexpression by HercepTest (grouped into Hercep 1+, 2+ and 3+). HER2 gene amplification (ratio $\geqslant$ 2.0) by FISH was found in 9/10, 10/30 and 0/6 in IHC 3+, 2+ and 1+/0 cases, respectively. Digitalized automated spot counting performed with recently developed CW4000 CytoFISH software was 100% concordant with manual FISH scoring. Using MLPA 18/46 samples showed a clear HER2 amplification. Comparing MLPA and IHC showed the same results as for FISH and IHC. All but one FISH positive cases (18/19) were confirmed by MLPA for the presence of the gene amplification. The overall concordance of detection of Her2 gene amplification by FISH and MLPA was 98% (45/46). Furthermore, both the level of amplification and equivocal results correlated well between both methods. In conclusion, MLPA is a reliable and reproducible technique and can be used as an either alternative or additional test to determine HER2 status in breast carcinomas.

Keywords: HER2, amplification, MLPA, FISH, automatic, quantification, spot counting

1. Introduction

The Human Epidermal Growth Factor Receptor-2 (HER2) oncogene, located on the long arm of chromosome 17 (17q21), encodes a transmembrane tyrosine kinase growth factor receptor and is involved in cell growth and development. Amplification of the HER2 gene can be detected in 20–30% of invasive breast carcinomas. Gene amplification results in overexpression of HER2 protein and is associated with poor prognosis and better response on Trastuzumab (Herceptin) and taxane based therapy [1,11,18,20].

Two techniques to determine the HER2 status are most frequently used: determination of overexpression of the protein by immunohistochemistry (IHC) and detection of gene amplification by Fluorescence In Situ Hybridization (FISH). For IHC and FISH, FDA approved tests are available but both techniques have their limitations. IHC is the most widely accepted test for the detection of HER2 protein expression in breast cancer specimens on formalin-fixed and paraffin embedded tissues. Advantages include direct detection of protein expression, ease of performance, cost and quick turnaround time. However, subjective grading can cause difficulty in interpretation [6,7,17].

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FISH is quantitatively more accurate as IHC and is regarded as the most predictive test for response to therapy, but more expensive, time-consuming and technically demanding. Although less inter-observer variation is reported as compared to IHC, the interpretation can be difficult. Both IHC and FISH can lead to inter-observer variability. Comparison studies of IHC and FISH have generally shown a high concordance rate in IHC negative (Hercep 0 and 1+) and IHC strong positive carcinomas (Hercep 3+). For routine applications current guidelines recommend after initial screening by IHC, FISH testing of IHC 2+ samples [7,10,17].

Since analysis of FISH is time-consuming, several systems for automatic image analysis and spot counting have been developed to determine the HER2 status [4,16]. Usually these are fully automated and expensive scanning systems, appropriate for high throughput testing. Recently, CytoFISH software (Leica) was developed, suitable for a low throughput setting.

In the CytoFISH software a "Systematic Sampling" of the images is performed. Spots are counted inside "Sampling Regions" which are square boxes that are placed on the DAPI-stained interphase cells. The Sampling Regions are placed on the brightest DAPI objects, in such a way that they do not overlap. Local thresholding of each sample region then ensures that spots are detected, and a ratio of the red and green spots is calculated.

Although FISH is regarded as the gold standard recently also alternative techniques were introduced for determination of HER2 status like quantitative real-time PCR assays and MLPA (Multiplex Ligation-dependent probe amplification). Several reports show good correlations between FISH and PCR based techniques as quantitative real-time PCR [5,14,15]. MLPA is a relative new and easy to perform PCR based technique first described by Schouten et al. [21]. This latter technique determines relative copy numbers in a quantitative way and recently many applications have been described in molecular diagnostics, as detection of large genomic deletions and insertions in genes and detection of aneuploidy [8,22,23]. Because only small DNA fragments are necessary for MLPA, this technique is very suitable for formalin fixed, paraffin embedded material [2].

In this study we compare manual FISH analysis with the automated CytoFISH HER2 spot counting application. Furthermore, we describe the evaluation of the HER2 MLPA on formalin fixed, paraffin embedded tissues and compare the performance of MLPA with FISH for detection of HER2 amplification on routinely IHC tested breast carcinomas.

2. Materials and methods

2.1. Clinical specimens

Formalin-fixed, paraffin-embedded material from 46 primary ductal breast carcinomas were used in this study. All tissues were routine diagnostic, surgical specimens that had been fixed, processed, and stored using standard protocols. Only invasive tumor areas were examined, all areas containing DCIS were excluded.

HER2 expression status was determined by routine IHC, using the HercepTest (DakoCytomation, Glostrup, Denmark). Two independent observers scored slides. In case of discrepancy, a consensus score was obtained. Samples were tested in routine diagnostics from 2003 to 2005. For some cases retrospective analysis of Her2 status was performed (25 tissues originating from 1992 to 2002 and one case from 1981). Formalin fixed, paraffin embedded non-tumor lymph node tissues were used as normal controls for MLPA.

2.2. FISH

Amplification of HER2 was evaluated using the FDA approved PathVysion HER2 DNA Probe assay (Vysis, Abbott Laboratories, Abbott Park, IL, USA), which uses a dual-color probe system for detection of both HER2 (spectrum orange) and the chromosome 17 centromeres (spectrum green) to obtain the actual HER2 gene amplification ratio. The assay was used following the manufacturer's instructions with a few modifications to enable optimal results for paraffin embedded tissues. Slides containing 4-μm thick paraffin embedded tissue sections were deparaffinized in xylene, followed by dehydration with absolute ethanol. Slides were subsequently pretreated with HCl, followed by incubation with citrate buffer for 40 minutes at 98°C and a protease treatment with pepsin 0.25% for 2 minutes at 37°C. After dehydration and drying of the slides at room temperature for one hour, 10-μl probe was applied. They were coverslipped and denatured at 88°C for 3 minutes. Hybridization was performed overnight at 37°C. Post-hybridization wash was applied using 2SSC/0.5% Tween20 at 72°C for 2 minutes. Slides were air-dried and counterstained with DAPI (4,6-diaminidino-2-phenylinodole).

2.3. FISH analysis

Quality of FISH was predominantly monitored by the use of internal controls. Normal cells should give 1–2 spots with both the HER2 probe as well as centromere probe, whereas background should be clear and morphology of nuclei preserved. Only those nuclei showing signals using the chromosome 17 centromere probe were analyzed for HER2 signals.

Fluorescence signals were counted in three representative areas with in total 60 nuclei signals, using a fluorescence microscope (Leica DMRXA), equipped with an appropriate filter set (Spectrum Green; Spectrum Orange). The ratio of HER2 signals (orange) to chromosome 17 centromere signals (green) was determined with ratios <2.0 considered nonamplified and those $\geqslant 2.0$ amplified, according to the manufacturer's guidelines for the PathVysion HER2 DNA Probe assay.

For automatic spot counting pictures were taken from three representative areas with DCF350F camera equipment (Leica, Solms, Germany). Automatic spot counting was performed with the recently introduced CW4000 CytoFISH software in collaboration with Leica Research (Cambridge, UK). With this system, square boxes so called sampling regions, were placed on the brightest DAPI objects in a defined area of the slide, representing interphase nuclei. Within these sampling regions probe signals were automatically counted and a HER2 to centromere 17 ratio was calculated. Nuclei without red and green signal were manually removed but no additional image analysis was performed before CytoFISH analysis. In total 100 to 150 nuclei were analyzed. Manual control was performed for the recognition of additional red and green signals, which were not counted automatically due to elevated background or overexpressed signals in case of strong HER2 gene amplification. In samples with an equivocal ratio and low level amplified samples (ratio 1.5–2.5) an additional 100 to 150 nuclei were counted.

2.4. MLPA

From each sample 5 μm tissue sections obtained from formalin fixed, paraffin embedded material were mounted on glass sides. An area with an invasive part of the tumor was selected by a pathologist on a haematoxylin and eosin stained slide and the percentage of tumor cells in this area was estimated. Most samples had a tumor content of 70–90%. A minimal tumor percentage of 50% was used to obtain reliable MLPA results. Subsequently, the selected area was manually dissected using a sterile scalpel or 18-gauge needle. DNA was isolated by overnight proteinase incubation at 56°C, followed by boiling of the samples for 5 minutes.

Before clinical evaluation the MLPA was first optimized and validated for use of formalin fixed, paraffin embedded material using non-tumor material from normal lymph nodes. Best MLPA results were obtained if only a simple ethanol precipitation was performed after an overnight Proteinase K treatment. The optimal DNA input was 150 ng (range tested 20–500 ng).

MLPA was performed with the HER2 kit P012 (MRC Holland, Amsterdam, The Netherlands) according to the manufacturers' guidelines. The P012 probe mix for detection of HER2 amplification contains three probes for the HER2 gene, one probe located <1 MB of the HER2 gene on chromosome 17q and eight control fragments, located on different chromosomes. Details on probe sequences, gene loci and chromosome locations can be found at www.mrc-holland.com. All incubations were performed in a PCR machine with heated lid (PTC-200 DNA Engine, MJ research, Waltham, MA, USA).

150–200 ng DNA (in 5 μl 10 mM Tris–0.1 mM EDTA, pH 8.2) was denatured at 98°C for 5 minutes. Subsequently 3 μl probe-mix was added. This mixture was heated at 95°C for 1 minute and incubated for 16 hours at 60°C. Ligation was performed using the heat stable ligase-65 enzyme at 54°C for 15 minutes, followed by inactivation at 98°C for 2 minutes. The ligation products were amplified by PCR using a single fluorescent labeled (6-FAM) primer pair. PCR products were analyzed on an ABI310 capillary sequencer (Applied Biosystems, Foster City, CA, USA) with a 36 cm capillary and POP4 polymer. Genescan-ROX500 was used as size standard. Fragment analysis was performed using Genescan Analysis software (Applied Biosystems, Foster City, CA, USA).

2.5. Interpretation of the MLPA

In each run formalin fixed, paraffin embedded non-tumor lymph node tissues were included as control samples. Samples with a strong HER2 amplification could easily be distinguished from non-amplified samples by visual, qualitative inspection of the MLPA peak profiles. For all samples quantitative analysis of the data was performed by calculating the relative copy numbers for each of the three HER2 fragments against the control fragments. The relative quantity of the probes in each sample was determined using an Ex-

cel template as described by MRC Holland (Amsterdam, The Netherlands; www.MLPA.com). In this way a relative copy number could be obtained, representing the gene dosage of a particular fragment. The relative probe signal was defined by dividing each measured peak area by the sum of all peak areas of that sample. The ratio of each individual probe relative area was subsequently normalized to the average ratio obtained with control DNA samples tested in the same experiment. Dosage results were grouped into three categories. The threshold was set at 1.2 and a value equal or higher as 2 was considered as amplification. A relative copy number of 2 is expected for a 2-fold gene amplification in tumors. An equivocal category with relative amplification values ranging from 1.2–2.0 remained. These equivocal samples were considered as non-amplified.

3. Results

3.1. Comparison of HER2 status by Immunohistochemistry and FISH

Forty-seven breast cancer samples previously tested with immunohistochemistry (eight cases scored 0 and 1+, 30 cases 2+ and nine cases scored as Hercep 3+) were tested for HER2 gene amplification by FISH (Table 1). HER2 FISH analysis was successfully performed on 46/47 breast cancer cases tested. In one case, a tissue block originating from 1981, no FISH results could be obtained probably due to alternative fixation of this sample. A representative result of an amplified and a non-amplified HER2 FISH is given in Fig. 1, showing a centromere 17 signal in green and a signal from the HER2 probe in red. Clearly 1–2 centromere 17 spots are present in diploid cases (29/46) as shown in Fig. 1 (2A), whereas in the remaining 17/46 FISH tested samples polyploidy is found (2B). For HER2 signals, 1–2 signals are present in the not amplified sample and a strong clustering of red signals is present in the amplified sample (2B). One sample of the Hercep 0/1+ group (1/8, 13%), 10/30 (33 %) of the 2+ cases and 7/9 (78%) of the Hercep 3+ cases showed clear HER2 amplifications. Most amplified samples showed bright and clustered HER-2 signals, although some cases showed a scattered signal pattern.

Several samples of Hercep 0/1+ and Hercep 2+ groups (1/8 and 4/30, respectively) showed an elevated number of HER2 signals in relation to the centromere 17 signals with ratios just below 2.0. These equivocal samples were considered as not amplified, according to the manufacturers instructions.

3.2. FISH analysis

For 41 samples manual determination of the HER2 amplification rate was compared with automated spot counting. Correction for the recognition of red and green signals was mainly necessary in amplified samples with clustered red signals resulting in overlapping signals. These samples were correctly identified as amplified but under representation of the HER2 amplification rate is possible without manual correction. All manual obtained data could be confirmed with the use of automatic spot counting. Automatic spot counting resulted in a decrease of analysis-time of about 50%.

3.3. Detection of HER2 gene amplification by MLPA

All 47 samples tested by IHC and FISH showed positive results by MLPA (Table 1). Also the FISH negative sample showed in MLPA a reliable result. This IHC 0/1+ sample had no HER2 amplification in MLPA. Representative MLPA peak profiles of both an amplified and a non-amplified sample are shown in Fig. 1. One Hercep 0/1+ sample (1/8, 13%), 10/30

Table 1

Prevalence of Her-2/neu amplification by FISH and MLPA in relation to immunohistochemical results for 47 breast cancer samples before discrepancy analysis

IHC	FISH			MLPA	
Score	Amplification	No amplification	Not interpretable	Amplification	No amplification
0/1+	1*	6	1	1*	7
2+	10	20	0	10	20
3+	7	2†	0	8	1

* After discrepancy analysis this sample showed to be IHC 3+; † one sample original FISH negative showed to be amplified (ratio 2.2) after retesting.

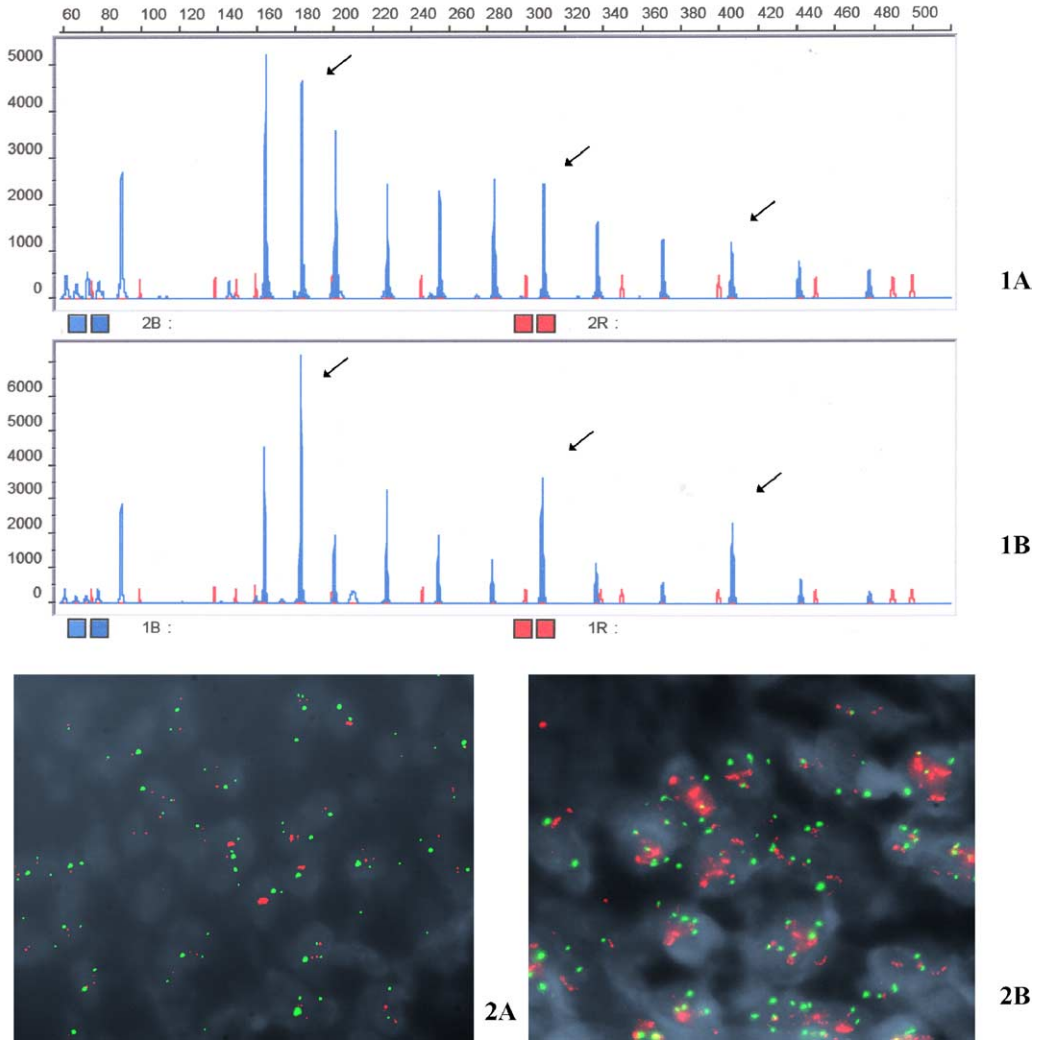

Fig. 1. Representative results of HER2 MLPA and FISH. 1: MLPA peak profile showing three HER2 probes (indicated by arrows), other peaks correspond to control probes. 2: Images of FISH results with HER2 signals in red and centromere 17 control signals in green, counterstained DAPI (magnification ×1000). 1A and 2A are showing a non-amplified sample (#20 IHC 2+) both in MLPA (ratio 1.2) and in ISH (ratio 1.0). In 1B and 2B a sample with a HER2 amplification is shown (sample #2 IHC 2+) in MLPA (amplification ratio 2.9) and in FISH ratio 2.2).

(33%) of the 2+ cases and 8/9 (89%) of the Hercep 3+ cases showed a clear HER2 amplification.

In these HER2 amplified samples we detect a large variation in relative copy numbers for the three individual HER2 probes. Both for samples with a strong HER2 amplification and samples from the equivocal category, there is a difference in the performance of the three individual probes. Amplification is not always detectable in all three HER2 probes. But the obtained relative copy numbers for the three individual HER2 probes are very reproducible. In non-amplified tumor samples none of the three HER2 probes did show an amplified signal and the variation in relative copy numbers is within the same range as for the control samples.

3.4. *Comparison of FISH and MLPA in relation to immunohistochemistry*

For 46 cases tested by FISH and MLPA results are compared in Table 2. Using MLPA 18/46 breast carcinomas showed a clear amplification with a relative copy number higher as 2.0, 19 samples had a normal amplification ratio below 1.2 for the HER2 probe and 11 samples (23%) showed equivocal results by MLPA

with a relative copy number higher as 1.2 but below 2.0. These samples were also considered as not amplified. At least 2-fold amplification of the HER2 gene was shown in 1/8 (13%) of the IHC 1+ tumors both by MLPA and FISH and in 9/30 (30%) of the IHC 2+ tumors by MLPA and 10/30 (33%) by FISH. Of the IHC 3+ tumors 8/9 (89%) showed amplification by MLPA whereas in FISH 7/9 (78%) samples were amplified. In 17/18 amplified cases in FISH the amplification could be confirmed with MLPA (Table 2).

In FISH five cases with equivocal results were obtained with a HER2/centromere 17 ratio just below 2.0. These samples (four Hercep 2+ and a Hercep 1+ sample) showed also equivocal results by MLPA. With MLPA six additional cases showed an elevated amplification rate. All the samples with a borderline amplification had an amount of tumor cells from at least 60%.

In FISH one sample showed tumor heterogeneity. About 10% of the tumor cells showed a very strong amplification with a HER2/centromere 17 ratio of 4.0 in contrast to the remaining majority of tumor cells in which the HER2 gene was found not to be amplified. According to the obtained average HER2/centromere 17 ratio, this sample was considered as not amplified. Also in MLPA no amplification could be detected. Immunohistochemistry showed a homogeneous staining, scored as IHC2+ for all tumor cells.

Table 2

Comparison of FISH and MLPA for detection of Her-2/neu amplification in 46 breast tumors

	FISH		
	Amplified	Not amplified	Total
MLPA			
Amplified	17	1*	18
Not amplified	1	27	28
Total	18	28	46

*One samples original FISH negative showed to be amplified (ratio 2.2) after retesting.

HER2 amplification ratios obtained by MLPA and CytoFISH analysis were very similar for samples lacking amplification. In amplified samples HER2 ratios showed large differences in MLPA and CytoFISH as presented in Table 3. 10/13 amplified samples tested by CytoFISH showed higher amplification rates in MLPA as in FISH. Three of these amplified samples showing weak amplifications in FISH with ratios equal or just above cut off value of 2.0 (2.0–2.2), were strongly amplified in MLPA with HER2 amplification ratios ranging from 2.9 to 5.2. Hercep 2+ category represents the IHC weak positive samples. But with MLPA no difference in relative amplification ratio in Hercep 2+ and 3+ cases was found. Both in the Hercep 2+ and 3+ groups, strong and borderline amplification could be detected whereas all samples were standardized for at least 50% tumor cells.

3.5. Analysis of equivocal and discrepant cases

All discrepant and equivocal cases were reanalyzed both with IHC, FISH and MLPA. Retesting of a single IHC 1+, FISH and MLPA positive sample by IHC resulted in a strong positive HercepTest (3+). All other

Table 3

HER2 ratios in FISH and MLPA in Amplified and Equivocal samples

Amplified				Equivocal in MLPA			
Sample nr	HerCep	FISH	MLPA	Sample nr	HerCep	FISH	MLPA
2	2+	2.2	2.9	4	2+	1.5	1.4
9	3+	4.6	3.9	5	2+	1.0	1.9
10	3+	2.7	5.6	12	3+	1.0	1.5
11	3+	2.9	4.6	23	1+	1.6	1.3
13	3+	2.7	5.6	40	2+	1.2	1.9
15	3+	2.0	5.2	41	2+	1.1	1.4
16	3+	3.3	3.3	43	2+	1.1	1.5
17	3+	2.5	3.6	44	2+	1.5	1.4
19	2+	2.4	6.9	45	2+	1.6	1.4
28	2+	2.2	4.0	46	2+	1.8	1.5
29	2+	3.8	8.5	55*	2+	3.7	1.5
30	2+	2.5	7.7				
32	2+	2.4	5.9				

The relative copy number for MLPA is the average of the normalized peak fractions. For both MLPA and FISH: N – non-amplified for a relative copy number <2.0, A – amplified for a relative copy number $\geqslant 2.0$.

*Sample #55 is the only discrepant sample in this study with a clear amplification detected in FISH and an equivocal HER2 ratio in MLPA.

samples retested by IHC resulted in the same Hercep score as from the original slide. Repeating the MLPA test for the discrepant and equivocal samples resulted for all cases in the same relative copy numbers as the first test. The test proved to be very reproducible. One discrepant sample, which was original, amplified in MLPA (HER2 ratio 2.5) but not in FISH, showed after retesting a weak amplification in FISH (ratio 2.2). All other samples retested by did not result in any differences.

Comparison of definitive FISH and MLPA data resulted in one discrepancy (Hercep 2+), showing a clear amplification in FISH (ratio 3.9) and an equivocal HER2 amplification ratio in MLPA of 1.7 (sample #55, Table 3). After retesting the discrepant cases the final overall concordance of detection of HER2 gene amplification by FISH and MLPA was 98%.

4. Discussion

In this study MLPA has shown to be a reliable method for the detection of HER2 gene amplification. A high degree of concordance of 98% (45/46) between FISH and MLPA was found for the samples tested. By FISH an amplification of the HER2 gene could be detected in none of the IHC 1+ cases, 30% of the IHC 2+ cases and in 89% of the IHC 3+ samples. In spite of the low numbers tested in this study, these data are in line with those found in literature; about 90–95% of the IHC 3+ samples and 5% of IHC 1+ are amplified. For IHC 2+ samples however large differences are described. A wide variation of amplification in IHC 2+ scored tumors is reported in different studies, ranging from 25–50% [3,6,13,17,19].

In literature limited data concerning digitalized spot counting is available. In this study comparison of FISH data obtained by manual determination of the HER2 amplification status with digitalized, automatically determined ratios did result in 100% concordance. Automatic spot counting software is an objective, more reliable, time-reducing and standardized supplement in detection of HER2 amplification by FISH. By digitalization of the FISH results, the images are more easily available for archival storage and reflex testing purposes.

Comparison of data obtained in MLPA and FISH resulted in a very good correlation of the discrimination of amplified and non-amplified samples. In this study only one discrepant sample is present between FISH and MLPA. Also in the obtained HER2 ratios, indicating the level of amplification a good correlation is found, but variation can occur. Independently of the level of HER2 protein overexpression as indicated by IHC, both weak and strong underlying gene amplifications were found. With both FISH and MLPA, samples with HER2 ratios close to 1.0 (amplification negative) and equivocal samples (ratio 1.2–2.0) were found. All FISH equivocal results were also determined as equivocal in MLPA. MLPA showed some additional samples with equivocal HER2 ratios.

Interestingly, in HER2 amplified samples lower ratios were obtained in FISH combined with CytoFISH spot counting as compared to MLPA. The differences observed might be caused by the different approach for normalization to obtain amplification ratios [12,15]. In FISH the ratio is based on the relation HER2 to chromosome 17 centromere probe, in MLPA normalization is performed with nine loci, located on different chromosomes. Furthermore the relationship between gene dosage and mean peak ratios is not completely linear in MLPA [21]. In addition, automated spot counting can lead to underestimation of HER2 signals, especially in samples with high level clustered HER2 amplification (overlapping FISH signals), resulting in lower HER2/centromere 17 ratios. However, accurate manual determination of HER2 amplification ratios can also be problematic in those samples. The correlation between the level of HER2 gene amplification and its clinical value (i.e. progression, response to therapy) is still unknown. This would be an interesting object for future studies.

The MLPA test used in this study is a small HER2 kit with only three HER2 and nine control probes. For a more accurate comparison with the FISH data, capillary electrophoresis is used in this study. All amplifications (relative copy number of >2) can be identified by visual inspection of the obtained peak profiles. For quantification, particularly interestingly in cases of weak and intermediate amplifications, spreadsheet analysis is necessary. In this study a simple method was used to compare the peak areas of the HER2 probes with the average peak area of all control probes. However for this small HER2 assay, containing 12 fragments ranging from 166 to 480 bp, capillary electrophoresis is not essential. Visual examination of ethidium bromide stained agarose gels permits detection of a two-fold amplification of the HER2 gene [21]. Just a PCR machine and standard electrophoresis equipment are sufficient for applying HER2 MLPA.

Three HER2 probes are present to determine the amplification status in MLPA. Differences in the relative

copy numbers in tumor-samples can be observed for individual probes and this emphasis the use of more than one probe. In this study the average peak ratio of the three HER2 probes is used resulting in a reliable amplification factor. Although the variation obtained in the relative copy number in MLPA is a little higher as reported for non-fixed cell material with various MLPA tests [8,23], the good correlation with FISH found in this study shows that the accuracy of the technique for this purpose is sufficient.

Whereas in normal control samples there is very little variation in the performance (range 0.75–1.21), the tumor samples show a lot of differences in individual control probes (range 0.42–4.98). This is due to other genetic changes present in the tumor cells. This variation can influence the obtained amplification rate but because nine control probes located on different chromosomes are used to determine the ploidy status of the cells, the effect of additional individual genetic aberrations is diminished and a reliable HER2 status will be obtained.

As the DNA sequence recognized by a MLPA probe is only 50–70 nucleotides long, this molecular technique is very suitable for the use in pathology where only formalin fixed, paraffin embedded tissue is available. Initial studies and several publications showed that these tests are as robust and reliable as performed on non-fixed cell material [2,24]. Although processing of clinical samples is standardized in our laboratory, differences in fixation resulting in differences in DNA quality might still occur. By using control samples treated on the same way, effects of poorer DNA quality are diminished. Multiplex Ligation-dependent probe amplification is a technique that allows the rapid and precise quantification of multiple (>40) sequences within a nucleic acid sample. It is a single tube, semi-quantitative method that is highly automated and suitable for high throughput testing. Furthermore, it is an efficient and cost effective method since up to 40 target sequences can be analyzed simultaneously. Because MLPA is flexible in its target loci, it is extremely versatile in its applications. However, a disadvantage of MLPA and other PCR based techniques is the necessity for selection of tumor cells. To detect low-level gene amplification, in real-time PCR a minimal amount of 30% tumor cells is necessary [5]. In the tested series of breast tumor samples with a minimal amount of 50% tumor cells all amplifications but one, detected by FISH could also be detected by MLPA.

For both FISH and MLPA the determination of the amount of tumor cells and selection of tumor cells in sample is important. FISH and MLPA have the advantage being a more quantitative method as IHC. Tumor heterogeneity can influence the obtained results for all mentioned techniques. With FISH analysis different regions of the tumor sample are thoroughly examined to exclude any effect of heterogeneity. In this study no heterogeneity in different areas of the tumor, could be detected. However, heterogeneity at the cellular level was observed in a single case where different amplification ratios were present in different tumor cells in the same tissue area.

A key advantage of both IHC and FISH for HER2 evaluation is that they are based on microscopic analysis having tissue architecture preserved, allowing a clear distinction between invasive and non-invasive components. Nevertheless inter-observer variation with respect to the interpretation can occur [9]. Manual determination of the HER2 amplification ratio with FISH can be very time-consuming but analysis time can be reduced by the use of spot counting software.

In conclusion, this study shows that assessment of HER2 gene amplification by MLPA correlates very well with the results of the more established assays of FISH and IHC. Although at present FISH is the gold standard technique for determination of HER2 amplification status, MLPA has several advantages. It is a fast, efficient, less expensive technique and very suitable for large series of samples. MLPA might serve as either an alternative or supplementary technique (reflex testing) for FISH analysis. Alternatively, easy-to-perform FISH or MLPA assays might replace IHC screening. If and how MLPA can be used in the determination of HER2 amplification status has to be determined in future evaluation studies.

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