Progress in HER2 testing in breast cancer:

*M*ultiplex *L*igation-dependent *P*robe *A*mplification
and
automated immunohistochemistry

Cathy Beatrice Moelans

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Nieuwe ontwikkelingen in de diagnostiek van HER2 bij borstkanker: multiplex ligation-dependent probe amplificatie en geautomatiseerde immuunhistochemie

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General Introduction

General introduction

Breast cancer: facts and figures

Despite significant clinical and laboratory progress, breast cancer remains a major global public health problem. One in ten of all new cancers diagnosed and almost one in four cancers diagnosed in women worldwide is a cancer of the breast [1]. Worldwide, over 1.1 million cases of breast cancer are diagnosed each year and the numbers of women being diagnosed annually worldwide has almost doubled since 1975 [2]. Breast cancer is the main cause of death from cancer in women globally. In The Netherlands there are about 11,500 new patients each year, and 3,500 breast cancer deaths, which comprise 5% of the overall female mortality. Men are generally at low risk for developing breast cancer: annually 70 men are diagnosed with breast cancer in The Netherlands. On average, one out of nine women will develop breast cancer during her lifetime. In women younger than 30 years, breast cancer is rare [5]. For women older than 30 years, the incidence rises with age. About 75% of women who are diagnosed with breast cancer are older than 50 years and the mean age at diagnosis is 60 years. The 5 and 10 year survival rates are 80% and 69% [3]. Although mortality trends have been declining since 1992, especially for women aged 65-74, the incidence of breast cancer has been rising since then, possibly due to the introduction of breast screening programs in 1988 and the proportional increase of the ageing population [4]. Genetic factors such as germline mutations in the two major susceptibility genes (BRCA1 and BRCA2), may account for up to 5% of breast cancer cases ("hereditary breast cancer") but the vast majority of cases are likely to be a consequence of the accumulation of genetic changes ("sporadic breast cancer"). The major risk factors associated with breast cancer are reproductive factors (low age at menarche, high age at menopause, few pregnancies, high age at first full pregnancy, little breast feeding), body size/obesity, alcohol consumption, physical activity, exogenous hormones (oral contraceptives, hormone replacement therapy) and exposure to ionizing radiation [6]. This thesis will focus on sporadic breast cancer.

Detection and treatment of breast cancer

Advances in diagnostic imaging during the past two decades have greatly changed detection and diagnostic strategies. Mammography screening, educational programs, and improved consciousness in the female population have contributed to earlier detection of breast cancer. If a patient presents with clinical signs of a tumor in the breast, the diagnostic work-up consists of clinical examination, mammography and/or ultrasonography, and fine-needle aspiration for cytology or core-needle biopsy for histopathologic examination. Based on the outcome of these tests a decision is made whether further examination and treatment is necessary. There are traditionally four major treatment modalities:

surgery (breast conserving or mastectomy), radiotherapy, chemotherapy and hormonal therapy. Based on the increasing knowledge on the genetic changes in tumors and their effects on protein expression, a fifth class of treatment has emerged based on antibodies that target tumor antigens like HER2 (Herceptin®, trastuzumab) and Vascular Endothelial Growth Factor (Avastin®, bevacizumab). It is common practice that women with breast cancer receive a combination of these treatments [7]. The choice and the order of the different treatments depend on different factors including the characteristics of the tumor, the stage of the disease and the patient's age. The increase in therapeutic options for individual patients is paralleled by diagnostic procedures to provide optimal patient tailored treatment of breast cancer based on prognostic and predictive factors.

Predictive factors in breast cancer

Many investigators have focused on finding molecular markers that may predict response to therapy (so-called "predictive factors") but most findings remain controversial and inconclusive. So far, the only established predictive factors in breast cancer are steroid hormone receptors, which predict response to endocrine (hormone) therapy, and HER2 and EGFR, which predict response to the respective antibody based therapies. Topoisomerase IIα has recently gained interest as a predictive marker for chemosensitivity.

Steroid hormone receptors

When analyzed with immunohistochemistry (IHC), approximately 70% of all breast cancers overexpress the estrogen receptor (ER), and 50% overexpress the progesterone receptor (PR). Both markers are used as predictive factors for response to endocrine therapy with both tamoxifen and aromatase inhibitors. Hormone therapy with tamoxifen is often given to patients with early stages of breast cancer and those with metastatic breast cancer (cancer that has spread to other parts of the body). Hormone therapy with an aromatase inhibitor is given to some postmenopausal women who have hormone-dependent breast cancer. Aromatase inhibitors decrease the body's estrogen by blocking an enzyme called aromatase from turning androgen into estrogen. Response rates to endocrine therapy range from 80% in ER+/PR+ patients, through 30% in ER+/PR- patients, to <10% in ER-/PR- breast cancers [8].

Human Epidermal growth factor Receptor 2

The HER-2/*neu* proto-oncogene located on chromosome 17 encodes a 185-kD transmembrane tyrosine kinase growth factor receptor belonging to the epidermal growth factor receptor family which is involved in cell growth and development [9]. HER-2/*neu* amplification has been reported to occur in 15% to 25% of breast carcinomas and is usually accompanied by overexpression of its protein [10,11], as determined by immunohistochemistry (IHC).

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The development of trastuzumab (Herceptin), a humanized monoclonal antibody that specifically targets the extracellular domain of HER-2/*neu* [12], offers a new therapeutic approach for women with HER-2/*neu* positive breast cancer. In addition, HER2 positive breast cancer patients respond better to taxane chemotherapy [13]. However, the costs for trastuzumab are € 30.000-40.000 per patient per year and adjuvant trastuzumab is associated with severe cardiac dysfunction in 2% (+ taxane) to 15% (+ anthracyclin) of all treated patients. The significant costs and toxicity of trastuzumab [14] and taxanes necessitate accurate determination of HER-2/*neu* status. The amplification of HER-2/*neu* is also known to confer a poor prognosis [15] and may predict worse response to hormonal therapy [16] and standard chemotherapeutic regimens [17].

Topoisomerase IIα

The topoisomerase IIα (TopoIIα) gene is located at chromosome 17q21.2 and encodes a 170 kD protein that plays a key role in cell division by controlling and modifying the topological status of DNA [18]. Furthermore, TopoIIα protein is the direct target of anthracyclines (TopoIIα inhibitors), one of the most powerful chemotherapeutic agents available for the treatment of breast cancer. The binding of anthracyclines to TopoIIα is believed to stabilize the DNA double-strand breaks created by TopoIIα [18], leading to apoptosis. The TopoIIα gene is located near HER2 and co-amplification of HER2 and TopoIIα is seen in approximately 40% of HER2-amplified breast cancer patients [19, 20]. Overall, TopoIIα amplification should be considered an uncommon event in breast cancer, with a prevalence of approximately 5-10% [21, 22]. TopoIIα but not HER2 overexpression is now believed to be the ultimate predictor of response to anthracyclines [19, 22, 23] and TopoIIα status is therefore increasingly considered to be of major importance in clinical practice for breast cancer patients. Expression of TopoIIα protein has not been shown to reliably predict response to anthracyclines, despite the fact that it is the direct target for these compounds [20, 22, 24, 25]. In contrast, TopoIIα gene copy number appears to be a more solid marker for favourable response to treatment with TopoIIα inhibitors [26, 27]. Furthermore, contrary to HER2, TopoIIα amplification has shown an inconsistent correlation with TopoIIα protein expression [28, 29], mainly because TopoIIα protein is highly dependent on the stage of the cell cycle and the proliferation rate.

Detection of HER2 amplification and/or overexpression

Accurate determination of the HER2 status is extremely important in guiding therapy, and the reliability of the diagnostic method used to determine HER2 status is critical in selecting the most appropriate patients for HER2-directed therapies. Currently, HER-2/*neu* status is usually determined using two methods: those that reveal gene amplification, and those aimed at detecting the overexpressed HER-2/*neu* protein [30, 31]. Although many efforts have been made to standardize HER2 protein immunohistochemistry procedure and

interpretation, there is still a lot of intra- and inter- laboratory variability in results. Consequently, there is a desperate need for a more reproducible test to detect overexpression of HER2. Some studies claim that gene amplification status better predicts response to trastuzumab therapy than protein overexpression does [32]. These methods will be further discussed in Chapter 2 of this thesis.

MLPA: Multiplex Ligation-dependent Probe Amplification

A relatively new PCR based technique to assess HER-2/*neu* amplification is Multiplex Ligation-dependent Probe Amplification (MLPA). MLPA is a multiplex PCR method detecting abnormal copy numbers of up to 50 different genomic DNA or RNA sequences in one reaction. The MLPA technique is easy to use and can be performed in many laboratories, as it only requires a thermocycler and capillary electrophoresis equipment. Up to 96 samples can be handled simultaneously, with results being available within 24 hours. The MLPA technique was first described in 2002 [33] and is further discussed in Chapter 2 of this thesis. MLPA has found applications in assessing gene copy number changes [34-36], gene expression [37, 38], and methylation [39-41]. Due to the short lengths of the target sequences of the probes, MLPA can not only be applied to fresh frozen material but is also suitable for paraffin embedded material. The ability to carry out a multiplex copy number assessment (e.g. HER2 and Topolla at the same time) on small amounts (50-200 ng) of DNA from paraffin embedded material makes MLPA a very attractive method in daily pathology practice. Also, MLPA is cheaper than most other gene amplification detection methods (35-110 €/patient depending on the amount of patients taken along in 1 MLPA run). Obviously, it remains a non-morphological method that requires proper morphological control of the input material.

Molecular biology of sporadic breast cancer

Most cases of breast cancer are 'sporadic', not hereditary, and are caused by accumulation of gene damage acquired to breast cells during a woman's lifetime ('somatic' changes). A wide variety of genes are commonly amplified, mutated, deleted or silenced in sporadic breast cancers and have been implicated in the development and progression of the disease. These include genes encoding growth factors and receptors, intracellular signaling molecules, cell cycle regulators, apoptosis (cell death) regulators, and adhesion molecules. This so-called "neoplastic progression" is generally characterized by the accumulation of multiple genetic alternations in the cells as the tumor progresses to advanced stages. Studies of these altered molecules are identifying new diagnostic and prognostic markers and unearthing new potential targets for therapy. Genome alternations in breast cancer can be subdivided into amplifications, losses, mutations, and chromosomal breaks.

Amplifications

Gene amplifications are essential features of advanced cancers and have prognostic as well as therapeutic significance in clinical cancer treatment. Several chromosomal regions are amplified in breast cancer and these amplifications often cover large regions of DNA although some are limited to a single locus. The drivers of large amplifications have been rarely identified. In each case several neighboring genes are co-amplified and their participation in the oncogenic process is sometimes evoked. The most probable model explaining the amplification process is the breakage-fusion-bridge cycle proposed by McClintock [42]. Amplifications manifest as extrachromosomal DNA fragments (double minutes, episomes), interspersed small insertions into the genome or homogeneously staining regions. The role of fragile sites is suspected in this matter [43, 44].

Table 1. Recurrent amplifications in breast cancer detected by comparative genomic hybridisation and in situ hybridization studies

Chrom. Position	% Amplifications	Genes involved	Remarks	REF
1q22	50%	RAB25		[45]
8p11-12	10-15%	FGFR1	Several amplicons Co-amplified with 11q13	[46]
8q24	10-20%	MYC	Co-amp lified with HER2	[47]
11q13	15%	CCND1, EMSY	Several amplicons ER+ Co-amplified with 8p12	[48, 49]
12p13	5%	EPS8, NOL1		[50]
12q14-15	5-10%	MDM2		[51]
17q12	15-20%	HER2	Co-amplified with 8p12	[15]
17q23	15%	RPS6KB1, PPM1D	BRCA-associated	[52, 53]
20q13	5-15%	ZNF217, AURKA		[54]

Comparative genomic hybridization (CGH) analyses and array-CGH have described whole-genome alterations in breast cancer. Many regions of gain have been found [55, 56]. Meta-analyses of amplifications have been published [47, 51, 54, 57]. Over 60% of breast tumors have at least one amplification. Several regions can be co-amplified in the same tumor. In breast cancer, more than 60% of the highly amplified genes is also significantly elevated at the protein level. On average, a 2-fold change in DNA copy number transcribes to a 1.5-fold difference in the mRNA level and at least 12% of all gene expression changes in breast tumors are directly associated with the gene copy number [58].

Table 1 summarizes the most common amplifications described so far. 8p12, 8q24 and 17q12/HER2 amplification are associated with poor prognosis while 20q13 is only variably associated with prognosis [59]. 11q13 amplification is associated with ER+ breast tumors [60]. Various other amplifications are not well

characterized or are detected with low frequency in breast cancer, e.g. 3q26 (PI3KCA), 6p [61], 6q25 (ESR1, 20% [62]), 7p12 (EGFR, 1%), 7q35-36 (EZH2, [63]), 8q13 (PRDM14, [64]), 10p [65], 13q21-31[66], 14q23 (SIX1, 5%) [67], 15q [68], 16p13 [69], 19p13 (INSR, 9%) [70], 19q12 (CCNE1, 6%, [71]) etc.

Mutations and losses

Mutations are found in many genes. These genes can code for oncogenes (activating mutations) or tumor suppressors (inactivating mutations). Wood et al showed that, in each breast tumor, there are approximately 14 mutated candidate genes [72]. Genes frequently mutated in this study were TP53 (17p), PIK3CA (3q), GAB1 (4q), IKBKB (8p), IRS4 (Xq), RPS6KA3 (Xp) and ATP8B1 (18q).

Loss of genomic regions is frequent in breast cancer. When recurrent, these regions are supposed to contain tumor suppressor genes (TSG). Many potential TSG have been proposed and among them p53 is probably the most important one. Mutations have been found in some TSG.. Table 2 gives an overview of important oncogenes and tumor suppressor genes that are mutated and/or lost in sporadic breast cancer.

Table 2. Oncogenes and tumor suppressor genes mutated or lost in sporadic breast cancer.

Pathway	Gene	Mutation%	Ref	Remarks
Oncogenes				
PI3K/AKT pathway	PI3KCA	20-40%	[73, 74]	Poor outcome Especially NFKB pathway
	AKT	8%	[75]	
	mTOR	-		
ERBB family	ERBB2	rare	[76]	
	ERBB3	rare, silent	[77]	
	ERBB4	rare	[78]	
	EGFR	rare, vIII	[79, 99,100]	Constitutively activated
RAS pathway	KRAS, HRAS	rare	[80]	
	BRAF	rare	[80]	

Location	Gene	Alternation %	Ref	Remarks
Tumor suppressor genes				
17p13	TP53	20-40% mutations + LOH	[81]	Worse outcome
10q23	PTEN	LOH frequent mutation rare	[82]	Herceptin resistance when lost
3p14.2	FHIT	LOH frequent mutation rare	[83]	Location of FRA3B
8q11	RB1CC1	20%	[84]	Truncating mutations
16q22	CDH1	50-60%	[85]	Truncating mutations in lobular carcinoma
13q14	RB1	20%	[86]	Mainly deletions not mutations

Other tumor suppressor genes of interest in breast cancer include p16 (CDKN2A), nm23 (metastasis suppressor), and maspin (SERPINB5) [87]. BRCA1, BRCA2, CHEK2, BRIP1, ATM and PALB2 are mutated in hereditary cancers but rarely in sporadic forms. However, loss of their expression is frequent. Inactivation of tumor suppressor genes can also occur by epigenetic mechanisms such as promoter hypermethylation (i.e. p16), and by modification of subcellular localization, accelerated degradation and/or aberrant splicing.

Chromosomal breaks and translocations

Chromosomal breaks occur frequently in breast cancer. Some are the consequence of overall genomic instability, others may represent oncogenic alterations. The latter are recurrent. Recurrent breaks can be observed at common fragile sites (for example FRA3B at 3p14 containing the FHIT gene and FRA16D at 16q23 containing the WWOX gene).

Two balanced translocations have been extensively described in breast cancer. A t(12;15)(p13;q25) translocation fuses the ETV6 gene to the NTRK3 gene and produces the ETV6-NTRK3 fusion and chimeric protein. This event occurs in a specific rare form of breast cancer, i.e. secretory breast cancer [88]. The gene fusion encodes a constitutively active tyrosine kinase with potent transforming activity. Also, t(1;16)(q10;p10) resulting in loss of 16q and gain of 1q, occurs frequently in low grade breast cancers, specifically in the lobular subtype (>60%), but also in pre-cancerous stadia like columnar cell lesions and low-grade ductal carcinoma *in situ* [89].

Polysomy of chromosome 17

Chromosome 17 is one of the smallest and most densely gene-loaded human chromosomes. It is rearranged in at least 30% of breast cancers with short and long arms differing in the type of events they harbor [90, 91]. 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. One of the genes located on chromosome 17q is HER2. As described above, amplification of the HER2 gene is present in about 15-25% of breast carcinomas, correlates with a poor outcome [92] and is an indication for treatment with trastuzumab [93, 94]. 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 critical for determination of true HER2 gene amplification as opposed to increased chromosome 17 copy number [95, 96]. 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 [97]. In a recent study by

Bartlett et al [98] it was suggested that the presence of polysomy 17 as established by CEP17 FISH, rather than HER2 and TOP2A alternations, could be predictive for response to anthracyclines. There has been a lot of speculation and discussion about the true prognostic and therapeutic meaning and the existence of true polysomy 17 in breast cancer but until now, the discussion continues.

In summary

Accurate determination of HER2 status is extremely important in guiding therapy, and the reliability of the diagnostic method used to determine HER2 status is critical in determining eligibility for HER2-directed therapies. Although many efforts have been made to standardize diagnostic tests such as HER2 protein immunohistochemistry, there is still a lot of variability in IHC procedure (choice of antibody, staining process) and interpretation. It is therefore essential that we look for ways to make HER2 IHC more reproducible.

Recent developments in molecular technologies have resulted in better understanding of the processes/pathways involved in tumorigenesis and cancer development, and it is now apparent that tumors with the same phenotype can actually be genetically quite different. The application of molecular diagnostics is beginning to show improvements over existing clinicopathological marker assessment. Single-marker diagnostics are already a reality in the clinical setting and the best examples are ER status and HER2 status. It is not very likely that analysis of a single marker will be sufficient for proper therapeutic choice-making (considering primary and acquired resistance, the existence of multiple pathways etc) and perhaps multiplex assays may bring us closer to a more accurate prediction of prognosis and response to specific therapy regimens. So, there is a desperate need for a reliable quantitative test to detect amplification of HER2 and even more, for a test that is able to analyze several prognostic and predictive genes simultaneously, facilitating oncologists in clinical decision making and allowing a more personalized treatment for breast cancer patients. Furthermore, several processes such as polysomy 17 and genetic variation are still not yet fully understood.

The primary aim of this thesis was therefore to explore MLPA as a new, cheap and easy method to simultaneously analyze the copy number status of a broad spectrum of genes including HER2, leading to a better understanding of several processes in breast cancer such as polysomy 17 and genetic heterogeneity in breast cancer patients. In the future, this knowledge could contribute to a patient-specific genetically based adaptation of therapy and to a better estimation of a patient's individual prognosis. Furthermore, we evaluated a new fully automated HER2 immunohistochemistry kit based on a monoclonal antibody as a possible more reproducible alternative to the currently used manual HER2 overexpression detection kit based on a polyclonal antibody.

Outline of this thesis

Despite the development of better diagnostic and therapeutic tools, breast cancer remains a major global health problem. More research is necessary to improve the current understanding of the molecular pathogenesis of breast cancer. New prognostic markers and diagnostic tools will help to better individualize treatment and guide the development of new therapeutic strategies. In *Chapter 2*, current amplification/overexpression detection methods for HER2, presently one of the most important prognostic and predictive markers in breast cancer, is discussed, with special focus on a new technique called MLPA. In *Chapter 3* we evaluated the suitability of a new fully-automatic HER2 protein staining system for use as an aid in determination of eligibility for trastuzumab therapy, by comparing it with other already established methods. In *Chapter 4* of this thesis we explored MLPA as a low cost, technically uncomplicated and quantitative method to detect amplifications of the HER2 gene in breast cancer in comparison with other techniques used for HER2 amplification or overexpression detection (IHC, FISH, CISH). Since MLPA is a non-morphological technique that relies on the amount of tumor cells in the sample, we further investigated in *Chapter 5* whether manual or laser microdissection is necessary for improvement of the sensitivity of this technique to detect HER2 amplifications. The second focus of this thesis was to explore MLPA as a multiplex technique in Chapters 6 to 8. In *Chapter 6* we analyzed a large set of patients for both HER2 and TOP2A copy number and protein expression by MLPA, CISH and IHC. In *Chapter 7* we investigated the presence of polysomy 17 in breast cancer by simultaneously analyzing a set of 17 chromosome 17 genes in 111 patients using MLPA. In *Chapter 8*, we investigated the frequencies of amplifications, co-amplifications and losses of multiple important or potential breast cancer (onco)genes and studied their association with each other and with clinicopathological parameters that are currently used to determine prognosis and therapy regimen.

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Chapter 2

Invasive breast cancer: overexpression of HER-2 determined by immunohistochemistry and Multiplex Ligation-dependent Probe Amplification

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Introduction

The HER-2/*neu* proto-oncogene located on chromosome 17 encodes a 185-kD transmembrane tyrosine kinase growth factor receptor belonging to the epidermal growth factor receptor family [1] which is involved in cell growth and development [2]. HER-2 is amplified in 20% to 30% of breast carcinomas. This amplification of HER-2/*neu* is now known to confer a poor prognosis [3, 4] and may also predict a worse response to hormonal therapy [5] and standard chemotherapy regimens [6]. HER-2/*neu* proto-oncogene amplification is usually accompanied by overexpression of its protein [7] as determined by immunohistochemistry (IHC). The recent development of trastuzumab (Herceptin), a humanized monoclonal antibody to the extracellular domain of HER-2/*neu*, offers a new therapeutic approach for women with HER-2/*neu* positive breast cancer. In addition, HER-2/*neu* positive breast cancer patients respond better to taxane chemotherapy [6]. However, the significant costs and toxicity of trastuzumab [8] and taxanes have raised attention with regard to accurate determination of HER-2/*neu* status. Currently, HER-2/*neu* status is determined using two methods: those that reveal gene amplification, and those aimed at detecting the overexpressed HER-2/*neu* protein [9-12]. Some studies claim that gene amplification status better predicts response to therapy than protein overexpression does [13].

Immunohistochemistry (IHC) is the most commonly used method to assess protein overexpression. It is a rather easy morphological method which has many advantages but it may be hampered by technical problems and requires strict quality control and standardization [14]. Moreover, the different IHC technical steps are highly dependent on fixation conditions that significantly modify membrane staining [12, 15-17]. Consequently, significant variability of IHC results has been demonstrated in inter-laboratory quality control studies. For scoring of IHC staining, the 0 to 3+ visual system developed for the HercepTest (Dako, Glostrup. Denmark) is widely in use (Figure 1). While there is little difficulty in assigning the 0 and 3+ scores, interpretation is more problematic for the two intermediate levels. For cases scoring 2+ (10%-15% of all breast cancers), the concordance with gene amplification by fluorescence *in situ* hybridization (FISH) is barely 25%, and yet a proportion of these 2+ cases are true HER-2/*neu* amplified tumors. These cases, therefore, require a second line amplification test.

Gene amplification can be assessed by different methods. Southern blotting is the gold standard, but is time consuming, complicated and requires a lot of DNA which makes it an unattractive method for daily pathology practice. Therefore, traditionally FISH has for long been the most popular method for HER-2/*neu* gene amplification testing. However, FISH is expensive, technically challenging and sensitive to differences in digestion methods, and the commercially available kits have a limited half life. This method is, therefore, not a practical primary screening tool [18], although it has been recognized as such by the United States Food and

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Drug Administration [19]. Its use, therefore, is usually limited to equivocal cases. Chromogenic ISH (CISH) overcomes many of the disadvantages of FISH but is less sensitive and quantitative [20, 21]. A relatively new PCR based technique to assess HER-2/*neu* amplification is Multiplex Ligation-dependent Probe Amplification (MLPA). In this chapter we review the value of MLPA for detection of HER-2/*neu* amplification in breast cancer in comparison with other available methods.
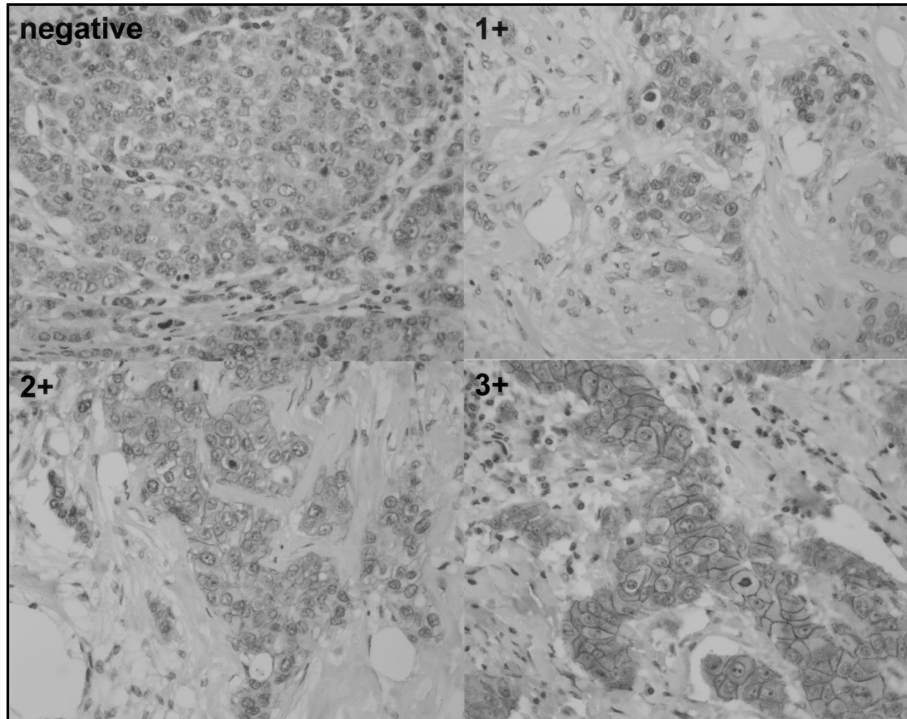
Figure 1. Examples of the scoring of HER-2/*neu* immunohistochemical staining. (<u>Top left</u>) Negative staining: no staining, only cytoplasmic staining or less than 10% cells with membrane staining. (<u>Top right</u>) 1+ staining: more than 10% cells with membrane staining which is however incomplete. (<u>Bottom left</u>) 2+ staining: more than 10% cells with complete membrane staining which is however not strong in intensity. (<u>Bottom right</u>) 3+ staining: more than 10% cells with complete intense membrane staining (reprinted with permission from Purmomosari et al [22])

Multiplex Ligation-dependent Probe Amplification

The MLPA technique was first described in 2002 by Schouten et al [23] and is summarized in Figure 2. This technique uses a mixture of hemi-probe sets that consist of two oligonucleotides, both having PCR primer sequences on the outer ends and a sequence complementary to a part of the target sequence on the inner ends. One of the primers has a spacer (in light gray) of variable length in between the PCR primer sequence and the complementary target sequence. When the complementary target sequences of both hemi-probes hybridize

adjacent to each other on the target sequence, they can be ligated to each other, and subsequently amplified using the PCR primer sequences. Because the PCR primers are the same for all hemi-probe sets, they can be amplified in a single PCR, which will provide amplicons of unique and defined lengths due to the specific spacer length within each probe set. Up to 40 probe sets can be run in one reaction. MLPA has found applications to assess gene copy number changes [24-26], gene expression [27, 28], and methylation [29-31]. Due to the short lengths of the target sequences of the hemiprobes, MLPA can not only be applied to fresh frozen material but is also suitable for paraffin embedded material. Depending on the quality of the DNA, 20-200 ng of DNA suffice, although reproducibility may be less with very small amounts of DNA. The ability to carry out a multiplex copy number assessment on small amounts of paraffin embedded material makes MLPA a very attractive method in pathology. Obviously, it remains a non-morphological method that requires proper morphological control of the input material. In cases with a low percentage of relevant material, meso- or microdissection may be necessary.

Multiplex Ligation-dependent Probe Amplification for detection of HER-2/neu amplification

A typical protocol comprises the following [22]: 50–500 ng target DNA/5 µl of 10 mM Tris (pH 8)-0.1 mM EDTA is denatured for 5 min at 98°C after which 3 µl of the probe mix is added. The mixture is heated at 95°C for 1 min and incubated at 60°C overnight (16 h). Ligation is performed with the temperature-stable Ligase-65 enzyme (MRC-Holland) for 15 min at 54°C. Next, the ligase is inactivated by incubation for 5 min at 98°C. Ten µl of this ligation mix is premixed with 30 µl of PCR buffer and placed in a PCR machine at 60°C. Subsequently, a 10-µl mix is added containing deoxynucleoside triphosphate, Taq polymerase, and one unlabeled and one carboxyfluorescein-labeled PCR primer, which are complementary to the universal primer sequences. PCR is carried out for 33 cycles (30 sec at 95°C, 30 sec at 60°C, and 1 min at 72°C). The fragments can be analyzed on an ABI model 310 capillary sequencer (Applied Biosystems) using Genescan-TAMRA 500 size standards (Applied Biosystems). Fragment analysis can be performed with Genescan software. DNA from Centre d'Etude Polymorphisme du Humain (CEPH) can be used as control sample and is analyzed simultaneously with breast cancer samples in every run. To objectify the interpretation of the fragment analysis, the relative quantity of the amplified probes in each sample needs to be determined using an Excel template. For this purpose, the relative peak areas for each probe are calculated as fractions of the total sum of peak areas in a certain sample. Subsequently, the fraction of each peak is divided by the average peak fractions of the corresponding probe in control samples. Finally, the values have to be normalized using the values obtained for the autosomal control probes, which serve as a reference for the copy number of 2.0.

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Cases that show a copy number above 2 for at least two of the probes on the HER-2/*neu* locus are considered to be amplified. Two further studies by Moerland et al [32] and Moelans et al [33] used similar protocols.
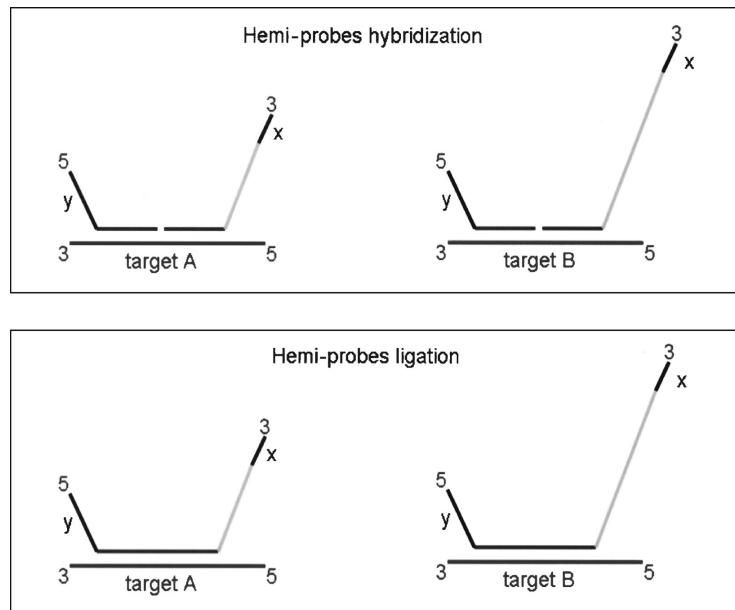
Figure 2. Principle of Multiplex Ligation–dependent Probe Amplification (MLPA). MLPA uses a mixture of hemi-probe sets that consist of two oligonucleotides, both having PCR primer sequences (x/y) on the outer ends, while both on the inner ends having a sequence complementary to a part of the target sequence (A or B). One of the primers has a spacer (in light gray) of variable length in between the PCR primer sequence and the complementary target sequence. When the complementary target sequences of both hemi-probes hybridize adjacent to each other on the target sequence (top figure), they can be ligated to each other (bottom figure), and subsequently amplified using the PCR primer sequences. Because the PCR primers are the same for all hemi-probe sets, they can be amplified in a single PCR, which will provide amplicons of unique and defined lengths due to the specific spacer length within each probe set.

Correlations between HER-2/neu Multiplex Ligation-dependent Probe Amplification and immunohistochemistry

In the study of Purnomosari et al [22], 60 frozen and accompanying formaldehyde fixed and paraffin embedded breast cancer samples were obtained from women treated at Sardjito Hospital, Yogyakarta, Indonesia. Table 1 shows the correlation between MLPA results in comparison with HER-2/*neu* immunohistochemistry (IHC) using the CB11 antibody from Novocastra, which was interpreted according to the DAKO scoring system. Of the 60 cases, 36 were HER-2/*neu* negative by IHC and 7, 8, and 9 of the cases showed 1+, 2+ and 3+ HER-2/*neu* overexpression, respectively. A total of 13/60 cases (22%) showed gene amplification by MLPA. Of

these amplified cases, 8 (62%) showed 3+ IHC, 5 (38%) 2+ IHC and no cases were 1+ or IHC negative. None of the 36 IHC negative and 0/7 1+ cases were amplified. Five of the eight (63%) 2+ cases were amplified, and as many as 8/9 (89%) IHC 3+ tumors showed gene amplification by MLPA assay. These data made clear that MLPA is a good method to identify the HER-2/*neu* amplified cases within the IHC 2+ group. On the other hand, not all IHC 3+ cases are HER-2/*neu* amplified. Figure 3 shows an example of an HER-2/*neu* MLPA test on an HER-2 amplified invasive breast cancer compared to a normal sample.

Table 1. Comparison between HER-2/*neu* immunohistochemistry (IHC) to assess protein overexpression and multiplex ligation-dependent probe amplification (MLPA) to detect HER-2/*neu* gene amplification (Purnomosari et al, 2006).

	MLPA	
IHC score	*Normal*	*Amplified*
0	36	0
1+	7	0
2+	3	5
3+	1	8
Total	47	13

In the study of Moerland et al [32], 47 breast cancers were studied by MLPA, of which 19 showed a clear amplification (40%). At least a 2-fold amplification of the HER-2 gene was shown in 1/8 (13%) of the IHC 0/1+ tumors and in 10/30 (33%) of the IHC 2+ tumors. Of the IHC 3+ tumors, 8/9 (89%) showed amplification (Table 2). Both in the Hercep 2+ and 3+ groups, strong amplification could be detected, whereas all samples were standardized for at least 50% tumor cells.

Table 2. Comparison between HER-2/*neu* immunohistochemistry (IHC) to assess protein overexpression and multiplex ligation-dependent probe amplification (MLPA) to detect HER-2/*neu* gene amplification (Moerland et al, 2006).

	MLPA	
IHC score	*Normal*	*Amplified*
0/1+	7	1
2+	20	10
3+	1	8
Total	28	19

In the study of Moelans et al [33], using the same MLPA kit and the DAKO Hercep test, 518 breast cancers were studied. IHC showed a 2+ score in 7% of patients,

and 9% of cases were IHC 3+. MLPA showed clear amplification in 11% and an intermediate amplification (significantly higher than controls but < 2) in 3.5% of cases. At least a 2-fold amplification of the HER-2 gene was shown in 6/434 (1%) of the IHC 0/1+ tumors and in 7/36 (19%) of the IHC 2+ tumors. Of the IHC 3+ tumors 47/48 (98%) showed amplification (Table 3).

Table 3. Comparison between HER-2/*neu* immunohistochemistry (IHC) to assess protein overexpression and multiplex ligation-dependent probe amplification (MLPA) to detect HER-2/*neu* gene amplification (Moelans et al, 2009).

	MLPA	
IHC score	*Normal*	*Intermediate/Amplified*
0/1 +	419	9/6
2 +	24	5/7
3 +	1	4/43
Total	**444**	**18/56**

Correlation between Multiplex Ligation-dependent Probe Amplification and other amplification detection methods

The study of Moerland et al [32] also compared HER-2 gene amplification by MLPA and FISH (PathVysion kit). In a series of 46 formaldehyde-fixed paraffin-embedded breast carcinomas, HER-2 gene amplification by FISH was found in 7/9, 10/30, and 1/7 in IHC 3+, 2+ and 0/1+ cases, respectively (Table 4). They also applied digitalized automated spot counting that was 100% concordant with manual FISH scoring. All but one FISH positive cases (17/18) were confirmed by MLPA for the presence of the gene amplification. The overall concordance of FISH and MLPA was 96% (44/46) (Table 5). Furthermore, both the level of amplification and equivocal results correlated well between both methods (Table 6). This underlines that MLPA is a reliable and reproducible technique that can be used either as an alternative or as an additional test to determine HER-2 status in invasive breast cancers.

Table 4. Comparison between HER-2/*neu* immunohistochemistry (IHC) to assess protein overexpression and FISH to detect HER-2/*neu* gene amplification (Moerland et al, 2006).

	FISH	
IHC score	*Normal*	*Amplified*
0/1 +	6	1
2 +	20	10
3 +	2	7
Total	**28**	**18**

The study of Moelans et al [33] also compared HER-2 gene amplification by MLPA and FISH (PathVysion kit). In a series of 67 formaldehyde-fixed paraffin-embedded breast carcinomas, HER-2 gene amplification by FISH was found in 18/20, 6/21 and 5/26 in IHC 3+, 2+ and 0/1+ cases, respectively (Table 7).

Table 5. Comparison between HER-2/*neu* amplification by MLPA and FISH in 46 cases of invasive breast cancer (Moerland et al, 2006).

	FISH	
MLPA	*Normal*	*Amplified*
Normal	27	1
Amplified	1	17
Total	28	18

Table 6. Comparison between HER-2/*neu* amplification ratios by MLPA and FISH in 13 cases of HER-2 amplified invasive breast cancer (Moerland et al, 2006).

		HER2 ratio	
Sample	**IHC score**	*FISH*	*MLPA*
1	2+	2.2	2.9
2	3+	4.6	3.9
3	3+	2.7	5.6
4	3+	2.9	4.6
5	3+	2.7	5.6
6	3+	2.0	5.2
7	3+	3.3	3.3
8	3+	2.5	3.6
9	2+	2.4	6.9
10	2+	2.2	4.0
11	2+	3.8	8.5
12	2+	2.5	7.7
13	2+	2.4	5.9

Table 7. Comparison between HER-2/*neu* immunohistochemistry (IHC) to assess protein overexpression and FISH to detect HER-2/*neu* gene amplification (Moelans et al, 2009).

	FISH	
IHC score	*Normal*	*Amplified*
0/1 +	21	5
2 +	15	6
3 +	2	18
Total	38	29

In the same series of 67 formaldehyde-fixed paraffin-embedded breast carcinomas, HER-2 gene amplification by FISH was found in 22/25, 6/13 and 1/29 MLPA amplified, intermediate and normal samples, respectively (Table 8). Similar data were obtained with CISH.

Table 8. Comparison between HER-2/*neu* amplification by MLPA and FISH in 67 cases of invasive breast cancer (Moelans et al, 2009).

	FISH	
MLPA	*Normal*	*Amplified*
Normal	28	1
Intermediate	7	6
Amplified	3	22
Total	**38**	**29**

Discussion

Several methods are in use for the detection of HER-2/*neu* gene amplification or protein overexpression, including immunostaining of the protein, FISH, quantitative Southern blotting, and real time PCR. The most widely applied test for HER-2/*neu* is IHC. Depending on the antibody and scoring system used, HER-2/*neu* overexpression rates in the literature vary between 14% [34] and 60% [35]. The subjectivity of IHC generally tends to decrease with increasing positivity, such that inter observer correlation is higher for strongly positive cases. Chromosome 17 polysomy has been postulated to play a role in other studies showing discrepancies between protein expression and gene amplification. Pauletti et al [36] attributed such 3+ positive, FISH-negative cases to chromosome 17 polysomy, and also found this subset of patients to have similar clinical outcomes to patients without the HER-2/*neu* gene alteration. In the literature, concordance rates between IHC and FISH range from 79% to 100% for 3+ cases [11, 37] and between 12% and 36% for 2+ cases [38, 39], demonstrating the importance of a gene amplification test.

Recently, two new methods have been described for the measurement of gene copy number; multiplex amplifiable probe hybridization (MAPH) [40] and MLPA [23]. Both techniques rely on comparative quantitation of specifically bound probes that are amplified by PCR with universal primers. The introduction of universal primers has advantages in that multiplexing numerous targets simultaneously becomes much easier, and when fluorescence detection of products is being used, only one fluorescent primer is required, thus reducing the cost compared to buying fluorescent probes for each target. Technically, FISH has disadvantages comparing to MLPA in determining part-gene deletions and remains a relatively low throughput when compared to other molecular genetic techniques available. The latter limitation also holds for Southern blotting, where only a few samples can be run per gel, a limited number of loci can be queried per

blot, and the tests may take several days [41]. Real-time PCR can also be used as a semi-quantitative technique when an internal amplification control is incorporated and has the advantage of not requiring post-PCR analysis. However, the number of targets that can be interrogated in a reaction is limited by the number of fluorophores available and the detection capabilities of the instrument. In general, PCR-based techniques for gene dosage determination can offer a less labor intensive alternative with higher throughput.
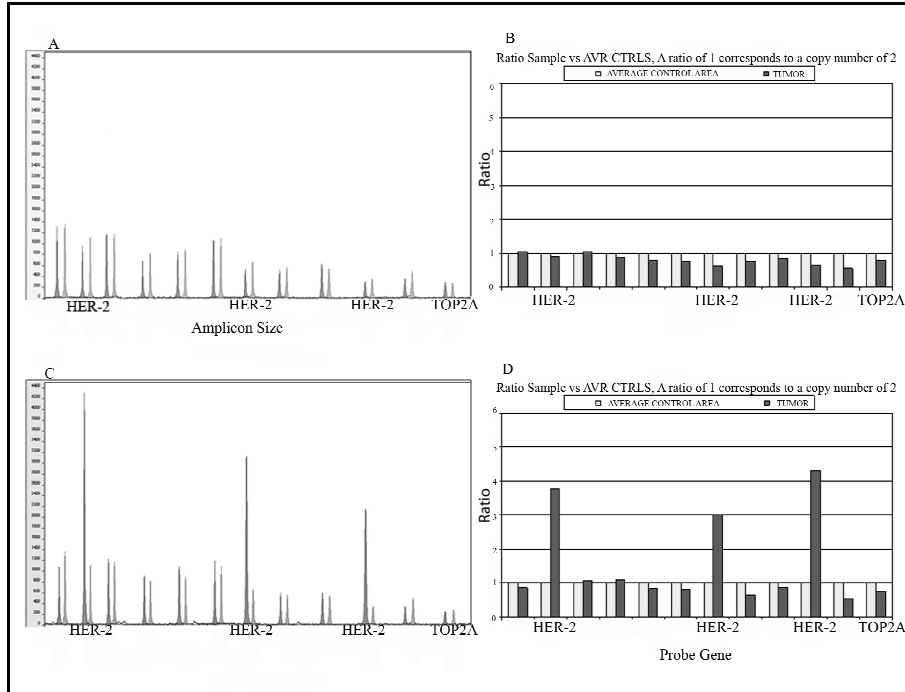
Figure 3. Examples of a HER-2/*neu* MLPA test in two invasive breast cancers and copy number calculations. (***A***) Breast cancer without HER-2 amplification showing on the left tumor peaks (dark gray) comparable to each other and to the control (light gray) peaks, further demonstrated by (***B***) copy number calculations yielding a ratio of around 1 for all probes. (***C***) Invasive breast cancer with HER-2/neu amplification as demonstrated by 3 dark gray HER-2 peaks which are clearly higher than the light gray autosomal control peaks for this sample and the light gray normal control peaks pointing to increased HER-2 gene copy number, further demonstrated by (***D***) copy number calculations yielding a ratio between 3 and 5 for the HER-2 probes compared to ratios around 1 for the control probes. Please note that the chromosome 17 located TOP2A gene (far right) shows normal copy number, denying chromosome 17 polysomy as an explanation for the HER-2 gene amplification.

Recent studies have evaluated MLPA for HER-2/*neu* gene amplification testing. In general, there is a good concordance between IHC and MLPA, and MLPA was clearly able to identify the amplified cases among the equivocal IHC 2+ cases. However, some IHC 0/1+ cases appear to be amplified, and some IHC 3+ cases lack amplification. In view of the very good concordance between MLPA and

FISH, it therefore seems that MLPA may at least have additional value to IHC, but may even be attractive for upfront amplification testing.

Compared to FISH, MLPA has many practical advantages. FISH probes loose their fluorescence in time, so the kits have a limited half life and stained slides cannot be kept indefinitely. Furthermore, interpretation has to be done under a fluorescence microscope in a darkened room, which is unpractical for the pathologist. The consequence of the fact that a 100x oil objective is needed to view the small spots is that overview is lost and heterogeneity may be easily missed. The probes for MLPA can well be kept, the method works well on small amounts of DNA extracted from paraffin-embedded tissue, and can be done in a high-throughput way. An additional advantage of the MRC-Holland kit is that a TOP2A probe is included. TOP2A is located close to the HER-2 locus, is often coamplified with HER-2, and this is related to response to HER-2 targeting therapies [42-44]. Besides being a chromosome 17 polysomy control, it may also help to predict response to therapy. A disadvantage is that MLPA is not a morphological method. Therefore, proper tissue selection guided by control H&E sections, and meso-/microdissection in cases with a low percentage of tumor epithelium may be necessary. The lower limit of tumor percentage still allowing meaningful MLPA analysis needs to be established. Obviously, the higher the amplification, the easier it will be to detect amplification in a background of non-amplified cells. As DCIS often shows HER-2 amplification while the invasive surrounding parts are negative, one has to be careful with blocks showing extensive DCIS which may yield false-positive results. Also in these cases, meso-/microdissection may be necessary. No studies have yet been published evaluating MLPA as a predictor or response to HER-2 targeting therapy. In view of the high correlation with FISH, we expect MLPA to have equal predictive power as FISH [45-47].

Chromogenic *in situ* hybridization, which has recently been introduced as an alternative to FISH, circumvents many of the disadvantages of FISH. The kits last, stained slides can be kept well, interpretation can be done with a light microscope using dry objective, making it easier to screen a full slide, skip DCIS and detect heterogeneity. The disadvantage of not having a chromosome 17 probe can be overcome using serial sections when deemed necessary. FISH and CISH have been proven to correlate well [20, 21, 48], but CISH is less quantitative than FISH and MLPA, and is not a high throughput method. Time and future comparative studies will tell whether CISH or MLPA will prevail as HER-2 amplification tests.

In view of the increasing appreciation of the high value of new methods such as MLPA and CISH, there will in the future probably be a discussion whether such methods need to be applied next to IHC in all cases. Although this will increase the costs of HER-2 testing, this will easily be compensated for when a small fraction of patients can be spared a very expensive therapy with significant side

effects. Even better, it may be that high throughput amplification tests completely replace IHC. Future studies need to address these cost-effectiveness issues. In conclusion, MLPA is a quick, cheap and easy method to detect HER-2/*neu* amplification in frozen and paraffin material in daily laboratory practice. MLPA is an attractive alternative to FISH for amplification testing in IHC equivocal cases, but may also be well suited for upfront HER-2 amplification testing in invasive breast cancer.

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Chapter 3

Validation of a fully automated HER2 staining kit in breast cancer

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Abstract

Background

Testing for HER2 amplification and/or overexpression is currently considered routine practice to guide Herceptin therapy in invasive breast cancer. At present, HER2 status is most commonly assessed by immunohistochemistry (IHC) and/or gene amplification tests such as chromogenic *in situ* hybridization (CISH). Standardization of HER2 IHC assays and slide interpretation are of utmost clinical and economical importance. At present, HER2 IHC is most commonly performed with the HercepTest which contains a polyclonal antibody and applies a manual staining procedure. Analytical variability in HER2 IHC testing could be diminished by a fully automatic staining system with a monoclonal antibody.

Materials and methods

200 invasive breast cancers were fully automatically stained with the monoclonal antibody based Oracle HER2 Bond IHC kit (Leica) and manually with the HercepTest (Dako). Discrepancies were tested for amplification with CISH.

Results

HercepTest yielded an overall sharper membrane staining, with less cytoplasmic and stromal background than Oracle in 19% of cases. Overall concordance between both IHC techniques was 94% (187/200) with a kappa value of 0.849 (95% CI: 0.773-0.925), indicating an excellent agreement. Most (11/13) discrepancies between HercepTest and Oracle showed a weaker staining for Oracle. Seven of the 13 discrepant cases were high level HER2 amplified by CISH, and in 6 of these HercepTest IHC better reflected gene amplification status although this was not significant (p=0.143). All 7 HER2 amplified discrepant cases were at least 2+ by HercepTest, in contrast with 5/7 that were at least 2+ for Oracle.

Conclusion

Fully-automated HER2 staining with the monoclonal antibody in the Oracle kit shows a high level of agreement with manual staining by the polyclonal antibody of the HercepTest. Although Oracle shows in general some more cytoplasmic staining and may be slightly less sensitive in picking up HER2 amplified cases, it may be considered as an alternative method to evaluate HER2 expression in breast cancer with potentially less analytical variability.

Introduction

HER-2/*neu* is a proto-oncogene located on chromosome 17q21 encoding a 185kD transmembrane tyrosine kinase receptor protein that is involved in signal transduction [1, 2]. HER2 belongs to the human epidermal growth factor receptor (EGFR) family and is amplified in about 15-25% of breast carcinomas causing an increased expression of its protein [3-5]. Patients having this overexpression respond well to treatment with trastuzumab (Herceptin®), a recombinant humanized monoclonal anti-HER2 antibody [6, 7]. Since the costs for trastuzumab therapy are high and side effects are significant, accurate selection of eligible patients for this therapy is crucial. Furthermore, amplification and overexpression of HER2 has also been shown to correlate with poor prognosis [8] and with resistance to conventional adjuvant chemotherapy and tamoxifen [9-13]. For these reasons, testing for HER2 amplification and/or overexpression is currently considered as routine practice in clinical pathology laboratories. At present, HER2 status is most commonly assessed by immunohistochemistry (IHC) and/or gene amplification tests such as fluorescence *in situ* hybridization (FISH) [14-16] or chromogenic *in situ* hybridization (CISH) [17]. Immunohistochemistry (IHC) is the most commonly used method to assess HER2 protein overexpression. It is a rather easy morphological method which has many advantages like its wide availability, relatively low cost, easy preservation of stained slides, and use of a familiar routine microscope. Disadvantages of IHC include the impact of pre-analytic issues including storage, duration and type of fixation, intensity of antigen retrieval, type of antibody (polyclonal versus monoclonal) [4], nature of control samples and the difficulties in applying a subjective semi-quantitative slide scoring system. For scoring of IHC staining, the 0 to 3+ visual system developed for the HercepTest (based on a polyclonal anti-HER2 antibody, clone A0485, Dako, Glostrup, Denmark) is widely in use. While there is little difficulty in assigning the 0 and 3+ scores, interpretation is more problematic for the two intermediate levels. For cases scoring 2+ (10-15% of all breast cancers), the concordance with gene amplification by FISH or CISH is barely 25%, and yet a proportion of these 2+ cases are true HER2 amplified tumors. These cases, therefore, require a second line gene amplification test. Because of its central importance in breast cancer therapy selection, standardization of HER2 IHC assays and slide interpretation are of utmost clinical and economical importance. Analytical variability in HER2 IHC testing can be minimized by the use of standardized tests, and by inter-laboratory quality control assessments. A fully automatic IHC staining system similar to the FDA-approved Ventana system (Pathway® anti-HER2 rabbit monoclonal antibody, clone 4B5, Ventana Medical Systems, Tucson, AZ) can improve the specificity, positive predictive value and efficiency of IHC [18] and can thereby produce a more consistent and reproducible result. The present study aimed to examine the suitability of the new Oracle HER2 Bond IHC System (Leica Microsystems, TA9145) for use as an aid in determination of eligibility for trastuzumab therapy. This fully automatic system is intended for use on Leica Microsystems' Bond-max™ devices and contains a ready-to-use mouse monoclonal anti-HER2 antibody (clone CB11)

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and a ready-to-use Compact Polymer™ detection system, both required to complete an immunohistochemical staining procedure for formalin-fixed paraffin-embedded tissues.

Materials and methods

Patient material

Tissue samples of 200 invasive breast cancer patients were retrospectively collected at the Department of Pathology of the University Medical Centre in Utrecht (UMCU) and at the Department of Pathology of the Laboratorium voor de Volksgezondheid Friesland (LVF). Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in both hospitals [19]. Biopsies were excluded from this study, and only whole sections were used. Both institutes separately carried out parallel manual and automated IHC stainings on their own tissue samples, using identical protocols and machines. All CISH stainings were performed at the UMCU.

Immunohistochemistry (IHC)

Manual IHC for HER2 was performed using the HercepTest (Dako, Glostrup, Denmark) according to the manufacturers' instructions on 4 μm thick sections from neutral buffered formaldehyde fixed tissue blocks. As control, a small tissue array containing a 0, 1+ 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.
Automated IHC for HER2 was performed on a Bond-max™ device using the Oracle HER2 Bond IHC System (Leica Microsystems, Newcastle, UK, TA9145). Staining was performed according to the manufacturers' instructions on 4 μm thick sections from neutral buffered formaldehyde fixed tissue blocks. Control slides with four (0, 1+, 2+ and 3+ intensity) formalin fixed, paraffin-embedded human breast cancer cell lines are provided to validate staining runs. In each run (i.e. slide tray) 4 tumor samples (primary monoclonal anti-HER2 antibody, clone CB11), 4 negative control samples (primary antibody is replaced by a supplied ready-to-use mouse IgG), a supplied HER2 positive control slide and an in-house positive control slide were analyzed. Also, at the UMCU, a small tissue array containing a 0, 1+ and 3+ breast tumor sample was taken along on the same slide as the tumor to be analyzed.

IHC membrane staining was semi-quantitatively scored as negative (0), weakly positive (1+), equivocal (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 all IHC stainings was done blinded by 1 experienced breast pathologist at the UMCU to exclude inter-observer variability.

Chromogenic in situ hybridization (CISH)

All CISH assays were run using the Zymed SPoT-Light HER2 CISH (Zymed, South San Francisco, CA) kit according the manufacturers' instructions. CISH was performed on 4μm thick paraffin sections and scoring was performed according the manufacturers' guidelines. Briefly, HER2 was scored high level amplified when large peroxidase-positive intra-nuclear gene copy clusters or >10 individual small signals were present in more then 50% of tumor cells. HER2 was scored low-level amplified when more than 50% of the tumor cells showed 6-10 dots per nucleus, or in the presence of small clusters. Tumors were scored as non-HER2 amplified when tumor cells showed 1- 5 dots per nucleus. A positive control was included in each CISH run and consisted of a paraffin section of a case known to be HER2 amplified by CISH. Scoring was done blinded to the IHC results by one experienced observer. Doubtful cases were evaluated together with another experienced observer until agreement was reached.

Statistics

Results obtained with manual and automated IHC techniques were compared by cross tables, and the concordance percentages and kappa-scores were calculated.

Results

Overall, HercepTest yielded a sharper membrane staining and showed less cytoplasmic and stromal background than Oracle in 37/200 (19%) of the patients, which is illustrated in figure 1. Table 1 shows the concordance between the HercepTest and Oracle HER2 staining. Overall concordance between both IHC techniques was 94% (187/200) with a kappa value of 0.849 (95% confidence interval 0.773-0.925) indicating an excellent agreement.

Table 1. Concordance between HercepTest and Oracle HER2 staining in 200 invasive breast tumors.

		Oracle			
		0/1+	*2+*	*3+*	*Total*
HercepTest	*0/1+*	142	1	0	143
	2+	6	11	1	18
	3+	0	5	34	39
Total		148	17	35	200

Table 2 shows the CISH results on all 13 discrepancies between HercepTest and Oracle. Of the six 2+ to 1+ discrepancies, two were high level amplified by CISH, 2 were low level amplified, and two tumors did not show HER2 amplification by CISH. The single case that was 1+ by HercepTest and 2+ by Oracle was low level

amplified by CISH. Of the 6 cases that were 2+ by HercepTest and 1+ by Oracle, 2 were not amplified, 2 low level amplified, and 2 high level amplified by CISH. The single case that was 2+ by HercepTest and 3+ by Oracle was high level amplified by CISH. Of the 5 cases that were 3+ by HercepTest and 2+ by Oracle, 1 was low level amplified and 4 high level amplified by CISH. Thereby, in the 7/13 discrepant cases that were high level HER2 amplified by CISH, HercepTest better reflected gene amplification status than Oracle in 6/7 cases, but this was not significant (p=0.143). All the 7 HER2 amplified discrepant cases were at least 2+ by HercepTest, in contrast with 5/7 that were at least 2+ for Oracle. Figure 2 shows two tumors with discrepant HercepTest and Oracle scores.

Table 2. HER2 chromogenic *in situ* hybridization (CISH) amplification scores for 13 discrepancies between HercepTest and Oracle HER2 staining from an original group of 200 breast cancer cases.

Hercep test score	Oracle score	CISH score
1+	2+	LA
2+	1+	NA
2+	1+	NA
2+	1+	LA
2+	1+	LA
2+	1+	A
2+	1+	A
2+	3+	A
3+	2+	LA
3+	2+	A
3+	2+	A
3+	2+	A
3+	2+	A

NA=not amplified, LA=low level amplified, A=amplified

Discussion

This study aimed to validate the Oracle HER2 Bond IHC System as an alternative to HercepTest for determination of eligibility of breast cancer patients for trastuzumab therapy. The Oracle system is based on a monoclonal antibody and automated staining, and is thereby potentially less liable to analytical variability than the polyclonal antibody and manual staining based HercepTest.

Overall, HercepTest yielded a sharper membrane staining with less cytoplasmic and stromal background than Oracle. This is theoretically a slight disadvantage since membrane staining may be more difficult to assess, and may lead to a higher interobserver variability as described in other studies comparing CB11 and other anti-HER2 antibodies [20-22]. Nevertheless, the clinically relevant 3+ staining is still well recognizable, and 2+/3+ discrepancies were not caused by background staining in the present study. In practice, this theoretical disadvantage is therefore probably not a big problem. We found an excellent

overall agreement (94%) between HercepTest and Oracle with a kappa score of 0.85. All discrepancies were tested for HER2 gene amplification by CISH as gold standard. In the vast majority of these discrepancies, Oracle showed a weaker staining than HercepTest. Further, in the 7/13 discrepant cases that were high level HER2 amplified by CISH, HercepTest better reflected gene amplification status (by higher IHC score) than Oracle in 6/7 cases. All the 7 HER2 amplified discrepant cases were at least 2+ by HercepTest, in contrast with 5/7 that were at least 2+ for Oracle. The 2 amplified cases with only 1+ Oracle staining are clinically most relevant, as the 1+ score would not have triggered a second line amplification test in daily practice, and these patients would not have received Herceptin therapy from which they may have benefited. It therefore seems that the clinical sensitivity of the HercepTest may be slightly better than that of the Oracle system.
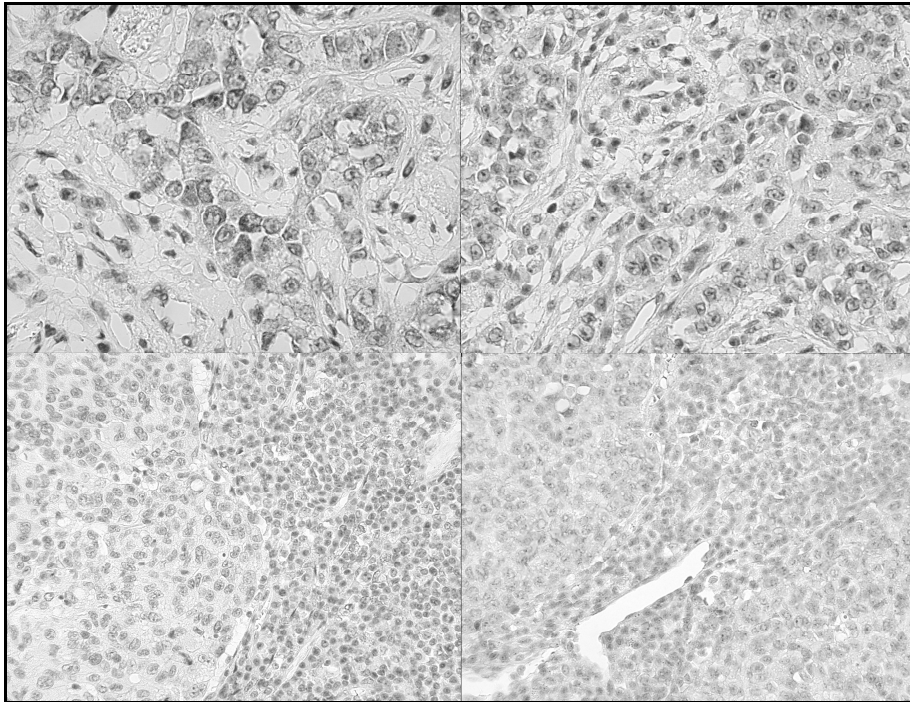
Figure 1. HER2 immunohistochemistry (IHC) by the Oracle HER2 Bond IHC detection system and HercepTest. <u>Top left</u>: Oracle shows cytoplasmic staining while <u>Top right</u>: HercepTest does not show cytoplasmic staining for the same tumor. <u>Bottom left</u>: Oracle shows aspecific staining of the surrounding tissue while <u>Bottom right:</u> HercepTest does not.

According to the current ASCO/CAP guidelines [23], initial test validation requires 25-100 samples tested by an alternative validated method in the same laboratory or by a validated method in another laboratory. There should be proof of initial testing validation in which positive and negative HER2 categories are at least 95%

concordant with an alternative validated method or same validated method for HER2. In our study, we validated the Oracle HER2 Bond IHC system with the FDA approved HercepTest in 200 breast tumors and found a 94% concordance. Only 6/148 (4%) of Oracle 0/1+ and 1/35 (3%) of Oracle 3+ scored tumors were discordant with HercepTest, thus, according to the ASCO/CAP guidelines, the Oracle HER2 Bond IHC system should be a suitable alternative for detection of HER2 overexpression in breast tumors. Other recommendations of the ASCO/CAP guidelines are exclusion criteria. Certain pre-analytical factors should result in the rejection of the specimen for IHC evaluation of HER2 status such as fixation longer than 48 h, tissues fixed in fixatives other than neutral-buffered formalin and the presence of severe edge or crush artifacts in core needle biopsies. Given this last recommendation we decided to exclude biopsies from this validation study. Further validation of the Oracle system for core needle biopsies will be the subject of future research.
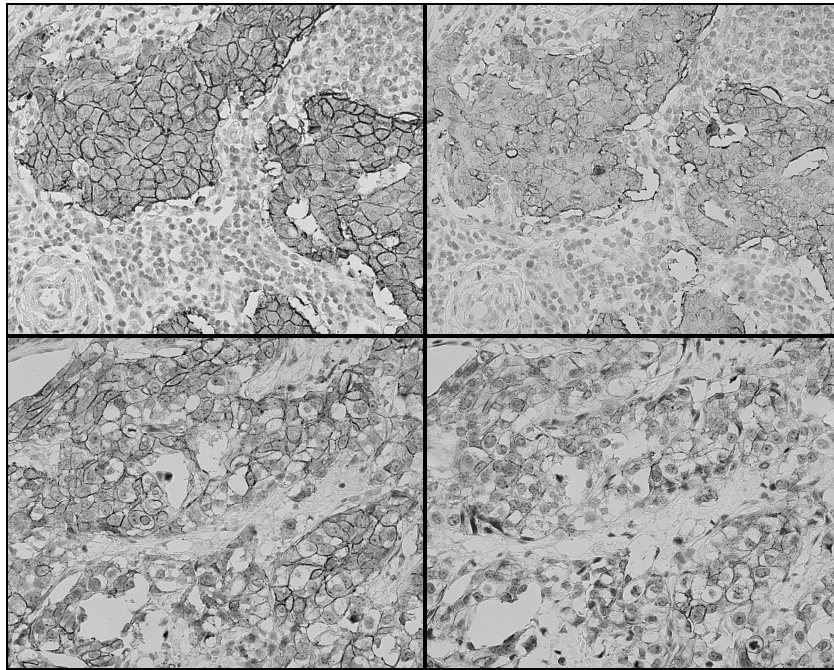
Figure 2. HER2 immunohistochemistry (IHC) discrepancies between Hercep test and the Oracle HER2 Bond IHC detection system. Top left: tumor with Hercep test 3+ score, Top right: same tumor with Oracle HER2 Bond IHC system 2+ score (strong membrane staining but not in 30% of cells); Bottom left: tumor with Hercep test 2+ score, Bottom right: same tumor with Oracle HER2 IHC system 1+ score (relatively strong membrane staining but not complete in > 10% of cells).

IHC assays are appealing for a number of practical perspectives including the lower cost, lower turn-around time and the adaptability to most pathology

laboratories. However, immunohistochemistry is a multi-step diagnostic process that requires specialized training in all aspects of the procedure including the selection of the appropriate reagents and tissue, fixation, processing and interpretation of the staining results. It is thus of utmost importance that all steps of the process are properly standardized. The development of fully automated systems can aid in the standardization of the IHC staining process, thereby potentially producing more consistent results. In conclusion, fully-automated HER2 staining with the monoclonal CB11 antibody in the Oracle kit shows a high level of agreement with manual staining by the polyclonal antibody in the HercepTest. Although Oracle shows in general some more cytoplasmic staining and may be slightly less sensitive in picking up HER2 amplified cases, it may be considered as an alternative method to evaluate the HER2 expression in breast cancer with potentially less analytical variability.

Acknowledgements

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Chapter 4

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.

Materials and 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%) IHC3+ 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.

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 185kD transmembrane protein that is involved in signal transduction [1, 2]. In about 20-30% of breast carcinomas HER2 is amplified and the expression of its receptor protein is increased [3-5]. Such patients respond well to treatment with trastuzumab, a recombinant humanized monoclonal anti-HER2 antibody [6 7]. 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 HER2 has also been shown to correlate with poor prognosis [8] and with resistance to conventional adjuvant chemotherapy and tamoxifen [9-13]. With the recognition of its prognostic, predictive and therapeutic implications, assessment of HER2 status has now become of major importance in clinical practice for breast cancer patients. At present, the most common method to assess HER2 status is immuno-histochemistry (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 [14-16], leading to both false negative and positive IHC results. In addition, there is some evidence that testing for HER2 gene amplification provides better predictive information than IHC [17-20]. 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, HER2 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, 22]. 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 x100 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 HER2 FISH in 2000 by Tanner et al. [23], 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

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in fluorescence microscopy and is less expensive than FISH. In several studies, HER2 CISH was demonstrated to be well correlated with FISH and IHC [23-28]. 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 HER2 amplification is multiplex ligation-dependent probe amplification (MLPA) [29]. 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 [30]. The aim of the present study was to compare MLPA as a new method to assess HER2 gene amplification in comparison with FISH and CISH data as gold standard in a large group of breast cancer patients.

Materials and Methods

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 [31]. All tissue samples were analyzed for IHC to assess HER2 protein expression and MLPA to determine HER2 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 [32]. 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 one pathologist (PvD).

Immunohistochemistry (IHC)

IHC was performed using the Hercep 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 but equivocal (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 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.

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 square cm tumor tissue) with a scalpel. DNA was isolated from these tissue fragments by 1 hr incubation in proteinase K (10 mg/ml; Roche, Almere, 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 3 probes for the HER2 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). HER2 gene copy number was determined by calculating the mean ratio of all three HER2 probe peaks in duplicate (6 values). If this mean value was below 1.5 the test was scored HER2 normal. A value 1.5-2.0 was scored as HER2 low level amplified, and values >2.0 as HER2 amplified. The 2.0 threshold was used in accordance with previous HER2 MLPA studies [30, 33], while the 1.5 threshold was empirically established during routine diagnostic application of MLPA kits for trisomy detection.

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 HER2 (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 2x 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 HER2 amplified by FISH. The FISH signals were visualized by using a fluorescence microscope.

Enumeration was done following the manufacturers' guidelines. HER2 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 HER2/CEP17 ratio >2.2 was considered HER2 gene amplified. All ratios < 1.8 were scored as HER2 non-amplified. Ratios between 1.8 and 2.2 were considered low-level amplified. Chromosome 17 polysomy was defined as >3 CEP17 signals. Borderline FISH/CISH was confirmed by counting additional cells. When FISH/CISH remained equivocal after recounting, FISH/CISH was repeated.

Chromogenic In Situ Hybridization (CISH)

All CISH assays were run using the Zymed SPoT-Light HER2 CISH (Zymed, South San Francisco, CA) kit according the manufacturers' instructions. CISH was performed on 4μm thick whole paraffin sections and/or on tissue microarray sections. First, sections were baked overnight at 56ºC 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. After 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 ºC) for 5 min and hybridization was carried out overnight at 37 ºC. After hybridization, appropriate stringency washes at 80 ºC 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, HER2 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 HER2 amplified by CISH.

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 a gold standard.

Results

<u>Table 1</u> shows the comparison between HER2 IHC and HER2 gene amplification by MLPA. About 53% of all patients tested negative for IHC, 30% was scored IHC 1+, 7% IHC 2+ (equivocal) and 10% IHC 3+ (strongly positive). HER2 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 1. Comparison of HER-2/neu protein overexpression by immuno-histochemistry (IHC) with gene amplification by multiplex ligation-dependent probe amplification (MLPA) in 518 invasive breast cancer patients.

		IHC				*Total*
		0	*1+*	*2+*	*3+*	
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

<u>Table 2</u> compares HER2 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.

<u>Table 3</u> 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%.

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

		IHC				
		0	1+	2+	3+	Total
Biopsy	**MLPA**					
	Normal	34	39	9	2	84 (86%)
	Low level	0	1	1	1	3 (3%)
	Amplified	0	0	1	10	11 (11%)
		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

To determine a tumor load cut-off from which MLPA results are reliable to detect amplification, we compared results from every tumor load group with IHC and FISH/CISH. When the tumor percentage was higher than 30%, the correlation between IHC and MLPA was best with 31/33 (94%) IHC 3+ cases showing amplification by MLPA. Both discrepant cases were low level amplified by MLPA. One of the discrepant cases that were MLPA low level amplified, was amplified by FISH/CISH and one was not.

Table 3. MLPA HER2 test results for 423 cases divided into groups according to tumor percentage.

	MLPA			Total
Tumor %	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

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%). HER2 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 (2%) 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%).

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
IHC	Normal	Low level	Amplified	Normal	Low level	Amplified	
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

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 HER2 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. For this calculation, low and high levels of amplification were taken together, as were 2+ and 3+ IHC scores.

Table 5. Concordance percentages between different techniques to detect HER2 overexpression and gene amplification as well as correlation by Spearman's rho. All correlations shown are significant.

	Agreement	Correlation
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
# smaller series of selected discrepant cases leading to lower agreement		

Table 6. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for IHC by HercepTest and MLPA as determined by analysis of 321 invasive breast cancer patients, considering CISH as gold standard.

	Sensitivity	Specificity	PPV	NPV
IHC	85%	92%	67%	92%
MLPA	90%	97%	84%	98%

Discussion

The aim of this study was to compare MLPA as a new method to assess HER2 gene amplification with FISH and CISH as gold standard in a large group of breast cancer patients. Gene amplification analysis by FISH and CISH was highly comparable, and MLPA correlated well with CISH. MLPA, FISH and CISH all detected amplification among cases without HER-2/neu overexpression, and all three techniques could not confirm amplification in a fraction of HER-2/*neu* over-expressors.

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 [3, 5, 9, 34] although several other studies have reported lower (10-18%) percentages [24, 35-38] as well. It is likely that many of the series in which higher HER2 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 [33]. 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 to 6 copies of HER2). MLPA also correlated well with IHC as in a previous smaller study [30].

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 [15, 16, 32, 35, 39, 40]. 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%) IHC3+ 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 IHC3+ cases that are generally considered to be eligible for HER2 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 IHC2+ cases. There are as yet only few data to indicate that amplified but not overexpressed cases respond to HER2 directed therapy [17, 41], but nevertheless one can wonder whether MLPA would be suitable as a pre-screening tool alternative to IHC. Indeed, MLPA is fast, easy, cheap, and more quantitative than IHC allowing more straight-forward interpretation. Furthermore, in the same analysis several genes that are important in therapy selection and/or prognosis like TopoIIα can be tested for 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, and perform careful microdissection before MLPA or use

CISH/FISH as an alternative. An important issue is how to deal with MLPA low level amplified 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.
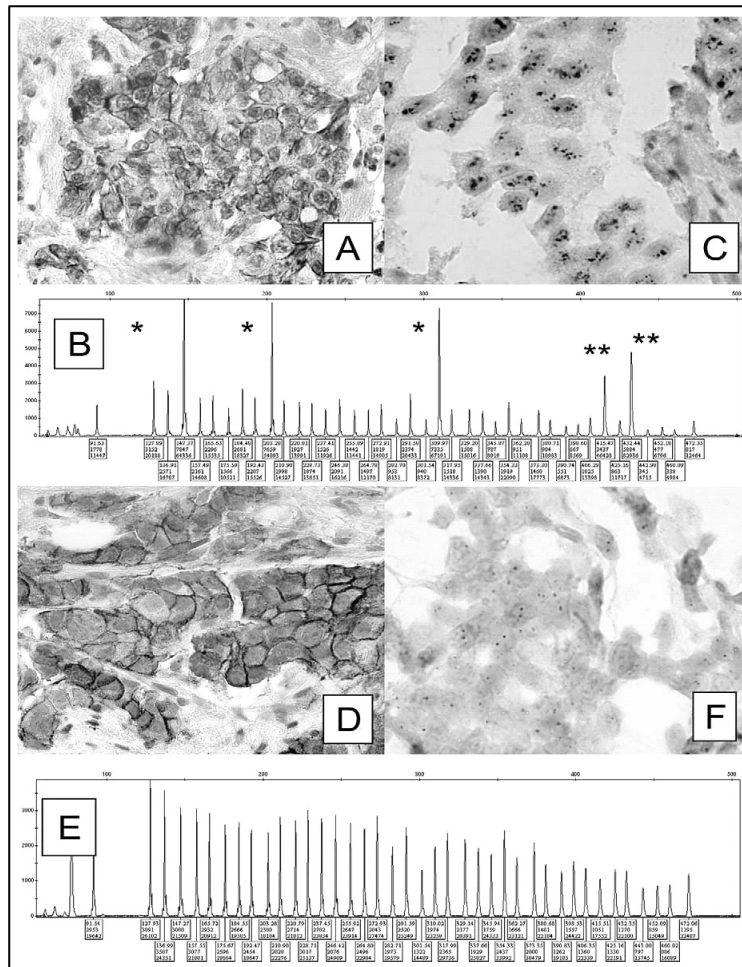
Figure 1. MLPA, CISH and IHC to detect HER2 amplification in invasive breast cancer. A) HER2 IHC 1+ case with in B) amplification by MLPA (see 3 HER2 probe peaks (*) way above the controls and two other chromosome 17 peaks (**) co-amplified) as confirmed by CISH in C). D) HER2 IHC 3+ case with in E) no amplification by MLPA (none of the 3 HER2 probe peaks above the controls) or CISH in F).

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 HER2. In cases that

receive neoadjuvant therapy [42], or where the primary tumor will not be resected but ablated [43], marker studies completely rely on the core biopsies. Studies comparing the HER2 status in needle biopsies and surgical resections have reported an overall concordance of 91-100% using IHC alone [44-49]. However some studies have also suggested that the validity of IHC score 3+ in core biopsies is limited [48], 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 [23-28]. 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 100x 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 [50]. 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 [50], and these low level amplified cases probably do not respond as well to HER2 directed therapy as high level amplified cases [41].

Concordance between ISH and IHC was high as expected [40, 51]. 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 [18, 52] 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 [53], 90% respectively 95% of IHC 0 and IHC 1+ tumors should show no HER2 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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Addendum Chapter 4

Multiplex ligation-dependent probe amplification to detect HER2 amplification in breast cancer: new insights in optimal cut-off value

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Dear Sir,

In issue 31 of Cellular Oncology, 2009, we published an article titled "HER2-*neu* amplification in breast cancer by multiplex ligation-dependent probe amplification in comparison with immunohistochemistry and *in situ* hybridization" [1]. To analyze our multiplex ligation-dependent probe amplification (MLPA) data we used a cut-off value of 1.5 to discriminate between HER2 non-amplified and low-level amplified patients. This cut-off was at that time empirically established in our lab during routine diagnostic application of MLPA kits for trisomy detection. However, based on currently published data [2-4], we now believe that a cut-off value of 1.3 (delta value 0.3) instead of 1.5 is better validated and more closely reflects the amplification status. We therefore re-analyzed our data with 1.3 as a cut-off value.

HER2 amplification status by MLPA was normal in 82% of cases, low level amplified in 7% and high level amplified, as before, in 11% of cases. Of all immunohistochemistry (IHC) negative cases, 95% were MLPA normal, and in the group of IHC 1+ cases, 88% were MLPA normal. In these IHC 0 and 1+ cases, 4% and 10% were MLPA low level amplified, respectively. In the IHC 3+ group there was no change in the percentage of MLPA normal and low-level amplified cases. In the IHC 2+ group discrepancies with MLPA were, as expected, most pronounced: 59% was not amplified, 22% low level amplified and 19% amplified. Overall, there was 87.5% agreement between both techniques, which is slightly lower than with the former 1.5 cut-off value (90%).
Correlation of MLPA with fluorescence *in situ* hybridization (FISH, selected cases) and chromogenic *in situ* hybridization (CISH, consecutive cases) was 73% and 91% respectively, with corresponding Spearman correlation coefficients of 0.78 and 0.83. None of the MLPA normal cases was amplified by FISH and 1/248 by CISH. MLPA low level amplified cases were high level amplified by FISH and CISH in 29% and 12% of cases, and low level amplified in 12% and 27% of cases, respectively.

With the new cut-off value (1.3 in stead of 1.5), using CISH as gold standard and considering CISH and MLPA low level amplified tumors as amplified, sensitivity of MLPA increased from 90% to 98%, specificity dropped from 97% to 92%, positive predictive value (PPV) dropped from 84% to 70% and negative predictive value (NPV) increased from 98% to 99.6%. When CISH and MLPA low level amplified tumors were considered not amplified, sensitivity, specificity, PPV and NPV remained 90%, 99%, 90% and 99% respectively. So, using the new cut-off value increases sensitivity and NPV of MLPA but decreases the specificity and PPV, given that all low level amplifications detected by MLPA are considered amplified or, even better, are reanalysed by CISH or FISH.

In conclusion, MLPA is a reliable and cheap high throughput method to detect breast cancer HER2 amplification in small quantities of DNA isolated from paraffin embedded material, and thereby a good alternative for FISH or CISH. Lowering the cut-off value for low level amplification to 1.3 increases the sensitivity of MLPA to detect HER2 amplification in breast cancer, indicating that this new experimentally validated cut-off value indeed improves the value of MLPA as a diagnostic test.

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Chapter 5

HER-2/*neu* amplification testing in breast cancer by
Multiplex Ligation-dependent Probe Amplification:
influence of manual- and laser microdissection

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Abstract

Background

Accurate assessment of HER-2/*neu* status is crucial for proper prognostic information and to offer direct appropriate treatment for breast cancer patients. Next to immunohistochemistry (IHC) to evaluate HER2 protein overexpression, a second line gene amplification test is generally deemed necessary for cases with equivocal protein expression. Recently, a new PCR based test, called Multiplex Ligation-dependent Probe Amplification (MLPA), was introduced as a simple and quick method to assess HER-2/*neu* gene amplification status in invasive breast cancer. MLPA was previously shown to correlate well with IHC and *in situ* hybridization (ISH), but a low tumor percentage in the tissue tested could negatively affect the accuracy of MLPA results.

Materials and methods

To examine this, MLPA was repeated in 42 patients after serial H&E section guided manual dissection with a scalpel and after laser microdissection of the tumor.

Results

Both dissection techniques led to higher HER2 gene copy number ratios and thereby made MLPA more quantitative. Concordance between MLPA and ISH improved from 61% to 84% after manual microdissection and to 90% after laser microdissection.

Conclusion

Manual and laser microdissection similarly increase the dynamic range of MLPA copy number ratios which is a technical advantage. As clinically a dichotomization between normal and amplified suffices and MLPA is relatively unsensitive to tumor content, microdissection before MLPA may not be routinely necessary but may be advisable in case of very low tumor content (≤30%), when MLPA results are equivocal, or when extensive ductal carcinoma *in situ* is present. Since differences between manual and laser microdissection were small, less time consuming manual microdissection appears to be sufficient.

Introduction

HER-2/*neu* is a proto-oncogene located on chromosome 17q21 encoding a 185 kD transmembrane protein that is involved in signal transduction [1,2]. HER2 belongs to the human epidermal growth factor receptor (EGFR) family and is amplified in about 10–20% of breast carcinomas causing an increased expression of its protein [3-5]. Patients having this overexpression respond well to treatment with trastuzumab, a recombinant humanized monoclonal anti-HER2 antibody [6,7]. 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 HER2 has also been shown to correlate with poor prognosis [8] and with resistance to conventional adjuvant chemotherapy and tamoxifen [9-13]. With the recognition of its prognostic, predictive and therapeutic implications, assessment of HER2 status has now become of major importance in clinical practice for breast cancer patients. At present, HER2 status is most commonly assessed by immunohistochemistry (IHC) and/or gene amplification tests such as fluorescence *in situ* hybridization (FISH) [14- 16] or chromogenic *in situ* hybridization (CISH) [17]. However, these techniques can only be assessed semiquantitatively, and amplification detection by easier quantitative PCR techniques has therefore been proposed as an alternative. One of the newly introduced techniques for detection of HER2 amplification is multiplex ligationdependent probe amplification (MLPA)[18]. In MLPA reactions, mixes composed of up to 45 probes can be used which makes it easy to quantitatively assess the copy number of different genes simultaneously, allowing for multiple target probes and controls. Moreover, this technique requires only minute quantities of short DNA fragments, which makes it very suitable for DNA isolated from paraffin embedded material. In previous studies using whole tissue sections we obtained very promising results with MLPA in comparison with IHC [19], FISH and CISH [20]. However, the dynamic range of MLPA copy number ratios was lower than with FISH. Furthermore, although results showed that amplification could even be detected in cases with a tumor percentage lower than 10%, the sensitivity of MLPA in these cases will depend on the degree of amplification, so lower levels of amplifications can be missed in case of a low tumor percentage. Laser-based tissue microdissection can potentially solve this issue [21]. However, it is relatively time consuming and therefore not very attractive as a routine test, so the question is whether faster H&E guided manual microdissection with a scalpel ("mesodissection") would suffice.

The aim of this study was therefore to determine to which extent manual and laser microdissection improve the dynamic range of copy number ratios and the sensitivity for amplification detection of HER2 by MLPA.

Materials and methods

Patient material

Resection specimens were chosen from a previously used series of 423 consecutive invasive breast cancer patients collected between November 2004 and June 2006 at the Department of Pathology of the University Medical Centre in Utrecht. This study using left over material was approved by the Tissue Science Committee of the UMC Utrecht. All tissue samples had already been analyzed by MLPA and IHC and a smaller fraction by *in situ* hybridization (ISH) for HER2 amplification status [20]. From this series, thirty one samples with low tumor content (< 60%) and/or discrepant results between MLPA and IHC/ISH were selected to study whether concordance with ISH (as gold standard) would improve after microdissection. In addition, 11 MLPA-amplified cases were selected to examine whether the dynamic range of HER2 gene copy number ratios increases after microdissection. Tumor percentages were between 10 and 90%. Although MLPA was shown to work well on biopsies in our previous study [20], we selected for this study only resection specimens to be sure to have sufficient material after recutting paraffin blocks.

Microdissection

Microdissection was performed on 4 μm thick paraffin sections. For manual microdissection, the relevant area was scraped off with a scalpel by comparing with a serial H&E stained slide where tumor tissue was marked and presence of ductal carcinoma *in situ* (DCIS) was noted. For laser microdissection, sections were baked at 56°C for 1 hour, deparaffinized in xylene for 10 minutes and rehydrated through graded alcohols (100%, 85% and 70% for 1 minute each). After staining with haematoxylin for 5 seconds, slides were rinsed in water and dipped in eosin for 5 seconds. Finally, slides were dehydrated in 100% ethanol for 1 minute and air dried. At this point PALM Liquid Cover Glass (LiquidCoverglass, PALM AG, Bernried, Germany) was applied by aerosol to improve morphology and to allow larger tissue areas to be laser pressure-catapulted [22], and sections were air dried for at least 30 minutes. A microdissection system with UV laser (PALM Microlaser Technologies AG, Bernried, Germany) was used to separate between 3 and 40 square mm of invasive tumor groups from their surrounding tissue. Subsequently, these groups were catapulted by laser pressure catapulting into a cap of a common microfuge tube moistened with a drop of mineral oil.

Multiplex ligation-dependent Probe Amplification(MLPA)

DNA from dissected tumor was isolated by 1 hour incubation in proteinase K (10 mg/ml; Roche, Almere, Netherlands) at 56°C followed by boiling for 10 minutes. 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 3 probes for the HER2 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 http://www.mrc-holland.com. All tests were performed in duplicate using an ABI 9700 PCR machine. PCR products were analyzed on an ABI310 capillary sequencer (Applied Biosystems, Foster City, CA, USA). HER2 gene copy number was determined by calculating the mean ratio of all three HER2 probe peaks in duplicate (6 values). If this value was below 1.5 (cut-off value) the test was scored non-amplified, values 1.5–2.0 were scored as a low level amplification, and values > 2.0 as HER2 amplified.

Statistical Analysis

Comparison between copy number ratios before and after microdissection were analyzed by a paired-samples t-test after testing for normal distribution. Association between difference in copy number ratio before and after microdissection and tumor percentage was tested by subtracting ratios and plotting them against tumor percentage, followed by linear regression analysis. All tests were done with SPSS software, regarding two-sided p-values < 0.05 as significant.

Results

Figure 1 shows the MLPA copy number ratios for 11 MLPA amplified cases before and after microdissection. Manual microdissection led to an increase in measured HER2 gene copy number (p = 0.001), with in most cases a further increase after laser microdissection (p = 0.007 vs nondissected MLPA), with no significant difference between manual and laser microdissection (p = 0.055). In two cases the presence of DCIS may have caused the laser microdissection value to be lower than the manual microdissection value. Figure 2 shows that there was no association between copy number ratios before and after microdissection and tumor percentage. Table 1 shows the amplification status of 42 breast cancer patients without and after manual and laser microdissection, in comparison with *in situ* hybridization (ISH) results. Tables 2, 3 and 4 show the concordance between ISH and MLPA without, after manual- and laser microdissection, respectively, with concordance percentages of 61%, 84% and 90%, respectively.

For 11/17 patients (65%) that showed discrepancies between MLPA (low or high level) amplified and IHC/ISH, manual or laser microdissection was able to adjust the original MLPA score (based on the whole slide). For 8/11 of these cases (73%), there was no obvious difference between laser microdissection and manual microdissection. However, in 3/11 cases (27%) only laser microdissection was able to change the MLPA outcome. Figure 3 shows that for all but one (11/12) MLPA non-amplified (9 of them IHC equivocal) cases, the MLPA score was unchanged after manual- and laser microdissection. For this case (tumor percentage 70%) the

MLPA score became low level amplified after laser microdissection. 12/31 (39%) samples contained DCIS. In 4/12 (33%) of these cases this could have contributed to biased MLPA results, which was circumvented by manual and/or laser microdissection.
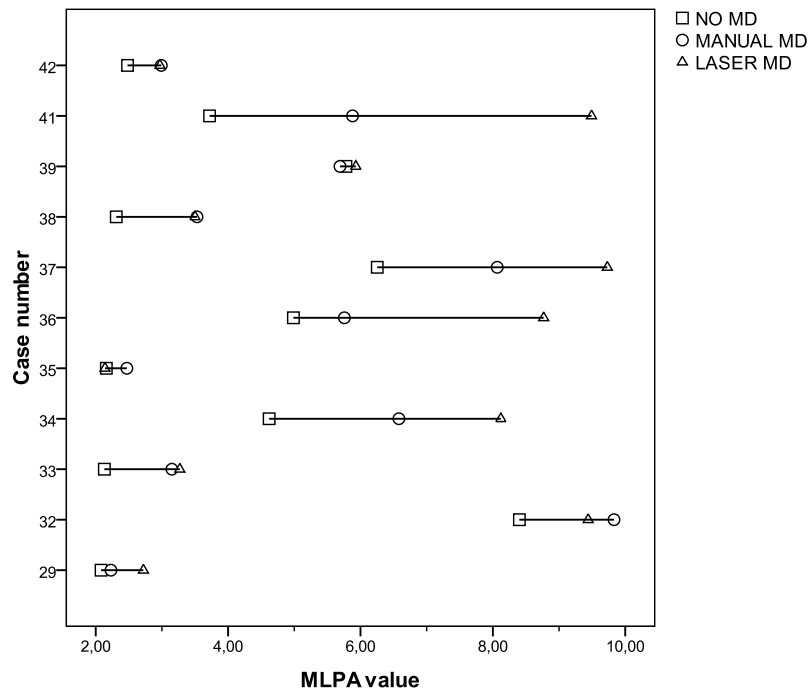
Figure 1. MLPA values (copy number ratios) for 11 HER2 amplified breast cancer cases before (no MD) and after manual and laser microdissection (MD).

As this paper is in part focussed on discrepant results we also evaluated concordance between the three HER-2/*neu* probes included in the MLPA kit in individual cases, as well as the variation of the control probes between the duplicate assessments.

There was full (amplification status) concordance for the three probes in 71% of cases. In the other 29% of cases, the discordant probe ratio value was close but just across the cut-off values. In 62% and 38% of the cases with a discordant probe this concerned a discrepancy between non-amplified/low level amplified and low level/high level amplification, respectively. The third probe (size 310 nt) accounted for most discordance (48% of discrepancies), possibly due to its lower ratio values compared to the other probes.

Table 1. HER-2/*neu* amplification status by multiplex ligation-dependent probe amplification (MLPA) of 42 breast cancer patients in undissected sections and after manual and laser microdissection, in comparison with *in situ* hybridization and immunohistochemistry.

			MLPA				
Case	IHC	ISH	Undissected	Manual MD	Laser MD	DCIS	Tumor%
1	0	NA	1	1	1	Yes	30
2	0	NA	1	1	1		30
3	0	NA	1	1	1	Yes	10
4	2	NA	1	1	1		10
5	2	NA	1	1	1	Yes	70
6	2	NA	1	1	2	Yes	70
7	2	NA	1	1	1		50
8	2	NA	1	1	1		70
9	2	NA	1	1	1		70
10	2	NA	1	1	1		60
11	2	NA	1	1	1		30
12	2	NA	1	1	1		60
13	1	LA	2	2	2		70
14	2	A	2	3	3		80
15	2	A	2	2	3	Yes	80
16	3	A	2	2	3		80
17	2	LA	2	2	3		80
18	0	NA	2	1	1	Yes	90
19	0	NA	2	1	1		80
20	1	A	2	3	3		70
21	1	LA	2	3	2	Yes	50
22	1	NA	2	2	1	Yes	80
23	1	NA	2	1	1	Yes	10
24	1	NA	2	1	1	Yes	60
25	0	NA	3	1	1		60
26	0	NA	3	1	1		80
27	1	A	3	3	3		70
28	1	NA	3	3	3	Yes	30
29	3	A	3	3	3		50
30	1	A	3	3	3		80
31	2	A	3	3	3	Yes	70
32	3	A	3	3	3	Yes	80
33	3	A	3	3	3		20
34	3	A	3	3	3		70
35	3	A	3	3	3		80
36	3	A	3	3	3		70
37	3	A	3	3	3		30
38	3	A	3	3	3		70
39	3	A	3	3	3		60
40	2	A	3	3	3	Yes	40
41	3	A	3	3	3	Yes	20
42	3	A	3	3	3		10

IHC = immunohistochemistry (Hercep test), ISH = *in situ* hybridization, NA = non-amplified, LA = low-level amplified, A= amplified, MD = microdissection, DCIS = ductal carcinoma *in situ*

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Between duplicate measurements, the three probes performed similarly with discrepancies of 9.5%, 11% and 12%, respectively. As to the 25 reference probes in the P004-A1 MLPA kit (data from the whole original group of unselected patients), amplifications were found in 0.6–10.8% of the patients. High level amplifications of the control probes were very rare, varying between 0 and 2%. Low level amplifications were however common for some probes (range 0.6–9.1%) with 5/25 probes (HIPK3, STCH, CCNB1, PTPN1 and IER3) showing low level amplification in more than 5% of patients.

Table 2. Concordance between HER2 ISH and MLPA without microdissection in 31 invasive breast cancer cases.
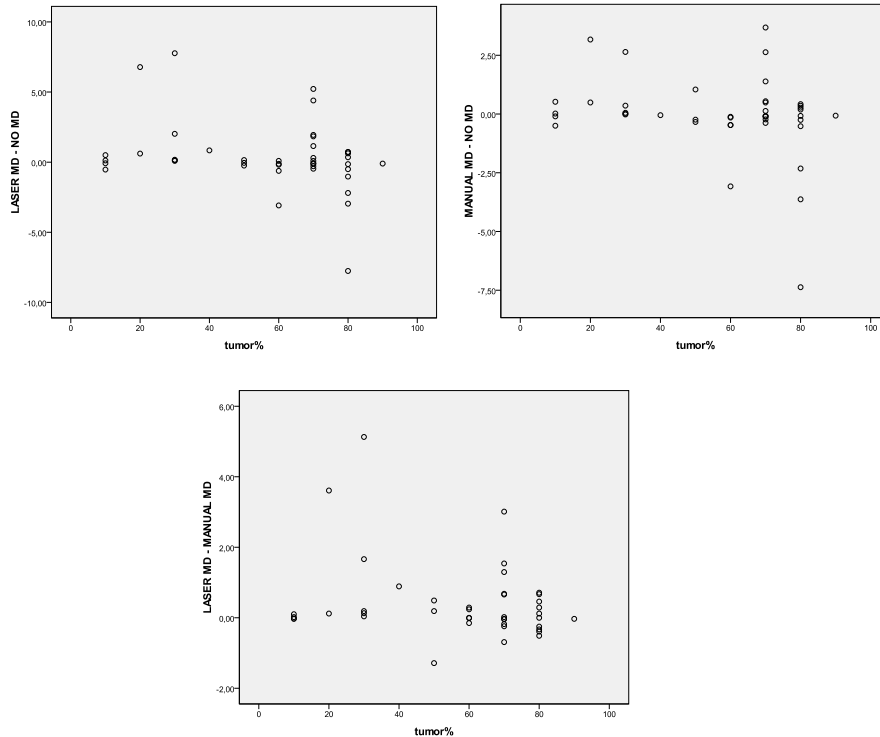
		MLPA			
		Not amplified	Low level amplified	Amplified	Total
ISH	Not amplified	12	5	3	**20**
	Low level amplified	0	3	0	**3**
	Amplified	0	4	4	**8**
	Total	**12**	**12**	**7**	**31**

Table 3. Concordance between HER2 ISH and MLPA after manual micro-dissection in 31 invasive breast cancer cases.

		MLPA			
		Not amplified	Low level amplified	Amplified	Total
ISH	Not amplified	18	1	1	**20**
	Low level amplified	0	2	1	**3**
	Amplified	0	2	6	**8**
	Total	**18**	**5**	**8**	**31**

Table 4. Concordance between HER2 ISH and MLPA after manual micro-dissection in 31 invasive breast cancer cases.

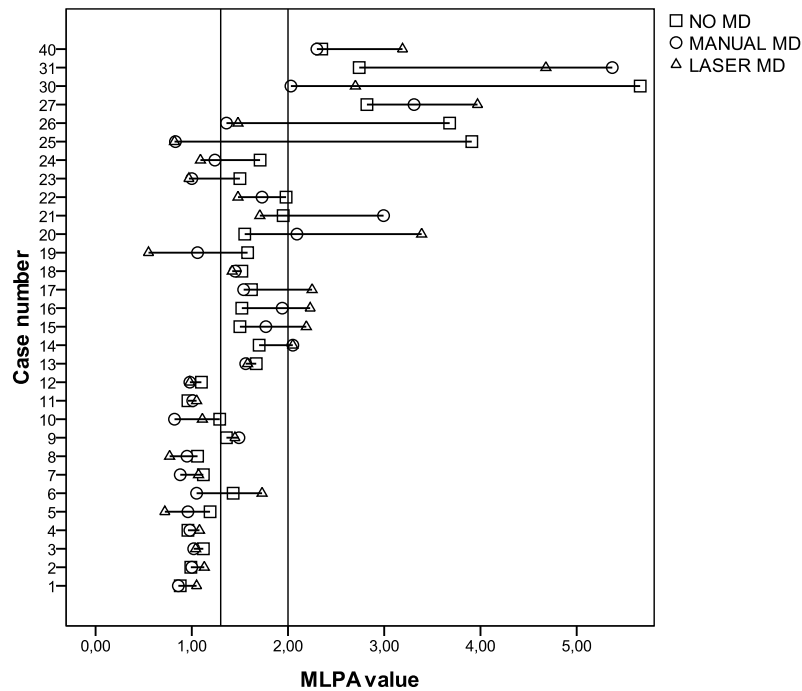|		MLPA			
		Not amplified	Low level amplified	Amplified	Total
ISH	Not amplified	18	1	1	**20**
	Low level amplified	0	2	1	**3**
	Amplified	0	0	8	**8**
	Total	**18**	**3**	**10**	**31**

Figure 2. Scatter plots showing no association between the MLPA ratio difference before (no MD) and after manual- or laser microdissection (MD) on the one hand and tumor percentage of the sample on the other.

Discussion

The goal of our study was to examine the effect of manual and laser microdissection on HER2 MLPA copy number ratios of 42 breast cancer samples with low tumor percentage and/or discrepancies between MLPA on the one hand and IHC and/or ISH on the other. As we wanted to simulate daily practice, we applied a crude method to obtain DNA and did not isolate DNA with more refined methods. We have as yet no indication that a more precise DNA isolation improves HER2 amplification detection by the MLPA technique. Copy number ratios increased after manual microdissection and even more after laser microdissection, indicating that the dynamic range of MLPA increases when the test sample is enriched for tumor cells, making this technique more quantitative. Nevertheless, the highest ratio observed was about 10, which is less than generally observed with FISH.

The fact that higher ratios are not observed even after maximal enrichment for tumor cells is probably inherent to the MLPA technique. However, since the copy

number ratio, once amplified, does not further contribute to clinical decision making this is not at all a problem in daily practice.

Figure 3. MLPA values (copy number ratios) for 30 patients before (no MD) and after manual and laser microdissection (MD). The vertical lines show the cut-off values (1.50 and 2.00) between an MLPA non-amplified, low-level amplified, and amplified outcome.

We showed in Figure 2 that there was no association between copy number ratios before and after microdissection and tumor percentage. This can be explained by the presence of background signal from non-tumorous cells (infiltrate/benign breast) and DCIS in non-dissected samples. DCIS can cause a false higher copy number ratio that becomes lower after performing microdissection to exclude the DCIS. Correlation between MLPA and ISH (as gold standard) improved after manual or laser microdissection, indicating that enrichment for tumor cells increases reliability of MLPA. However, manual or laser microdissection had only an effect on MLPA score in 1/12 non-amplified patients. In this study and in our previous study amplification was detected by MLPA even in cases with a tumor percentage below 10% [20], indicating that MLPA is relatively insensitive to tumor percentage (although sensitivity of MLPA will likely depend on the degree of amplification in case of low tumor percentage) and that

routine microdissection may not be required in daily practice. However, also some undissected samples with high tumor % and without DCIS showed amplification by MLPA that could not be confirmed by ISH. Although we cannot exclude that tumor heterogeneity plays a role here, MLPA may occasionally provide false positive results. This concerned however only 3/423 cases, so the rate of false positivity may only be in the range of 1%. Nevertheless, patients with especially a low level amplification MLPA result seem to benefit most from microdissection. Manual microdissection seems to suffice in most cases as differences between manual and laser microdissection results were small. The amplification and overexpression of HER2 is seen more frequently in DCIS (50–60%) [23] than in invasive ductal carcinoma of the breast (10–20%) and the presence of DCIS can thereby bias MLPA results. In our study the presence of DCIS probably contributed to biased MLPA outcome as circumvented by manual and/or laser microdissection in 33% (4/12) of (selected) cases. Therefore, if MLPA results are equivocal or extensive DCIS is present, we advise to perform careful manual (or laser) microdissection before MLPA or to use CISH/FISH as an alternative.

When comparing performance of the three HER2 probes, the third probe (peak 21) showed most frequently a discordance with the other two probes, possibly due to its lower ratio values. In duplicate assessments, however, all probes performed about equally well, indicating that reproducibility is good. Some of the 25 reference probes that were originally chosen for their supposed lack of amplification in breast cancer were nevertheless amplified. Therefore, at least the five probes (HIPK3, STCH, CCNB1, PTPN1 and IER3) that were (low level) amplified in more than 5% of cases may need to be replaced.

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. Amplification can be detected even in cases with very low tumor percentages. Manual or laser microdissection of breast cancer slides before HER2 MLPA may hence not be routinely necessary. However, the dynamic range of the technique improves after manual and laser microdissection and may therefore at least be advisable in case of very low tumour content (≤30%), when the MLPA outcome is equivocal or when extensive DCIS is present. Since differences between manual and laser microdissection were small, less time consuming manual microdissection seems to suffice then.

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Addendum Chapter 5

Influence of microdissection on multiplex ligation-
dependent probe amplification to detect HER2
amplification in breast cancer: new insights in optimal cut-
off value

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Dear Sir,

In issue 9 of BMC Cancer, 2009, we published an article titled "HER-2/*neu* amplification testing in breast cancer by Multiplex Ligation-dependent Probe Amplification: influence of manual- and laser microdissection" [1]. To analyze our multiplex ligation-dependent probe amplification (MLPA) data we used a cut-off value of 1.5 to discriminate between HER2 non-amplified and low-level amplified patients. This cut-off was at that time empirically established in our lab during routine diagnostic application of MLPA kits for trisomy detection. However, based on currently published data [2-4], we now believe that a cut-off value of 1.3 (delta value 0.3) instead of 1.5 is better validated and more closely reflects amplification status. We therefore re-analyzed our data with 1.3 as cut-off value.

MLPA was previously shown to correlate well with immunohistochemistry (IHC) and *in situ* hybridization (ISH) [5], but a low tumor percentage in the tissue tested could negatively affect the accuracy of MLPA results. To examine this, MLPA was repeated in 31 patients after serial H&E section guided manual dissection with a scalpel and after laser microdissection of the tumor. The concordance percentages between ISH and MLPA without, after manual- and after laser microdissection were 58%, 74% and 77%, respectively (see Tables 1, 2 and 3). For 10/17 patients (59%) that showed discrepancies between MLPA (low or high level) amplified and IHC/ISH, manual or laser microdissection was able to adjust the original MLPA score (based on the whole slide). For 8/10 of these cases (80%), there was no obvious difference between laser microdissection and manual microdissection. However, in 2/10 cases (20%) only laser microdissection was able to change the MLPA outcome.

For all but one (10/11) MLPA non-amplified (9 of them IHC equivocal) cases, the MLPA score was unchanged after manual- and laser microdissection. For this case (tumor percentage 70%) the MLPA score became low level amplified after laser microdissection. 12/31 (39%) samples contained ductal carcinoma *in situ* (DCIS). In 2/12 (17%) of these cases this could have contributed to biased MLPA results, which was circumvented by manual and laser microdissection.

Table 1. Concordance between HER2 ISH and MLPA without microdissection in 31 invasive breast cancer cases.

		MLPA			
		Not amplified	Low level amplified	Amplified	Total
ISH	Not amplified	11	6	3	*20*
	Low level amplified	0	3	0	*3*
	Amplified	0	4	4	*8*
	Total	*11*	*13*	*7*	*31*

Table 2. Concordance between HER2 ISH and MLPA after manual microdissection in 31 invasive breast cancer cases.

		MLPA			
		Not amplified	Low level amplified	Amplified	Total
ISH	Not amplified	15	4	1	**20**
	Low level amplified	0	2	1	**3**
	Amplified	0	2	6	**8**
	Total	**15**	**8**	**8**	**31**

Table 3. Concordance between HER2 ISH and MLPA after laser microdissection in 31 invasive breast cancer cases.

		MLPA			
		Not amplified	Low level amplified	Amplified	Total
ISH	Not amplified	14	5	1	**20**
	Low level amplified	0	2	1	**3**
	Amplified	0	0	8	**8**
	Total	**14**	**7**	**10**	**31**

In conclusion, with the revised cut-off value of 1.3 in stead of 1.5, the influence of manual or laser microdissection on MLPA results decreased slightly. As clinically a dichotomization between normal and amplified suffices and MLPA is relatively unsensitive to tumor content, microdissection before MLPA is not routinely necessary but may be advisable in case of very low tumor content (≤30%), when MLPA results are equivocal, or when extensive DCIS is present. Since differences between manual and laser microdissection were small, less time consuming manual microdissection appears to be sufficient.

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Chapter 6

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

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Abstract

Background

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, that 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.

Materials and methods

To this end, multiplex ligation-dependent probe amplification results were compared with *TopoIIα*, *HER2* and CEP17 chromogenic *in situ* hybridization. We also assessed *TopoIIα* protein expression by immunohistochemistry.

Results

Of 353 patients, 9% showed *TopoIIα* amplification by multiplex ligation-dependent probe amplification 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 multiplex ligation-dependent probe amplification and chromogenic *in situ* hybridization 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.

Conclusion

Multiplex ligation-dependent probe amplification 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 chromogenic *in situ* hybridization.

Introduction

The topoisomerase IIα (*TopoIIα, TOP2A*) gene is located at chromosome 17q21.2 and encodes a 170 kD protein that plays 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-KD transmembrane glycoprotein and overexpression of the protein is associated with poor prognostic factors as a consequence of increased cell proliferation, angiogenesis and invasive growth, and resistance to apoptosis. The *HER2* gene is amplified and overexpressed in 10-30% of breast cancers in which it plays 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. Since 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 the *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 [25]. Since the applied *HER2* kit also contains a *TopoIIa* 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 chromogenic *in situ* hybridization in a subgroup of these patients. In addition, we investigated the correlation between *TopoIIa* protein expression levels and gene amplification status on tissue micro arrays, using immunohistochemistry and chromogenic *in situ* hybridization, 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 *TopoIIa* gene amplification status. For *TopoIIa* chromogenic *in situ* hybridization 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 (MLPA)

Invasive tumor areas were harvested from 4 μm thick paraffin sections by dissection with a scalpel (using at least 1 square cm tumor tissue) and DNA was isolated by 1 hr incubation in proteinase K (10 mg/ml; Roche, Almere, Netherlands) at 56ºC 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 3 probes for the *HER2* gene, a probe for *TopoIIa*, 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 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 *TopoIIa* 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 *TopoIIa* peak in duplicate (2 values) was calculated. If this mean value was below 0.7, *TopoIIa* or *HER2* was considered deleted, values between 0.7-1.5 were considered normal, values between 1.5-2.0 as low level amplified, and values >2.0 as *HER2* or *TopoIIa* amplified. The 2.0 threshold was used in

accordance with previous *HER2* MLPA studies [24, 27], while the 1.5 threshold was empirically established during routine diagnostic application of MLPA kits for trisomy detection.

Chromogenic in situ hybridization (CISH)

HER2 and *TopoIIa* chromogenic *in situ* hybridization assays were performed on 4 µm thick paraffin serial tissue array sections using the SPoT-Light *HER2* or *TopoIIa* kits (Zymed, San Francisco, CA) according the manufacturers' instructions. First, sections were baked overnight at 56ºC and deparaffinized in xylene and alcohol 100%. For *HER2* and *TopoIIa*, 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. After 20 min of air drying, the digoxigenin-labeled *TopoIIa* or *HER2* probes were applied to the slides. Then, the sections were denatured on a hot plate (95 ºC) for 5 min and hybridization was carried out overnight at 37 ºC. After hybridization, appropriate stringency washes at 80 ºC 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 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 chromogenic *in situ* hybridization run and consisted of paraffin sections of a case known to be *TopoIIa*/*HER2* amplified by chromogenic *in situ* hybridization. At least 30 preferably non-overlapping nuclei in every tumor sample were scored by two blinded observers to determine the number of *HER2* and *TopoIIa* 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 5 individual small signals was scored as *HER2*/*TopoIIa* non-amplified.

Immunohistochemistry

Immunohistochemistry was performed using a mouse monoclonal antibody against the *TopoIIa* 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 56ºC, 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 room temperature. 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 chromogenic *in situ* hybridization 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 Chi square test. P-values below 0.05 were considered significant.

Results

Multiplex ligation-dependent probe amplification (MLPA)

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* and two of these deletions were accompanied by HER2 amplification (one high level and one low level).

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)	*Amplification*
HER2	10/353 (3%)	34/353 (10%)	44/353 (13%)
TopoIIα	25/353 (7%)	8/353 (2%)	33/353 (9%)

Chromogenic in situ hybridization (CISH)

Topolla and *HER2* chromogenic *in situ* hybridization were performed on 284 patients that were analyzed by MLPA (see Table 2). For *Topolla* 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 *Topolla* amplified by chromogenic *in situ* hybridization, and the other twenty cases were scored normal by chromogenic *in situ* hybridization. All MLPA highly amplified cases were confirmed by chromogenic *in situ* hybridization, although two of these cases only showed a low level amplification by chromogenic *in situ* hybridization. Of the non-amplified cases by MLPA, 249 (99%) were concordant with chromogenic *in situ* hybridization, while two non-amplified cases were scored low level amplified by chromogenic *in situ* hybridization.

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

Sensitivity, specificity, positive predictive value and negative predictive value of MLPA for *HER2* and *Topolla* were calculated and depicted in Table 4 using chromogenic *in situ* hybridization 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 chromogenic *in situ* hybridization (as gold standard) for *Topolla*. Nevertheless, increasing the cut-off value lead to a decrease in sensitivity of MLPA for both genes.

Table 2. Comparison between multiplex ligation-dependent probe amplification (MLPA) and chromogenic *in situ* hybridization (CISH) results for *Topolla* 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
Topolla 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

Immunohistochemistry

From tissue arrays containing cores of 315 patients, information for both immunohistochemistry and chromogenic *in situ* hybridization was obtained for 265 patients. 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 CISH (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 chromogenic *in situ* hybridization 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 chromogenic *in situ* hybridization (n=14, mean 28% immunohistochemistry positive) versus 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).

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*

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 chromogenic *in situ* hybridization 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 level 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. Further, methodological variation is an unlikely explanation as the fraction of HER-2/*neu* amplified cases by immunohistochemistry (10%, [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 chromogenic *in situ* hybridization. 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 like *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 unclear. Also, to which extent most of these co-amplified genes have an impact on response to *HER2* targeted treatment with trastuzumab is unknown at this time.

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 two of these deletions were 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 4 cases displayed low level amplification by chromogenic *in situ* hybridization, and 2 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 since 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 chromogenic *in situ* hybridization, 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 easy, but also cheap. Consumables costs are €11 per reaction compared to €70 per reaction for HER2 chromogenic *in situ* hybridization and €56 per reaction for Topollα chromogenic in situ hybridization.
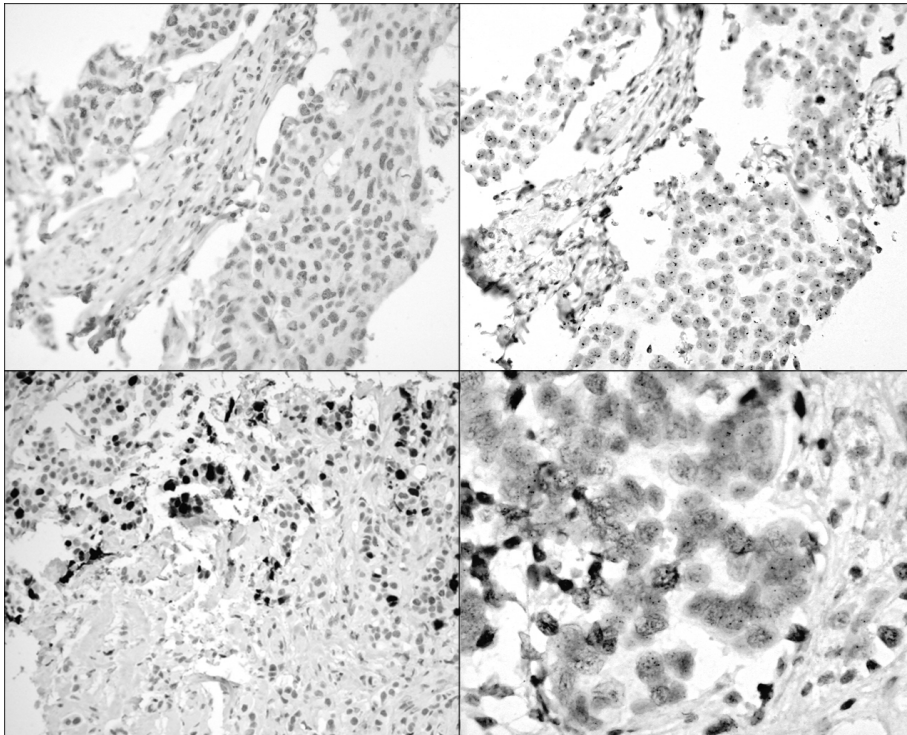
Figure 1. Correlation between gene amplification and protein expression in breast cancer as determined by chromogenic *in situ* hybridization and immuno-histochemistry. <u>Top left</u>: Almost no *Topollα* protein expression is present. <u>Top right</u>: Large chromogenic *in situ* hybridization clusters indicate *Topollα* gene amplification in the same patient. <u>Bottom left</u>: strong *Topollα* protein expression is present in 5% of tumor cells. <u>Bottom right</u>: chromogenic *in situ* hybridization shows less than 5 signals per cell indicating no *Topollα* gene amplification

Furthermore MLPA is more quantitative than immunohistochemistry allowing more straight-forward interpretation, and in the same analysis several genes that are important in therapy selection and/or prognosis like *Topollα* 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 chromogenic *in situ* hybridization for *Topollα* 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 *Topollα* in the current kit,

indicating that the kit would benefit from more *Topolla* probes. More MLPA probes for *Topolla* could make a more accurate estimate of whether a sample is low level or not amplified. Concordance between MLPA and chromogenic *in situ* hybridization for *HER2* and *Topolla* 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 = .03) from adjuvant trastuzumab [40].

Nevertheless we 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 for discrepancies could be a lack of sensitivity by chromogenic *in situ* hybridization for low level amplification.

Also, the non-morphological aspect of MLPA could play a role in some of the discrepancies. 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].

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

	Sensitivity	Specificity	PPV	NPV	Concordance
Topolla 1.5	86.7	92.6	39.4	99.2	91.2
Topolla 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

These data show that MLPA is suited to detect amplification (as well as deletion) of *HER2* and *Topolla* in breast cancer patients in one test. Both *HER2* and *Topolla* gene alternations have independently been associated with an increased responsiveness to anthracycline-containing chemotherapy regimens relative to non-anthracycline regimens [42, 43], indicating that measurements of alternations of both genes can guide in the selection of anthracyline-containing regimens.
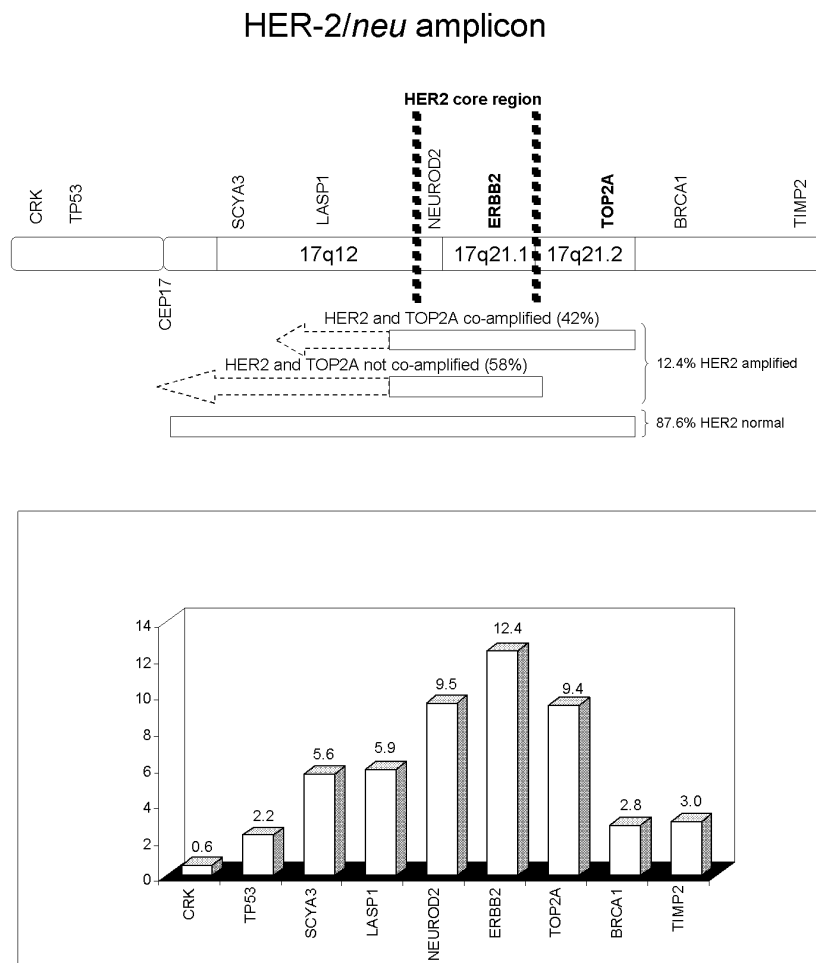
HER-2/*neu* amplicon

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-A1 kit are depicted above the chromosome and their (low plus high level) amplification frequencies (in %) are depicted in the chart below.

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].

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This is even more an advantage since recently the definition of chromosome 17 polysomy based on the CEP17 only is found most questionable [45].

In conclusion, MLPA is an easy and cheap method to simultaneously detect breast cancer polysomy 17, *HER-2/neu* and *TopoIIa* 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.

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Addendum Chapter 6

Simultaneous detection of TOP2A and HER2 gene amplification by multiplex ligation-dependent probe amplification in breast cancer: new insights in optimal cut-off value

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Dear Sir,

In issue 23 of Modern Pathology, 2009, we published an article titled "Simultaneous detection of *TOP2A* and *HER2* gene amplification by multiplex ligation-dependent probe amplification in breast cancer" [1]. To analyze our multiplex ligation-dependent probe amplification (MLPA) data we used a cut-off value of 1.5 to discriminate between *HER2* non-amplified and low-level amplified patients. This cut-off was at that time empirically established in our lab during routine diagnostic application of MLPA kits for trisomy detection. However, based on recently published data [2-4], we now believe that a cut-off value of 1.3 (delta value 0.3) instead of 1.5 is better validated and more closely reflects amplification status. We therefore re-analyzed our data with 1.3 as a cut-off value.

Table 1 shows that, with the new cut-off value, the *Topoɪɪɑ* gene was low level amplified in 22% of cases and highly amplified in 8 cases (2%), adding up to a total of 84/353 (24%) cases with amplification. *HER2* was low level amplified in 26/353 cases (7%) and highly amplified in 34/353 cases (10%), adding up to a total of 60/353 (17%) of amplified cases. Co-amplification with *Topoɪɪɑ* was seen in 45% of *HER2* amplified cases (including both low and high levels, 27/60).

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

Gene	Low level amplification (target/control ratio 1.3-2.0)	High level amplification (target/control ratio > 2.0)	*Total*
HER2	26/353 (7%)	34/353 (10%)	60/353 (17%)
Topoɪɪɑ	76/353 (22%)	8/353 (2%)	84/353 (24%)

Topoɪɪɑ MLPA was concordant with Topoɪɪɑ chromogenic *in situ* hybridization (CISH) in 232 out of 284 (82%) of these patients (see Table 2). Most discordance was found in cases scored as low level by MLPA. Only 7/56 of these cases were confirmed to be *Topoɪɪɑ* amplified by CISH, and the other 49 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. All non-amplified cases by MLPA were concordant with chromogenic *in situ* hybridization.

For HER2, 265/284 (93%) cases were concordant between MLPA and chromogenic *in situ* hybridization. Concordance was highest in MLPA amplified (27/28) and non-amplified (233/234) cases, while 14/22 MLPA low level cases were scored normal by CISH.

Table 2. Comparison between multiplex ligation-dependent probe amplification (MLPA) and chromogenic *in situ* hybridization (CISH) results for *Topollα* and *HER2* on 284 breast cancer patients when a cut-off value of 1.3 between normal and low level amplified was applied.

	MLPA (cut-off = 1.3)			
	Not amplified	Low level amplified	Amplified	Total
Topollα CISH				
Not amplified	220	49	0	269
Low level amplified	0	6	2	8
Amplified	0	1	6	7
HER2 CISH				
Not amplified	233	14	1	248
Low level amplified	1	7	2	10
Amplified	0	1	25	26

When the cut-off was set at 1.3, the number of low level amplified patients increased significantly, thereby decreasing the concordance between MLPA and CISH (as gold standard) for Topollα. Nevertheless, decreasing the cut-off value lead to an increase in sensitivity (from 87% and 89% to 100% and 97% for *Topollα* and *HER2*, respectively) of MLPA to detect amplification for both genes, at the cost of specificity (from 93% and 98% to 82% and 94% for Topollα and HER2 respectively) and the positive predictive value of the technique (see Table 3).

Table 3. Sensitivity, specificity, positive-predictive value (PPV), negative- predictive value (NPV) and concordance of MLPA with chromogenic in situ hybridization for Topollα and HER2 using cut-off values of 1.3, 1.5 and 1.8 to discriminate between no amplification and low level amplification status.

	Sensitivity	Specificity	PPV	NPV	Concordance
Topollα 1.3	100	81.8	23.4	100	81.7
Topollα 1.5	86.7	92.6	39.4	99.2	91.2
Topollα 1.8	73.3	99.6	91.7	98.5	97.5
HER2 1.3	97.2	94.0	70.0	99.6	93.3
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

Topoisomerase IIα overexpression was significantly associated with Topollα amplification by MLPA (p=0.044) although 22/50 (44%) of amplified tumors did not overexpress the Topollα protein.

In conclusion, MLPA is a reliable and cheap high throughput method to simultaneously detect breast cancer *HER2* and *Topollα* amplification in small

quantities of DNA isolated from paraffin embedded material. Lowering the cut-off value for low level amplification to 1.3 increases the sensitivity of MLPA to detect *HER2* and *TopoIIa* amplification in breast cancer at the cost of the specificity.

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Chapter 7

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

Background

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.

Materials and methods

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).

Results

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.

Conclusion

In conclusion, 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 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.

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 immuno-histochemistry, 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 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 could be 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 the neutral buffered formaldehyde fixed tissue blocks. IHC membrane staining was semiquantitatively scored as negative (0), weakly positive (1+), positive but equivocal (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 2 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 approximately 1 cm2 tumor tissue) with a scalpel. DNA was isolated from these tissue fragments by 1 hr

incubation in proteinase K (10 mg/ml; Roche, Almere, The Netherlands) at 56ºC 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 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 0.7-1.3 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].

Table 1. Genes on chromosome 17 targeted by probes in the P004-B1 MLPA kit.

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

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 4μm 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 3 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 over 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 Figure 1. There were 4 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 3 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).
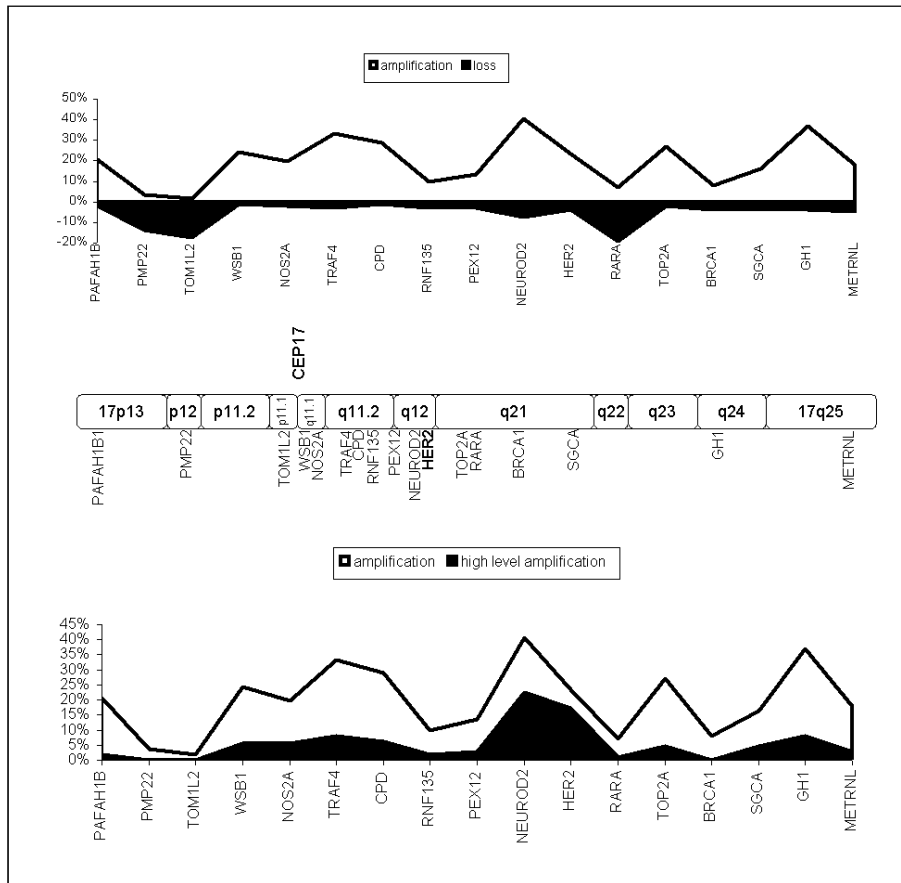
Figure 1. Chromosome 17 copy number aberrations by MLPA in 111 breast cancer patients. <u>Top</u>: percentage of patients showing amplifications (ratio > 1.3, white) and losses (ratio < 0.7, black) for all the chromosome 17 probes of the MLPA kit. <u>Bottom</u>: percentage of patients showing amplifications (> 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 analysis in 104 invasive breast cancer patients (concordance 92.3%).

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

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 3 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 3 17p probes. This patient had a normal 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 and 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. Fifty five % (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.

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

		CEP17 CISH		**WSB1 MLPA**	
		≤ 2 copies	> 2 copies	Ratio ≤ 1.3	Ratio > 1.3
HER2 status	Normal	74	8	74	8
	Amplified	10	12	14	8

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 (Figure 2) showed that only HER2 and NEUROD2 were frequently clustered together which is consistent with other studies [21]. WSB1, NOS2A and TRAF4 seemed to form a second small gene cluster independent of the HER2/NEUROD2 cluster.
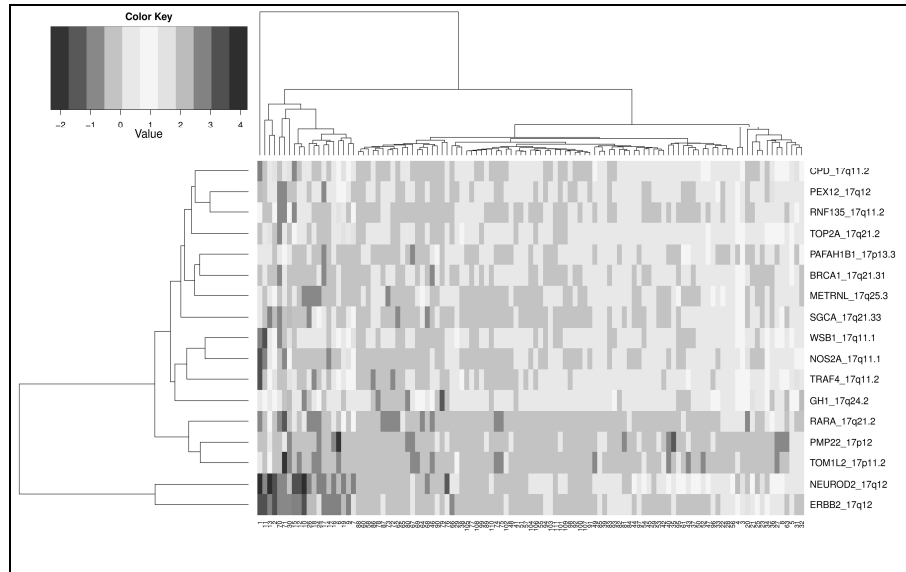
Figure 2. Hierarchical cluster analysis of 111 invasive breast cancer patients analyzed for copy number status of 17 chromosome 17 genes by MLPA

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 Figure 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 as compared to the other chromosome 17 amplicons.

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This study identified 3 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 a 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 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 [24]. 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, that 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 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.

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Supplementary Table 1. Copy number aberrations for 17 genes along chromosome 17 by MLPA analysis in 111 invasive breast cancer patients. From left to right, all analyzed chromosome 17 genes are depicted in the order in which they reside on chromosome 17 (p → q). The centromere region is indicated with a vertical line. Amplifications (>1.3) are depicted in light gray, and losses (<0.7) in dark gray.

Patient	PAFAH1B1 17p13.3	PMP22 17p12	TOM1L2 17p11.2	WSB1 17q11.1	NOS2A 17q11.1	TRAF4 17q11.2	CPD 17q11.2	RNF135 17q11.2	PEX12 17q11.2	NEUROD2 17q12	ERBB2 17q12	RARA 17q12	TOP2A 17q21.2	BRCA1 17q21.2	SGCA 17q21.31	GH1 17q24.2	METRNL 17q25.3

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Chapter 8

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

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Abstract

Background

Several oncogenes and tumor suppressor genes have been shown to be implicated in the development, progression and response to therapy of invasive breast cancer. The phenotypic uniqueness (and thus the heterogeneity of clinical behavior) among patients' tumors may be traceable to the underlying variation in gene copy number of these genes.

Materials and methods

In order to obtain a more complete view of gene copy number changes and their relation to phenotype we analyzed 20 breast cancer related genes in 104 invasive breast cancers with the use of multiplex ligation-dependent probe amplification (MLPA).

Results

We identified *MYC* gene amplification in 48% of patients, *PRDM14* in 34%, *TOP2A* in 32%, *ADAM9* in 32%, *HER2* in 28%, *CCND1* in 26%, *EMSY* in 25%, *IKBKB* in 21%, *AURKA* in 17%, *FGFR1* in 17%, *ESR1* in 16%, *CCNE1* in 12% and *EGFR* in 9% of patients. There was a significant correlation between the number of amplified genes and the histological grade and mitotic index of the tumor. Gene amplifications of *EGFR*, *CCNE1* and *HER2* were negatively associated with estrogen receptor (ER) status while *FGFR1*, *ADAM9*, *IKBKB* and *TOP2A* revealed a positive association. Amplifications of *ESR1*, *PRDM14*, *MYC* and *HER2* were associated with a high mitotic index, and *PRDM14* and *HER2* amplifications with high histological grade. *MYC* amplification was detected more frequently in ductal tumors and high level *MYC* amplifications were significantly associated with large tumor size. *HER2/MYC*, *HER2/CCNE1* and *EGFR/MYC* co-amplified tumors were significantly larger than tumors with either of these amplifications. Gene loss occurred most frequently in *CDH1* (20%) and *FGFR1* (10%).

Conclusion

Multiplex ligation-dependent probe amplification analysis with this "breast cancer kit" allowed to simultaneously assess copy numbers of 20 important breast cancer genes, providing an overview of the most frequent (co)amplifications as well as interesting phenotypic correlations, and thereby data on the potential importance of these genes in breast cancer.

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Introduction

Several genes have been shown to be involved in the development, progression and response to therapy of invasive breast cancer. Among these, HER-2/neu is likely the most important proto-oncogene. Amplification of the HER2 gene is present in about 15-30% of breast carcinomas and leads to protein overexpression, which correlates with a poor outcome [1-3] and is associated with a good response to treatment with trastuzumab, a recombinant humanized monoclonal anti-HER2 antibody [4, 5]. Furthermore, amplification of HER2 has also been shown to correlate with resistance to conventional adjuvant chemotherapy and tamoxifen [6-10]. Topoisomerase IIα (TOP2A) gene amplification seems to be predictive of response to a class of cytostatic agents called TopoII inhibitors, that include the anthracyclines [11-16]. Recently, estrogen receptor alpha (ESR1) gene amplification has been implicated in response to tamoxifen therapy [17], but its significance was doubted by others [18]. Amplification of MYC has been associated with poor prognosis and resistance to anti-estrogen therapy [19]. Therapeutic or prognostic significance of other frequently amplified genes such as cyclin D1 (CCND1) [20] or frequent loss of genes such as E-Cadherin (CDH1) is less clear, and comparative genomic hybridization studies have pointed to many more genes and chromosomal loci with potentially important copy number changes [21-23].

Nevertheless, no single gene copy number seems to completely explain prognosis or response to therapy of individual breast cancer patients. A simultaneous analysis of copy number changes of a variety of genes involved in prognosis and therapy response may thus be very useful for molecular profiling of individual breast cancer patients. This can be achieved by array comparative genomic hybridization (CGH) but this is still a costly and labor intensive technique that requires a relatively large amount of sample DNA and specialized personnel to deal with the complexity of the data. In the present study we used an easier and faster high-throughput PCR based technique, called multiplex ligation-dependent probe amplification (MLPA) [24]. This assay determines relative gene copy numbers in a quantitative way and requires only minute quantities of small DNA fragments, which makes it very suitable for DNA isolated from paraffin embedded material. In previous studies we obtained promising results with HER2 MLPA in comparison with immunohistochemistry [25] and in situ hybridization [26] and evaluated this technique to simultaneously determine copy number changes of HER2 [27] and TOP2A [16]. The goal of the present study was to apply MLPA as a technique to simultaneously detect amplifications and/or losses of a large set of breast cancer related genes. These genes (including HER2, EGFR, TOP2A, MYC, CCND1, CCNE1, ESR1, AURKA, EMSY, CDH1, FGFR1, PRDM14, ADAM9, IKBKB) were selected based on their prognostic and/or therapeutic implications in breast cancer, or their proven frequent copy number change by comparative genomic hybridization. We sought to obtain a more complete view of the clinical significance of MLPA-detected gene copy number alternations and

therefore investigated their mutual interactions as well as their associations with common prognostic factors such as age, histological type and grade, HER2 immunohistochemistry, estrogen and progesterone receptor (ER, PR) status, mitotic index [28] and tumor size.

Materials and Methods

Patient material

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

Immunohistochemistry

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

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 square cm tumor tissue) with a scalpel. We have estimated the tumor percentages of all samples prior to MLPA and used samples with tumor percentages of at least 70%.

Table 1. Contents of the P078-A1 MLPA kit (MRC Holland, the Netherlands). For each gene the chromosomal position, mapview position, the number of probes present in the MLPA kit, a description of the transcript protein and if possible a relevant (breast cancer) reference is given.

Gene	Chrom	Mapview	# Probes	Transcript description	Ref
ESR1	06q25	06-152.307247 06-152.423838 06-152.457215	3	Transcription factor	[17]
EGFR	07p11	07-055.191055 07-055.196767 07-055.233957	3	Receptor tyrosine kinase involved in signal transduction	[50]
FGFR1	08p12	08-038.391533 08-038.434092	2	Receptor tyrosine kinase involved in signal transduction	[37, 40]
ADAM9	08p11	08-038.998319	1	Metalloproteinase associated with protein metabolism	[37, 40]
IKBKB	08p11	08-042.292902 08-042.302676	2	Serine/threonine kinase associated with signal transduction	[37]
PRDM14	08q13	08-071.130073	1	Transcription regulatory protein	[43]
MYC	08q24	08-128.821796 08-128.822001 08-128.822151	3	Transcription factor involved in apoptosis and cell proliferation	[51]
CCND1	11q13	11-069.165399 11-069.167779 11-069.175089	3	Cell cycle control protein involved in signal transduction	[47]
EMSY	11q13	11-075.902087 11-075.926543	2	Transcription regulatory protein	[47]
CDH1	16q22	16-067.328716 16-067.404826 16-067.419579	3	Adhesion molecule associated with signal transduction	[52]
NOS2A	17q11	17-023.114082	1	Enzyme with oxidoreductase activity involved in metabolism	-
TRAF4	17q11	17-024.098403	1	Adaptor molecule involved in signal transduction, cell proliferation and apoptosis	[53]
CPD	17q11	17-025.795018	1	Carboxypeptidase involved in protein metabolism	-
LASP1	17q12	17-034-308187	1	Cytoskeletal associated protein involved in signal transduction	[54]
PPARBP	17q12	17-034.840858	1	Transcription regulatory protein involved in signal transduction	[55]
HER2	17q12	17-035-118101 17-035-122165 17-035.127183	5	Receptor tyrosine kinase associated with signal transduction	[26]
CDC6	17q21	17-035.699283	1	Cell cycle control protein involved in signal transduction	[56]
TOP2A	17q21	17-035.812698 17-035.816651 17-035.818297	3	DNA topoisomerase protein involved in regulation of the topological status of DNA	[14]
CCNE1	19q12	19-034.999920 19-035.000150 19-035.005214	3	Cell cycle control protein involved in signal transduction	[46, 57]
AURKA	20q13	20-054.389980	1	Serine/threonine kinase involved in signal transduction	[58, 59]

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In a previous study [26] we showed that tumor percentages higher than 30% are already sufficient for reliable MLPA performance and that more than half of the tumors show a tumor percentage >60%. DNA was isolated from these tissue fragments by 1 hr incubation in proteinase K (10 mg/ml; Roche, Almere, The Netherlands) at 56ºC followed by boiling for 10 min. This DNA solution (50-100 µl) was, after centrifugation, used in the MLPA analysis according the manufacturers' instructions, using the P078-A1 kit (MRC Holland, Amsterdam, The Netherlands). The contents of this kit are depicted in Table 1. All tests were performed in duplicate using an ABI 9700 PCR machine (Applied Biosystems, Foster City, CA, USA). PCR products were analyzed on an ABI310 capillary sequencer (Applied Biosystems). Gene copy numbers were analyzed using Genescan (Applied Biosystems) and Coffalyser (version 7.0) software (MRC-Holland). For genes with more than one probe present in the kit, the mean of all the probe peak ratios 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 0.7-1.3 was defined as normal, 1.3-2.0 as low level amplification, and values >2.0 as high level amplified, as previously established [31, 32].

Statistics

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

Results

Amplifications and losses

Frequencies of gains and losses for the 20 analyzed genes in 104 invasive breast cancers are depicted in figure 1 and table 2. All analyzed regions were involved in amplification with varying frequencies. Most amplifications (low and high level) were found on chromosome 8 (particularly MYC, PRDM14 and ADAM9 in 48%, 34% and 32% of the patients respectively) and on chromosome 17 (particularly TRAF4, CDC6, TOP2A and HER2 in 36%, 35%, 32% and 28% of the patients, respectively). Although MYC showed amplification in almost half the patients, only 16% of these amplifications were high level (ratio > 2.0). For HER2, in contrast, most amplifications (72%) were high level. In a previous study we already established a good correlation between HER2 gene amplification by MLPA and HER2 gene amplification by in situ hybridization [26]. Of 56/104 (54%) patients we had previously determined HER2 chromogenic in situ hybridization

data: of 11/21 MLPA amplified patients there were CISH data available and all 11 patients showed CISH amplification. Of 41/75 MLPA HER2 normal patients, CISH data were available and all 41 tumors were normal by CISH. Of 4/8 HER2 MLPA low level amplified patients, CISH data were available: 3/4 were normal and 1/4 was amplified by CISH. CCND1 amplification was found in 26% of the patients, and 56% of these amplifications were high level. ESR1 amplification was found in 16% of the patients although most were low level and only rarely high level (2% of all patients).

Several regions showed loss by MLPA. The two regions with the most frequent loss were CDH1 on chromosome 16 (20% of patients: 13/21 of ductal and 6/21 of lobular type) and FGFR1 on chromosome 8 (10% of the patients).
On average, this study found 5 amplifications per patient (range 0-17 of the 20 analyzed genes) of which 2 were high level amplifications (range 0-10). Only 5 patients (5%) did not show any amplification or loss for the analyzed regions. Five other patients showed no amplifications but did show loss of one or more genes. Of these 5 patients, there were three patients with only loss of CDH1 (2/3 were ductal carcinomas). One other patient with a lobular carcinoma showed a loss of CDH1 accompanied by IKBKB, CCND1 and LASP1 loss, and the fifth patient presented with a PRDM14 and FGFR1 loss. Ten patients showed amplifications of just one gene: PRDM14 (3/10), MYC (3/10), EMSY (2/10) and a high level amplification of AURKA and EGFR in one patient each.

Co-amplified regions, loss of regions and their association

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

Fifteen percent of all patients showed a co-amplification of HER2 and MYC, 13% of HER2 and TOP2A, 9% of HER2 and CCND1 and 7% of HER2 and CCNE1. Eighteen percent of all patients showed a co-amplification of MYC and TOP2A and 16% of MYC and CCND1. Figure 2 shows the percentage of co-amplifications of HER2, MYC, CCND1, CCNE1 and TOP2A amplified breast tumors. Of the 27 HER2 amplified breast cancers, 52% and 45% were MYC and TOP2A co-amplified, respectively. Of the 12 CCNE1 amplified patients, 10 were also MYC amplified (83%). When only high level amplifications (MLPA ratio >2.0) were considered relevant, 5/21 HER2 high level amplified patients were also CCND1 (high level) amplified, 4/21 TOP2A co-amplified, 1/21 MYC co-amplified and 1/21 CCNE1 co-amplified. Of the 15 CCND1 high level amplified patients, 5/15 were HER2 co-

amplified, 2/15 TOP2A co-amplified and 1/15 was MYC amplified. Patients with an amplification of EGFR had an increased likelihood to also have CCNE1 amplifications (p<0.001) and tumors with a TOP2A amplification had an increased probability of EMSY amplification (p=0.004). Furthermore, patients with high level HER2 amplifications had an increased probability to have high level TOP2A amplifications (p=0.017) and patients with high level EGFR amplification a higher risk of having high level MYC amplifications (p=0.023).
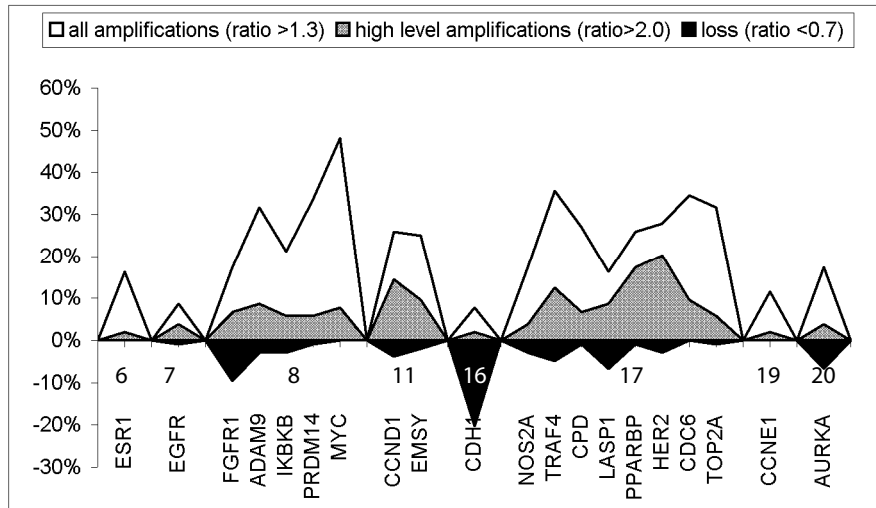
Figure 1. Amplifications (green) and losses (red) for 20 oncogenes and tumor suppressor genes as found by analysis of 104 invasive breast cancer patients with the P078-A1 breast cancer dedicated MLPA kit. The chromosome numbers of the genes are shown on the horizontal axis.

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

Association between amplified regions and clinical characteristics

There was a significant correlation between the number of amplifications per tumor and grade (p=0.030) and even more between the number of high level amplifications per tumor and grade (p<0.001). There was a significant association between the number of amplifications and HER2 immunohistochemistry status. Also, there was a significant correlation between the number of amplifications and high level amplifications per tumor and mitotic index (p=0.015 and p=0.004 respectively) but there was no association with tumor size, with hormone receptor status nor with the patient's age. We also found significantly more high

level amplifications per tumor for tumors of the ductal subtype than for tumors of the lobular subtype (p<0.001) but not for all amplifications (p=0.083). Although not significant, we did find more CDH1 loss in lobular tumors (36%) than in ductal tumors (17%).
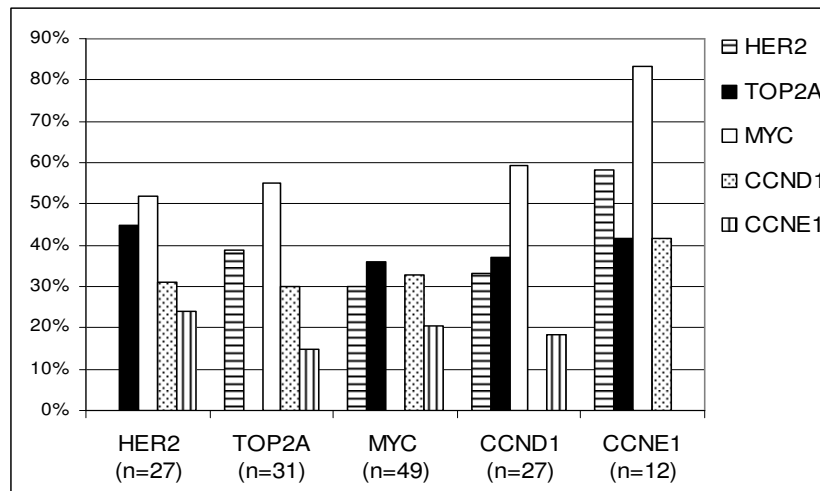
Figure 2. Co-amplifications of *HER2*, *TOP2A*, *MYC*, *CCND1* and *CCNE1* amplified breast tumors in a series of 104 invasive breast cancers analyzed by multiplex ligation-dependent probe amplification.

Table 3 shows the association of several amplified regions with clinico-pathological characteristics. There was, as expected, a significant association between HER2 immunohistochemistry and HER2 gene amplification (p<0.001), but there was also a significant association with PRDM14 gene amplification (p=0.027). ESR1 amplification was significantly associated with higher MAI (p=0.007, 14/16 MAI ≥13) and showed a trend towards association with higher grade (p=0.054). EGFR amplification was significantly associated with negative ER status (p=0.005) and showed a trend towards association with negative PR status (p=0.052). FGFR1 and ADAM9 amplifications were significantly associated with positive ER status (p=0.032 and p=0.019, respectively). IKBKB was significantly associated with positive ER and PR status (p=0.026 and 0.015, respectively). PRDM14 amplification was correlated with higher grade (p=0.049) and MAI (p=0.010). MYC amplification was significantly associated with higher MAI (p=0.040) and with the ductal subtype (p=0.011). High level MYC amplifications were significantly associated with a larger tumor size (p=0.045). HER2 amplification was associated with higher grade (p=0.040) and MAI (p=0.036) and showed a trend towards association with ER status (p=0.060, for high level amplifications p=0.004). TOP2A amplification was significantly associated with positive ER status of the tumor (p=0.045), in contrary to CCNE1 which was

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significantly associated with ER negativity (p=0.004). CCND1, EMSY and AURKA amplification did not show any significant associations with clinical-pathological features. We also did not find any association between amplified regions and age.

Table 2. Frequencies of amplification (ratio > 1.3), high level (HL) amplification (ratio > 2.0) and loss (ratio < 0.7) for all 20 genes analyzed by multiplex ligation-dependent probe amplification in 104 invasive breast cancer patients. The two last columns represent published amplification (or loss of *CDH1*) frequencies and corresponding references respectively.

Gene	Chr	All amps (%)	High level amps (%)	Loss (%)	Expected amps/loss in % (range)	Ref
ESR1	06q25	16	2	0	0-20.6	[17, 18]
EGFR	07p11	9	4	1	5-10 (7-65)	[19]
FGFR1	08p12	17	7	10	9	[35, 60]
ADAM9	08p11	32	9	3	-	-
IKBKB	08p11	21	6	3	-	-
PRDM14	08q13	34	6	1	-	-
MYC	08q24	48	8	0	9-15 (1-94)	[19, 57]
CCND1	11q13	26	14	4	15 (0-27)	[19, 57]
EMSY	11q13	25	10	2	7-13	[47, 48]
CDH1	16q22	8	2	20	50 LOH 16q	[52, 61]
NOS2A	17q11	17	4	3	-	-
TRAF4	17q11	36	13	5	-	-
CPD	17q11	27	7	1	-	-
LASP1	17q12	16	9	7	-	-
PPARBP	17q12	26	17	1	-	-
HER2	17q12	28	20	3	15-30	[1, 3]
CDC6	17q21	35	10	0	-	-
TOP2A	17q21	32	6	1	5-10	[62, 63]
CCNE1	19q12	12	2	0	3-6	[46, 57]
AURKA	20q13	17	4	7	14 (5-20)	[35]

Chr=chromosome position, amps=amplifications, Ref=reference

Tumors with HER2 and MYC co-amplification were significantly larger in size (p=0.030) than tumors with amplification of only one or neither of these genes, as were HER2-CCNE1 co-amplified tumors (p=0.017). Tumors with HER2-MYC co-amplification were also significantly associated with higher HER2 immuno-histochemistry status (p<0.001). There was also a trend towards an association between tumor size and HER2-TOP2A co-amplification (p=0.061). Tumors with EGFR-MYC co-amplification were significantly associated with ER negativity

(p=0.023), were significantly larger (p=0.017) and showed a trend towards higher MAI (p=0.059) than tumors with either or neither of these amplifications.
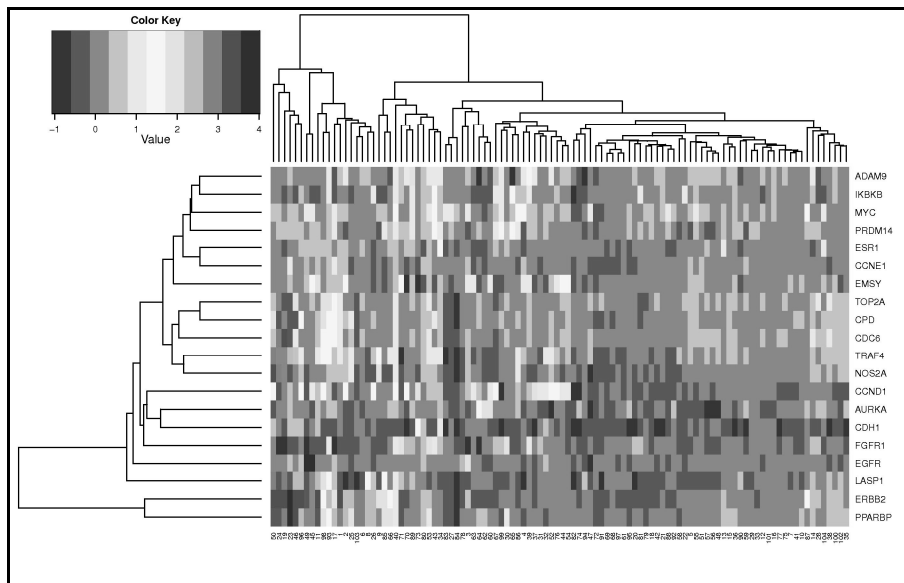
Figure 3. Hierarchical cluster analysis of 104 invasive breast cancer patients (horizontal axis) analyzed by multiplex ligation-dependent probe amplification for 20 breast cancer related genes (vertical axis).

Discussion

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

It has long been known that the more advanced a cancer is, the more rearranged the genome is. We were therefore interested in verifying whether there was an association between the number of genetic alterations observed in a tumor and worse clinical-pathological tumor characteristics. On average, this study found 5 amplifications of the 20 analyzed genes per patient of which 2 high level amplifications. Ten patients (10%) showed single amplifications (of which 60% involved MYC or PRDM14 amplifications), and interestingly these tumors were all grade 2 or 3 and were often highly proliferative with MAI>13.

Only 5 patients (5%) did not show any amplification or loss in the analyzed regions. In these 5 tumors the MAI was smaller than 5, and three of them were grade 1 while two were grade 2. Although grade 1 tumors generally displayed

fewer genomic events than grade 2/3 tumors, they also rarely showed more complex genomic patterns associated with more advanced tumors indicating that there is not a strict relation between genomic state and histological grade. Nevertheless, this study found a significant correlation between the number of (high level) amplifications and the histological grade and MAI. Presence of gene amplifications may not only be important because of the resulting overexpression of the oncogenes, it may also serve as a surrogate parameter for increased genetic instability of a cancer and, as such, represent an indicator of poor patient prognosis. Indeed, an association between patient survival and the number of amplifications was described by some studies [33, 34].

Table 3. Association of amplified regions with clinicopathological characteristics. For significant associations, the corresponding p-values following Chi-square statistics are depicted in the table. Trends are mentioned between brackets.

		ER	PR	Grade	Age	MAI	Tumor size	HER2 IHC	Type
	n	31/70	48/53	19/72	23/81	50/43	35/60	82/21	76/11
ESR1	16			(0.054)		0.007			
EGFR	9	0.005	(0.052)						
FGFR1	17	0.032							
ADAM9	32	0.019							
IKBKB	21	0.026	0.015						
PRDM14	34			0.049		0.010		0.027	
MYC	48					0.040			0.011
CCND1	26								
EMSY	25								
HER2	28	(0.060)		0.040		0.036		<0.001	
TOP2A	32	0.045							
CCNE1	12	0.004							
AURKA	17								

ER 0/1, PR 0/1, Grade 1/2-3, Age <50/≥50, MAI <13/≥13, Tumor size pT1/pT2-3,
HER2 IHC (immunohistochemistry) 0-1+/2+-3+, Type ductal/lobular

Amplifications and losses

Amplifications involving chromosomes 8p (FGFR1, ADAM9, IKBKB), 11q (CCND1, EMSY) and 17q (NOS2A, TRAF4, CPD, LASP1, PPARBP, HER2, CDC6, TOP2A) are among the most common high-level copy number aberrations in breast tumors, occurring, for example, in one study, in 22.8%, 19.6% and 9.9% of tumors, respectively [35]. Table 2 shows that the frequencies of amplification observed by MLPA for all analyzed genes in this study are in line with other studies. Most MYC amplifications observed in this study were low level (84%), which is consistent with published results [36]. High level amplifications of ESR1, a gene that is possibly involved in tamoxifen response [17], were rare although we did find 16% of patients with increased ESR1 copy numbers. In a study by Chin et al [37], low-

level copy number aberrations by array comparative genomic hybridization were not associated with reduced survival and they hypothesized that these aberrations are presumably selected during tumor development because they increase basal cell metabolism.

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

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

Association of genomic regions with clinicopathological parameters

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

HER2 and PRDM14 amplifications were associated with positive HER2 immunohistochemistry. ESR1, PRDM14, MYC and HER2 amplifications were associated with a higher MAI and PRDM14 and HER2 amplifications were also correlated with higher grade. For ESR1, we found a trend towards association with higher grade, which is in strong contrast to a previous study [17] that associated ESR1 amplification with low grade. For PRDM14, one study found no correlation between its expression levels and clinicopathological characteristics, which was assumed to reflect the small number of samples analyzed [43]. MYC amplification was more likely to be present in tumors of the ductal subtype

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compared to lobular ones, and high level MYC amplifications were significantly associated with a larger tumor size.

Co-amplified regions

Patients with more amplified loci had a significantly higher grade and MAI. As not only the number of amplified loci but also the function of the genes involved determine tumor characteristics, we selected pairs of frequently co-amplified genes and studied their relation to clinicopathological features. HER2-MYC co-amplification, for example, was present in 15% of the tumors which could indicate the existence of a selective advantage associated with their co-amplification. This hypothesis is supported by our findings showing that concomitant amplification of HER2 and MYC is associated with a significant larger tumor size and higher HER2 IHC status, and by other studies that found a relationship between HER2-MYC co-amplification and reduced survival [33, 34]. From preliminary analyses from the NSABP B-31 trial, it was suggested that tumors that are HER2-MYC co-amplified have a remarkably favorable prognosis with adjuvant trastuzumab treatment [44]. Although not as frequent (7%) as HER2-MYC co-amplification, the present study found that HER2-CCNE1 co-amplified tumors were significantly larger than tumors with either of these amplifications. CCNE1 protein overexpression has previously been associated with positive HER2 status and poor prognosis [45], but CCNE1 amplification on itself was shown to have no prognostic role in breast cancer so far [46]. Tumors with EGFR-MYC co-amplification (7%) were larger and showed a trend towards higher MAI than tumors with either or neither of these amplifications. Several other frequent co-amplifications in this study (e.g, 18% MYC-TOP2A, 16% MYC-CCND1, 12% TOP2A-EMSY, 9% HER2-CCND1) did not show any association with clinicopathological characteristics. These data imply that there is no relationship between the frequency of the co-amplification and the association with current prognostic markers and that the type of genes involved in the co-amplifications determines the association with prognostic factors.

Co-amplification of 8p12 (FGFR1) has been reported in 30-40% of tumors with CCND1 (11q13) amplification. In our study 33% (9/27) of CCND1 amplifications were concomitant with FGFR1 amplifications. Co-amplification of these genes is associated with significantly reduced survival [34], but in our study this co-amplification was not associated with any clinicopathological characteristics.
In this study 50% of EMSY amplifications were also CCND1 amplified which is less than the 70% described by another study [47]. CCND1 and EMSY amplifications have both been associated with poor overall survival [47, 48], but there is no straightforward association between CCND1 amplification and expression, and CCND1 expression has been associated with ER and good survival [49]. The mechanism for the frequent co-amplification of genes spread over different chromosomes is yet unclear.

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

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Chapter 9

Summary and General Discussion

Summary and general discussion

One of the most frequent genetic changes in sporadic breast cancer is amplification of the HER2 gene, usually resulting in protein overexpression on the cell membrane. Intracellular tyrosine kinase activation then results in a growth activation of the cells. Breast cancer patients that have this HER2 amplification have a worse prognosis but can be treated with an antibody (trastuzumab or Herceptin®) directed against the HER2 receptor which results in a survival benefit. However, this treatment is expensive and is associated with potentially severe side effects such as cardiotoxicity and should thus only be administrated to patients that are likely to show a response. It is therefore very important that a reliable diagnostic test exists that selects patients for this targeted treatment. In *chapter 2*, we reviewed current diagnostic tests to detect HER2 amplification (with special focus on MLPA) and overexpression and discussed their advantages and disadvantages. At present, the most common method to assess HER2 status is immunohistochemistry (IHC), which is a relatively cheap routine technique available in all pathology laboratories to detect protein expression levels. However, although staining and scoring methodology has been better standardized with the introduction of the FDA approved Hercep®test, there are still problems with reproducibility and interpretation of IHC assays. The same is true for other (mostly second-line) diagnostic tests to detect amplification of HER2 such as chromogenic and fluorescence *in situ* hybridization. Furthermore, given the inherent molecular complexity of the malignant process, it seems unlikely that the assay of a single marker such as HER2, regardless of methodology, will ever give us the complete answer as to the response to targeted therapeutics.

The primary aim of this thesis was therefore to search for new, easy, cheap and easy-to-interpret alternate methods to reliably select patients for trastuzumab therapy. We validated a new PCR based technique, called multiplex ligation-dependent probe amplification (MLPA), for HER2 amplification detection in breast cancer and applied this same technique to simultaneously analyze the copy number status of a broad spectrum of genes, leading to a better understanding of several genetic changes in breast cancer such as polysomy 17 and thus possibly to a more patient tailored treatment in the future. Furthermore, we evaluated a new fully automated IHC assay based on a monoclonal antibody which can possibly reduce the analytical variability in HER2 IHC results by standardizing the IHC staining process.

Since a fully automated procedure based on a monoclonal antibody could increase the reproducibility of HER2 IHC, we validated a new fully automated HER2 overexpression detection kit (Oracle HER2 Bond IHC System) in *chapter 3* by comparing its results in 200 invasive breast cancer patients with the currently most frequently used and FDA approved manual HercepTest. Fully-automated HER2 staining with the monoclonal antibody in the Oracle kit showed a 94%

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agreement (kappa 0.85) with manual staining by the polyclonal antibody in the HercepTest. Although Oracle showed in general some more cytoplasmic staining and was possibly slightly less sensitive in picking up HER2 amplified cases, it may be considered as an alternative method to evaluate the HER2 expression in breast cancer with potentially less analytical variability.

One antibody?

There are currently many antibodies on the market to detect HER2 overexpression. The percentage of positive (3+) IHC scores obtained with these different antibodies varies between 17 and 36% in the same group of patients. The analytical variation is so large that sometimes the FISH/CISH safety net may not work: a sample that is scored as 2+ with antibody A can be scored 0 with antibody B. In The Netherlands 48 labs use 7 different antibodies. A survey performed by Roche showed a variation of 7 to 16% in the 3+ score and of 5-25% in the 2+ score. The variations in the outcomes of the CISH/FISH tests are also relatively large (VAP Visie 4, 2009). Of course, for now, the variability between the outcomes with different antibodies is unacceptable and must at least be guarded by good internal and external quality control. However, in the long run there may be 2 possible more definite solutions: (1) Choose 1 antibody that is used by all pathology labs in The Netherlands. The question then of course remains which antibody to choose, and a "monopoly" will most likely lead to higher prices and lower quality. (2) Search for a different diagnostic test that is easy, reliable and more quantitative leading to more consistent results.

IHC for HER2 is generally evaluated according to the DAKO scoring system which is graded from 0 to 3+. Patients with 3+ scored tumors are eligible for trastuzumab therapy while patients with a 0 or 1+ score are not. For IHC equivocal cases (score 2+), a second-line HER2 gene amplification test is deemed necessary. There are currently two FDA-approved gene amplification tests available: fluorescence *in situ* hybridization (FISH) and chromogenic *in situ* hybridization (CISH). FISH testing is a cumbersome and expensive technique and the fluorescently labelled HER2 probe fades over time. CISH results, in the contrary, can be interpreted using a conventional bright field microscope and a permanent staining record can be retained. However, interpretation of IHC, FISH and CISH remains semi-quantitative, unavoidably leading to discrepancies between observers. Of 29 labs participating inthe Dutch quality sendings organised by SKML (Stichting Kwaliteitsbewaring Medische Laboratorium-diagnostiek) in 2009, 45% used CISH, 42% FISH and 13% used a different technique to asses HER2 amplification.

Centralize?

Each year approximately 12,000 women are diagnosed with breast cancer in The Netherlands and 2,400 (up to 20%) of them have an amplification/overexpression of HER2. Assuming that 10% of these results are false positive, this will lead to an unnecessary, cardiotoxic and expensive treatment in 240 patients per year. Probably, there are also approximately 2% false negative test results, meaning that about 200 patients mistakenly will not receive trastuzumab, with up to 20 unnecessary deaths as a result. In a French investigation where IHC was checked with FISH, there were 2.9% false positive and 2.2% false negative IHC results in the high-volume central labs, versus 13.8% false positive and 4.5% false negative results in the local labs. So, there may be three possibilities:
- Centralize testing in reference labs that perform many tests per year. This may increase quality but will lead to delays in completing tumor profiling.
- Expand the safety net of gene amplification tests to all cases. This may serve as quality control
- Search for a better alternative primary diagnostic test that produces consistent, reliable, affordable and easy-to-interpret results.

Dual-colour FISH (Pathvision) was first FDA approved in 1998 and in 2008 CISH (Invitrogen) was also approved. CISH has the obvious advantage of being able to work with bright field microscopy but many of the CISH assays available are still monoprobe assays, meaning that only the presence of more copies of HER2 is verified. High level HER2 amplifications are easily recognisable, only low level amplifications - which are less frequent - could be confused with polysomy. However, as we showed in chapter 7, true polysomy is very rare or even non-existent in breast cancer and what co-amplification of HER2 and CEP17 means in terms of response to trastuzumab is not clear yet. There is now also a modern CISH (Dako DuoCISH) version which is a duo-probe assay containing a centromeric 17 probe. Furthermore, Ventana has now requested FDA approval for the fully automated INFORM™ HER2 Silver in situ Hybridization (SISH) technology which makes a 6-hour time to diagnosis possible and uses metallography where the bound HRP catalyses the reduction of silver acetate to produce a black signal. There is at this time no consensus on which technique is the better one, or on the use of mono- or duoprobe systems and manual or automated systems. For a long time FISH has been the gold standard but CISH is taking over in many labs although recent reports of the Dutch SKML quality control showed that CISH results showed more variability between (especially small) labs than FISH results.

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Because of the need for a more quantitative and thus easy to interpret technique, we introduced a new diagnostic test for HER2 gene amplification detection in ***chapter 4***, called multiplex ligation-dependent probe amplification (MLPA), and compared this new test with IHC and CISH in 321 breast tumors. Concordance between MLPA and CISH was 94% and between MLPA and IHC 90% (later adjusted to 91% and 88% using a refined MLPA threshold). Both sensitivity and specificity of MLPA was similarly good as that of IHC, when CISH was considered as gold standard. We concluded that MLPA is a cheap and accurate method to detect breast cancer HER2 amplification in small quantities of DNA extracted from paraffin blocks, and thereby a good alternative or supplementary technique to other gene amplification detection methods like FISH and CISH. An amplification test such as MLPA could even be suitable as a pre-screening tool, alternative to IHC. Indeed, MLPA is easy, cheap and more quantitative than IHC allowing a more straightforward interpretation, although the dynamic range seems to be less than FISH. Furthermore, since MLPA can analyze up to 45 genes in one assay, several genes that are important in therapy selection and/or prognosis can be tested for amplification in the same analysis as shown in chapter 8. However, MLPA has the disadvantage of being a non-morphological technique that can result in the overlooking of heterogeneity and DCIS which can 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.

Why is our percentage of HER2 amplification and overexpression so small?

The percentage of HER2 amplified and overexpressed tumors found in our studies is in the lower range of the 20-30% positivity that has generally been described in the literature. Methodological variation is an unlikely explanation as the fraction of HER2 amplified cases by IHC, MLPA and CISH was similar and as our study groups concerned consecutive patients, selection bias could be excluded. It is likely that many of the studies in which higher HER2 overexpression/amplification frequencies were described were not unselected. Furthermore, several other studies and other hospitals in The Netherlands have reported lower (10-18%) percentages as well. One study [1] investigated the percentage of HER2 positive tumors in the mid-north and the south-west of the Netherlands. There was a significant difference in the percentage of positivity in both regions. Possible explanations by oncologists were a relatively old population (north) and an often non-caucasian population (mid). This study showed that on average 15% of the patients had a HER2 positive tumor; in the mid-north this percentage was even smaller in most hospitals. What causes these regional differences is still unknown and requires further research.

Although we already proved in ***chapter 4*** that MLPA is a reliable and sensitive technique that can detect amplifications in samples with tumor percentages as low as 10%, the higher the tumor percentage is, the more reliable the results may be, since also smaller or low level amplified clones, will then be picked up.

In ***chapter 5*** we further evaluated the reliability of MLPA for HER2 testing, especially when analyzing samples that have a lot of normal breast tissue and/or *in situ* components resulting in a low tumor content. We investigated the influence of performing manual (with a scalpel) or laser-based microdissection on the sensitivity of MLPA to detect HER2 amplification. Concordance between MLPA and ISH improved from 61% to 84% after manual microdissection and to 90% after laser microdissection (later adjusted to 58%, 74% and 77% respectively using a refined MLPA threshold). Since the best correlations between MLPA and ISH were obtained in cases with a tumor percentage higher than 30%, we concluded that microdissection before MLPA may not be routinely necessary but may be advisable in case of very low tumor content (≤30%), but also when MLPA results are equivocal, or when extensive ductal carcinoma *in situ* is present. Since differences between manual and laser microdissection were small, less time consuming manual microdissection appears to be sufficient in those cases.

In chapters 2 to 5, we focussed on HER2 amplification in breast cancer. Of course, HER2 is not the only gene that plays a role in breast tumor development and growth. Many other genes are currently under investigation, and some of them could also be possible therapeutic targets (for example TOP2A, see general introduction). In the future this will bring along a need for a simple and affordable diagnostic test that is able to detect genetic changes of several genes simultaneously. In ***chapter 6***, we evaluated MLPA as a technique to simultaneously detect genetic changes of HER2 and TOP2A. Of 353 patients analyzed, 9.4% showed TOP2A amplification by MLPA and 12.4% of patients were HER2 amplified (later adjusted to 24% and 17% respectively using a refined MLPA threshold). TOP2A amplification was seen in 42% of HER2 amplified cases (later adjusted to 45%) and showed no high level amplification without HER2 amplification, which is in line with other studies. Eleven patients displayed TOP2A loss (3.1%) which is consistent with literature: overall prevalence of TOP2A deletions in breast cancer has varied from 2% to 11% in different studies. Concordance between MLPA and CISH was 91% for TOP2A and 96% for HER2 (later adjusted to 82% and 93% respectively using a refined MLPA threshold). MLPA is thus able to simultaneously detect breast cancer HER2 and TOP2A copy number. Correlation between amplification and overexpression of TOP2A was significant (p=0.035), but amplification did not predict protein overexpression well. Other factors, specifically the tumor proliferation status, may interfere with the TOP2A protein status since TOP2A is a marker of proliferation and its expression depends on the cell cycle status. Loss of the TOP2A gene was almost never associated with loss of its protein.

Clinical meaning of more or fewer TOP2A gene copies?

Over the past 15 years, a substantial amount of clinical data from multiple individual studies has indicated that the incremental benefit from adjuvant anthracycline-based therapies is largely restricted to the HER2-positive subgroup of human breast cancers (15-20%) but the vast majority of currently used adjuvant regimens for almost *all* breast cancer patients worldwide, both on and off study, are anthracycline based. A recent meta-analysis demonstrated that there was little or no benefit for patients whose cancers were HER2 normal [2], although these patients remain at risk for all the attendant toxicities associated with anthracyclines. The absence of any effect of anthracyclines observed in patients with HER2-negative disease suggests that this group of patients could be spared unnecessary toxic effects related to the use of this class of agent. The majority of the published data from at least 10 large studies indicate that the bulk of the TOP2A amplification events occur in a subset of the HER2-amplified breast cancers and represent a co-amplification phenomenon. The association of TOP2A deletions with anthracycline responsiveness by some studies conflicts with *in vivo* and *in vitro* findings that TOP2A deletions are associated with anthracycline resistance [3]. Further research is required to resolve this latter issue. It is likely that most of the various discrepancies between studies concerning altered TOP2A gene prevalence are due to technical differences in measuring those alterations, that is, different ratios and/or cut-offs used for calling a tumor amplified or deleted as well as very different technologies and/or reagents used to determine alteration rates including use of non-cell-based assays.

Currently, the overwhelming bulk of the published and/or reported data indicate that TOP2A alterations are important predictive factors for determining the likelihood of incremental benefits from anthracyclines in the adjuvant treatment of human breast cancers.

HER2 and TOP2A are both located on chromosome 17. In tumors there are often three or more copies of a chromosome rather than the expected two copies. This phenomenon is called polysomy and in breast cancer chromosome 17 polysomy has extensively been described to be of importance in therapy selection (see general introduction). In **chapter 7** we applied the multiplex aspect of MLPA to study polysomy 17 and showed that this phenomenon is not at all as common as described in literature based on the amplification of the centromere of chromosome 17 (CEP17). None of the 111 patients analyzed by MLPA showed a true polysomy of chromosome 17, as reflected by a copy number increase of all 17 probed genes along chromosome 17. Only 2% of the patients showed amplification of all the probed genes on the 17q arm, pointing to a gain of the long arm of chromosome 17. Chromosome 17 usually shows a complex pattern of gains and losses, rather unrelated to the copy number status of the centromere

(CEP17) and, at least in some cases, correction with CEP17 probes may provide misleading HER2 gene status assessment results.

Except for only a few studies, it is presently unknown whether patients with amplicons spanning HER2 and CEP17 will respond better or worse to trastuzumab. Two studies [4, 5] showed that "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 used by FISH (and to a lesser extent by CISH) 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. These data are intriguing but preliminary, and caution should be exercised in interpreting the results until further data on more patients can be analyzed. In our study up to 55% of HER2-amplified patients were polysomic based on CEP17 or MLPA WSB1 analysis. Several of these patients would probably not be eligible for trastuzumab based on the HER2/CEP17 ratio. Of course, most laboratories do not perform FISH but rather IHC as primary test and patients with an IHC 3+ score would not require a gene amplification test according to the current guidelines.

Next to HER2 and TOP2A, many other genes have been found to be implicated in therapy response, for example estrogen receptor alpha (ESR1) gene amplification in the response to tamoxifen therapy and MYC amplification in resistance to anti-estrogen therapy. Therapeutic or prognostic significance of other frequently amplified genes such as cyclin D1 (CCND1) or frequent loss of genes such as E-Cadherin (CDH1) is less clear. Classical and cytogenetic molecular genetic approaches have demonstrated that in breast cancer, almost every chromosome has at least one site involved in cancer-related genetic alterations (loss, amplification, mutation, or altered DNA methylation). As a consequence, the number of genes identified as being altered in breast cancer has been rising over the years. Tumor heterogeneity (and thus the heterogeneity of clinical behaviour) may be the consequence of an underlying variation in gene copy number and gene interactions. In **chapter 8** we took advantage once again of the multiplex aspect of MLPA and simultaneously studied copy number changes of 20 genes located on different chromosomes (6, 7, 8, 11, 17, 19 and 20) and evaluated their co-occurrence and their association with clinicopathological characteristics in 104 breast cancer patients. There was a significant correlation between the number of amplified genes and the histological grade and mitotic index of the tumor. Amplifications of ESR1, PRDM14, MYC and HER2 were associated with a high mitotic index, and PRDM14 and HER2 amplifications with high histological grade. HER2/MYC, HER2/CCNE1 and EGFR/MYC co-amplified tumors were significantly larger than tumors with either of these amplifications. Gene loss occurred most frequently in CDH1 and FGFR1.

MLPA: research or diagnostic tool?

The number of genes identified as being altered in breast cancer has been rising over the years. Tumor heterogeneity (and thus the heterogeneity of clinical behaviour) may be the consequence of an underlying variation in gene copy number and gene interactions. To be able to provide an optimal patient-tailored treatment in the future, it will thus be of outmost importance that techniques such as comparative genomic hybridization (CGH), microarray and MLPA continue their search for oncogenes and tumor suppressor genes and evaluate gene-gene interactions. Although CGH has the advantage of a genome wide scope, it is a more costly and labour intensive technique that requires a relatively large amount of sample DNA and trained personnel to deal with the complexity of the data. MLPA is certainly a promising research tool (as shown in chapters 7 and 8) that, at this moment, is being used by research groups all over the world and has found applications in assessing gene copy number changes, gene expression and methylation.

One of the main advantages of MLPA is its multiplex aspect: one can analyze 45 MLPA probes simultaneously, so several predictive and prognostic genes can be tested for amplification in the same analysis, and several probes can be used for the same gene providing an extra control for amplification/loss. The first step to take for MLPA to become clinically validated as a diagnostic tool (at first for HER2 amplification) is to couple MLPA results to response data. This will certainly be the subject of future research. At this time many pathologists will probably be reluctant to embrace MLPA, especially as a primary screening test, because it is a non-morphological technique. Nevertheless we have shown that, at least for HER2, there was a good correlation between MLPA, IHC and ISH and that in approximately 85% of the cases there is no need to perform manual microdissection. However - from experience - we do think that, for analyzing many other genes that (often) show lower levels of amplification than HER2, it is advisable to perform H&E-guided manual microdissection prior to MLPA. This, of course, would make MLPA somewhat less easy accessible but scraping off the enriched tumor area with a scalpel is only a small effort and is already standard practice in routine diagnostic mutation analysis (EGFR, K-RAS) in our lab.

Interpretation of MLPA is much more straightforward than for IHC or ISH, but cut-off values between no and low-level amplification and between low- and high-level amplification have to be chosen carefully. In our first MLPA studies, we used 1.5 as a cut-off value to discriminate between no and low level amplification, based on our experience with MLPA kits for trisomy detection. However, a cut-off value of 1.3 (delta value 0.3) instead of 1.5 was better validated by several other studies and seemed to more closely reflect the amplification status. Furthermore, the new manufacturer's software program (Coffalyser) used to analyze MLPA data also advised 1.3 as cut-off for low level amplification. We therefore re-analyzed our data with cut-off 1.3 (see addenda chapters 4, 5 and 6) which increased the

sensitivity of MLPA to detect HER2 amplification in breast cancer, although this was at the cost of the specificity. Of course, the optimal cut-off value can only be determined with certainty when clinical response data are available.

There is also the need for well chosen control probes in each type of MLPA kit. As tumors have many gene copy number aberrations spread over different chromosomes, it is often difficult to find control probes that are located in non-affected stable areas of the genome. The more stable the control probes, the more reliable MLPA results will be since the robustness of the normalization depends on the number of reference probes and their chromosomal locations. It will require some time and feedback to the manufacturer (MRC Holland) before each MLPA mix contains the most stable control probes. Figure 1 shows an example of the stability of control probes in the P004-B mix used in chapter 7. The control probe at 10q22b (VCL gene, vinculin) seems to be a reliable probe in breast cancer, with a small standard deviation (mean 0.98 +/- 0.10) and almost all ratios between 0.7 and 1.3 (minimum ratio 0.755 and maximum ratio 1.315). The control probe at 22q13 (SBF1 gene), for example, has to be replaced or removed (0.75 +/- 0.18).

Given its low costs, its reproducibility, reliability (as shown in the previous chapters) and its ease of implementation and interpretation, MLPA will undoubtedly play an important role in diagnostics in the future. However, before this is possible, MLPA mixes need to be optimized and clinically further validated.

Major conclusions of this thesis

- For HER2 detection, there is currently no gold standard. Every lab uses the IHC and/or ISH tests that are the most convenient and/or affordable in their situation. This may lead both to under-treated and over-treated patients. Good quality control and an adequate validation of new techniques are crucial for every lab. Centralisation and further standardization are both an option.
- Fully-automated HER2 IHC staining with the monoclonal antibody in the Oracle kit showed a 94% agreement (kappa 0.85) with manual staining by the polyclonal antibody in the HercepTest and may be considered as an alternative method to evaluate the HER2 expression in breast cancer with potentially less analytical variability.
- MLPA can reliably and simultaneously detect copy number changes of HER2, TOP2A and many other genes located on different chromosomes.
- Laser microdissection is not necessary before MLPA, and manual microdissection is advisable for gene copy number assessment of low level amplified genes, for samples with a very low tumor content (≤30%) and for tumors with extensive DCIS.

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- Polysomy 17 is very rare in breast cancer. It remains to be seen what implications this finding will have on diagnostic tests using dual-probe systems (HER2 and CEP17) such as FISH and CISH. Furthermore, the effect of centromere (co-) amplification on the response to trastuzumab and anthracyclins requires further investigation.
- The breast cancer dedicated MLPA kit can simultaneously detect copy number changes of several genes implicated in breast carcinogenesis and progression.
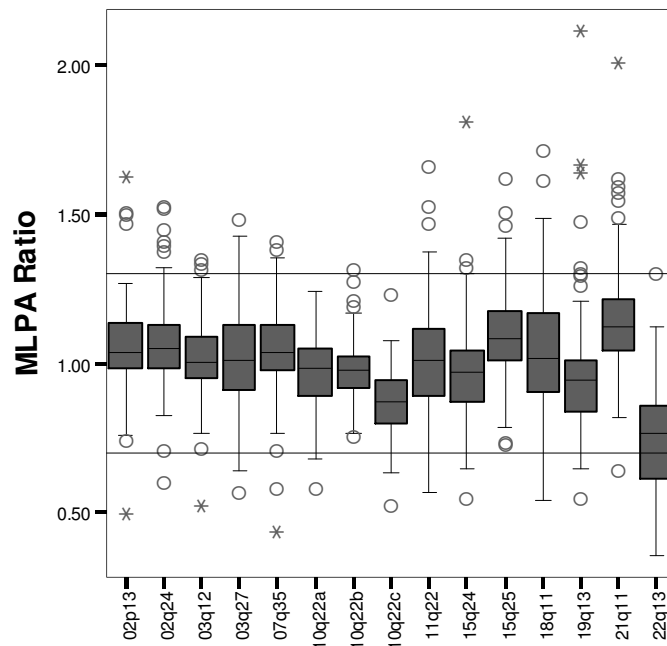
Figure 1. MLPA ratio results for all 15 control probes in the P004-B1 breast cancer chromosome 17 MLPA mix in 112 breast cancer patients.

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Color Figures
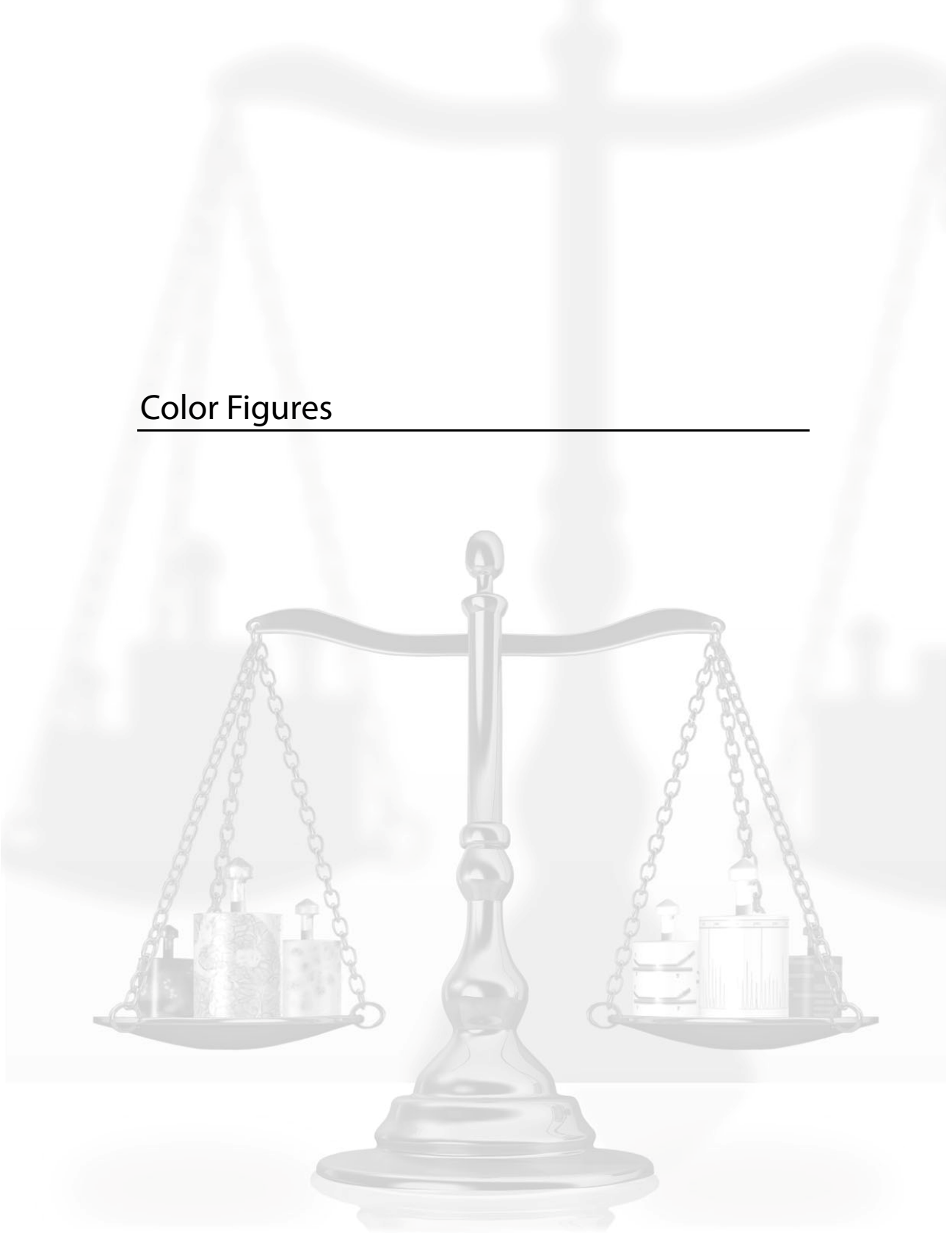
Chapter 2, Figure 1.

Examples of scoring of HER-2/*neu* immunohistochemical staining. (<u>Top left</u>) Negative staining: no staining, only cytoplasmic staining or less than 10% cells with membrane staining. (<u>Top right</u>) 1+ staining: more than 10% cells with membrane staining which is however incomplete. (<u>Bottom left</u>) 2+ staining: more than 10% cells with complete membrane staining which is however not strong in intensity. (<u>Bottom right</u>) 3+ staining: more than 10% cells with complete intense membrane staining (reprinted with permission from Purmomosari et al)
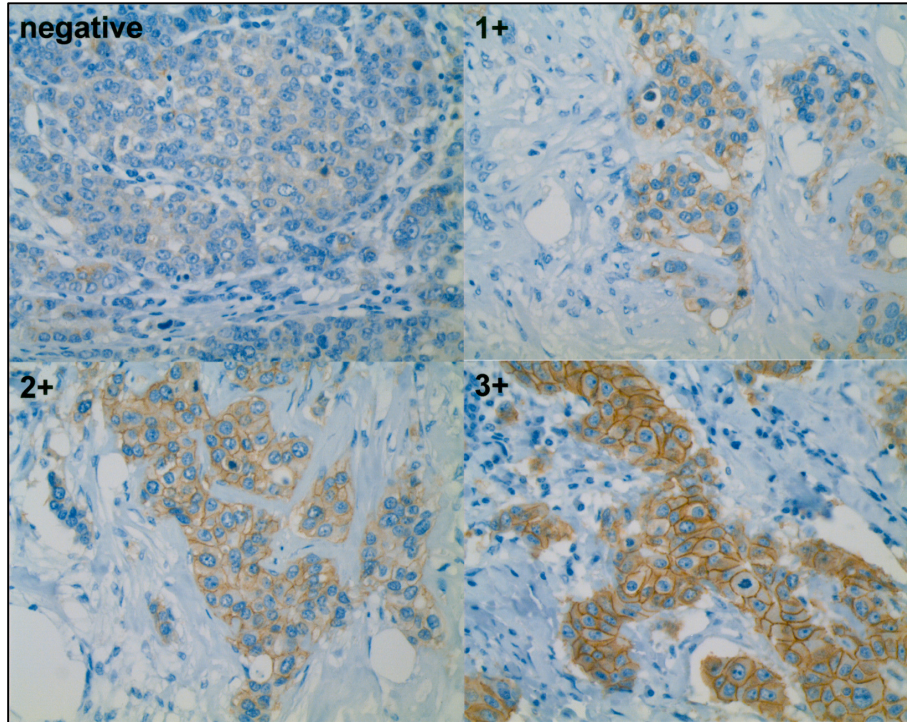
**Chapter 3, Figure 1.** HER2 immunohistochemistry (IHC) by the Oracle HER2 Bond IHC detection system and HercepTest

<u>Top left</u>: Oracle shows cytoplasmic staining while <u>Top right</u>: HercepTest does not show cytoplasmic staining for the same tumor. <u>Bottom left</u>: Oracle shows aspecific staining of the surrounding tissue while <u>Bottom right:</u> HercepTest does not
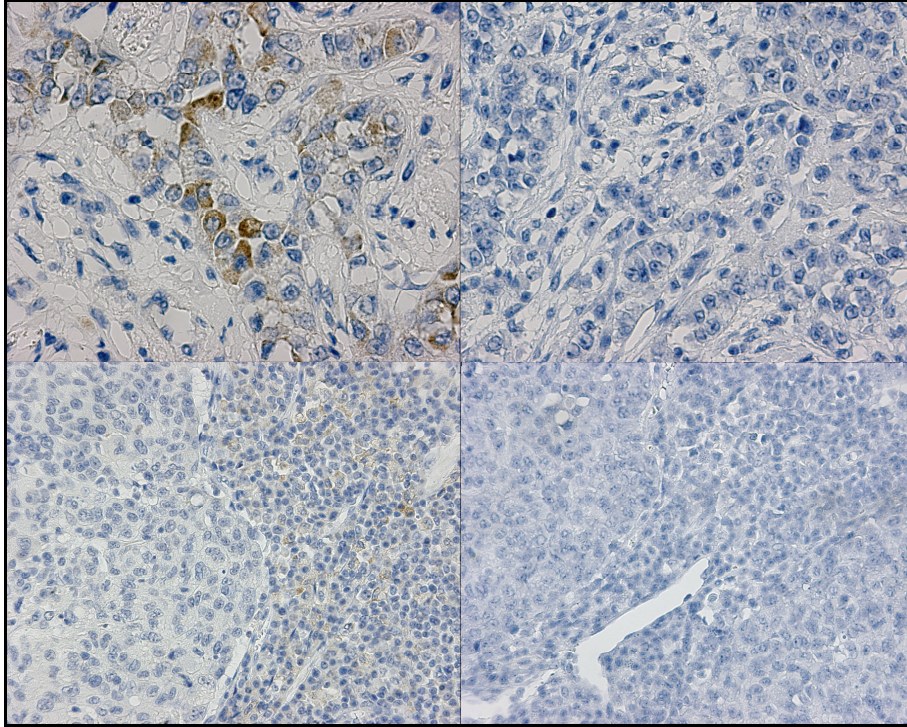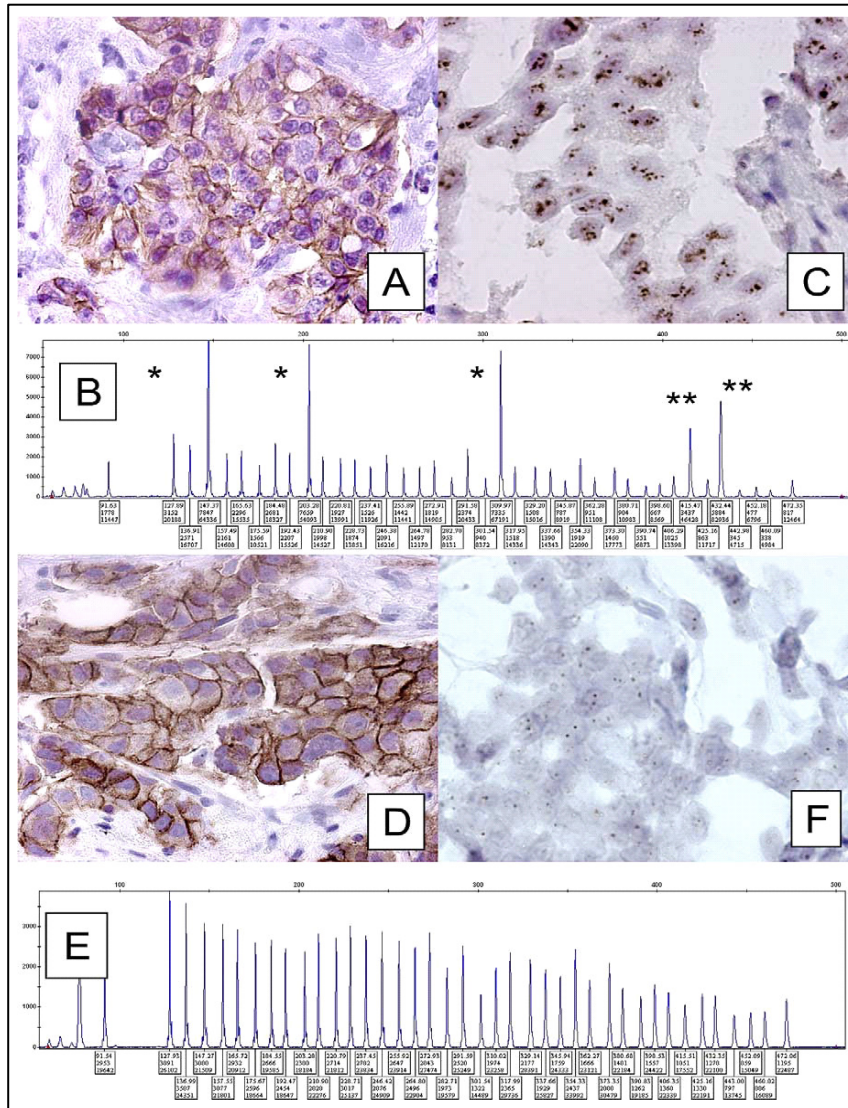
Chapter 3, Figure 2. HER2 immunohistochemistry (IHC) discrepancies between Hercep test and the Oracle HER2 Bond IHC detection system

<u>Top left</u>: tumor with Hercep test 3+ score, <u>Top right</u>: same tumor with Oracle HER2 Bond IHC system 2+ score (strong membrane staining but not in 30% of cells); <u>Bottom left</u>: tumor with Hercep test 2+ score, <u>Bottom right</u>: same tumor with Oracle HER2 IHC system 1+ score (relatively strong membrane staining but not complete in > 10% of cells).
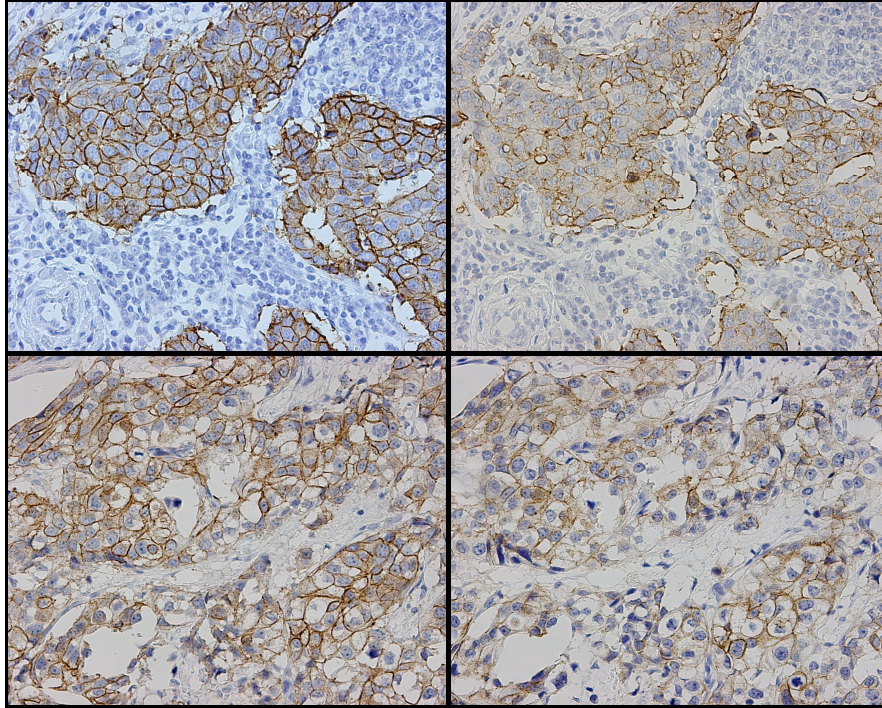
Chapter 4, Figure 1. MLPA, CISH and IHC to detect HER2 amplification in invasive breast cancer.

A) HER2 IHC 1+ case with in B) amplification by MLPA (see 3 HER2 probe peaks (*) way above the controls and two other chromosome 17 peaks (**) co-amplified) as confirmed by CISH in C). D) HER2 IHC 3+ case with in E) no amplification by MLPA (none of the 3 HER2 probe peaks above the controls) or CISH in F).
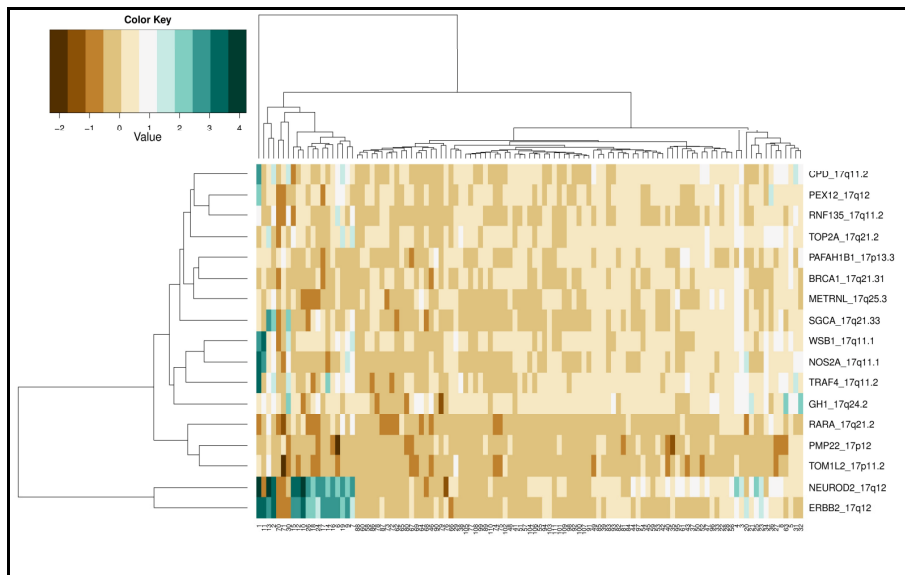
Chapter 6, Figure 1. Correlation between gene amplification and protein expression in breast cancer as determined by chromogenic *in situ* hybridization and immunohistochemistry

Top left: Almost no *TopoIIa* protein expression is present. Top right: Large chromogenic *in situ* hybridization clusters indicate *TopoIIa* gene amplification in the same patient. Bottom left: strong *TopoIIa* protein expression is present in 5% of tumor cells. Bottom right: chromogenic *in situ* hybridization shows less than 5 signals per cell indicating no *TopoIIa* gene amplification

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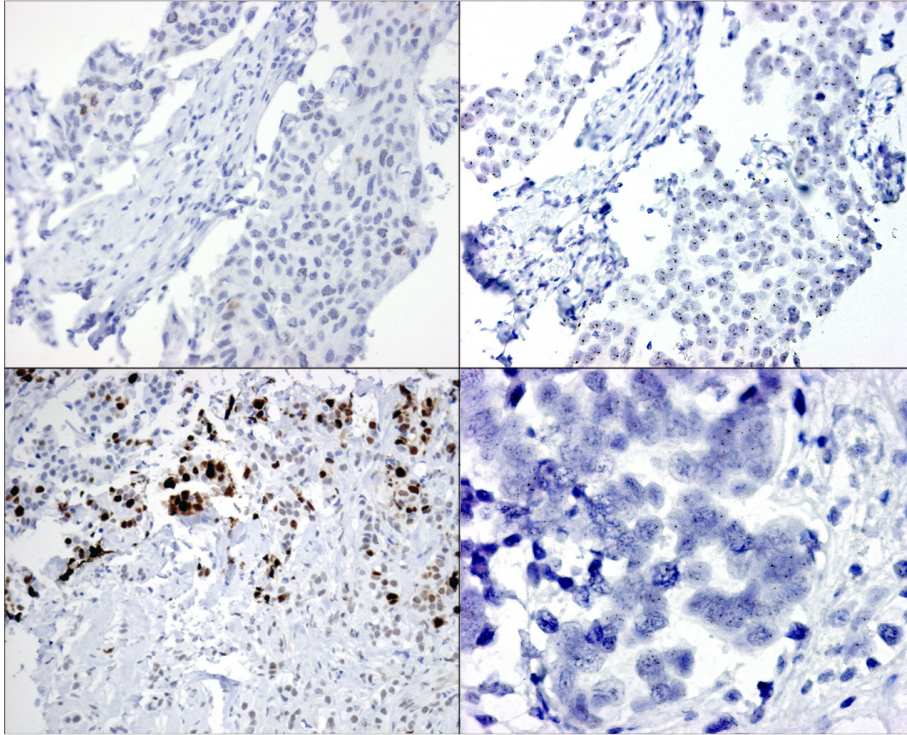
Chapter 7, Figure 2. Hierarchical cluster analysis of 111 invasive breast cancer patients analyzed for copy number status of 17 chromosome 17 genes by MLPA

Chapter 8, Figure 3. Hierarchical cluster analysis of 104 invasive breast cancer patients (horizontal axis) analyzed by multiplex ligation-dependent probe amplification for 20 breast cancer related genes (vertical axis).
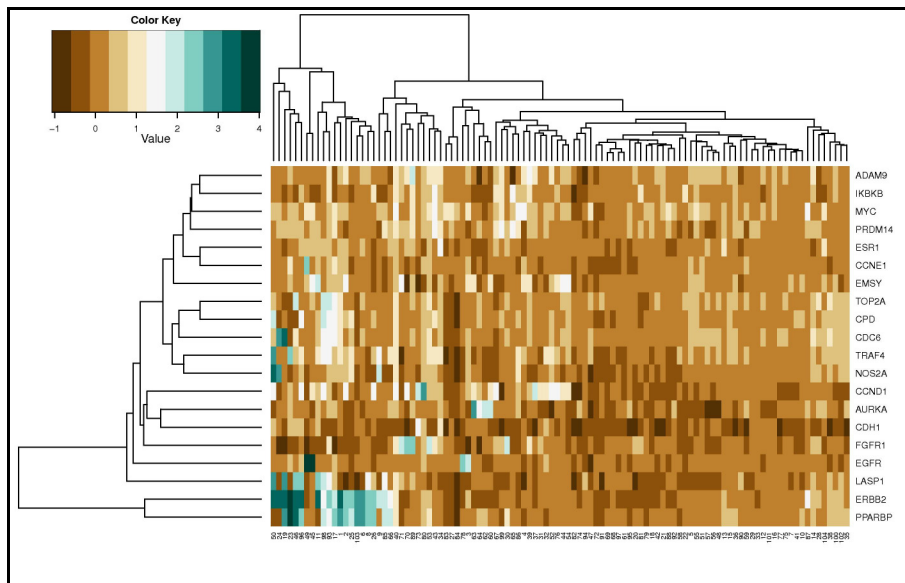
Chapter 10

Nederlandse Samenvatting voor niet-ingewijden

Nederlandse samenvatting voor niet-ingewijden

Naar schatting krijgen in Nederland elk jaar meer dan 11,500 vrouwen de diagnose borstkanker en sterven meer dan 3,500 vrouwen aan deze ziekte. Eén op de 9 vrouwen heeft of zal borstkanker ontwikkelen tijdens haar leven. Borstkanker is een ongecontroleerde groei van cellen in de borst. Om borstkanker beter te begrijpen, is het belangrijk te weten hoe kanker kan ontstaan. Kanker ontstaat als resultaat van mutaties, of abnormale veranderingen, in genen die verantwoordelijk zijn voor het regelen van de celgroei en voor het gezond houden van de cellen. Deze genen bevinden zich in de nucleus van elke cel, die fungeert als het "controle centrum". Normaal vervangen de cellen in onze lichamen zichzelf door een geordend proces van celgroei: gezonde nieuwe cellen nemen het over van oude cellen die afsterven. Maar gedurende het verouderingsproces kunnen mutaties bepaalde genen "aan zetten" of andere genen juist "uitschakelen" in een cel. Die veranderde cel verkrijgt dan de mogelijkheid om ongecontroleerd te blijven delen, en dus om nog meer zulke cellen te produceren wat leidt tot de vorming van een tumor. De term "borstkanker" verwijst naar een tumor die vanuit cellen in de borst is ontstaan. Gewoonlijk begint borstkanker bij de cellen van de lobulen, de melk-producerende klieren, of bij de melkgangen (ducten) die de melk vanuit de klieren tot bij de tepel brengt. Minder vaak voorkomend begint borstkanker bij het bind- en vetweefsel in de borst. Borstkanker ontstaat altijd door een genetische abnormaliteit (een "fout" in het genetisch materiaal). Nochtans zijn maar 5-10% van alle borsttumoren te wijten aan een abnormaliteit die wordt overgeërfd van een van onze ouders. Ongeveer 90% van de borsttumoren ontstaat doordat genetische abnormaliteiten zich opstapelen als gevolg van het verouderingsproces en de "slijtage" gedurende het leven. Deze laatste groep, ook wel sporadische borstkanker genoemd, vormt het onderwerp van dit proefschrift.

Een van de genetische veranderingen die vaak voorkomt bij sporadische borstkanker is de aanwezigheid van te veel kopieën van het HER2 gen (=een stukje DNA dat de informatie bevat om een eiwit te vormen binnenin de cel). Deze zogenaamde "genamplificatie" heeft tot gevolg dat er te veel HER2 receptoren op het membraan rondom de cellen in de borst aanwezig zijn (= "overexpressie"). Deze receptoren verzenden signalen ("boodschappen") naar de nucleus van de cel waardoor de cel kopieën van zichzelf gaat maken. Bovendien voorkomen deze signalen dat de cel dood gaat – en wetende dat bij elke celdeling fouten kunnen worden gemaakt tijdens het kopieer proces met als gevolg nieuwe genetische afwijkingen – resulteert dit in de opeenstapeling van genetische abnormaliteiten die aan de basis liggen van de tumorgroei. Borstkanker patiënten die deze HER2 amplificatie hebben een slechtere prognose maar komen in aanmerking voor behandeling met een antilichaam (genaamd trastuzumab of Herceptin) dat gericht is tegen de bovengenoemde receptoren wat een verhoogde overlevingskans met zich meebrengt. Maar deze behandeling is erg duur (30,000 - 40,000€ per patiënt per jaar) en kan gepaard gaan met

ernstige bijwerkingen (toxisch voor het hart). Het is dus essentieel dat er een betrouwbare diagnostische test bestaat die patiënten accuraat kan selecteren voor deze behandeling. In **hoofdstuk 2** werden de bestaande diagnostische testen voor HER2 amplificatie en overexpressie beschreven (met speciale aandacht voor MLPA, een recente techniek die het belangrijkste deel van dit proefschrift vormt) en werden hun voor- en nadelen besproken. Er bestaan 2 verschillende soorten diagnostische testen voor HER2: testen gebaseerd op het meten van HER2 eiwit overexpressie (zoals immuunhistochemie: een antilichaam bindt aan het HER2 eiwit) en testen gebaseerd op het meten van HER2 gen amplificatie (*in situ* hybridisatie technieken zoals CISH en FISH, en ook MLPA: een klein stukje DNA, een "probe", bindt aan het HER2 gen).

Op dit moment is de meest gebruikte methode om de HER2 status te bepalen immuunhistochemie (IHC). Deze techniek is relatief goedkoop en eenvoudig, en wordt routinematig in de meeste pathologie laboratoria als primaire test gebruikt om naar de receptor eiwitexpressie van HER2 te kijken. Nochtans, hoewel het kleurproces en het scoren beter gestandaardiseerd zijn door de introductie van de FDA (Food and Drug Administration) goedgekeurde Hercep®test, zijn er nog steeds problemen met de reproduceerbaarheid en de interpretatie van de IHC techniek. Hetzelfde is waar voor andere diagnostische testen die gebruikt worden om naar de amplificatie van HER2 te kijken zoals chromogene *in situ* hybridisatie (CISH) en fluorescente *in situ* hybridisatie (FISH). Bovendien lijkt het onwaarschijnlijk, gezien de complexiteit van het kwaadaardige proces, dat het kijken naar 1 enkele "marker" zoals HER2, onafhankelijk van de methodologie, ons ooit het volledige antwoord zal geven op de vraag of een patiënt zal reageren op een bepaalde gerichte therapie.

Het primaire doel van dit proefschrift was dan ook het zoeken naar nieuwe, goedkope en gemakkelijk interpreteerbare alternatieve methoden om patiënten op een betrouwbare manier te kunnen selecteren voor trastuzumab/herceptin therapie. We valideerden een nieuwe techniek (genaamd multiplex ligation-dependent probe amplification of MLPA, gebaseerd op het vele malen vermenigvuldigen van kleine stukjes DNA die we willen "meten") voor HER2 amplificatie detectie bij borstkanker en bovendien gebruikten we deze techniek om gelijktijdig naar het aantal kopieën van een groot aantal genen te kijken, waardoor we bepaalde genetische processen bij borstkanker zoals polysomie 17 (zie verder) beter kunnen begrijpen en mogelijk een verbeterde patiëntgerichte therapie kunnen aanbieden in de toekomst. Bovendien evalueerden we een nieuwe volautomatische IHC test gebaseerd op een monoklonaal antilichaam (=afkomstig van slechts 1 plasmacel), die mogelijk de analytische variabiliteit zou kunnen reduceren door het IHC kleurproces te standaardiseren.

Aangezien een volledig automatische procedure gebaseerd op een monoklonaal antilichaam de reproduceerbaarheid van de HER2 IHC techniek zou kunnen verbeteren, valideerden we een nieuwe volautomatische HER2 overexpressie

detectie kit (Oracle HER2 Bond IHC Systeem) in ***hoofdstuk 3*** door de resultaten van deze kit te vergelijken met resultaten van de huidige FDA goedgekeurde manuele HercepTest. De resultaten van de Oracle kit vertoonden 94% overeenkomst met de HercepTest die gebaseerd is op een polyklonaal antilichaam. Hoewel de Oracle kit meer cytoplasmatische aankleuring (= niet op de membraan maar binnenin de cel) vertoonde en iets minder gevoelig was voor het oppikken van HER2 amplificaties, kan het beschouwd worden als een alternatieve methode om HER2 eiwitexpressie te evalueren bij borstkanker met mogelijk minder analytische variabiliteit.

IHC voor HER2 wordt doorgaans geëvalueerd met het DAKO score systeem wat gegradeerd is van 0 tot 3+. Patiënten met een 3+ score komen in aanmerking voor trastuzumab therapie terwijl patiënten met een 0 of 1+ score niet in aanmerking komen. Voor tumoren met een IHC score 2+ is een HER2 gen amplificatie test nodig (zoals FISH of CISH).

Eén antilichaam?

Antilichamen zijn eiwitten die door de mens en andere gewervelde dieren worden geproduceerd als antwoord op het binnendringen in het lichaam van een lichaamsvreemde stof of lichaamsvreemde cellen. De binnendringende deeltjes, die door het lichaam als gevaarlijk worden beschouwd, heten antigenen. Tegen elk antigen kan een antilichaam gemaakt worden. Momenteel zijn er veel verschillende antilichamen op de markt die HER2 eiwit overexpressie kunnen detecteren. Het percentage van positieve (3+) IHC scores (die aangeven dat een patiënt in aanmerking komt voor trastuzumab therapie) dat met deze verschillende antilichamen gevonden wordt varieert tussen 17 en 36% in dezelfde groep patiënten. Deze variatie is soms zo groot dat het veiligheidsnet van FISH/CISH (bij 2+ score) gemist kan worden: een sample dat een score 2+ krijgt met antilichaam A kan een score 0 krijgen met antilichaam B. In Nederland gebruiken 48 labs 7 verschillende antilichamen. Een enquête uitgevoerd door Roche toonde een variatie van 7 tot 16% voor de 3+ score en van 5 tot 25% voor de 2+ score. De variaties in de resultaten van FISH/CISH zijn ook relatief groot (VAP Visie 4, 2009). Uiteraard is deze variatie onacceptabel en moet er op zijn minst een goede interne en externe kwaliteitscontrole uitgevoerd worden. Maar op lange termijn zouden er mogelijk twee meer permanente oplossingen kunnen zijn:

- 1 antilichaam kiezen dat door alle pathologische laboratoria gebruikt wordt in Nederland. De vraag blijft dan natuurlijk welk antilichaam te kiezen, en een "monopolie" zal waarschijnlijk leiden tot hogere prijzen
- Zoeken naar een andere betrouwbare diagnostische test die gemakkelijk uit te voeren en te interpreteren is, met als gevolg meer reproduceerbare resultaten

Centraliseren?

Elk jaar krijgen ongeveer 12,000 vrouwen de diagnose borstkanker in Nederland en 2,400 (tot 20%) van deze vrouwen hebben een amplificatie/overexpressie van HER2. Als we aannemen dat gemiddeld 10% van deze resultaten fout-positief zijn, kan dit leiden tot een onnodige, cardiotoxische (= gevaarlijk voor het hart) en dure therapie bij 240 patiënten per jaar. Waarschijnlijk zijn er ook ongeveer 2% fout-negatieve testresultaten, wat betekent dat ongeveer 200 patiënten ten onrechte geen trastuzumab zullen krijgen, met onnodige sterfgevallen tot gevolg. In een Frans onderzoek waar IHC resultaten met FISH vergeleken werden, waren er 2.9% fout-positieve en 2.2% fout-negatieve IHC resultaten in de grotere laboratoria, tegenover 13.8% fout-positieve en 4.5% fout-negatieve resultaten in kleinere locale laboratoria. Er zouden dus drie mogelijkheden kunnen zijn:

- Alle IHC testen centraliseren in referentie laboratoria die vele testen per jaar uitvoeren. Dit zou de kwaliteit kunnen opvoeren maar zal waarschijnlijk ook leiden tot een zekere vertraging bij het vervolledigen van het tumorprofiel.
- Het veiligheidsnet van genamplificatie testen vergroten naar alle samples (niet alleen 2+). Dit kan als kwaliteitscontrole dienen en zou mogelijke fout-positieve en -negatieve uitslagen kunnen verhelpen. Uiteraard is dit een duurdere oplossing.
- Op zoek gaan naar een betere alternatieve primaire diagnostische test die meer consistente, betrouwbare, betaalbare maar ook gemakkelijk te interpreteren resultaten oplevert.

Er zijn momenteel twee FDA goedgekeurde genamplificatie testen beschikbaar: fluorescentie *in situ* hybridisatie (FISH) en chromogene *in situ* hybridisatie (CISH). FISH is een relatief moeilijke en dure techniek, en de fluorescent gelabelde anti-HER2 probe (= bindt aan een stukje DNA zoals bijvoorbeeld een gen) vervaagt met de tijd. CISH resultaten, daarentegen, kunnen onbeperkt bewaard blijven net als bij IHC. Van 29 laboratoria die meededen aan de kwaliteitsrondzendingen georganiseerd door SKML (Stichting Kwaliteitsbewaring Medische Laboratorium-diagnostiek) in 2009, gebruikten 45% van de labs CISH als genamplificatie techniek, 42% FISH en 13% gebruikten een andere techniek. De interpretatie van IHC, FISH en CISH is semi-kwantitatief, wat onvermijdelijk leidt tot interpretatieverschillen onder pathologen.

Twee-kleuren FISH (Pathvision) werd goedgekeurd door de FDA in 1998, en in 2008 kreeg ook CISH (Invitrogen) een FDA goedkeuring. CISH het voor de hand liggende voordeel dat de signalen beoordeeld kunnen worden met een

lichtmicroscoop maar de meeste beschikbare CISH assays op dit moment zijn nog steeds monoprobe assays (= bevatten maar 1 probe), wat betekent dat alleen naar de aanwezigheid van meerdere kopieën van HER2 gekeken wordt. Dit betekent dat low-level amplificaties – dewelke minder frequent voorkomen dan high-level amplificaties – zouden verward kunnen worden met polysomie (= bij tumoren zijn vaak drie of meer kopieën van een chromosoom aanwezig in plaats van de verwachte twee kopieën, zie verder). Nochtans, zoals we in hoofdstuk 7 aantoonden, is echte polysomie van chromosoom 17 erg zeldzaam bij borstkanker. Er bestaat nu ook een moderne CISH (Dako DuoCISH) versie die bestaat uit zowel een HER2 probe als een CEP17 (=centrale deel of centromeer van chromsoom 17) probe. Bovendien heeft Ventana nu ook een FDA goedkeuring aangevraagd voor hun volautomatische INFORM™HER2 Silver in situ Hybridization (SISH) technologie die binnen 6 uren een diagnose mogelijk maakt en gebruik maakt van metallografie waarbij het gebonden HRP (horseradish peroxidase, een enzym dat een zwak signaal kan vermenigvuldigen om de detecteerbaarheid van het signaal te versterken) de reductie van zilveracetaat katalyseert om een zwart signaal te produceren. Op dit moment is er geen consensus over welke techniek nu de betere is, noch over het gebruik van mono- of duoprobe systemen, noch over het gebruik van manuele of geautomatiseerde systemen. Voor een lange tijd is FISH de "gouden standaard" geweest om HER2 amplificaties te beoordelen maar op dit moment gaan meer en meer laboratoria CISH gebruiken hoewel recente rapporten van de SKML kwaliteitscontrole aangaven dat CISH resultaten toch meer variabiliteit vertoonden dan FISH resultaten.

Omdat er een noodzaak is aan een meer kwantitatieve en dus gemakkelijk interpreteerbare techniek om HER2 amplificatie te beoordelen, introduceerden we een nieuwe diagnostische test in **hoofdstuk 4**, MLPA genaamd, en vergeleken we deze nieuwe test met IHC en CISH bij 321 borsttumoren. De overeenkomst tussen MLPA en CISH was 94% en tussen MLPA en IHC 90% (later aangepast naar 91% en 88% gebruik makend van een aangepaste MLPA interpretatie grenswaarde). Zowel de sensitiviteit als de specificiteit van MLPA was vergelijkbaar met die van IHC, wanneer CISH als gouden standaard beschouwd werd. We concludeerden dat MLPA een goedkope en accurate manier is om HER2 amplificatie te detecteren bij borstkanker en dus een goed alternatief of een supplementaire techniek zou kunnen zijn voor andere genamplificatie testen zoals FISH of CISH. Een amplificatie test zoals MLPA zou misschien zelfs geschikt kunnen zijn om als pre-screening tool te gebruiken, als alternatief voor IHC. MLPA is een gemakkelijke, goedkope en meer kwantitatieve techniek dan IHC die een vanzelfsprekende interpretatie toelaat hoewel de dynamische range (het maximaal aantal kopieën dat nog onderscheiden kan worden) wat minder lijkt te zijn dan bij FISH. Bovendien, aangezien MLPA tot wel 45 genen kan analyseren in 1 assay, kunnen verschillende genen die belangrijk zijn voor therapie selectie en/of prognose gelijktijdig getest worden voor amplificatie in dezelfde analyse, zoals aangetoond in hoofdstuk 8. Nochtans heeft MLPA het nadeel dat het een

niet-morfologische (visueel ontoegankelijke) techniek is die kan resulteren in het over het hoofd zien van heterogeniteit en DCIS (ductaal carcinoma *in situ* = nog ingekapseld). Dit kan deels opgevangen worden door een sequentiële H&E (haematoxyline en eosine, kleurt de kernen en het bindweefsel aan) coupe te maken. Een ander nadeel van MLPA is dat de resultaten afhangen van het tumor percentage van het te analyseren sample. Hoewel we al in hoofdstuk 4 aantoonden dat MLPA een betrouwbare en gevoelige techniek is die amplificaties kan detecteren in samples met een tumor percentage van maar 10%, kan het tumor percentage toch bepalend zijn voor de betrouwbaarheid van de resultaten aangezien kleine low-level geamplificeerde klonen (= ontstaan uit 1 cel) onopgemerkt zouden kunnen blijven bij lage tumorpercentages.

Waarom is het percentage HER2 amplificatie en overexpressie bij ons zo klein?

Het percentage van HER2 amplificatie en overexpressie in onze studies ligt in de lagere range van de 20-30% positiviteit die beschreven wordt in de literatuur. Methodologische variatie is een onwaarschijnlijke verklaring aangezien de fractie van HER2 geamplificeerde tumoren bij IHC, MLPA en CISH vergelijkbaar was, en omdat onze studie groep successieve patiënten betrof kan selectie bias uitgesloten worden. Het is mogelijk dat vele van de studies waarin hogere HER2 overexpressie/amplificatie frequenties beschreven werden toch geselecteerde patiëntengroepen gebruikt hebben. Bovendien hebben verschillende andere studies en andere ziekenhuizen in Nederland lagere (10-18%) percentages beschreven. Eén studie [1] deed onderzoek naar het percentage HER2 positieve tumoren in het midden-noorden en het zuidwesten van Nederland. Er was een significant verschil tussen het percentage positiviteit in beide regio's. Mogelijke verklaringen die gegeven werden door oncologen waren "een relatief oude populatie (noorden)" en "een vaak niet-caucasische populatie (midden)". Diezelfde studie vond een gemiddeld percentage van 15% HER2 positieve tumoren in Nederland; in het midden-noorden was dit percentage zelfs kleiner in de meeste ziekenhuizen. Wat deze regionale verschillen veroorzaakt is nog onduidelijk en vereist verder onderzoek.

In **hoofdstuk 5** evalueerden we de betrouwbaarheid van MLPA om HER2 amplificatie testen uit te voeren, voornamelijk bij het analyseren van samples die veel normaal borstweefsel en/of *in situ* componenten (= nog ingekapseld en dus niet invasief) bevatten - resulterend in een laag tumor percentage. We onderzochten de invloed van het uitvoeren van manuele (met een scalpel) of laser microdissectie op de sensitiviteit van MLPA om HER2 amplificaties te detecteren. De overeenkomst tussen MLPA en ISH verbeterde van 61% naar 84% na manuele microdissectie, en naar 90% na laser microdissectie (later aangepast

naar respectievelijk 58%, 74% en 77%, gebruik makend van een verbeterde MLPA cut-off waarde). Aangezien de beste correlaties tussen MLPA en ISH gezien werden bij tumor percentages hoger dan 30%, concludeerden we dat microdissectie voorafgaand aan MLPA niet routinematig nodig is maar wel aan te raden is bij heel lage tumor percentages (≤30%), wanneer de MLPA resultaten niet eenduidig zijn, en wanneer uitgebreid ductaal carcinoma *in situ* aanwezig is.

In hoofdstukken 2 tot 5 hebben we de nadruk gelegd op HER2 amplificatie bij borstkanker. Maar natuurlijk is HER2 niet het enige gen dat een rol speelt bij de ontwikkeling en groei van een tumor. Vele andere genen worden op dit moment wereldwijd onderzocht en sommige onder hen zouden ook mogelijke therapeutische targets kunnen zijn (bijvoorbeeld TOP2A, zie introductie). In de toekomst zal dit een behoefte aan een eenvoudige en betaalbare diagnostische test met zich meebrengen die gelijktijdig genetische veranderingen van verscheidene genen kan detecteren. In **hoofdstuk 6** evalueerden we MLPA als techniek om gelijktijdig naar genetische veranderingen van HER2 en TOP2A te kijken. Van de 353 geanalyseerde patiënten toonden 9% een TOP2A amplificatie met MLPA en 12% van de patiënten had een HER2 amplificatie (later aangepast naar 24% en 17% respectievelijk, gebruik makend van een aangepaste MLPA cut-off waarde). TOP2A amplificatie werd gezien bij 42% van de HER2 geamplificeerde tumoren (later aangepast naar 45%) en, zoals reeds in andere studies beschreven, werden er geen TOP2A amplificaties gezien zonder het optreden van HER2 amplificatie. Bij elf patiënten werd een verlies van het TOP2A gen (3%) gedetecteerd wat ook door andere studies bevestigd wordt: de prevalentie van TOP2A deleties bij borstkanker varieert tussen 2% en 11% in verschillende studies. Concordantie tussen MLPA en CISH was 91% voor TOP2A en 96% voor HER2 (later aangepast naar 82% en 93% respectievelijk, gebruik makend van een gereviseerde MLPA cut-off waarde). MLPA kan dus gelijktijdig HER2 en TOP2A kopie aantallen nagaan. De correlatie tussen amplificatie en overexpressie van TOP2A was significant (p=0.035), maar amplificatie voorspelde niet altijd een overexpressie van het eiwit. Andere factoren, voornamelijk de tumor proliferatie status (hoe meer celdelingen, hoe hoger de proliferatie status) kunnen de TOP2A eiwit status beïnvloeden aangezien TOP2A een marker voor proliferatie is en de expressie ervan afhangt van de celcyclus. Verlies van het TOP2A gen correleerde bijna nooit met een verlies van het eiwit.

Wat is de klinische betekenis van meer of minder TOP2A genkopieën?

Anthracyclines zijn een groep zeer effectieve anti-tumor geneesmiddelen die gepaard kunnen gaan met hartschade. Gedurende de afgelopen 15 jaren hebben grote hoeveelheden klinische data van vele individuele studies aangetoond dat het extra voordeel van adjuvante (= ter ondersteuning van een behandeling) anthracycline-gebaseerde therapieën voornamelijk beperkt blijft tot de HER2-positieve subgroep van humane borstkankers (15-20%), maar dat de overgrote meerderheid van de huidige adjuvante regimens voor bijna *alle* borstkanker patiënten wereldwijd anthracycline gebaseerd zijn. Een recente meta-analyse toonde aan dat er weinig of geen voordeel was voor patiënten wiens tumoren HER2 normaal waren [2], hoewel deze patiënten een risico blijven lopen op de toxiciteit geassocieerd met anthracycline gebruik. Het feit dat anthracyclines geen effect hebben bij HER2-negatieve patiënten suggereert dat deze groep patiënten van de onnodige bijwerkingen gespaard zou kunnen blijven. Minstens 10 gepubliceerde studies geven aan dat bijna alle TOP2A amplificaties voorkomen in de subgroep van de HER2 geamplificeerde borsttumoren en dus een co-amplificatie fenomeen zijn. De associatie van TOP2A deleties met een betere respons op anthracyclines gevonden door sommige studies spreekt andere *in vivo* en *in vitro* studies tegen die aantoonden dat TOP2A deleties juist gepaard gaan met anthracycline resistentie [3]. Verder onderzoek is nodig om dit uit te zoeken. Het is zeer waarschijnlijk dat de meeste verschillen tussen studies die gekeken hebben naar de prevalentie van TOP2A genkopie wijzigingen te wijten zijn aan de technische verschillen bij het meten van deze veranderingen, namelijk de verschillende ratio's en/of cut-off waarden die gebruikt werden voor het indelen van tumoren in "amplificatie"en "deletie", en de verschillende technologieën en/of reagentia gebruikt om deze veranderingen te meten.
Op dit moment geeft de grote meerderheid van de data aan dat TOP2A gen veranderingen belangrijke predictieve (=voorspellende) factoren zijn voor het bepalen van de kans op het ondervinden van een extra voordeel door anthracyclines toe te dienen bij de adjuvante behandeling van borstkanker.

HER2 en TOP2A liggen beide op chromosoom 17. Een chromosoom is een stuk opgerold DNA dat vele genen bevat. Humane cellen hebben 22 verschillende "autosomen", elk aanwezig in 2 kopieën, en twee geslachtschromosomen. Dit geeft een totaal van 46 (=22x2 +2) chromosomen. In tumoren zijn vaak drie of meer kopieën van een chromosoom aanwezig in plaats van de verwachte twee kopieën. Dit fenomeen wordt polysomie genoemd en bij borstkanker is polysomie van chromosoom 17 uitgebreid beschreven en van belang bij therapiekeuze (zie introductie). In *hoofdstuk 7* gebruikten we het multiplex

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aspect van MLPA om polysomie van chromosoom 17 te bestuderen en toonden we aan dat polysomie 17 helemaal niet zo frequent is als beschreven in de literatuur gebaseerd op de amplificatie van het centromeer van chromosoom 17 (CEP17, = centrale deel van het chromosoom, scheidt de lange "q" en de korte "p" arm van elkaar). Geen van de 117 patiënten die d.m.v. MLPA geanalyseerd werden vertoonde een volledige chromosoom 17 polysomie op basis van een verhoogd kopie aantal van alle 17 geanalyseerde probes gelegen op chromosoom 17. Maar 2% van de patiënten had een amplificatie van alle geanalyseerde probes op de lange (q) arm van chromosoom 17, wijzend op een "gain" van de 17q arm. Chromosoom 17 toont meestal een complex patroon van "gain" en "loss", niet gerelateerd aan het kopie aantal van het centromeer (CEP17).

Uitgezonderd van enkele studies is het op dit moment niet geweten of patiënten met amplicons (= regio van amplificatie) die HER2 en CEP17 overbruggen beter of slechter zullen reageren op trastuzumab. Twee studies [4, 5] toonden aan dat patiënten met "polysomie" die FISH negatief waren (maar wel IHC3+) een respons vertoonden op trastuzumab wat aangeeft dat FISH kan leiden tot vals negatieve bevindingen gebaseerd op de CEP17 amplificatie. De HER2/CEP17 ratio gebruikt door FISH (en in mindere mate door CISH) zou dus wel eens niet de beste manier kunnen zijn om de HER2 status te evalueren in alle gevallen en het absolute HER2 kopie aantal (of dit nu door amplificatie of polysomie veroorzaakt wordt) zou wel eens de belangrijkere determinant kunnen zijn voor trastuzumab respons bij sommige patiënten. Deze data zijn zeker intrigerend maar nog preliminair, en er moet voorzichtig omgegaan worden met het interpreteren van deze resultaten totdat data van meer patiënten onderzocht kunnen worden. In onze studie hadden tot 55% van de HER2-geamplificeerde patiënten een polysomie gebaseerd op CEP17 CISH of MLPA. Verscheidene van deze patiënten zouden waarschijnlijk niet in aanmerking gekomen zijn voor trastuzumab gebaseerd op de HER2/CEP17 ratio. Uiteraard gebruiken de meeste laboratoria IHC en niet FISH als primaire test en zouden patiënten met een IHC 3+ score geen genamplificatie test vereisen volgens de huidige richtlijnen.

Naast HER2 en TOP2A zijn er nog vele andere genen gevonden die een rol spelen bij de respons op therapie zoals bijvoorbeeld de oestrogeen receptor (ESR1) amplificatie bij de respons op tamoxifen therapie en MYC genamplificatie en resistentie bij anti-oestrogeen therapie. De therapeutische significantie van andere frequent geamplificeerde genen zoals Cycline D1 (CCND1) of frequent verlies van genen zoals E-cadherine (CDH1) is minder duidelijk. Klassieke en cytogenetische moleculair genetische toepassingen hebben aangetoond dat bij borstkanker bijna elk chromosoom tenminste 1 gebied heeft dat betrokken is bij kanker-geassocieerde genetische veranderingen (loss, amplificatie, mutatie, of veranderde DNA methylatie). Dit heeft tot gevolg dat het aantal genen met veranderingen die geïdentificeerd worden elk jaar toeneemt. Tumor heterogeniteit (en dus de heterogeniteit van het klinisch gedrag) zou het gevolg

kunnen zijn van een onderliggende variatie in genkopie aantallen en gen-interacties. In **hoofdstuk 8** gebruikten we alweer het multiplex aspect van MLPA om te kijken naar veranderingen in het aantal kopieën van 20 genen die op verschillende chromosomen gelegen zijn (6, 7, 8, 11, 17, 19 and 20) en bestudeerden we hun co-amplificaties en hun associatie met klinisch/pathologische karakteristieken van de tumoren (tumorgrootte, graad van de tumor, leeftijd van de patiënt, …) bij 104 patiënten. Er was een significante correlatie tussen het aantal genen met amplificaties per tumor en de histologische graad en mitose index (= aantal celdelingen) van de tumor. Amplificaties van ESR1, PRDM14, MYC en HER2 waren geassocieerd met een hogere mitose index, en PRDM14 en HER2 amplificaties met een hogere histologische graad. Tumoren met HER2/MYC, HER2/CCNE1 en EGFR/MYC co-amplificaties waren significant groter dan tumoren met maar 1 van deze amplificaties. Genkopie verlies werd vooral gevonden voor CDH1 en FGFR1.

MLPA: voor onderzoek of diagnostiek?

Het aantal nieuwe genen geïdentificeerd als zijnde "abnormaal" bij borstkanker blijft stijgen. Tumor heterogeniteit (en dus ook klinische heterogeniteit) zou het gevolg kunnen zijn van een onderliggende variatie in genkopie aantallen en genetische interacties. Om een zo optimaal mogelijke patiëntgerichte therapie te kunnen bieden in de toekomst, zal het dus van uiterst belang zijn dat technieken zoals Comparative Genomic Hybridization (CGH), microarray en MLPA hun zoektocht naar oncogenen en tumor suppressor genen doorzetten en hun gen-gen interacties evalueren. Hoewel CGH het voordeel heeft dat het een genoom-wijde scope heeft, is het dure en arbeidsintensieve techniek die een relatief grote hoeveelheid DNA vereist en opgeleid personeel om met de complexiteit van de data om te kunnen gaan. MLPA is zonder twijfel een veelbelovende techniek voor het onderzoek (zoals aangetoond in hoofdstukken 7 en 8) die op dit moment wereldwijd wordt gebruikt en toepassingen heeft gevonden in het bepalen van genkopie aantallen, genexpressie en methylatie (= een natuurlijk controle mechanisme dat de werking van genen regelt).

Eén van de grote voordelen van MLPA is het multiplex aspect: het is mogelijk om 45 MLPA probes gelijktijdig te analyseren zodat verschillende predictieve en prognostische genen getest kunnen worden tijdens dezelfde analyse, en bovendien kunnen verscheidene probes gebruikt worden voor hetzelfde gen als extra controle voor amplificatie/loss. De eerste stap die zal moeten genomen worden om MLPA klinisch te valideren als diagnostische test (allereerst voor HER2 amplificatie) is het koppelen van MLPA resultaten aan klinische respons data. Dit zal zeer zeker het onderwerp zijn van verder onderzoek. Op dit moment zullen veel pathologen waarschijnlijk moeite hebben om MLPA te aanvaarden als diagnostische test, zeker als primaire screening test, omdat het een niet-morfologische techniek is. Nochtans hebben we aangetoond dat, tenminste voor

HER2, er een goede correlatie was tussen MLPA, IHC en ISH en dat bij ongeveer 85% van de tumoren er geen noodzaak is om manuele microdissectie uit te voeren voorafgaand aan MLPA. Toch denken we uit plaatselijke ervaring dat, voor het analyseren van vele andere genen die vaak lagere amplificaties vertonen dan HER2, het aan te raden is om toch manuele microdissectie te gebruiken. Dit zou MLPA uiteraard iets minder toegankelijk maken maar het afschrapen van de tumor met een scalpel is maar een kleine moeite en is in ons laboratorium standaard voor routine diagnostische mutatie analyse.

Het interpreteren van MLPA resultaten is veel eenvoudiger dan voor IHC of ISH, tevens kunnen MLPA data van verschillende labs opgeslagen worden in databases wat verder en uitgebreider onderzoek naar borstkanker eenvoudiger mogelijk maakt. Nochtans moeten bij de interpretatie de cut-off waarden tussen "geen"en "low-level" amplificatie en tussen "low-level" en high-level" amplificatie zorgvuldig gekozen worden. Voor onze eerste MLPA studies hebben we een cut-off waarde van 1.5 gebruikt om een onderscheid te maken tussen "geen" en "low-level"amplificatie, gebaseerd op onze eigen ervaring met MLPA kits die gebruikt worden voor trisomie (= aanwezigheid van 3 kopieën van een bepaald chromosoom) detectie. Nochtans bleek een cut-off waarde van 1.3 (delta waarde 0.3) in plaats van 1.5 beter gevalideerd te zijn door verscheidene andere studies en bleek deze waarde een betere schatting te geven van de amplificatie status. Bovendien adviseert het software programma van de fabrikant (Coffalyser) ook een cut-off waarde van 1.3 voor low-level amplificatie. Daarom hebben we onze data opnieuw geanalyseerd met 1.3 als nieuwe experimenteel gevalideerde cut-off waarde (zie addenda hoofdstuk 4, 5 en 6) wat de sensitiviteit van MLPA om HER2 amplificaties te detecteren verbeterde. Uiteraard kan de optimale cut-off enkel bepaald worden wanneer klinische respons data beschikbaar zijn. Bovendien zijn ook goed gekozen controle probes in elke MLPA kit van cruciaal belang. Omdat er bij tumoren veranderingen optreden in de kopie aantallen van genen die verspreid zijn over verschillende chromosomen, is het vaak moeilijk om nog controle probes te vinden die zich in stabiel gebleven gebieden van het genoom bevinden. Hoe stabieler de controle probes, hoe betrouwbaarder de MLPA resultaten zullen zijn, aangezien de robuustheid van het normalisatie proces afhangt van het aantal referentie probes en hun chromosomale locatie. Het zal enige tijd en feedback naar de fabrikant (MRC Holland) vergen vooraleer elke MLPA mix de meest stabiele controle probes bevat. Figuur 1 toont een voorbeeld van de stabiliteit van de referentie probes in de P004-B1 mix die gebruikt werd in hoofdstuk 7.

De controle probe gelegen op 10q22b (VCL gen, vinculine) lijkt een betrouwbare probe te zijn bij borstkanker met een kleine standaarddeviatie (gemiddelde 0.98 +/- 0.10) en bijna alle ratio's liggen tussen 0.7 en 1.3 (minimum ratio 0.755 en maximum ratio 1.315). De controle probe gelegen op 22q13 (SBF1 gen), bijvoorbeeld, dient wel vervangen te worden (0.75 +/- 0.18).

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Gezien de lage kosten, de reproduceerbaarheid, de betrouwbaarheid (zoals aangetoond in de vorige hoofdstukken), het gebruiksgemak, en de eenvoud bij interpretatie, zal MLPA in de toekomst zonder twijfel een rol gaan spelen in de diagnostiek. Maar voordat dit echt mogelijk is, moeten MLPA mixen verder geoptimaliseerd en verder klinisch gevalideerd worden.
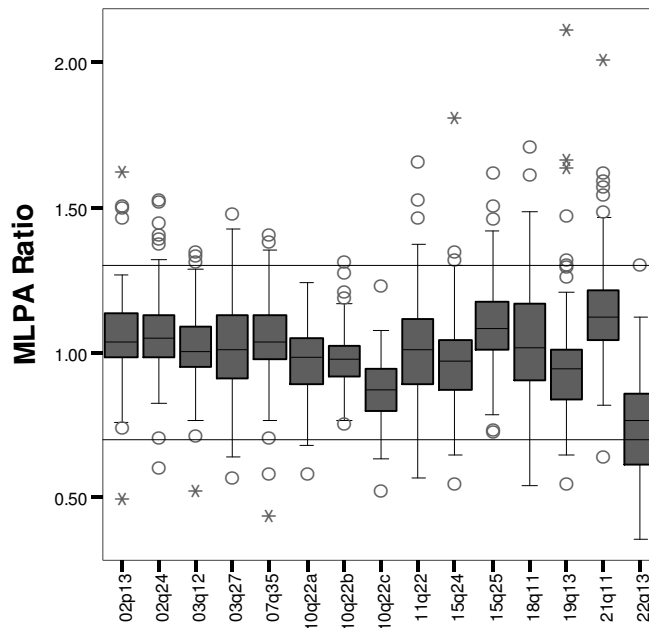
Figuur 1. MLPA ratio resultaten voor alle 15 controle probes in the P004-B1 borstkanker chromosoom 17 MLPA mix bij 112 borstkanker patiënten. Alle ratio's horen eigenlijk tussen 0.7 en 1.3 te liggen.

Conclusies van dit proefschrift

- Er bestaat op dit moment geen gouden standaard voor HER2 detectie. Elk lab gebruikt de IHC en/of ISH testen die het meest voor de hand liggen en/of het minst duur zijn. Dit kan leiden tot zowel overbehandeling als onderbehandeling van patiënten. Een goede kwaliteitscontrole en een adequate validatie van nieuwe technieken zijn cruciaal voor elk laboratorium. Centralisatie en verdere standaardisatie zijn beide een mogelijkheid.
- Een volledig automatische HER2 IHC aankleuring met het monoclonale antilichaam van de Oracle kit toonde een 94% overeenkomst met de manuele aankleuring met het polyclonale antilichaam van de HercepTest en kan dus gebruikt worden als een alternatieve methode om HER2 expressie te evalueren bij borstkanker met mogelijk minder analytische variabiliteit.

- MLPA kan gelijktijdig en betrouwbaar de kopie aantallen van HER2, TOP2A en vele andere genen die op verschillende chromosomen liggen, analyseren.
- Laser microdissectie is niet noodzakelijk voorafgaand aan de MLPA, en manuele microdissectie is aan te raden voor het bepalen van kopie aantallen van genen met low-level amplificaties, voor het analyseren van samples met een erg laag tumor percentage (≤30%) en voor borsttumoren met een uitgebreide DCIS component.
- De borstkanker-specifieke MLPA kit kan gelijktijdig genkopie aantallen bepalen van verschillende genen die belangrijk zijn gebleken bij de carcinogenese en de progressie van borstkanker.
- Polysomie 17 is erg zeldzaam bij borstkanker. Het blijft af te wachten wat de implicaties zijn van deze bevinding voor de diagnostische testen die gebruik maken van twee-kleuren systemen (HER2 en CEP17) zoals FISH en CISH. Bovendien vereist het effect van centromeer (co-)amplificatie op de respons op trastuzumab en anthracylines verder onderzoek.

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Curriculum vitae

Curriculum vitae

The author of this thesis, Cathy Moelans, was born on November 3, 1979, Belgium. In 1997 she graduated from the Sancta Maria Institute in Deurne with a specialisation in Latin and Mathematics. Directly after her graduation, she started to study Biomedical Science at the University of Antwerp, leading to her bachelor diploma in 1999. She completed her study in Medical Biochemistry at the University of Antwerp and received her master diploma with great honour (magna cum laude) in 2001. Immediately after receiving her diploma, she started her function as assisting academic personnel at the department of Cell- and Tissue Research of the University of Antwerp. Next to her research concerning the functional histology of the enteric nervous system, she was involved in the practical education of students in cell- and tissue structure. In 2003, Cathy moved to the Netherlands which made her decide to end her job at the University of Antwerp in 2004. In 2005 she found a new job as an immunohistochemical technician at the department of Pathology of the University Medical Center of Utrecht. In 2007 she decided to start a PhD next to her function as technician and in 2008, Cathy was given the opportunity to do one year of fulltime research so that she could pursue her PhD. In this year, Cathy worked for 5 months at MRC Holland in Amsterdam to design new probes that play an important role in breast cancer. After her promotion, Cathy will start a research project at the department of Pathology on formalin-free fixation which could improve laboratory efficiency by eliminating any pathogenic and carcinogenic risks of formalin.

Curriculum vitae

De auteur van dit proefschrift, Cathy Moelans, werd op 3 november 1979 geboren te Wilrijk, in België. In 1997 behaalde zij haar middelbare school diploma aan het Sancta Maria Instituut te Deurne met als specialisatie Latijn en Wiskunde. Direct daarna startte zij de studie Biomedische Wetenschappen aan de Universiteit Antwerpen, leidend tot haar kandidatuur (bachelor) diploma in 1999. Zij vervolgde haar studie in de richting Medische Biochemie aan de Universiteit Antwerpen en behaalde haar licentiaat (master) diploma in 2001 met grote onderscheiding. Onmiddellijk hierna startte ze haar baan als assisterend academisch personeel bij de dienst Cel- en Weefselleer van de Universiteit Antwerpen. Naast het verrichten van functioneel histologisch onderzoek met betrekking tot de darmwand innervatie, was zij ook betrokken bij het begeleiden van het praktisch onderwijs in de cel- en weefselleer. In 2003 verhuisde Cathy naar Nederland en besloot in 2004 om haar functie aan de Universiteit Antwerpen neer te leggen. In 2005 vond zij een baan bij de afdeling Pathologie van het Universitair Medisch Centrum Utrecht en in 2007 besloot zij om te gaan promoveren naast haar functie als specieel analist. In 2008 kreeg Cathy de mogelijkheid om een jaar deze functie naast haar neer te leggen, zodoende kon zij fulltime aan haar promotie onderzoek werken. Bovendien werkte zij in dit jaar 5 maanden bij MRC-Holland te Amsterdam waar zij nieuwe probes ontwikkelde tegen genen die een belangrijke rol spelen bij borstkanker. Na haar promotie zal Cathy bij de afdeling Pathologie een onderzoek starten naar het formaline-vrij werken op het lab.

List of publications

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C.B. Moelans, K.P.M. Suijkerbuijk, P. van der Groep, E. van der Wall, R.A. de Weger en P.J. van Diest
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Submitted for publication

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Dankwoord

Dankwoord

Een promotie is een traject met veel ups en downs waar veel mensen aan bijdragen en 1 ding is zeker: iedereen die het ver gebracht heeft, dankt dit meer aan zijn kennissen dan aan zijn kennis. Toen ik als schuchter Belgje kwam werken bij de afdeling pathologie, was een promotie onderzoek absoluut niet wat ik in gedachten had. Daar ben ik min of meer ingerold, met een duwtje van Prof. Dr. PJ van Diest natuurlijk. Bedankt, Paul, voor het eerste zetje. Ik denk dat de meesten onder ons intussen wel weten dat je probeert uit iedereen het beste te halen, met als gevolg dat je dit jaar volgens mij een record aantal promoties onder je naam hebt staan? Ondanks je drukke agenda wist je altijd wel ergens een gaatje te vinden om nog wat te verbeteren onder het motto "too many words". Je betrokkenheid en motivatie bij het hele proces is bewonderenswaardig, ik denk dat elke promovendus heel erg blij mag zijn met jou als promotor. Ook Dr. RA de Weger mag ik niet in dit rijtje vergeten. Roel, bedankt voor het vele en vaak erg snelle lees- en verbeter werk, voor alle goede raad en omdat je ondanks je soms drukke dagschema toch steeds tijd voor me maakte om coupes te kijken of om even wat te bespreken. Ik kon altijd bij je binnenvallen – wat het ook was; met jou als co-promotor en Paul als promotor heb ik me nooit verloren gevoeld. Bedankt!

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