RESEARCH ARTICLE

Detection of Gene Amplification by Multiplex Ligation-Dependent Probe Amplification in Comparison with In Situ Hybridization and Immunohistochemistry

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Abstract

Gene amplification is an important mechanism in the development and progression of cancer. Currently, gene amplification status is generally determined by in situ hybridization (ISH). Multiplex ligation-dependent probe amplification (MLPA) is a PCR-based method that allows copy number detection of up to 50 nucleic acid sequences in one reaction. The aim of the present study was to compare results for *HER2*, *CCND1*, *MYC* and *ESR1* gene amplification detected by MLPA with fluorescent in situ hybridization (FISH) and chromogenic in situ hybridization (CISH) as clinically approved methods. Tissue samples of 170 invasive breast cancers were collected. All were ER positive. Tissue samples had previously been tested for *HER2* using immunohistochemistry. Amplification of the selected genes were assessed using MLPA, FISH and CISH and results were compared. *HER2* MLPA and ISH results were also compared with *HER2* immunohistochemistry (IHC) which detects protein overexpression. Amplification of *HER2*, *CCND1*, *MYC* and *ESR1* by MLPA were found in 9%, 19%, 20% and 2% of samples, respectively. Amplification of *HER2*, *CCND1*, *MYC* and *ESR1* by FISH was noted in 7%, 16%, 16% and 1% of samples, respectively. A high level of concordance was found between MLPA/ FISH (*HER2*: 88%, *CCND1*: 88%, MYC: 86%, ESR1: 92%) and MLPA/ CISH (*HER2*: 84%). Of all IHC 3+ cases, 91% were amplified by MLPA. In IHC 2+ group, 31% were MLPA amplified. In IHC 1+ group, 2% were MLPA amplified. None of the IHC 0 cases were amplified by MLPA. Our results indicate that there is a good correlation between MLPA, IHC and ISH results. Therefore, MLPA can serve as an alternative to ISH for detection of gene amplification.

Keywords: Amplification - MLPA - FISH - CISH - breast cancer

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Introduction

Gene amplification is an important mechanism in the development and progression of cancer. Many genes undergo amplification during development of breast cancer, including *HER2*, *MYC*, *CCND1*, *AIB1*, *ESR1*, *EGFR* and *FGFR1* (Courjal et al., 1996; Anzick et al., 1997; Ross and Fletcher, 1998; Deming et al., 2000; Al-Kuraya et al., 2004; Park et al., 2007; Holm et al., 2012; Holst et al., 2012). Human epidermal growth factor receptor 2 (*HER2*) oncogene is located on chromosome 17q12 and codes for a 185 kd transmembrane protein with tyrosine kinase activity (Schechter et al., 1985). *HER2* gene is amplified and/or overexpressed in 15% to 20% of primary breast cancers and is regarded as an established prognostic and predictive biomarker for breast cancer (Slamon et al., 2001; Owens et al., 2004; Wolff et al., 2013). *CCND1* gene is located on chromosome 11q13 and encodes for cyclin D1 protein which is central to the regulation of G1-S phase transition (Santarius et al., 2010; Musgrove et al., 2011). *CCND1* gene is amplified in 15% to 20% and overexpressed in 50% to 70% of breast cancers (Musgrove et al., 2011). A number of studies have reported *CCND1* gene amplification to be a predictor of worse prognosis in breast cancer (Elsheikh et al., 2008; Lundgren et al., 2012). *MYC* oncogene, which is located on chromosome 8q24, encodes for a transcription factor that has an integral role in cell cycle progression, differentiation and apoptosis (Chen and Olopade, 2008; Xu et al., 2010). The frequency of *MYC* gene amplification in breast cancer is 12% to 19% (Deming et al., 2000). Amplification of *MYC* gene has been shown to be indicative of poor prognosis in breast cancer (Deming et al., 2000, Schlotter et al., 2003). *ESR1* gene is located on chromosome 6q25 and encode for estrogen receptor α (ERα). Approximately, 70% of breast tumors express ERα which regulates the transcription of estrogen responsive genes (Harvey et al., 1999). Recently, some investigators have suggested that *ESR1* amplification occurs frequently in breast cancer (13% to 20%) and has predictive value for

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hormone therapy (Holst et al., 2007; Ejlertsen et al., 2012), although, others have reported a much lower frequency (2% to 6%) (Moelans et al., 2010; Ooi et al., 2012).

Currently, gene amplification status is determined by immunohistochemistry (IHC) or by in situ hybridization (ISH). IHC is the most common method to assess *HER2* status, since there is a strong correlation between *HER2* overexpression determined by IHC and the presence of *HER2* amplification (Jimenez et al., 2000; Pauletti et al., 2000; Lebeau et al., 2001; Ghaffari et al., 2011). IHC is a routine method available in all pathology laboratories, but IHC analysis is based on subjective interpretation of staining intensity to assign a protein expression score of 0/1+ (regarded as IHC negative), 2+ (regarded as equivocal) and 3+ (regarded as positive) (Wolff et al., 2013). This method is liable to poor tissue fixation and there are some problems with reproducibility of IHC results. Only a minority of IHC 2+ cases have actually *HER2* amplification by FISH and even among IHC 3+ cases there are some false positive results (Tsuda et al., 2001, Tubbs et al., 2001, Bartlett et al., 2003, Dowsett et al., 2003). Most of the commercially available antibodies against *HER2* have a wide range of sensitivity and specificity (Press et al., 1994). Furthermore, there is some evidence that *HER2* amplification may serve as a better predictive marker than IHC results (Pauletti et al., 2000; Bartlett et al., 2001).

Fluorescence in situ hybridization (FISH) is considered the gold standard method for detection of gene amplification. This method has several advantages over IHC assay: internal controls are included in each assay and results are quantitative. In addition, DNA is less subject to effects of tissue fixation and processing than protein (Schnitt and Jacobs, 2001). Nevertheless, FISH method is expensive, time-consuming and requires highly trained personnel. FISH signals fade over time and it is not possible to analyze the detailed morphologic features of the tumor.

Chromogenic in situ hybridization (CISH) is an alternative in situ hybridization method to analyze gene amplification. CISH allows detection of gene amplification and simultaneous histologic examination by ordinary bright field microscopy. Furthermore, CISH slides can be archived permanently. Several studies have demonstrated good correlation between CISH and FISH results (Gong et al., 2009; Garcia-Caballero et al., 2010). Nevertheless, CISH is still fairly difficult and time-consuming.

Multiplex ligation-dependent probe amplification (MLPA) is a PCR-based method that allows copy number detection of up to 50 nucleic acid sequences in one reaction (Schouten et al., 2002). MLPA requires only 50 ng DNA and can be used on partially degraded DNA extracted from formalin-fixed paraffin embedded (FFPE) tissue. In addition, MLPA results are quantitative and do not depend on subjective interpretation. The aim of the present study was to compare the results of *HER2*, *CCND1*, *MYC* and *ESR1* gene amplification detected by MLPA with FISH, CISH and IHC as clinically approved gene amplification methods.

Materials and Methods

Patient selection

Tissue samples of 170 breast cancer patients were collected in Tehran, Iran. All patients had undergone breast cancer surgery between 2004 and 2011. Written informed consent was obtained from all patients. This study was approved by the local ethical committee. For each patient, FFPE tumor blocks with at least 50 percent invasive tumor component were selected. All of the tumor samples were ER positive. All tissue samples had previously been tested for *HER2* using immunohistochemistry. IHC 3+ reactivity was defined as *HER2* positive, IHC 2+ was regarded as equivocal and IHC 1+ and IHC 0 were defined as *HER2* negative according to ASCO/CAP guideline (Wolff et al., 2013).

Multiplex ligation-dependent probe amplification (MLPA)

All of the samples were analyzed by MLPA. Briefly, hematoxylin and eosin slides were reviewed to find invasive tumor areas. Using a scalpel, tumor areas (approximately 1 cm^2) from 10-μm thick unstained tissue sections were macrodissected. DNA isolation was performed using two 10-μm thick tissue sections by QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. In Brief, tumor sections were dewaxed using deparaffinization solution (Qiagen, Hilden, Germany). Samples were lysed under denaturing conditions using proteinase K, followed by incubation at 90°C to reverse formalin crosslinking. Then DNA was purified by silica-based membranes. DNA quantitation was performed by Biowave II & Biowave II spectrophotometer (Biochrom, Cambridge, UK). DNA purity was determined by measuring the *A260/280* absorbance ratios.

The isolated DNA was used for multiplex ligation-dependent probe amplification (MLPA) analysis using the MLPA P078-C1 Breast tumor kit (MRC Holland, Amsterdam, The Netherlands), according to manufacturer's instructions. This kit contains 41 probes for 22 genes (including *HER2*, *MYC*, *CCND1*, *ESR1*). All MLPA experiments were performed in duplicate in a FlexCycler PCR Thermal Cycler (Analytikjena, Jena, Germany). Three reference samples (normal breast tissue) were included in each MLPA experiment. MLPA PCR products were separated on an ABI 3730XL sequencer and interpretation of results was performed using Coffalyser. Net software (MRC Holland). For genes with more than one probe in the kit, the mean of probe values in duplicate were used. Peak values less than 0.7 were regarded as loss, between 0.7 and 1.3 as normal, more than 1.3 as amplification and peak values more than 2 as high level amplification as previously reported (Bunyan et al., 2004; Moelans et al., 2010; Tabarestani, et al., 2014).

Fluorescence in situ Hybridization (FISH)

FISH was performed using Zyto*Light* SPEC *HER2*/CEN 17 Dual Color Probe kit, Zyto*Light* SPEC *ESR1*/CEN 6 Dual Color Probe kit, Zyto*Light* SPEC C*MYC*/CEN 8 Dual Color Probe, Zyto*Light* SPEC *CCND1*/CEN 11 Dual Color Probe (ZytoVision, Germany), according

to the manufacturer's instructions. Samples which were detected as amplified by MLPA method (either high level or low level amplified), were also analyzed by FISH. For each gene, ten samples which were detected as normal by MLPA were also analyzed by FISH.

Briefly, slides were deparaffinized in xylene, rehydrated in graded ethanol, then incubated for 15 min in Heat Pretreatment Solution Citric at 98°C. Subsequently, pepsin solution was applied to slides and they were incubated for 11 min at 37°C, washed in Wash Buffer SSC for 5 minutes, and dehydrated in graded ethanol. Next, 10 µl Zyto*Light* SPEC *HER2*/CEN 17 Dual Color Probe (or other probes) was pipetted onto slides, then slides were denatured at 75°C for 10 min and incubated overnight at 37°C in a hybridizer (Dako, Denmark). After hybridization, coverslips were removed by submerging slides in Wash Buffer A at 37°C for 1 min. Subsequently, slides were washed in Wash Buffer A at 37°C for 2×5 min, dehydrated in graded ethanol. Next, 30 µl DAPI/DuraTect Solution was applied to the slides, followed by incubation in the dark for 15 min. FISH signals were visualized using a fluorescence microscope (Nikon Eklipse E600, Japan) and XCytoGen software (VISIA Imaging, Italy). The *HER2*, *ESR1*, C*MYC* and *CCND1* probes were labeled with ZyGreen (excitation at 503 nm and emission at 528 nm) and CEN 17, 6, 8 and 11 probes were labeled with ZyOrange (excitation at 547 nm and emission at 572 nm).

Interpretation of results was based on counting at least 20 cells in at least two different areas of the slide. *HER2* FISH result was considered positive if: *HER2*/CEP 17 ratio ≥ 2, or *HER2*/CEP 17 ratio< 2 with an average *HER2* copy number ≥ 6 signals/cell. *HER2* FISH result was considered equivocal if: *HER2*/CEP 17 ratio< 2 with an average *HER2* copy number ≥ 4 and < 6 signals/cell. *HER2* FISH result was considered negative if: *HER2*/CEP 17 ratio< 2 with an average *HER2* copy number < 4 signals/cell, according to ASCO/CAP guidelines (Wolff, Hammond et al. 2013). For other genes under study, FISH results were considered positive if gene of interest (GOI)/CEP ratio ≥ 2; FISH results were considered negative if GOI/CEP <2.

Chromogenic in situ Hybridization (CISH)

CISH was performed on all samples using Zyto*Dot* 2C SPEC *HER2*/CEN 17 Probe kit (ZytoVision, Germany), according to the manufacturer's instructions. Briefly, slides were deparaffinized in xylene, rehydrated in ethanol, then immersed in 3% H2O2 for 5 min. Subsequently, slides were incubated for 15 min in Heat Pretreatment Solution EDTA at 95°C. Pepsin solution was applied to slides and they were incubated for 11 min at room temperature, then dehydrated in graded ethanol. Next, 10 µl Zyto*Dot* 2C SPEC *HER2*/CEN 17 Probe was pipetted onto slides, then slides were denatured at 78°C for 5 min and incubated overnight at 37°C in a hybridizer (Dako, Denmark). After hybridization, coverslips were removed by submerging slides in Wash Buffer SSC at room temperature for 5 min. Subsequently, slides were washed in Wash Buffer SSC at 75°C for 5 min. Anti-DIG/DNP-Mix was applied to slides and they were incubated for 15 min at 37°C, then they were washed in Wash Buffer TBS for 3×1 min. Subsequently, HRP/AP-Polymer-Mix was applied to slides and they were incubated for 15 min at 37°C, then they were washed in Wash Buffer TBS for 3×1 min. AP-Red Solution was applied to slides and they were incubated for 10 min at room temperature. Then, HRP-Green Solution was applied to slides and they were incubated for 10 min at room temperature. Next, the slides were counterstained for 2 min with Nuclear Blue Solution, and dehydrated in ethanol. CISH signals were visualized using a light microscope (Nikon, Japan). The *HER2* probes were labeled with digoxigenin and CEN 17 probes were labeled with DNP. Interpretation of results was based on counting at least 20 cells and was similar to FISH, according to ASCO/CAP guidelines.

Statistical analysis

Statistical analyses were performed using SPSS version 20. Results from various techniques were compared using cross tables. For *HER2* gene, sensitivity, specificity, positive and negative predictive value (PPV and NPV, respectively) were calculated using CISH as the gold standard.

Results

Amplification of *HER2*, *CCND1*, *MYC* and *ESR1* by MLPA were found in 15 (9%), 32 (19%), 33 (20%)

Table 1. Comparison of Gene Amplifications Detected by MLPA with FISH, CISH and IHC

	FISH				CISH			IHC			
MLPA	Positive	Equivocal	Negative	Total	Positive	Equivocal	Negative	3+	2+	1+	0
	(%)	(%)	(%)	Total	(%)	(%)	(%)	(%)	(%)	(%)	(%)
HER2, amplification											
High level	10 (100)	0	0	10	10 (100)	0	0	9 (90)	1 (10)	0	0
Low level	2 (40)	2 (40)	1 (20)	5	1 (20)	2 (40)	2 (40)	1 (20)	3 (60)	1 (20)	0
Amplification	12 (80)	2 (13)	1 (7)	15	11 (74)	2 (13)	2 (13)	10 (66)	4 (27)	1 (7)	0
CCND1, amplification											
High level	17 (100)	0	0	17							
Low level	10 (67)	3 (20)	2 (13)	15							
Amplification	27 (85)	3 (9)	2 (6)	32							
MYC, amplification											
High level	16 (100)	0	0	16							
Low level	11 (65)	4 (23)	2 (12)	17							
Amplification	27 (82)	4 (12)	2 (6)	33							

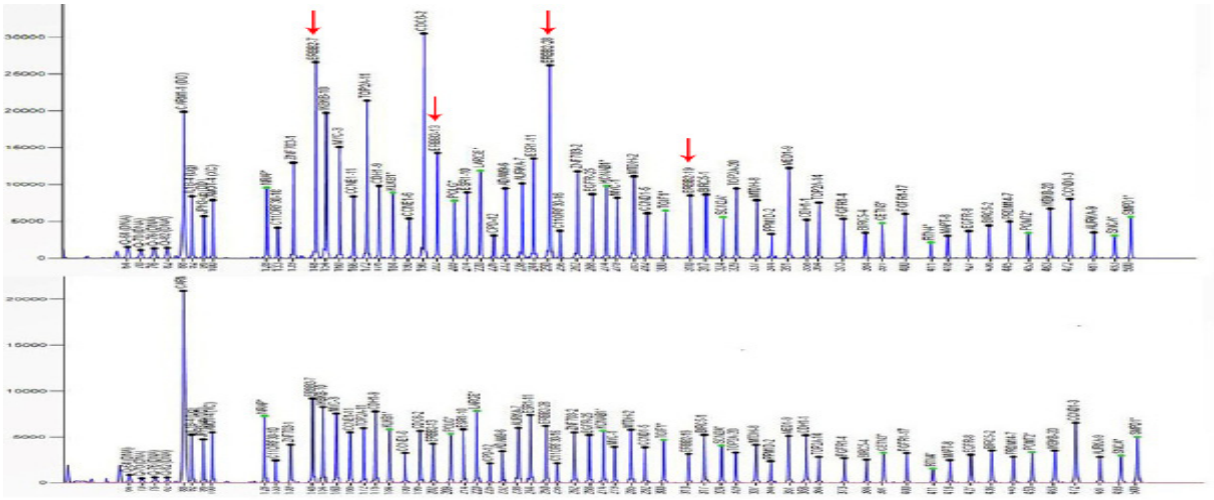

Figure 1. Representative Case Harboring *HER2* (*ERBB2*) Gene Amplification by MLPA. The top peak pattern shows *HER2* amplification in a breast cancer patient, compared to a normal breast (bottom). Arrows mark the four *HER2* (*ERBB2*) peaks

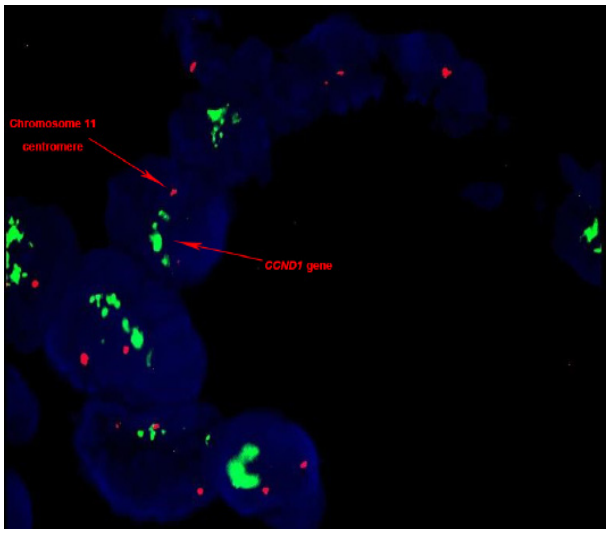

Figure 2. Representative Case Harboring *CCND1* Gene Amplification by FISH. Green signals show *CCND1* gene and red signals show chromosome 11 centromere. DAPI (blue) has delineated the nucleus boundaries

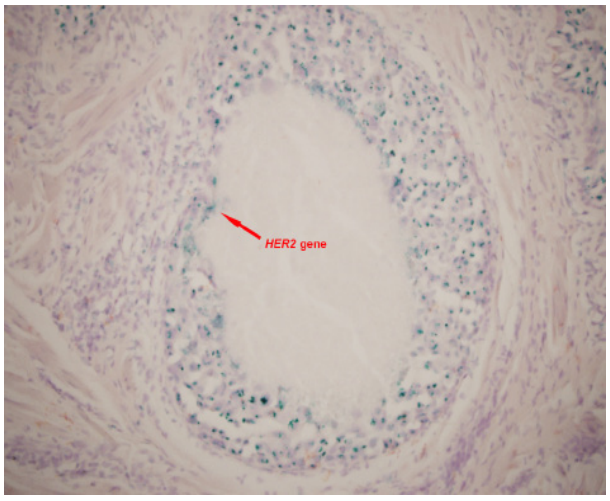

Figure 3. Representative Case Harboring *HER2* Gene Amplification by CISH. Green signals show *HER2* gene

and 3 (2%) of samples, respectively. Figure 1 shows a sample with *HER2* gene amplification by MLPA method. Amplification of *HER2*, *CCND1*, *MYC* and *ESR1* by FISH were found in 12 (7%), 27 (16%), 27 (16%) and 2 (1%) of samples, respectively. Figure 2 shows a sample with *CCND1* gene amplification by FISH method.

All of the high level amplified samples with MLPA were also amplified with FISH. All of the normal samples with MLPA were also normal with FISH. FISH analysis of 5 *HER2* low level amplified (peak value between 1.3 and 2) tumors showed that 2 were equivocal and 1 was not amplified. FISH analysis of 15 *CCND1* low level amplified tumors showed that 3 were equivocal and 2 were not amplified. FISH analysis of 17 *MYC* low level amplified tumors showed that 4 were equivocal and 2 were not amplified. In general, a high level of concordance was found between MLPA/FISH (*HER2*: 88%, *CCND1*: 88%, *MYC*: 86%, *ESR1*: 92%) (Table 1).

Amplification of *HER2* by CISH was found in 11 (6%) of samples. Figure 3 shows a sample with *HER2* gene amplification by CISH method. One of the samples which was low level amplified with MLPA and amplified with FISH, was not detected as amplified by CISH method. A high level of concordance was found between *HER2* MLPA/CISH (84%) and CISH/FISH (96%) (Table 1).

Among 11 IHC 3+ samples, 9 were high level amplified and 1 was low level amplified with MLPA. Of all IHC 3+ cases, (10/11) 91% were amplified by MLPA. Among 13 IHC 2+ samples, 1 was high level amplified and 3 were low level amplified with MLPA. In IHC 2+ group, (4/13) 31% were MLPA amplified. Among 67 IHC 1+ samples, none of them were high level amplified and 1 was low level amplified with MLPA. In IHC 1+ group, (1/67) 2% were MLPA amplified. None of the IHC 0 cases were amplified by MLPA (Table 1).

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of MLPA for *HER2* gene were, 100%, 97%, 73% and 100%, respectively.

Discussion

The purpose of this study was to compare MLPA as a method for gene amplification detection with FISH, CISH and IHC as clinically approved tests. Gene amplifications detected by FISH and CISH were highly comparable. All of the high level amplified samples with MLPA were also amplified with FISH, but analysis of some of the low level amplified samples with MLPA showed that some of them were equivocal and some were negative with FISH method. Our results are in line with studies of Moelans et al. , Farshid et al. and Pazhoomand et al. which reported considerable correlation between MLPA and ISH results (Moelans et al., 2009; Farshid et al., 2011; Pazhoomand et al., 2013).

In the present study, there was some discrepancy between cases of low level amplification with MLPA technique and those with positive results with FISH method. This discrepancy may be due to the fact that we used a cut-off value of 1.3 to discriminate between low level amplified and non-amplified cases. Using a cut-off value of 1.3 results in increased sensitivity and NPV of MLPA, but also leads to its decreased specificity and PPV , as previously reported (Moelans et al., 2010). Increasing the cut-off value would result in decreased sensitivity of MLPA and loss of detection of some of the amplified cases. Therefore, it seems that MLPA can be used as a gene amplification screening method and low level amplified samples need to be validated by ISH methods.

In conclusion, our results indicate that there is a good correlation between MLPA, IHC and ISH results. The low cost, high throughput, robustness and rapid turnaround time of MLPA implies that it can serve as an alternative to ISH for detection of gene amplification.

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