RESEARCH ARTICLE

Detection of HER2 Status in Breast Cancer: Comparison of Current Methods with MLPA and Real-time RT-PCR

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Abstract

Human epidermal growth factor receptor (HER) status is an important prognostic factor in breast cancer. There is no globally accepted method for determining its status, and which method is most precise is still a matter of debate. We here analyzed HER2 mRNA expression by quantitative reverse transcription-PCR (qRT-PCR) and HER2 DNA amplification using multiplex ligation-dependent probe amplification (MLPA). In parallel, we performed a routine evaluation of HER2 protein by immunohistochemistry (IHC). To assess the accuracy of the RT-PCR and MLPA techniques, a combination of IHC and fluorescence in situ hybridization (FISH) was used, substituting FISH when the results of IHC were ambiguous (2+) and for those IHC results that disagreed with MLPA and qRT-PCR, this approach being termed IHC-FISH. The IHC results for four samples were not compatible with the MLPA and qRT-PCR results; the MLPA and qRT-PCR results for these samples were confirmed by FISH. The correlations between IHC-FISH and qRT-PCR or MLPA were 0.945 and 0.973, respectively. The ASCO/CAP guideline IHC/FISH correlation with MLPA was (0.827) and with RT-PCR was (0.854). The correlations between the IHC results (0, 1+ as negative, and 3+ as positive) and qRT-PCR and MLPA techniques were 0.743 and 0.831, respectively. Given the shortcomings of IHC analysis and greater correlations between MLPA, qRT-PCR, and FISH methods than IHC analysis alone with each of these three methods, we propose that MLPA and real-time PCR are good alternatives to IHC. However a suitable cut-off point for qRT-PCR is a prerequisite for determining the exact status of HER2.

Keywords: Breast cancer - HER2 status- MLPA - IHC - FISH - RT-PCR - method comparison

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Introduction

Breast cancer is responsible for 28% of the newly diagnosed cancer cases in women worldwide (Siegel et al., 2013). Iranian female breast cancer patients are affected at least 10 years earlier (average age of diagnosis, 47.1-48.8 years old) than those in Western countries (Babu et al., 2011).

The treatment regimens for breast cancer patients are selected according to the biology and behavior of the tumor and depends on several factors such as: the human epidermal growth factor receptor 2 (HER2) status and the status of hormone (estrogen and progesterone) receptors. Despite the advances in targeted therapies, the disease recurs in approximately 30% of patients diagnosed with early stage breast cancer (Gonzalez-Angulo et al., 2007).

The HER2 (ERBB2) proto-oncogene is located on chromosome 17 and encodes a 185-kD transmembrane tyrosine kinase, which has a critical role in determining patient prognosis and the treatment of breast cancer (O'Malley et al., 2001; Rosa et al., 2009). Approximately 20-30% of breast cancer patients show HER2 amplification or over-expression. $HER2^+$ status is an unfavorable prognostic factor that is associated with worse overall survival of patients. Moreover, $HER2^+$ patients cannot benefit from adjuvant hormonal therapy (Blackwell et al., 2010; Hurvitz et al., 2013). Recent studies have proposed a strong correlation between HER2 status and the expression of MED1, another gene that plays a critical role in anti-hormonal therapy resistance (Cui et al., 2012). The first-line treatment for HER2+ patients is Herceptin (trastuzumab), a humanized monoclonal antibody against

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HER2 (Del Mastro et al., 2012).

The importance of precise detection of HER2 amplification is illustrated by the difficulties imposed by a 1-year course of treatment with Herceptin; this drug carries a substantial financial burden and, perhaps even more importantly, introduces the risk of cardiotoxicity (Keyhani et al., 2012; Criscitiello and Curigliano, 2013; Farolfi et al., 2013). HER2 alterations can be measured at the DNA, mRNA, and protein levels. However, according to the most recent guideline from the American Society of Clinical Oncology and College of American pathologists (ASCO/CAP), there is controversy over which method is the best for determining HER2 status (Wolff et al., 2007). Southern blotting is a gene amplification determining method that has lost its role in assessing HER2 status in favor of more modern methods, in part because it is labor-intensive, requires a large amount of DNA, and is not quantitative.

Currently, one widely accepted method to determine HER2 status is immunohistochemistry (IHC). This semi-quantitative method detects the protein expression according to percentage of stained tumor cells, and samples can be scored as 0, 1+, 2+ or 3+, where 0 and 1+ are interpreted as HER2-and 3+ as HER2+.The samples scored as 2+ require confirmation by another method, such as fluorescence in situ hybridization (FISH) (Wolff et al., 2007). Although ASCO/CAP has improved some pitfalls of IHC assay and its scoring system, some issues like subjective interpretation and problems imposed by fixation process has persisted (Sauter et al., 2009).

FISH is a Food and Drug Administration-approved method for the detection of HER2 gene amplification (Ross et al., 2009). This technique is accredited as the gold standard for assessing HER2 status because it offers standardized cut-off values (Schnitt and Jacobs, 2001), has more reproducibility (because DNA is resistant to the effects of tissue fixation and processing) (Ross et al., 2009), includes internal positive control, grants the possibility of quantitative evaluation and has greater sensitivity and specificity than IHC (Mass et al., 2005; Valabrega et al., 2007). A combined IHC/FISH approach has become more popular than approaches using FISH alone (according to ASCO/CAP guidelines) because FISH is a high-tech, time-consuming, and expensive method.

The FISH method is time-consuming, requiring more than 2 days to complete. In addition, the results of this method can be ambiguous within the score range of 1.8-2.2 (Gutierrez and Schiff, 2011).

Real-time quantitative reverse transcription-PCR (qRT-PCR) and multiplex ligation-dependent probe amplification (MLPA) are two PCR-based methods that are currently being used for HER2 status testing. qRT-PCR can be used to evaluate the status of HER2 at both the gene copy number and RNA expression levels (Gjerdrum et al., 2004). This rapid, simple and quantitative method offers an appropriate alternative for the assessment of HER2 gene alteration in routine clinical practice. MLPA is a newly developed technique for quantitative assessment of gene copy number variation. MLPA overcomes the variations in interpretation caused by suboptimal pretreatment processing or technician dependent result. Moreover, this method can analyze the copy number of up to 50 genes in one reaction with a minute amount of DNA.

Our objectives were, first, to compare MLPA to qRT-PCR as a method for evaluating the expression of HER2, and second, to evaluate the concordance of these techniques with the currently accepted method.

Materials and Methods

Tissue sampling

Tissue samples were selected from patients referred to Mehrad hospital of Tehran between Jan 2011 and Dec 2012, without regard to age or histopathological subtype. The patients and participants signed approved institutional review board consent forms before inclusion in the study. All the tumor tissue samples were obtained from macroscopically visible tumor regions and placed in two separate vials containing either RNAlater stabilization reagent or formalin. Samples were transferred to the Genetic Research Center of the University of Social Welfare and Rehabilitation Sciences within 24 hours post-surgery to avoid over-fixation of the tissues in formalin. One hundred thirteen invasive breast cancer specimens without history of radiotherapy or chemotherapy before surgery were selected, 95 of which were ductal and 10 were lobular breast cancer. Twenty specimens were excluded from the study due to document insufficiency and suboptimal tissue for DNA extraction. Since pattern of gene expression varies between lobular and ductal carcinoma (Zhao et al., 2004) we selected more ductal tissue for normal control. A total of 20 non-tumor control tissue samples were obtained: 17 were obtained from adjacent tissue of ductal tumors and 3 from excised tissues from cosmetic and breast reduction surgeries.

RNA extraction and cDNA synthesis

Total RNA of samples was extracted using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNAs were evaluated quantitatively and qualitatively using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and gel electrophoresis, respectively. cDNA was synthesized from1 μg RNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas, Thermo Fisher Scientific) according to the supplier's protocol.

Real-time PCR

The real-time qPCR mixes were prepared according to the Takara SYBR Master Mix instructions (Shiga, Japan) and carried out using an ABI7500 PCR machine (Applied Biosystems, Foster City, CA).

One of the critical prerequisites for the quantification of mRNA expression via qRT-PCR is an optimized primer design and a single peak on a dissociation curve (Figure 1). Data were analyzed by the LinRegPCR (version 12.18) software. PCR efficiencies per sample were calculated as the mean PCR efficiency for each amplicon (Ramakers et al., 2003). Following correction of cross points, the results were transferred to REST-2009 (Pfaffl et al., 2002; Ruijter

et al., 2009). The relative gene expression was estimated by comparing to normal breast tissue controls. Lyng et al suggested that specific reference gene selection is critical for every experimental set-up (Lyng et al., 2008). We evaluated three previously used housekeeping genes in breast tissue (Table 1), and HPRT showed the least variation in gene expression between normal and tumor samples in our tests.

Table 1. Primers Used for PCR

Target gene	Primer sequences	Product length (bp)
HPRT	F: 5`- ATTGTAATGACCAGTCAACAGGG -3`	117
	R: 5`- GCATTGTTTTGCCAGTGTCAA--3`	
B2m	F: 5`- TGGAGGCTATCCAGCGTACT -3`	111
	R: 5`- TGTCGGATGGATGAAACCCAGA -3`	
Pum1	F: 5`- AGGGAAGAGCGATGGGAGAGCA -3`	162
	R: 5`- TCTGCACCATGATTGGCTGGGA -3`	
HER2	F: 5`- CATCAACTGCACCCACTCCT -3`	207
	R: 5`- AGCTCCGTTTCCTGCAGC -3`	

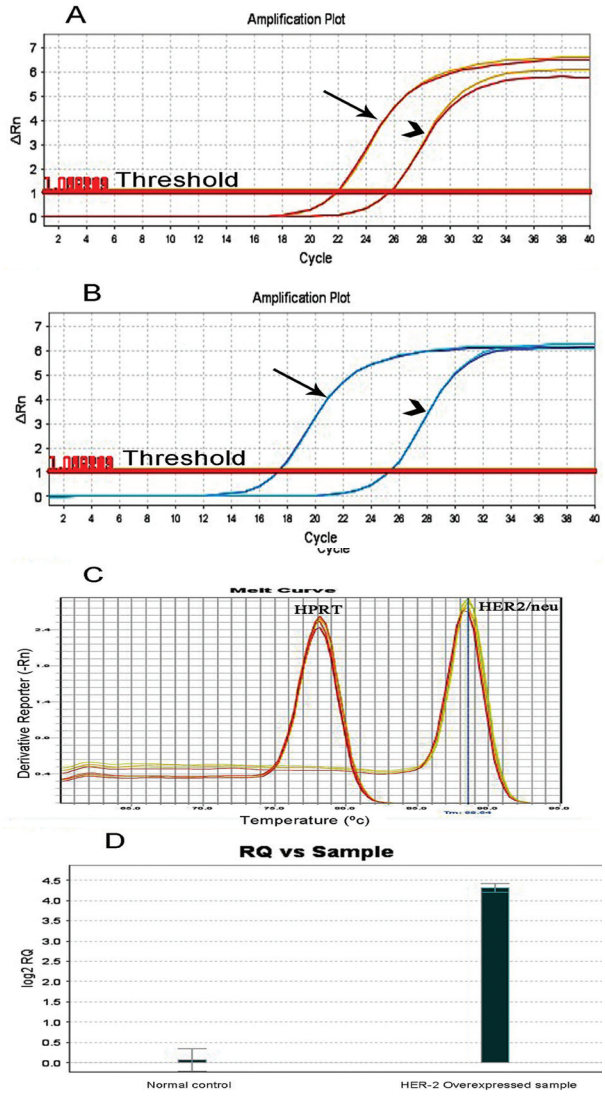

Figure 1. Real-time PCR was Performed in Duplicate. Amplification plot in an overexpressed sample (A). Amplification plot in normal-expression control (B). The Ct values for the HPRT gene are nearly the same in two plots (A=25.58 and B=25.77, arrows), indicating good standardization of RNA amounts. The Ct values for the HER2 gene are 17.3 and 21.9 (arrow heads) in the control and overexpressed samples, respectively. Melting peak of HPRT gene, left peak and HER2 gene, right peak (C). Log2 RQ for sample B compared with control A (D). NTC: no-template control

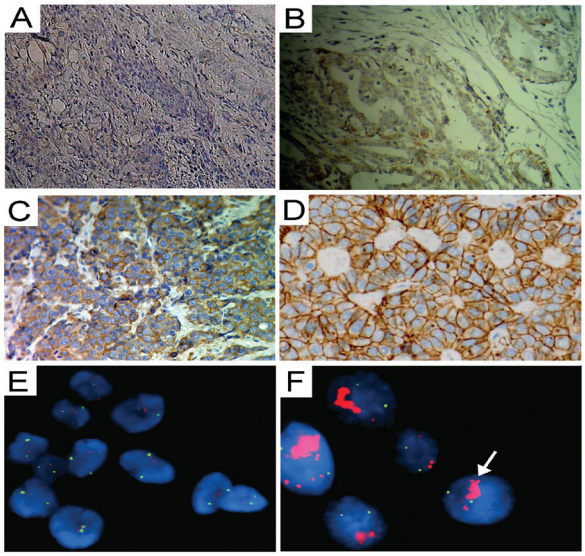

Figure 2. *HER2* Protein Expression by Immunohistochemistry (IHC) and *HER2* Gene Detection by Fluorescence in Situ Hybridization (FISH). Score of 0 by IHC (a), 1+ by IHC (b), 2+by IHC (c), and 3+by IHC (d). *HER2* Detection by FISH in non-HER2-Amplified (e) and *HER2*-Amplified Samples (f). The *HER2* gene is shown in Red, and Centromere 17 Control Signals are Shown in Green. Cluster formation is Indicated with a White Arrow

The qRT-PCR data were classified into three expression levels: normal, low overexpression, and overexpression. For this aim, we defined the normal cut-offs for real-time PCR output according to the t-distribution, mean, and standard error of mean of qRT-PCR results in 20 non-tumor controls. Cases with real-time values between the range of a (meancontrols±t0.975, 19 std errcontrols)=1.187 and b (meancontrols±t0.995, 19 std errcontrols)=1.53 were considered to have low overexpression, and the cases with the values greater than b were considered to have overexpression. According to the threshold that was selected for classification of HER2 mRNA expression, the overexpressed samples were grouped in two main classes; samples showing $a<\Delta\Delta Ct<b$ were grouped and referred to as low-over expression, and the cases with $\Delta\Delta Ct>b$ were deemed to show overexpression. The cases with $\Delta\Delta Ct<a$ were considered as normal expression.

MLPA

DNA was extracted from 93 fresh tumor tissues and 20 normal breast tissue samples using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's protocol.

The quality and integrity of DNAs was evaluated by agarose gel electrophoresis. The concentration of high-quality extracted DNA was standardized to the final amount of 125 ng per reaction using a NanoDrop ND-2000 spectrophotometer. Each PCR reaction contained 20 tumor samples, and 5 of the 20 normal samples were used simultaneously for normalization. SALSA MLPA P078-c1 (Breast Tumor) probe kit was used which contains four specific probes for the HER2 gene. Briefly, the DNA

extracted from the dissected tumors and normal samples was preheated to 98°C, and then salt solution and probe mix were added to the DNA. After the ligation of annealed nucleotides, PCR was performed to amplify target genes. PCR products were separated on an ABI3730-XL capillary sequencer (Applied Biosystems, Foster City, CA, USA). HER2 gene copy number was determined using GeneMarker (ver. 1.95). Cut-off values between 0.7 and 1.3 were considered normal. Results below 0.7 or 1.3-2 were interpreted as deletion and low level amplification of HER2, respectively. Besides values over 2 were referred to as high level amplification.

Immunohistochemistry

IHC for HER2 was performed with the HercepTest kit according to the manufacturer's protocol (DAKO, Glostrup, Denmark). Briefly, 4-μm-thick mounted sections on slides from formaldehyde-fixed paraffin-embedded tissue blocks were deparaffinized and rehydrated in graded alcohols. The slides were then incubated with pre-diluted anti-HER2 antibody. After incubation, the sections were washed in PBS and incubated with horseradish peroxidase-conjugated secondary antibody. Color development was performed using 3,3'-diaminobenzidine, and the tissue samples were counterstained with hematoxylin. Negative and positive control slides were included in each assay. Samples were interpreted according to the ASCO/CAP guidelines: negative (0, 1+), weakly positive (2+), and strongly positive (3+) (Figure 2 A-D). The results were interpreted by two independent pathologists.

FISH analysis of HER2 status

The Cytocell HER2 probe kit (LPS001) was used to analyze samples by FISH according to the supplier's instructions. First, the sections were baked overnight. Following deparaffinization by xylene, the slides were dehydrated and air-dried. After protease digestion, the slides and probes for HER2 and the centromere of chromosome 17 (internal control) were subjected to denaturation simultaneously. The probes and target DNA were hybridized in a humidified chamber at 37°C overnight. The slides were washed with post-hybridization wash buffer and counterstained with 4,6-diamidino-2-phenylindole. The number of chromosome 17 and HER2 signals were scored for 20 tumor cell nuclei in the invasive tumor region which had been previously marked by the pathologist. Samples were reported as amplified when the ratio of HER2 signals to chromosome 17 centromere signals was equal to or greater than 2.2. A HER2 to chromosome 17 centromere ratio of 1.8-2.2 was considered ambiguous (ASCO/CAP) (Figure 2E-F).

In routine practice equivocal IHC (2+) require retest or confirmation by FISH according to ASCO/CAP suggestion. In this study two groups of IHC were replaced by FISH results: 1_ The weakly positive IHC (2+), 2_ Some unequivocal results of IHC (0, 1+, or 3+) which were in disagreement with RT-PCR and MLPA while these two methods were identical in results. This protocol referred to as IHC-FISH in this study was applied as gold standard for better evaluation of the other two methods (RT-PCR and MLPA).

Statistical analysis

The sensitivity, specificity, concordance, negative predictive value (NPV), positive predictive value (PPV), and kappa value were calculated for each method, using IHC-FISH as the gold standard. The rate of concordance in HER2 results between RT-PCR, IHC-FISH, and MLPA was analyzed using crosstab. A p value <0.05 was considered to indicate statistical significance. The statistical software package used for these analyses was SPSS for Windows (version 18.0). The cut-off point for assigning negative and positive status for each method was established as previously described in the Materials and Methods. The correlation between the methods was determined by calculating the Spearman correlation coefficient.

Results

Of the 113 invasive breast tumor samples included in this study, 93 samples were selected for further evaluation of HER2 status using MLPA, qRT-PCR, and IHC.

HER2 status by IHC and FISH

The ASCO/CAP scoring system for IHC was applied for interpretation of HercepTest results. Using the HercepTest kit, 54 of the 93 tumors (58%) were negative for HER2, 25 (27%) scored 2+, and 14 (15%) scored 3+. Thirty-five cases were selected for FISH analysis from three classes: weakly positive IHC (2+) (25 cases), quality control (6 cases), and cases where the qRT-PCR and MLPA results disagreed with IHC (4 cases). Interestingly, the FISH results of the four disagreeing samples were in complete agreement with the qRT-PCR and MLPA results.

All 35 cases were interpretable for HER2 gene amplification by FISH. Nine out of 25 (36%) samples in the weakly positive group had positive FISH results. The entire quality control group showed complete concordance between FISH and IHC results. The average number of HER2 signals per nucleus in the amplified samples ranged from 4.8-18.0 (median 9.0). In non-amplified samples (62.9%), the mean number of fluorescent signals per nucleus ranged from 1.8-3 (median 2.2). There was no ambiguity in our results.

HER2 status by real-time qRT-PCR

Ninety-three samples were evaluated for HER2 mRNA expression using the SYBR green method. All reactions were performed in duplicate. Overexpression and low overexpression of HER2 mRNA was observed in 2 (2.1%) and 23 (24.7%) tumor samples, respectively. Among all the tumor samples, 68 cases (73.3%) showed normal expression of HER2.

Genomic alteration of HER2 assessed by MLPA

MLPA analysis was carried out successfully in all 93 tumor samples (Figure 3). The mean of four HER2 peak values was calculated and interpreted as discussed above. In the 25 HER2- positive samples (26.9% of all samples), 21 (22.6% of all samples) showed high amplification, and the remaining 4 (4.3%) belonged to the low amplification

Table 2. Comparison of IHC, MLPA, and qRT-PCR

IHC	MLPA			qRT-PCR			Row total
	No amplification	Low amplification	High amplification	Normal expression	Low overexpression	Overexpression	
0	8	0	0	7	0	1*	8
1+	43	1**	2**	43	1**	2**	46
2+	15	1*	9**	16	0	9**	25
3+	1*	2	11	2(1**+1*)	1	11	14
Column total	67	4	22	68	2	23	93

*FISH negative; **FISH positive

Table 3. Comparison of IHC-FISH, MLPA and qRT-PCR for HER2 Gene

qRT-PCR	IHC-FISH* Negative	Positive	Total
Normal expression	67	1	68
Low overexpression+Overexpression	(0+1)=1	(1+23)=24	25
MLPA			
Normal	67	0	67
Low amplification+High amplification	(1+0)=1	(3+22)=25	26

*Substituting FISH when the results of IHC were ambiguous(2+)andforthoseIHC results that disagreed with MLPA and qRT-PCR, qRT-PCR, quantitative reverse transcriptase PCR, MLPA, multiplex ligation-dependent probe amplification, IHC, immunohistochemistry, FISH, fluorescence in situ hybridization

Table 4. Spearman Correlation (Spearman's rho) between the Applied Methods to Detect HER2 Overexpression and Gene Amplification (all the p-values were <0.001)

	qRT-PCR	MLPA	FISH*
IHC-FISH [1]	0.95	0.98	-
IHC/FISH [2]	0.85	0.83	-
IHC **	0.74	0.83	-
qRT-PCR	1	0.92	1
MLPA	0.92	1	0.94

*FISH correlations were calculated using 35 cases, **IHC2+ excluded, [1]Substituting FISH when the results of IHC were ambiguous (2+) and for those IHC results that disagreed with MLPA and qRT-PCR, [2]Substituting FISH when the results of IHC were ambiguous (2+) (ASCO/CAP guideline) IHC, Immunohistochemistry qRT-PCR, quantitative reverse transcriptase PCR MLPA, multiplex ligation-dependent probe amplification FISH, fluorescence in situ hybridization

group. Of the four samples in the low amplification group, two samples had low overexpression by qRT-PCR and amplification by FISH, one sample had normal expression and no amplification, and the final sample had normal expression and amplification. Eighteen out of the 26 cases that showed HER2 amplification also had MED1 amplification, but none of the HER2-negative cases showed MED1 gene amplification. Furthermore, the correlation between the numerical values of HER2 and MED1 amplification was significant ($p<0.05$) (Figure 3A).

In Table 2, the results of the comparisons between IHC, MLPA and RT-PCR are displayed. All cases scored zero by IHC lacked amplification by MLPA whereas one case showed an overexpression by RT-PCR. Most of the IHC 3+ cases were amplified by MLPA (13/14, 92.8%) and overexpressed by RT-PCR (12/14, 85.7%). Of the IHC 1+ cases, 3/46 (6.5%) were amplified by MLPA and 3/46 (6.5%) overexpressed by RT-PCR. The IHC 2+ cases showed amplification by MLPA in 10/25 cases (40%) and by FISH in 9/25 cases (36%) and RT-PCR showed overexpression in 9/25 cases (36%).

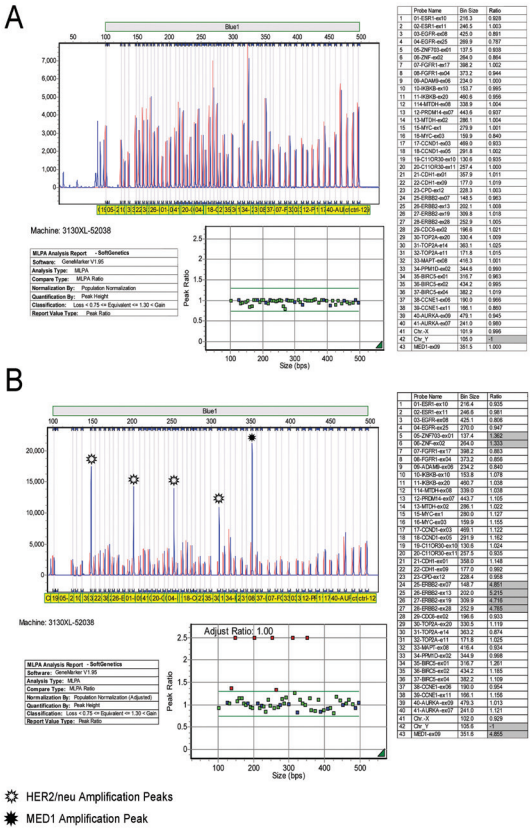

Figure 3. MLPA Analysis Report by GeneMarker Software. (A): no amplification; (B): co-amplification of *HER2* and MED1

HER-2 amplification by MLPA in 1/26 cases (3.8%) and HER-2 overexpression by RT-PCR in 1/25 cases (4%) were not confirmed by FISH. Of the 67 MLPA normal cases, no sample was amplified by FISH and 1/68 (1.5%) was overexpressed by RT-PCR. All IHC 2+ cases that were overexpressed by RT-PCR and 9/10 cases that were amplified by MLPA were also amplified by FISH. Results for different levels of HER2 amplification or overexpression (by MLPA or qRT-PCR respectively), as compared to IHC-FISH results, are summarized in table 3.

Correlation testing of IHC-FISH, RT-PCR, MLPA, and IHC/FISH

The Spearman's correlations of RT_PCR and MLPA with IHC-FISH and the other applied methods in this study are listed in Table 4. MLPA and IHC-FISH showed the highest correlation (0.973). IHC method showed lower correlation with MLPA (0.831) and RT-PCR (0.743) even after exclusion of equivocal results (Table 4). The

Table 5. Different Measures to Evaluate the Performance of Predictive Algorithms for MLPA and qRT-PCR in 93 Invasive Breast Cancer Patients

	Sensitivity	Specificity	Positive PV	Negative PV	Concordance	Kappa
qRT-PCR	0.985	0.96	0.985	0.96	0.978	0.945
MLPA	1	0.961	0.985	1	0.989	0.973

PV: predictive value, qRT-PCR, quantitative reverse transcriptase PCR MLPA, multiplex ligation-dependent probe amplification IHC-FISH was considered the gold standard

Table 6. Comparison of FISH, MLPA, and qRT-PCR in 35 cases

		MLPA		qRT-PCR		Total
		No amplification	Amplification	Normal expression	Overexpression	
FISH	Negative	21	1	22	0	22
	Positive	0	13	0	13	13
Total		21	14	22	13	35

Spearman's correlation between MLPA & FISH=0.940, p<0.001, Spearman's correlation between qRT-PCR & FISH=1.000, p<0.001, qRT-PCR, quantitative reverse transcriptase PCR, MLPA, multiplex ligation-dependent probe amplification

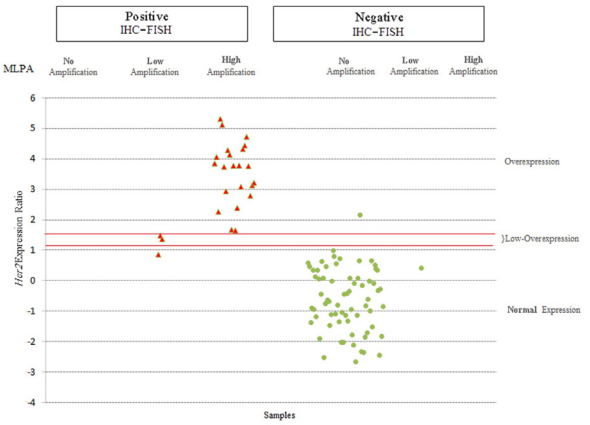

Figure 4. Scatter Plot of MLPA Ratio Compared to IHC/FISH Results. The HER2-Amplified and Non-HER2-Amplified Samples by IHC/FISH are Indicated by Triangle and Circle, Respectively

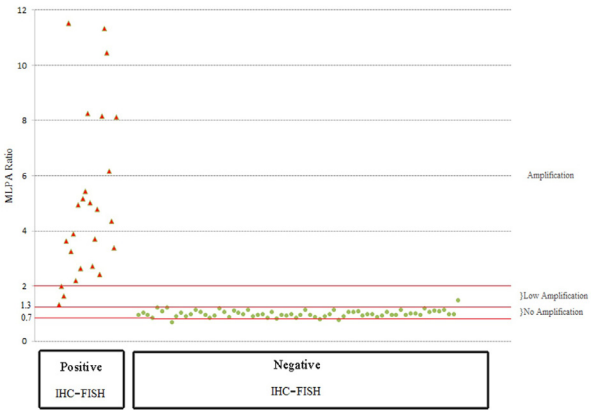

Figure 5. Scatter Plot of Concordance between Different Methods (qRT-PCR, IHC/FISH and MLPA). The *HER2*-Amplified and Non-*HER2*-Amplified Samples by IHC/FISH are Indicated by Triangle and Circle, Respectively. The Transcript Expression values Determined by qRT-PCR are Indicated in a Log Scale

concordance rate between MLPA and IHC-FISH was 0.973, where this rate between RT-PCR and IHC-FISH was 0.945. The MLPA showed more sensitivity than RT-PCR, 1 versus 0.985, while the specificities of these two methods were almost the same. More over PPV, NPV, and kappa were calculated for RT-PCR and MLPA methods; for these comparisons, IHC-FISH was considered the gold standard (Table 5).

The distribution of the MLPA ratio in figure 4 scatter plot shows that 3 positive cases by MLPA had low amplification (1.3<ratio<2) while the MLPA ratio of most positive cases were between 2 and 6.This ratio for 3 cases were over 10. Just one sample with low amplification by MLPA was categorized as negative using IHC-FISH. The sensitivity and specificity of MLPA were 100% and 96%, respectively. The positive MLPA results were in total agreement with those from IHC-FISH.

Among the 93 cases evaluated by all three methods (IHC-FISH, qRT-PCR, MLPA), the results of two samples showed a discrepancy between qRT-PCR and IHC-FISH (one sample as false positive, the other as false negative). In addition, the results from three samples showed discrepancies between qRT-PCR and MLPA (Figure 5).

Discussion

HER2, as a prognostic and predictive marker, has been widely accepted in the management and treatment of breast cancer (Rosa et al., 2009). The high cost and side effects of trastuzumab therapy demand that highly accurate, robust, sensitive, and cost-effective testing protocols be used in clinical settings (Barberis et al., 2008; Criscitiello and Curigliano, 2013). Previous studies have suggested that a reliable and precise method for the analysis of HER2 status would be beneficial to patients and clinical oncologists (Perez et al., 2006; Barrett et al., 2007). ASCO/CAP guideline and other studies have warned that approximately 20-26% of current HER2 test results may be inaccurate. Several studies have proposed IHC as the first-line screening method for HER2 status and suggested that FISH must be performed in weakly positive samples (Masood, 2006; Wolff et al., 2007). IHC staining is easy to perform and relatively inexpensive as a method for evaluating HER2 status. However, there is a wide range of inter- and intra-laboratory variation in its sensitivity and specificity (Vanden Bempt et al., 2005). A growing body of evidence has emphasized that the exclusion of subjective errors and general improvements in IHC testing using the Automated Cellular Imaging System have led to more agreement between IHC and FISH results (Wang et al., 2001; Bloom and Harrington, 2004; Tawfik et al., 2006). Despite these improvements in IHC, some shortcomings still exist. Factors such as tissue fixation (and its impact on HER2 protein antigenicity), the scoring method, and the choice of antibody may contribute to a lower specificity and sensitivity of IHC (Vanden Bempt et al., 2005; Collins et al., 2012).

Sauter et al have stated that HER2 amplification at the DNA level and protein overexpression are generally associated and that the disagreement between FISH and IHC methods is due to a lack of standardized protocols for

sample preparation and interpretation of IHC results. To better assign patients to adjuvant trastuzumab therapy, it seems that a precise alternative method is needed (Sauter et al., 2009). We compared HER2 status using two alternative methods at the DNA and mRNA levels: MLPA and qRT-PCR, respectively.

The sensitivity, accuracy and high-throughput potency of qRT-PCR has made it an appropriate candidate for HER2 status determination (Rosa et al., 2009; Baehner et al., 2010). Based on our results, 36% of samples with a score of 2+ by IHC had HER2 amplification. Among these samples, one sample presented contrary results (2+ IHC, normal expression by qRT-PCR, low amplification by MLPA, and positive by FISH). Only one sample with 3+ IHC showed neither HER2 overexpression nor gene amplification. FISH analysis in this sample confirmed the MLPA and qRT-PCR outcomes. One of the possible causes of the discrepancy between IHC and the other methods is the error-prone stage of sample preparation in IHC (crushing and retraction) (Bloom and Harrington, 2004). The concordance between IHC-FISH and qRT-PCR was 0.978, and the concordance between IHC-FISH and MLPA was 0.989.

Only one sample of 93 that had low amplification did not show amplification by FISH. Moelon et al reported lower specificity and sensitivity in their comparison between MLPA and FISH results (Moelans et al., 2009). This could have been due to their performing MLPA in formalin-fixed paraffin-embedded tissues because MRC highly recommends and emphasizes high-quality DNA for the MLPA procedure.

Some studies have shown high concordance between IHC and FISH (Dolan and Snover, 2005; Jorgensen et al., 2011). In the present study among 93 cases studied by IHC, four cases showed disagreement with qRT-PCR and MLPA results. Three cases detected as 1+ by IHC were positive by both qRT-PCR and MLPA, and one case determined to be 3+ was negative by the other two tests. The FISH results for these four samples with discordant results were in agreement with the qRT-PCR and MLPA outcomes.

In our study, 4 of 68 cases that, in routine practice, would not require further complementary testing (scored 0, 1+, or 3+ by IHC) were in disagreement with the other three methods.

We observed high agreement between qRT-PCR and FISH, similar to some other studies (Kostopoulou et al., 2007; Baehner et al., 2010). The qRT-PCR method showed normal amplification for only one sample that was positive by both FISH and MLPA, while one sample with overexpression detected by qRT-PCR had negative results in the other two tests (Table 3). One case showed low amplification by MLPA in spite of negative FISH and qRT-PCR. It is noteworthy that in this case, only two out of four probes showed amplification (with 1.57 and 1.65 scores), and the other two had 1.2 and 1.24 scores, resulting in a mean value of 1.415. Considering the MED1 amplification in this case, and with no such amplification having been observed in any of the HER2-negative cases in our study, this discrepancy could have been due to partial amplification of the HER2 gene in this sample.

In 35 cases that were subjected to FISH analysis, rather good correlations were observed between the FISH results and both MLPA and RT-PCR (Table 6). The concordance between both RT-PCR and MLPA and IHC-FISH in this study was substantially higher than the concordance of these two methods with IHC alone.

In conclusion, both MLPA and RT-PCR are accurate and can be used as alternatives to IHC protocols for evaluating the status of HER2 breast cancer. In addition, MLPA and RT-PCR are accessible for most laboratories. Compared to IHC, these methods are easily standardized and less dependent on individual interpretation. However, further studies with larger sample size in trastuzumab-treated patients are recommended.

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