Application of Multiplex Ligation-Dependent Probe Amplification in Determining the Copy Number Alterations of *HER* Gene Family Members in Invasive Ductal Breast Carcinoma

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Abstract

Background: The aim of this study was to assess the usability of multiplex ligation-dependent probe amplification (MLPA) for copy number determination of *HER* gene family members (*ERBB1-4*) in invasive breast carcinoma and to explore the association of *ERBB1-4* gene copy numbers with clinicopathological characteristics of breast cancer (BC) patients.

Methods: Clinical and immunohistochemical characteristics were assessed in 104 BC patients and the molecular subtype was determined for each tumor sample. Furthermore, HER-2/neu status was assessed by immunohistochemistry (IHC) and equivocal results were confirmed by Fluorescent in situ hybridization (FISH). The copy numbers of *ERBB1-4* genes were determined by MLPA.

Results: Twenty-five percent of all patients showed *ERBB2* gene-amplification by MLPA, whereas 14.4% of cases showed ERBB-2/neu overproduction at the protein level (IHC). Moreover, only 2.9% and 1.9% of patients showed amplification in *ERBB1* and *ERBB4*, respectively. No copy number changes were observed in *ERBB3*. Our results indicated a significant association between *ERBB2* copy number gain and histological grade (*p value*= 0.01), stage (*p value*= 0.02), and tumor subtypes (*p value*= <0.001). In addition, we found MLPA more accurate in assessing *HER2* status with 15.4% and 9.6% gene amplification detection in early stages (1, 2A and 2B) and advanced tumor stages (3A, 3B, and 4), respectively, compared to IHC (early stages= 13.5% and advanced stages= 4.7%).

Conclusions: According to our findings, MLPA is a fast, precise and low-cost technique to detect *ERBB2* amplification, especially in advanced tumor stages. However, due to infrequent amplification found in *ERBB1* and *ERBB4* as well as the lack of amplification in *ERBB3*, their importance in the prognostic evaluation of BC patients remains controversial.

Keywords: Breast Cancer, Copy number variation, *ERBB1-4*, IHC, MLPA.

Introduction

Breast cancer (BC) is considered as one of the most common types of cancer, with an estimated 2.09 million incident cases in 2018. In addition, BC is the chief cause of cancer death in women and the fifth major cause of cancer mortality for both sexes (1). Advances in diagnosis and management of BC have

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led to a decline in morbidity. However, the decrease can change widely around different geographic locations (2, 3). Targeted therapies seem to be very promising by keeping side effects as low as possible. Despite recent advancements in BC management, many of patients are unable to benefit from prognostic biomarkers, and several efforts have been accomplished to appreciate prognostic and predictive factors in BC (4, 5).

Various biologic responses such as cell propagation, differentiation, and survival are mediated by growth factors which in turn with receptor tyrosine kinases activity, activate cell-surface receptors by attaching to them (6). *ERBB* oncogene family including *ERBB1* (also called as *HER1* or *EGFR*), *ERBB2* (also known as *HER2*), *ERBB3* (also known as *HER3*) and *ERBB4* (also known as *HER4*), are subclass I receptor tyrosine kinases. Members of this family can be aberrantly activated by genomic mutations such as genomic copy number gain and as a result, immanent tumorigenic processes, can appear as pathogenesis cause of BC (7). ERBB2 overproduction is a decisive molecular target for tumor therapy by Trastuzumab, but, a therapeutic benefit is estimated to influence approximately 50% of patients (8, 9). In line with this, *ERBB2* amplification alone is insufficient to predict patient responsiveness to drug therapy. The direct interaction of other *ERBB* family members with *ERBB2* can also be considered as a potential prognostic factor or therapeutic marker due to their significant structural and functional homology (10). Owing to the fact that the functionality of ERBB receptors effective on one another, it seems the communication of these receptors have a major impact on tumor growth.

ERBB1 overexpression is detected in BC patients and seems to play an important role during malignant transformation. Likewise, ERBB1 overexpression has been correlated with higher histological grade and the involvement of lymph node (11, 12). However, the prognostic value of *ERBB1* remains unresolved in BC patients. No active kinase has been generated by ERBB3, thus its activation needs to be dimerized with other ERBB members (13, 14). The association of ERBB3 overexpression with BC is not in agreement in all studies. Several studies elucidated

a poor prognosis, while, others represented a good prognosis (15, 16). ERBB4 stimulates multiple downstream proteins such as STAT5, SHC, and CBL. It also has a great impact on activation of PI3K signaling (17). Regarding functionality of ERBB4 in cell apoptosis, it may be considered as a good prognostic marker in BC (18, 19).

Copy number variations (CNVs) are particularly recognized to be associated with phenotypic and genetic multiplicity among cancers. CNVs can lead to the loss of tumor suppressors or activation of oncogenic drivers (17, 20). In this line, in the current study, the copy number of ERBB family members oncogenic genes were scrutinized using MLPA technique in Iranian sporadic invasive ductal BC patients and the influence of copy numbers was considered on clinical and pathological features. Furthermore, the accuracy of MLPA was compared with IHC technique as the most conventional detection method for evaluating the situation of *ERBB2*.

Materials and methods

DNA and tissue samples

With the approval of ethics committee of the university of Social Welfare and Rehabilitation Sciences, Tehran, Iran, tissue samples were obtained from patients with breast invasive ductal carcinoma. Biological materials were provided by Mehrad hospital, Tehran, Iran and the Iran National Tumor Bank which is funded by Cancer Institute of Tehran University of Medical Sciences, for Cancer Research. 124 invasive ductal BC specimens were selected and written consent was obtained from all the patients. Twenty specimens were excluded from the study due to the document inadequacy and insufficient tissue for DNA extraction. The patients were sporadic cases with no family history of cancer, excluding those with other malignancies or bilateral BC and those who have any history of radiotherapy or chemotherapy before surgery. At the time of pretherapeutic biopsy or surgery, Samples were snap frozen in liquid nitrogen and stored at -80 ˚C until genomic DNA was extracted.

Progesterone receptor (PR) and estrogen receptor (ER) were previously tested for all tissue samples using immunohistochemistry (IHC). ER and PR positivity were defined as more than 1 %

ER/PR positive cells. Likewise, the IHC was applied to assess ERBB2/ neu protein production. Fluorescence in situ hybridization (FISH) was used to confirm equivocal results of IHC. Clinicopathological information like lymph node metastasis, tumor size, histological and nuclear grade, stage, TP53 mutation and Ki-67 were obtained from the pathology reports.

DNA extraction
All reagents and biochemicals used in this study The genomic DNA of Patients was extracted from formalin-fixed paraffin-embedded (FFPE) tissue sections (10-µm) using a QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Agarose gel electrophoresis was used to evaluate the quality of DNAs. The concentration of high quality extracted DNA was standardized to the final amount of 75 ng per µl using a NanoDrop ND-2000 spectrophotometer.

MLPA
Every *ERBB* gene copy number variation was analyzed with MLPA gene copy number assay SALSA MLPA probemix X026-A1 HER (MRC-Holland, Amsterdam, the Netherlands) which contains 23 probes for the *ERBB* gene family: six probes for *ERBB1*, eight probes for *ERBB2*, four probes for *ERBB3* and five probes for *ERBB4.* Furthermore, it also contains one flanking probe for each arm of chromosomes 2, 7, 12 and 17, where the *ERBB* family member genes are located. Healthy reference samples were applied in each MLPA experiment to be used for intersample normalization of copy number. We used 3 different references to estimate the reproducibility of each probe within each MLPA run and when testing more than 21 samples simultaneously, we added 1 additional reference sample for every 7 additional test samples and they were distributed randomly over the experiment to minimize variation. Reference samples were obtained from healthy individuals and they were as similar as possible to test samples in all other aspects. MLPA was performed according to the manufacturer's protocol available online at www.mlpa.com. Briefly, 75 ng per µl tumoral DNA and reference DNA samples were warmed in advance to 98°C to denature, followed by addition of MLPA buffer and probemix. After overnight hybridization of probes and the ligation of hybridized probes, PCR was carried out to amplify probes using a universal primer pair. PCR products were separated on an ABI3730-XL capillary sequencer (Applied Biosystems, Foster City, CA, USA). *ERBB1-4* gene copy numbers were analyzed using Coffalyser.Net software (MRC-Holland, Client version v. 140721. 1958). Normal results were considered between 0.7 and 1.3 Cut-off values. Results lower down 0.7 were interpreted as deletion and values over 1.3 were referred to as gain or amplification. The MLPA test using X026 probemix was repeated for all the abnormal results.

IHC
The IHC was performed on sections of paraffin blocks (4 µm thick), utilizing the Hercep test (Dako, Glostrup, Denmark) according to the manufacturers' instructions. The score of 0 to 3+ was given by IHC test which evaluates the distribution of ERBB2 receptor protein on the surface of the cells in a BC tissue sample. The score from 0 to 1+, were defined as "ERBB2 negative." The score of 2+, was called "equivocal", and the score of 3+ was defined as "ERBB2 positive". Two skilled pathologist were interpretating staining. Negative controls were achieved without primary antibody. A small tissue array containing a 0, 1+, 2+ and 3+ breast tumor sample, as control, was put along on the same slide as the tumor to be tested.

FISH
Using The Cytocell HER2 probe kit (LPS001) the FISH tests were carried out which contained probes for determining the copy number of both *ERBB2* (red) and the chromosome 17 centromere (CEP17, green).

Statistical analysis
Following the overnight incubation, 4500 Logistic regressions was used to find relationship between *ERBB2* copy number and clinicopathological parameters. To analyze the association between tumor subtypes, histological garde, and tumor stage and *ERBB*

gene family copy numbers, the nonparametric multiple comparison Steel test was used. A p value <0.05 was considered to indicate statistical significance. The statistical software package used for these analyses were SPSS (version 25.0) and R package (version 3.5.2). For assigning positive and negative status for each method, the cut-off point was considered as aforementioned in the Materials and Methods.

Results

The age of patients in this study ranged between 30 and 71 years (mean 49.5 ± 10.7). Right breast (51.9%) was more involved compared to the left breast (48.1%). In terms of tumor size, the group 2 (>2 cm) with 68.3% involvement had a higher occurrence than the group 1 (≤2 cm). In terms of axillary lymph node metastasis, 31.7% of the patients were affected. Regarding the tumor stage, 25%, 51.9%, 18.3% and 4.8% of the patients were in stage I, stage II, stage III and stage IV, respectively. For histological grading, grade II with 64.4% was located in the first rank and from the point of nuclear grading, 58.6% were located in grade 2. Based on IHC and FISH results for *ERBB2,* it was elucidated that overproduction occurred in 14.4% of the patients and they needed to receive Trastuzumab. *TP53* mutation was found in 35.5%

of the patients. It is previously revealed that a suitable prognostic cut-off for P53 is 50% production (21). In our cohort, the frequency of patients with <50% and ≥50% were estimated about 95.2% and 4.8%, respectively. Ki-67 prognostic cut-off was considered 15% cell staining and Ki-67 IHC results showed that 35.6% of the cases were in the Ki-67 group 2, ≥15%. In terms of hormone receptor status, 81.7% were ER positive and 76.9% were PR positive, while 76.9% of the patients were both ER and PR positive. In terms of molecular subtypes, 69.2%, 11.5%, 6.73% and 12.5% of the patients were in luminal A, luminal B, ERBB2 and Triple negative (TN), respectively (Table 1). In our cohort study *ERBB2* had the highest copy number variation, 26/104 (25%) increased amplification levels. 3/104 (2.9%) and 2/104 (1.9%) of our patients have amplification of *ERBB1* and *ERBB4*, respectively. We did not find increased copy number of *ERBB3* in our dataset (Figure 1A). According to our results, *ERBB1* and *ERBB2* co-amplification was observed in 2/104 (1.9%) of patients. We did not find co-amplification in any other ERBB family members in our dataset (Data not shown). The distribution of *ERBB 1-4* amplification for the four main tumor subtypes (luminal A, luminal B, triple-negative, and ERBB2-enrich) is presented in figure 1B.
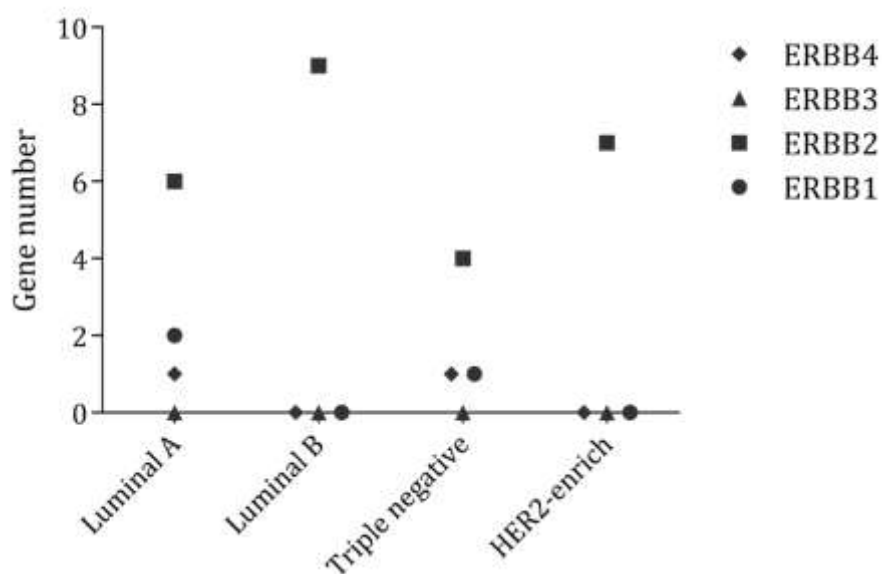
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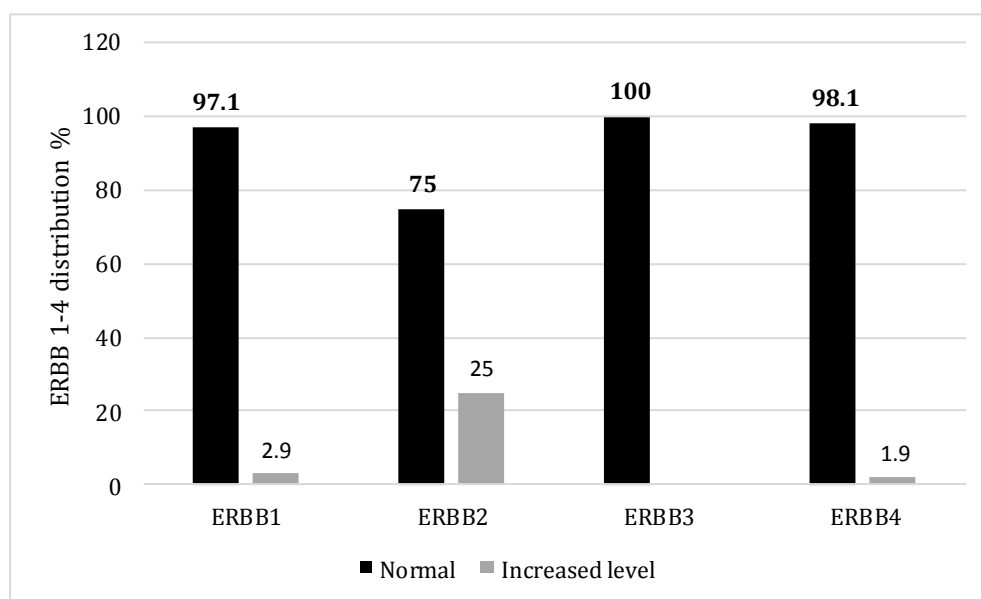
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Fig.1. Distribution of amplification level of *ERBB* family. **A)** The frequency of increased copy number among *ERBB 1-4* genes; **B)** The distribution of *ERBB 1-4* increased level regard to tumor subtypes.

Table 1. Clinical and pathological characteristics of patients.

Variable	n	%	Variable	n	%
Age			**Lymph node**		
< 50	54	51.9	**Negative**	71	68.3
≥50	50	48.1	**Positive**	33	31.7
Breast			**Menopause**		
Right	54	51.9	**No**	55	52.9
Left	50	48.1	**Yes**	49	47.1
Marital			**Smoking**		
Married	92	88.5	**No**	92	88.5
Single	12	11.5	**Yes**	12	11.5
Nulliparity			**P53**		
No	92	88.5	**< 50%**	99	95.2
Yes	12	11.5	**≥50%**	5	4.8
Tumor size			**Ki.67**		
≤2	33	31.7	**< 15%**	67	64.4
>2	71	68.3	**≥15%**	37	35.6
ER			**Nuclear grade**		
Positive	85	81.7	**1**	31	29.8
Negative	19	18.3	**2**	61	58.7
PR			**3**	12	11.5
Positive	80	76.9	**Histologic grade**		
Negative	24	23.1	**1**	19	18.3
HER-2/neu			**2**	67	64.4
Positive	19	18.3	**3**	18	17.3
Negative	85	81.7	**Stage**		
Tumor subtype			**I**	26	25
Luminal A	72	69.2	**II**	54	51.9
Luminal B	12	11.5	**III**	19	18.3
Triple negative	13	12.5	**IV**	5	4.8
ERBB2-enrich	7	6.7			

Correlation between clinicopathological parameters and copy number variation of ERBB1–4

ERBB2 amplification showed a significant correlation with age (*p value*= 0.01), location of the affected breast (*p value*= 0.01), nulliparity (*p value*= 0.08), menopause (*p value*= 0.008), negative ER (*p value*= 0.02), positive ERBB2 status (*p value*= 0.004), high histological grade (*p value*= 0.05), and high nuclear grade (*p value*= 0.02 (Table 2). Because of low number of patients with amplified *ERBB1*, *ERBB3*, and *ERBB4*, we were not able to observe a typical correlation between them and clinicopathological features. Nonparametric Steel Multiple Comparison Wilcoxon Test showed that *ERBB2* copy number amplification represented a significant association with BC tumor subtypes in comparison with luminal A subtype (*P*= <0.001). Moreover, by doing the same nonparametric test, histological grade and tumor stage also showed a significant association with *ERBB2* copy number amplification (*P*= 0.01 and *P*= 0.02, respectively (Table 2).

Table 2. The relationship between *ERBB2* copy number and clinicopathological parameters based on logistic regression calculation.

Marital			Smoking		
Married	92	Ref*	No	92	Ref*
Single	12	0.26	Yes	12	0.38
Nulliparity			P53		
No	92	Ref*	< 50%	99	Ref*
Yes	12	0.08	≥50%	5	0.46
Tumor size			Ki.67		
≤2	33	Ref*	< 15%	67	Ref*
>2	71	0.61	≥15%	37	0.94
Estrogen receptor			HER-2/neu		
Positive	19	Ref*	Positive	85	Ref*
Negative	85	0.02	Negative	19	0.004
Progesterone receptor					
Positive	24	Ref*			
Negative	80	0.99			

Tumor subtypes	n	ERBB1	ERBB2	ERBB3	ERBB4
Luminal A	72	---	---	---	---
Luminal B	12	0.68	<0.001	NA	0.51
Triple-negative	13				
ERBB2-enrich	7				
Histological grade					
I	19	---	---	---	---
II	67	0.58	0.01	NA	0.61
III	18				
Nuclear grade					
I	31	---	---	---	---
II	61	0.61	0.16	NA	0.47

Comparison of MLPA and IHC for copy number assessment of ERBB2

According to MLPA results, 75% of patients had normal copy number status for ERBB2, and 25% showed amplification. The FISH test was performed on the samples that showed IHC equivocal results, samples which were IHC negative/MLPA amplified and samples which were IHC positive/MLPA normal (Table 3). ERBB2 overproduction status by IHC in our

cohort study represented that in ERBB2 IHC score 0 group, 77.8% of cases were CNV normal and in the +1 group, 94.3% were CNV normal. 22.2% and 5.7%, in IHC 0 and 1+ cases, respectively, had CNV increased level. In IHC 2+ group, 38.9% had CNV increased level and 61.1% were CNV normal. Finally, in IHC 3+ group, 80% showed MLPA amplification and 20% were CNV normal (Table 3).

In addition, MLPA and IHC techniques were both compared by dividing the cases into two groups according to the tumor stage; early stages (1, 2A, and 2B) and advanced stages (3A, 3B, and 4). In early stages, 64/104 (61.5%) and 16/104 (15.4%) of patients were categorized into CNV normal and amplified, respectively. While, for

ERBB2 IHC, 66/104 (63.5%) and 14/104 (13.5%) were classified into IHC negative and positive, respectively. In advanced stages, 14/104 (13.5%) and 10/104 (9.6%) of patients were categorized into CNV normal and amplified, respectively. While, for ERBB2 IHC, 19/104 (18.3%) and 5/104 (4.7%) were classified into IHC negative and positive, respectively (see table 4). Our results also indicated that MLPA is more sensitive than IHC in both early and advanced tumor stages. The power of amplification detection for MLPA was 15.4% in early stages while for IHC was 13.5% (1.2-fold more sensitive). Moreover, in advanced stages, the power of amplification detection for MLPA was 9.6% while for IHC was 4.7% (2-fold more sensitive).

Table 3. Comparison of ERB*B*2 gene amplification by MLPA with HER-2/neu protein overexpression by IHC.

		IHC				
		0	**1+**	**2+**	**3+**	**Total**
MLPA	**Normal**	14 (77.8%)	50 (94.3%)	11** (61.1%)	3** (20%)	78
	Amplified	4* (22.2%)	3* (5.7%)	7* (38.9%)	12 (80%)	26
Total		18	53	18	15	104

*These cases were amplified by FISH. **These cases were not amplified by FISH.

Table 4. MLPA and IHC ERBB2 test results for 104 cases divided into two groups regard to tumor stages.

Stage category	MLPA		IHC	
	Normal	**Increased amplification level**	**Negative**	**Positive**
Early stage (1, 2A, 2B)	64 (61.5%)	16 (15.4%)	66 (63.5%)	14 (13.5%)
Advanced stage (3A, 3B, 4)	14 (13.5%)	10 (9.6%)	19 (18.3%)	5 (4.7%)

Discussion

One of the conventional mechanisms for oncogene overexpression is *genomic amplification* which is frequently found in human cancers including BC (22-24). According to the literature, significant role of the ERBB family of receptor tyrosine kinases has been demonstrated in the tumorigenesis of various cancers (25). The amplification of ERBB family members has been reported in different cancer types including BC (26, 27). However, except *ERBB2*, their clinical significance in the prognostic evaluation of BC patients remains mainly obscure (28-30).

Various techniques have been employed to detect *HER2* amplification or protein overproduction including IHC, FISH, quantitative Southern blotting, and real-time quantitative PCR. The most widely applied technique for determining HER-2/neu status is IHC. However, due to technical issues and equivocal results gained by IHC, it seems that PCR-based techniques offer a more straightforward alternative with higher throughput for gene dosage determination (31).

COSMIC is the largest source of somatic mutation information for human cancers which

includes over 32000 genomes. It also consists of information from other databases such as the cancer genome atlas (TCGA) and international cancer genome consortium (ICGC), that from a somatic perspective provides comprehensive coverage of the cancer genomic landscape. In addition, CCLE is a public access database for visualization of genomic alteration in about 1100 cancer cell lines. By using the information provided through these two databases, the distribution pattern of ERBB 1-4 in different types of cancer was found, including BC. Our data had concordance with the information provided by COSMIC and CCLE and showed that among all ERBB family members, *ERBB2* gene amplification was highly observed in BC patients. Moreover, this was consistent with the most of previous studies that reported *ERBB2* amplification being frequently found in BC and could be used as a precious prognostic biomarker for BC (26, 32).

In the current research, protein overproduction by IHC is lower than the values described in the literature, namely 20–30% positivity (33-35). The 25% amplification occurred in *ERBB2* MLPA results which is more likely consistent with standard production of HER-2/neu as mentioned before. This confirms results from similar studies (36, 37). This also validates MLPA as a good alternative assay for detection of HER-2/neu amplification in BC.

Consistent with a previous study (31), we found that MLPA had a good correlation with IHC. The obtained MLPA results showed amplification in 38.8% of the IHC 2+ cases that are generally regarded as equivocal and necessitating a second line amplification test, which is in accordance with previous studies (38-41). This represented that MLPA can aid therapeutic decision in these equivocal cases. In our dataset, a high concordance between amplification by MLPA and FISH was observed. In this regard, MLPA seems to be suitable for detection of HER-2/neu amplification in perhaps all BC cases, not just restricted to the IHC 2+ cases. There are as yet only few available data indicating that both amplified and not overexpressed cases respond to ERBB-2 directed therapy (42, 43). Nevertheless, MLPA might be suitable as an alternative pre-screening tool to IHC. In addition, our results indicated that by comparing the percentage of positive amplification detection, MLPA is more sensitive than IHC in early stages (1, 2A, and 2B) and in advanced stages (3A, 3B, and 4). In this line, MLPA seems more valuable than IHC for subcategorizing in tumor stages. Altogether, MLPA as a PCR-based technique is fast, easy to implement specially on FFPE samples, cheap, and more quantitative than IHC and consequently allowing more straightforward interpretation.

In a larger study by Rimawi MF et al (11) it was concluded that *ERBB1* expression in patients receiving adjuvant treatment has correlation with higher risk of relapse and blocking ERBB1 may improve outcome in patients. In contrast with our findings, a recent study reported that *ERBB1* amplification occurred in 25.2% of 114 studied cases and suggested that *ERBB1* and *ERBB2* co-amplification favor distant metastasis following initial surgery and are remarkably correlated with poor clinical outcome in BC patients (44). This implies that, maybe there are enormous geographic variations in *ERBB1* amplification status. The favorable effect of *ERBB4* on patient outcome has been demonstrated in multiple studies that assessed all family members simultaneously (28, 45-47). In current research, no association between *ERBB4* and patient outcome was found because of data insufficiency.

In addition, *ERBB3* was the only member of ERBB family which no amplification was observed for this gene in the studied subjects. This observation is in agreement regarding the negative prognostic value of ERBB3 which previously reported by other studies (45, 48, 49). On the other hand, several studies have suggested that ERBB3 expression is associated with a favorable clinical outcome (28, 46). Furthermore, a positive correlation between ERBB3 and ERBB4 along with a negative association with ERBB1 expression has been found in a number of studies evaluating the ERBB family members (28, 46, 48).

During last years, it is pointed that the increasing impact of oncogenic *ERBB2* activation due to mutation in somatic genes has become an important issue. It is elucidated that an important

subgroup of *ERBB2*-activated cancers has not been correlated with simultaneous *ERBB2* amplification and thus, they may be examined by standard analyses of *ERBB2* positivity based on FISH, MLPA, and IHC techniques. Accordingly, to analyze the interactions between alternative somatic mutations and amplifications of *ERBB* family members, further be studies with larger cohort sample sizes.

Acknowledgment

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