MOLECULAR TESTING OF HER2 OVEREXPRESSION IN BREAST TUMORS

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ABSTRACT

HER-2 is amplified in approximately 15–20% of all breast cancers this results in overexpression of the HER-2 protein. The HER-2 gene and its protein have been utilized for disease diagnosis and treatment response. This study was conducted for quantitative analysis of HER-2 in breast cancer patients by the quantitative Real Time- Polymerase Chain Reaction (qPCR) technique. The qPCR technique was performed on DNA extracted from 30 fresh frozen breast cancer tissue samples collected from National Cancer Institute, Misurata (NCI), The relative quantification of HER-2 gene amplification was measured in normal and tumor breast tissues and compared with the amplification of IGF-1 gene. The results of HER-2 amplification fold changes were analyzed and compared with Immunohistochemistry (IHC) tests of the same tissues. The results of our study demonstrated that 20% of the studied breast tissues had high levels of HER-2 gene expression and their fold change ranged from 2.3 to 25 folds using the qPCR method. The breast tissues with high expression of HER2 gene were scored by immunohistochemistry analysis as +2, and +3. All breast tissues with low level of expression of HER-2 gene determined by qPCR had negative or low score of HER-2 tested by IHC with 100% agreement in the two methods. However, only 38.46% of breast tumor tissues with high score of HER-2 by IHC, had high amplification fold change by qPCR. In our data, HER-2 amplification detected by qPCR was significantly associated with the absence of estrogen and progesterone receptors. The gene amplification of HER-2 had no association with stage, age, lymph node status, site of tumor, blood group, and the tumor type. qPCR method may play a significant role in diagnostics of HER-2 status in breast cancer tissues.

KEY WORDS: Breast cancer, HER-2 gene, qPCR, DNA amplification, Immunohistochemistry (IHC), Estrogen receptor, Progesterone receptor.

INTRODUCTION

Breast cancer is the most common type of cancer and the most common cause of cancer-related mortality among women worldwide[(1)]. In women, breast cancer comprises for 29 % of new cases of cancer and 14% of cancer deaths, according to American Cancer Society (ACS)[(2)]. Genetic alteration in gene copy number by amplification or deletion is a common mechanism that leads to deregulation of gene expression and finally to neoplastic transformation. Gene amplification plays an important role in the initiation and progression of many tumors[(3-5)]. HER-2 gene is a proto-oncogene localized on chromosome 17q and known as ERBB2, NEU, NGL, TKP1, CD340, and HER-2/neu. It is a member of the ErbB protein family, and recognized as the epidermal growth factor receptor family, which includes four transmembrane receptor tyrosine kinases (HER family) involved in signal transduction pathways that regulate cell growth and proliferation. HER-2 gene amplification is found in 10–34% of breast carcinomas[(6,7, 8,9)] and clinically, HER2 is important as the target of the monoclonal antibody trastuzumab (Herceptin). Trastuzumab is only effective in breast cancer where the HER2 receptor is over-expressed[(10)]. The most common detection methods for *HER-2/neu* include measurement of protein overexpression by the immunohistochemical assay HERCEPTest[(11)] and the detection of gene amplification by fluorescence in situ hybridization (FISH) techniques [(1)], both approved by the US Food and Drug Administration. Recently, PCR based methods have become important in clinical analyses, such as conventional competitive PCR[(13)] and competitive reverse transcription-PCR (RT-PCR) methods[(14)] or more advanced, quantitative real-time PCR methods for RNA[(15,16)] and DNA[(17,18,19)] analysis. Several methods can be used to determine the HER-2/neu DNA amplification and protein overexpression including fluorescence in situ hybridization (FISH)، immunohistochemistry (IHC)[(20)], chromogenic in situ hybridization (CISH) which are semi-quantitative[(21,22)]. In addition, qPCR can be used for quantitative measurement of the HER-2 DNA/RNA[(23)]. However, FISH and IHC are the most common methods used and have been approved by the US Food and Drug Administration (FDA)[(24)].

This study used qPCR analysis of DNA from fresh frozen breast cancer tissues to examine HER-2/neu gene amplification and to correlate the findings with patients' clinical data. In addition, the obtained data of qPCR compared with IHC results.

MATERIALS AND METHODS

The study was carried out on thirty female patients diagnosed with breast cancer in National Cancer Institute, Misurata, Libya. The mean age of the patients was 45.89 years, ranging from 32 to 70 years. Fresh

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tumor samples were obtained from both mastectomy and excision biopsy specimens. Samples were collected in the period between 2015 and 2016, and stored at -80ºC.To evaluate the status of HER-2, a piece from the tumor and paired normal tissues (about 10 samples) were collected. For these cases, sampling was cut at least 2 cm away from the tumor margins the rest of the specimen was placed in formalin and send to pathology laboratory at NCI, Misurata for routinely analysis by immunohistochemistry (IHC). Thirteen of these samples were evaluated as IHC 3+, two were evaluated as IHC 2+ and 15 of them were negative for HER-2 using the same methodology. All the subjects provided written informed consent and the study was approved by NCI ethical committee.

DNA extraction:

The genomic DNA was extracted in Biotechnology Research Center (Misurata, Libya) from fresh frozen sections of breast tissues using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, tissues were lysed enzymatically in optimized buffers that stabilize nucleic acids, and then the lysates loaded onto the QIAmp spin column in which the DNA binds specifically to the silica gel membrane while contaminants pass through. After washing steps, the DNA eluted in TE buffer (Tris-HCL+EDTA). The extracted DNA stored at -20 ^{0}C. The quality and concentrations of all isolated DNA samples were estimated based on their ultraviolet absorbance spectra between 260 and 280 nm on a QIA xpert machine (Qiagen company), and gel electrophoresis. The DNA was of high purity as indicated by the ultraviolet absorbance spectrum at ratio A260/280 between 1.8 and 2.0.

Immunohistochemistry:

IHC for HER-2/neu was performed in neutral-buffered formalin-fixed paraffin-embedded tissue (FFPE) from all breast cancer patients. Sections with 5mm thickness were cut from the tissue blocks and transferred them to glass slides (ChemMate capillary gap microscope slides, 75 µm; Dako). The paraffin sections were placed in an oven overnight at 37 °C. After deparaffinization for 10 min in xylene, acetone, and acetone/Tris-buffered saline (100 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5), the sections were washed twice with Tris-buffered saline. Staining was performed with the HERCEPTest reagent set (Dako) exactly as specified by the manufacturer. Epitope retrieval was performed at 95 °C in a water bath for 40 min. Positive and negative controls from the reagent set were used. Counterstaining was performed with hematoxylin. The slides were dehydrated in graded alcohol and cleaned in xylene before being cover slipped in gelatin. Invasive breast cancer cells were scored as 0, +1, +2 or+ 3 according to the HERCEPTest guidelines. Scores of 0 or +1 were regarded as immunohistochemically negative, whereas scores of +2 and +3 were regarded as immunohistochemically positive.

Quantitative Real-Time PCR (qPCR):

The SYBR Green chemistry used to perform the qPCR on a Roter-gene Q real-time PCR cycler (Qiagen, Germany) with HER-2 primers (HER2F: ACAACCAAGTGAGGCAGGTC, HER2R: GTATTGTTCAGCGGGTCTCC. Insulin like growth factor 1 (IGF-1) was used as a reference gene (endogenous control) for data normalization of qPCR. IGF-1 primers were: IGF-1 F: AGCTCGGCATAGTCTT, and IGF-1 R: CCAAGTGAGGGGTGTGA. The PCR amplification was performed using a 36-well tray with 1x SYBR Green qPCR Master Mix, 0.3 µM of each primer, 100 ng of DNA sample, and water in a final volume of 25µl for each reaction. The initial activation of the reaction was at 95^{0}C for 5 min followed by 40 cycles of 95^{0}C for 5 sec, and annealing /extension at 60^{0}C for 10sec. To rule out DNA cross contamination of reagents and formation of primer dimers; no-template control included in each run. Each DNA sample analyzed in triplicates and the means were calculated.

Standard curve preparation:

A dilution series of the DNA from normal samples were prepared ranging from 0.0002 ng to 20 ng to test the linearity of the measurement achieved by the qPCR threshold cycle. Ct value from each dilution was plotted versus the log DNA initial concentration. The optimum DNA concentration value was established in 0.2 ng because it gives the lower standard deviation SD. So all DNA was diluted to the same concentration (0.2 ng). The relative gene copy number was determined based on estimating the threshold cycles Ct of HER-2. Calculating the amplification fold change was carried out by following the steps [25]:

ΔCt = (Ct sample – Ct reference gene)

$\Delta\Delta Ct$ = (ΔCt sample – ΔCt calibrator)

Where Ct sample = Ct (target gene) – Ct (reference gene), Ct calibrator = Ct (target gene in normal (non-cancerous) tissue – Ct (reference gene).

Expression fold change = $2^{-\Delta\Delta Ct}$

Statistical analysis:

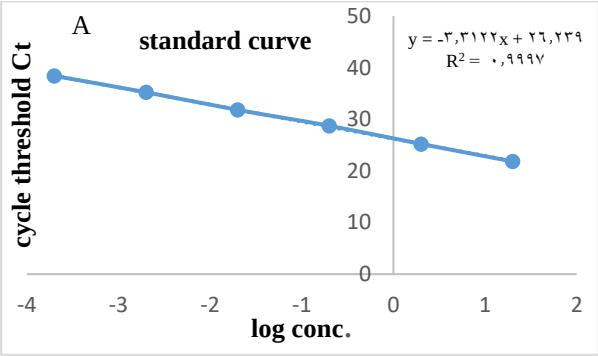
The Chi-square test was used to determine the association between HER-2 amplification and clinical data of patients. A value of $p<0.05$ was considered statistically significant. All the analyses were performed using IBM SPSS 21 statistical software.

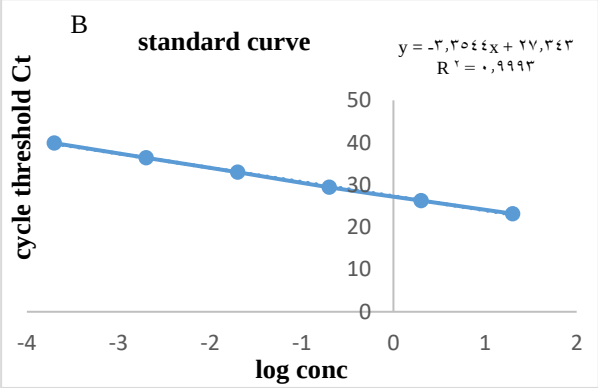
RESULTS

qPCR amplification of HER-2 and its correlation with clinical data:

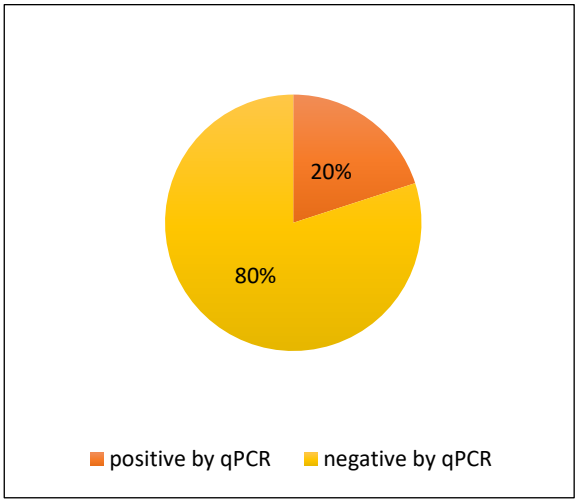
The standard curves of HER-2 and IGF-1 amplifications are shown in (figure1), A and B, respectively. To consider a case positive by qPCR, we set a cutoff for

the amplification of HER-2 to be higher than 2 as reported in previous studies[26, 27]. From the 30 patients, 6 (20%) showed positive amplified of HER-2 gene. The amplification range was between 2.37 to twenty-six-fold. The remaining 24 (80%) of patients were negative for HER-2 amplification and their ranges were less than 2.0 as shown in (figure 2).



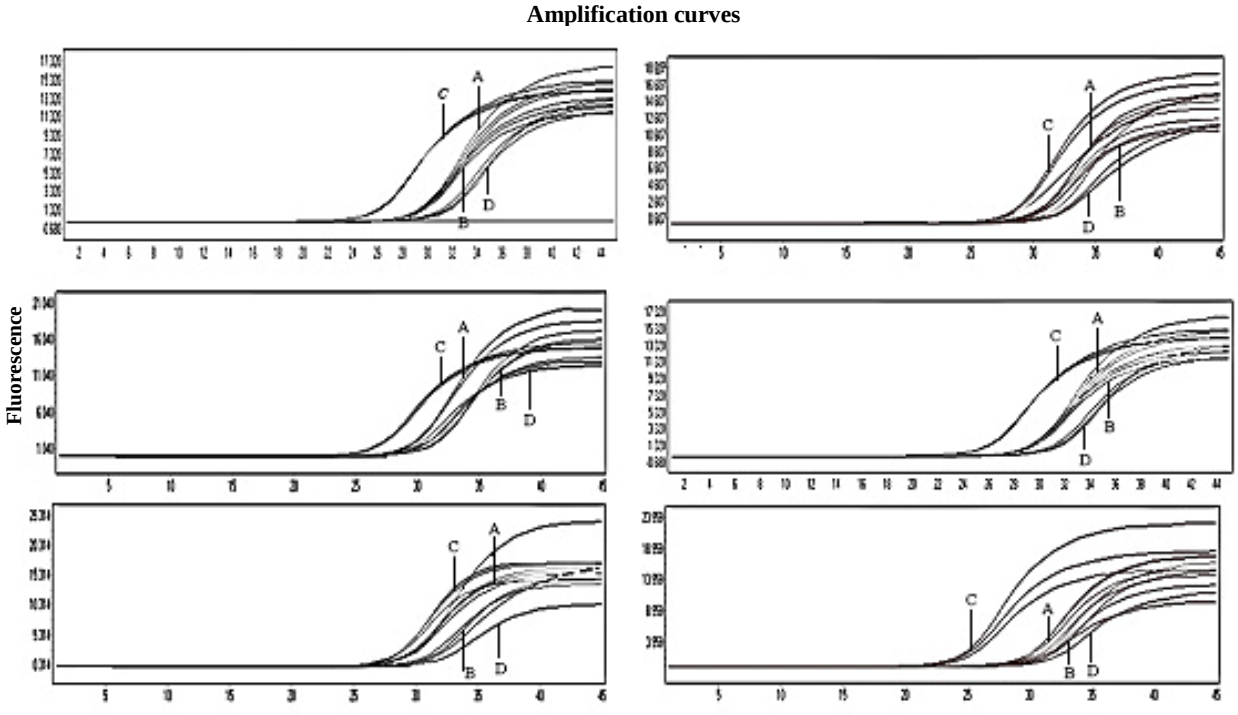


(Figure 1) Mean standard curve of triplicate quantification experiments for HER-2 gene(A), and IGF-1 gene (B) 1:10 dilutions.



(Figure 2) qPCR amplification of HER-2.

The results of the typical signal *curves for patients with significant HER-2 gene amplification were shown in figure 3. The amplification of reference gene* IGF-1 is similar in both tumor breast tissues and normal breast tissues (A and B curve). On the other hand, the amplification of HER-2 gene is different in tumor breast tissues (C) and in normal breast tissues (D) (figure 3).



(Figure 3) Comparative quantification of HER-2 and IGF-1 in breast tissues.

In each qPCR experiment 0.2 ng of genomic DNA was analyzed in triplicates

The qPCR data was correlated to ER, PR status, lymph node status, age, stage, type of breast cancer, site of tumor, and blood groups. The positive HER-2 patients showed strong association with negative ER, PR status (P = 0.000, 0.001 respectively), and no significant association was found between positive HER-2 patients with lymph node status (P=0.361), age (P=0.439), stage (P=0.744), site of tumor (P=0.223), blood groups (P=0.732), and the tumor type (P=0.138) (table 1).

The comparison between qPCR and IHC

To determine the strength of potential agreement between both qPCR and IHC, we calculated the percent positive /negative agreement as follows: the number of positive /negative cases detected by both techniques divided by the number of positive/negative cases identified by IHC [27, 28]. The percent positive agreement was 38.46% (5/13) while the percent negative agreement was 100 % (15/15). qPCR and IHC identified the tumors as belonging to the same group in 71% of the cases. Furthermore, 94% of IHC negative tumors (0, +1, +2) were also negative by real time PCR. In comparison between the HER-2 status determined by qPCR and IHC, the tumors that were positive by qPCR (fold change > 2) had positive scores by IHC: +2, +3. While the tumors that had less fold change by qPCR <2, they had scores by IHC: 0, +1, +2, +3. Our results showed that 8 breast tumors were positive by IHC but negative by qPCR and had high scores +3 by IHC, while qPCR analysis revealed that they had fold change < 2. The results of comparison are presented in (table 2).

(Table 1) Association between HER-2 amplification by qPCR and clinical data (n=30).

	qPCR (negative)	qPCR (positive)	P value
ER status positive negative	 22 2	 1 5	 0.000
PR status positive negative	 22 2	 2 4	 0.001
Lymph node status Positive negative	 21 3	 0 6	 0.361
Age <50 years >50years	 17 7	 5 1	 0.439
Stage Stage I Stage II Stage III	 2 10 10	 0 2 4	 0.744
site of tumor left side right side	 11 11	 4 1	 0.223
Blood Groups A B O AB	 9 5 8 2	 1 0 3 0	
Type of tumor IDC ILC IDC+ILC IDC+DCIS Comedo carcinoma	 19 3 1 0 1	 4 0 1 1 0	 0.138

ER estrogen receptor, PR progesterone receptor, IDC invasive ductal carcinoma, ILC invasive lobular carcinoma, IDC+ILC combined invasive ductal carcinoma with invasive lobular carcinoma, IDC+DCIS combined invasive ductal carcinoma with ductal carcinoma *in situ*. qPCR quantitative real time PCR.

(Table 2) Comparison of HER-2 status detected by qPCR and IHC (n=30).

IHC	qPCR	
	Amplified HER-2 (fold <2)	**Amplified HER-2 (fold > 2)**
0	12	0
+1	3	0
+2	1	1
+3	8	5
Total	24	6

DISCUSSION

The incidence of breast cancer is increasing worldwide. Human epidermal growth factor receptor 2 (HER-2) has high impact on the diagnosis, and eventually choosing the right therapy of cancer. Therefore, it is important to use simple, accurate and reproducible method to determine HER-2 status. HER-2/neu gene amplification and protein overexpression have been found in about 20-30% of breast cancers. In approximately 90% of these cancers, the over expression of HER-2 protein is attributable to gene amplification [29]. The IHC score +2 needs further confirmation by FISH which is considered a high cost procedure. We have used qPCR of DNA amplification as an alternative method to determine HER-2 status. The development of real-time PCR techniques makes it possible to perform more precise quantitative analysis of gene amplification. These techniques are easy and quick to perform. qPCR can be used to run multiple samples, and can be automated, making them potentially useful technique for screening tumors for HER-2/neu amplification in a routine clinical setting[30, 31].

In our study, 20% of 30 breast tumors showed high HER-2 gene amplification using qPCR. Our finding is similar to other studies, which reported that HER-2 amplification was found in 20-40% of breast cancer patients[30, 32, 33, 27]. Some studies planned to evaluate the concordance between qPCR for HER-2/neu testing and the conventional tests such as immunohistochemistry and FISH[34, 35]. The negative agreement between

qPCR and IHC results were high at 100% as previously reported[27, 36]. However, the percent positive agreement was lower at 38%. About 38% of score +3 of tumors showed positive HER-2 amplification; whereas the remaining 61% of these tumors were negative. The cause of discordance between results of IHC and qPCR may due the sensitivities of the two methods including post-transcriptional regulation of the gene that results in receptor overexpression without gene amplification. Or may because intra observer error due to subjectivity of IHC interpretation and qPCR analysis.

Moreover, the present study showed significant association between HER-2 gene amplification by qPCR and ER, PR status. This has been explained by hormone dependent down regulation of HER-2 involving a complex molecular interaction. Estrogen and its receptor are required to suppress HER-2[31] as reported previously[27, 30]. The gene amplification of HER-2 had no significant association with stage, age, lymph node status, site of tumor, blood group, and the tumor type. Many authors reported that HER-2 gene had no association with histological type[27, 32] age[37] stage[34, 27, 37-40], and lymph nodes involvement[27, 39, 41, 42]. In contrast, some authors, reported an association of HER2 over-expression with age[41], lymph nodes involvement[27, 29] and histological type[29, 43, 44]. Dissimilarities in the results might depend on the use of different antibodies in IHC, different primers, probes, more than one reference gene and different DNA binding dyes in the real time PCR.

CONCLUSION

We conclude that qPCR is a sensitive and promising method to evaluate the level of HER-2 gene. HER-2 gene amplification is associated with the low level of ER and PR expression. qPCR method could serve as a complementary screening tool for detecting the HER-2 gene level in breast tissues.

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