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Detection of *HER2* gene amplification in formalin-fixed and paraffin-embedded breast cancer tissues by quantitative real time polymerase chain reaction in ER and PR-positive *HER2* immunohistochemistry 0 or ≥ 2+ breast cancer

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ABSTRACT

Amplification of the human epidermal growth factor receptor 2 (*HER2*) genes and overexpression of its gene product, the *HER2* protein was found in 20% of breast cancers. Genetic instability and hence aberrant *HER2* amplification may be associated with breast cancer progression. *HER2* status determination plays an important role in selecting the best treatment options in early and advanced breast cancer. This is a cross-sectional clinical genetic study of breast cancer patients in Kuwait. The aim of this study is to detect *HER2* gene amplification in Formalin-Fixed Paraffin Embedded (FFPE) breast cancer tissue samples with IHC 0 or ≥ 2+ and were ER and PR positive.

Quantitative real time Polymerase Chain Reaction (qPCR) was performed on 44 FFPE breast cancer tissue samples with IHC 0 or ≥2+ and were ER and PR positive. DNA was isolated using NucleoSpin®DNA FFPE XS kit. Real time PCR was carried out using TaqMan™ Fast advanced Master Mix. The beta actin gene was used as a reference gene to the *HER2* gene investigated. The 7500 fast real-time PCR system was used.

Samples that were evaluated as *HER2* equivocal and were negative by FISH presented high qPCR values of 10 and 28. A sample that was equivocal and then negative by FISH presented a qPCR value of 28 and was from a grade 3 patient. On the contrary, one *HER2* equivocal sample by IHC and positive by FISH presented a qPCR value of 16 which was from a grade I patient. Seven samples that were grade I and II were evaluated as *HER2* negative by IHC presented high qPCR values of 9, 12,13, and 15.

Conclusion: Quantitative PCR is a molecular method that is more sensitive than FISH or IHC. The screening for the status of the *HER2* gene alongside the protein status by IHC provides thorough evaluation especially in the higher grades of breast cancer who might get more accurate evaluation and might benefit from better treatment options.

Keywords: *HER2* gene; Amplification; Formalin-Fixed Paraffin Embedded (FFPE); Breast cancer; Polymerase chain reaction

INTRODUCTION

The human epidermal growth factor receptor 2 gene (*HER2*, also known as *erbB2*) has been regarded as a critical factor for diagnosis and effective treatment of breast cancer patients [1]. The gene encoding *HER2* protein is located on chromosome 17. Amplification of the *HER2* gene and overexpression of its gene product, the *HER2* protein was found in 20% of breast cancers [2,3]. The signaling pathways of the Estrogen Receptor (ER) and the *HER2* are the dominant drivers of cell proliferation and survival in the majority of breast cancers [1,4]. Genetic in-stability, and hence aberrant *HER2* amplification may be associated with breast cancer progression [5].

Progesterone Receptors (PR), (ER) and *HER2* are the most routinely used basic, prime molecular markers for detection of breast cancer worldwide. These markers provide the suitable prediction of the prognosis of cancer recurrence after an initial remedial treatment. These three markers indicate eventual future appropriate treatment; and thus, they play a key role in the management of breast cancer. Molecular techniques can be implemented to detect ER, PR and *HER2* levels. The RT-PCR technique can detect low concentrations of ER/PR/*HER2* mRNA. If the molecular techniques such as RT-PCR and qPCR are utilised in the routine diagnosis of best cancer a new breast cancer scoring system based on these newer expeditious technologies will be established, then grading and management of breast cancer will be easier, enhanced, and quicker. Hormone receptors and *HER2* as molecular markers are of prime therapeutic importance and have the capability to take part in future drug development techniques [6].

Amplification and/or over expression of the *HER2* oncogene is found in many different types of human cancers, including breast, ovarian, lung, gastric and oral cancers. It is the second member of the epidermal growth factor receptor family. A correlation was found between the degree of gene amplification and aggressive potential of the tumour and its proliferative activity. It was found that amplifications and deletions are the most common mechanisms leading to gene deregulation [7].

HER2 gene amplification was found in Estrogen Receptor (ER)-positive, *HER2* Immuno-Histo-Chemistry (IHC) 0 or 1+ breast cancer in patients who developed early distant metastasis [8]. This indicates that *HER2* gene amplification detection can play an important role in the prognosis of the disease and the design of treatments plans. An association between *HER2* amplification and poor prognosis was found to be attributed to global genomic instability. This is due to the association that exists between cells with high frequencies of chromosomal alteration and increased cellular proliferation and aggressive behaviour [9]. Tumor grade and/or Ki67 expression are predictors of early recurrence in ER-positive, *HER2*-nagative breast cancer [10-12]. Endocrine therapy is the most important treatment option for women with ER-positive breast cancer [13]. The clinical significance in breast cancer for *HER2* gene amplification in *HER2* scores 0 or 1+, with no gene overexpression, was identified. In addition, some patients with *HER2*-IHC 0 or 1+ primary tumors develop early relapse with *HER2*-positive metastasis, which might be due to the existence of *HER2* gene amplification in the primary tumors [8]. Moreover, *HER2* mRNA detection could potentially serve as a quantitative and reliable method for identifying *HER2*-low breast cancer [14].

HER2 status determination plays an important role in selecting the best treatment options in early and advanced breast cancer [15,16]. The qPCR technique is based on the detection of DNA amplification. Accordingly, using a quantitative method such as qPCR will be complementary to IHC and FISH in determining *HER2* status in breast cancer patients and give them a better treatment opportunity. The sensitivity of the real-time PCR (RT-PCR) technique can provide the information related to small alterations such as point mutation, gene expression, gene loss or amplification and the analysis of cancer markers. Accordingly, RT-PCR plays an important role in clinical testing [17]. The implementation of molecular classifications for malignancies provides powerful tools for diagnosing and treating cancer. *HER2* gene quantification could benefit cancer patients if they will make them eligible for *HER2* targeted medications.

In this study qPCR was used to detect the losses or amplifications if any are found in the *HER2* gene in 44 FFPE breast cancer tissue samples with IHC 0 or ≥2+, and were ER and PR positive. Real time PCR is used for sensitive detection and quantification of the gene fold levels [19]. Breast cancer in Kuwait contributes for 49.4% from all types of cancers that were reported at Kuwait cancer registry for all adults. Kuwaiti breast cancer patient's ages were 15-99 years [20]. Across the Gulf cooperation countries, the GCC breast cancer stands as the prevailing malignancy [21]. In the GCC geographic region, there are distinctive features that the breast cancer manifests with, including an early onset, typically occurring before the age of 50, an advanced stage at presentation, and a higher pathological grade. In addition, in the GCC region more aggressive features such as *HER2* positivity, or the presence of Triple-Negative (TN) attributes were observed, particularly among younger patients [20].

MATERIALS AND METHODS

In this study Forty-four 44 FFPE breast cancer tissue samples from Royal Hayat hospital in Kuwait were studies. For all patients' tissues, IHC was performed to verify the expression of *HER2*. IHC test for the ^{HER2} protein over expression was performed using HercepTestTM kit (Dako). The IHC test for *HER2* protein was performed and validated at Royal Hayat hospital. DNA isolation from FFPE samples was performed using the NucleoSpin®DNA FFPE XS kit. According to the histopathology reports obtained from Royal Hayat hospital, 8 samples were grade I, 21 samples were grade II and 15 samples were grade III. The grading was made using the tumour size, Lymph Node involvement and Metastasis (TNM) grading system.

Quantitation of the *HER2* gene was done by real time PCR. Samples with no DNA and with normal DNA were included. The primes used for the *HER2* gene were as follows: Forward primer for *HER2* gene, 5'-AGCCTCTGCATTTAGGGATTCTC, reverse primer for the *HER2* gene, 5'-TAGCGCCGGGACGC, and the probe used was 6FAM5'-TGAGAACGGCTGCAGGCAACCC-3'TAMRA.1 For the quantitation of the β actin gene the following forward and reverse primer sequences were used respectively: ACTCCTATGTGGGCAACGAG, and AGGTGTGGTGCCAGATCTTC [22].

For real time PCR TaqMan™ fast advanced master mix (Applied bio-systems, Thermo Fisher Scientific Baltics UAB) was used. The beta actin gene was used as a reference gene to the *HER2* gene investigated. The 7500 fast real-time PCR system (Applied bio-systems) was used. Amplification conditions were 50ºC for 2 min, 95ºC for 10 min, 40 PCR cycles at 95 ºC for 15 s, and 60ºC for 1 min.

The qPCR for the *HER2* gene was set up in 96-well plates, in a final volume of 20 µL containing of up to 1 µL of isolated DNA, 10 µL of TaqMan™ gene expression master mix, 1 µL of forward gene primer, 1 µL of reverse gene primer, 0.5 µL of *HER2* gene probe, and 6.5 µL of molecular water. For the β actin gene qPCR reaction the following mixture was used: 1 µL of isolated DNA, 10 µL of PowerUpTM SYBR®Green Master Mix (appliedbiosystems Thermo Fisher Scientific Baltics UAB), 1 µL of forward gene primer, 1 µL of reverse gene primer and 8 µL of free molecular water.

Calculation of the relative fold values of the *HER2* gene

Averages for the Ct values were calculated for the *HER2* gene. The *HER2* gene was normalized with β actin gene. The ΔCt value which is the difference between the average of the Ct value of the target and the Ct value of the reference gene was calculated. Then the ΔΔCt value was calculated by subtracting the ΔCt value of the samples from that ΔCt value of the control which was the normal DNA sample. Then the 2^ΔΔCt value was determined. Finally, the relative fold change of the target gene in relation to the control sample was determined by dividing the sample 2^ΔΔCt value by that of the control normal DNA.

Converting the Ct values to a linear form using the 2^ΔΔCt value more accurately depicts the individual variation among replicate reactions [23].

Statistical analysis

A One-Way Analysis of Variance (ANOVA) test was used to analyse the data taken from the breast cancer samples to determine whether the three groups of patients; grade I, II and III *HER2* qPCR relative values are statistically different by calculating the means of the three groups. Then the means of the three patients' groups were tested for their difference from the mean of the dependent variable. The one way-ANOVA test will show if the dependent variable changes according to the level of the independent variable [23]. The breast cancer is considered as the independent variable. The qPCR values in the three groups were considered as the dependent variables. The null (H_0) hypothesis of the one way-ANOVA is that there is no difference among group means. The alternative hypothesis is that one group differs significantly from the overall mean of the dependent variable. One way-ANOVA test was applied to the data using Microsoft Excel. The significance threshold in the analysis was .05. The P value was interpreted to be significant if it is ≤ 0.05.

RESULTS

The qPCR values for the *HER2* gene were obtained. The Ct values for each breast tumor sample were normalized with β actin gene Ct values. The results were compared with normal DNA sample and the 1 fold change was obtained for normal sample. The fold change that is above 1 was considered an up-regulation and the fold change below 1 was considered a down-regulation. Regarding the *HER2* protein status of the samples determined by IHC; 32 samples were negative for *HER2*, 6 samples were equivocal, and 6 were positive.

The ages of the patients where the samples were investigated were 30-80 years. The highest number of patients was in the 50 years old group (Figure 1).

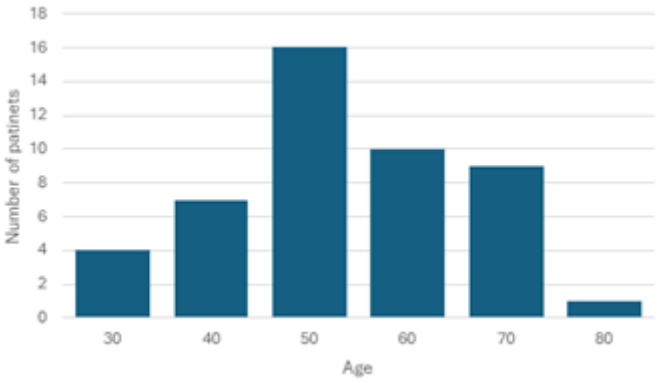

Figure 1. Number of patients related to age.

All the samples showed abnormal qPCR relative values compared to the normal. The relative fold values of qPCR of the *HER2* gene in grade I patients were all abnormally elevated compared to the normal sample (Figure 2). Similarly, the relative fold values of qPCR of the *HER2* gene in grade II patients were abnormally up-regulated (Figure 3).

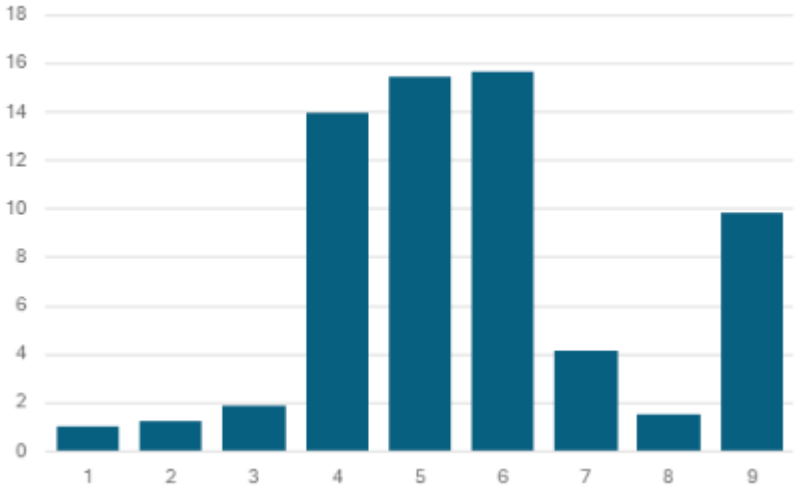

Figure 2. The relative fold values of qPCR of the *HER2* gene in grade I patients (Y axis) versus the patient number (X axis). Number 1 is the control sample.

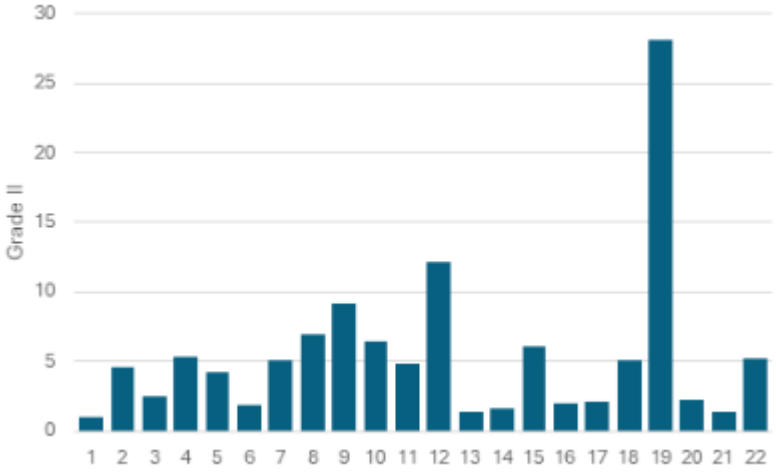

Figure 3. The relative fold values of qPCR of the *HER2* gene in grade II patients (Y axis) versus the patient number (X axis). Number 1 is the control sample.

One sample in the grade III group was down-regulated concerning the *HER2* qPCR relative value while all the remining samples showed variable degrees of up-regulation (Figure 4).

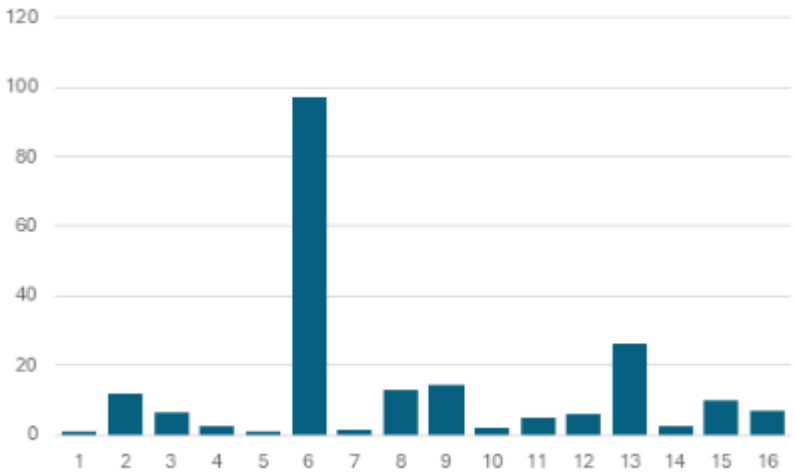

Figure 4. The relative fold values of qPCR of the *HER2* gene in grade III patients (Y axis) versus the patient number (X axis). Number 1 is the control sample.

With a P-value of .251138 for the one way-ANOVA test (Table 1), there is no significant association between the grade of the breast cancer tumor and the qPCR relative values. On the other hand, an amplified qPCR value for the *HER2* gene was observed in nearly all the samples, including the *HER2* negative samples. The *HER2* negative samples were the majority.

Table 1. ANOVA: Single factor results.

Summary						
Groups	**Count**	**Sum**	**Average**	**Variance**		
Column 1	8	52.84328	6.605409	34.5265		
Column 2	21	118.3349	5.634996	34.1284		
Column 3	15	207.42	13.828	575.6986		
ANOVA						
Source of variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between groups	626.4219	2	313.211	1.429386	0.251138	3.225684
Within groups	8984.033	41	219.1228			
Total	9610.455	43				

From the 44 archival samples previously evaluated by IHC the qPCR results showed variable relative values. Samples reported as *HER2* positive (3+) (Figure 5), presented high relative values. Two samples qPCR relative values were 15 and the others were 26, 97, 13. The sample with the 97 qPCR relative value was from a grade III patient. On the other hand, one *HER2* positive (3+) evaluated sample presented a qPCR value=2 which was taken from a grade I patient. Samples evaluated as *HER2* equivocal and were negative by FISH presented high qPCR values of 10 and 28 (Figure 6). The sample that presented a qPCR value=28 was from a grade 3 patient. On the contrary, one HER2 equivocal sample by IHC and positive by FISH presented a qPCR value of 16 which was from a grade I patient. Seven samples were evaluated as *HER2* negative by IHC (Figure 7) presented high qPCR values, being 2 samples with a relative value of 9, two other samples yielded a relative qPCR value =12, and 2, others gave a relative value =15, and 1 sample with qPCR value=13. Samples with an evaluation of *HER2* negative by IHC and high qPCR relative values were from grades I and II patients.

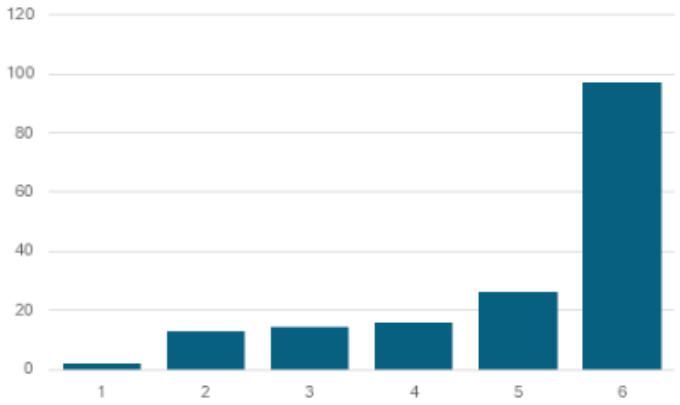

Figure 5. The distribution of *HER2* positive cases as detected by IHC related to the *HER2* qPCR relative value (y axis).

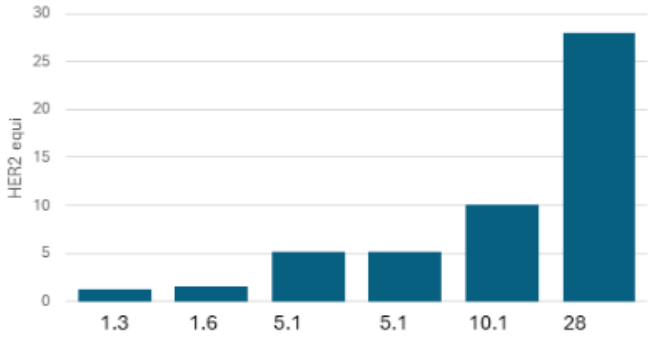

Figure 6. The distribution of *HER2* equivocal cases as detected by IHC related to the *HER2* qPCR relative value.

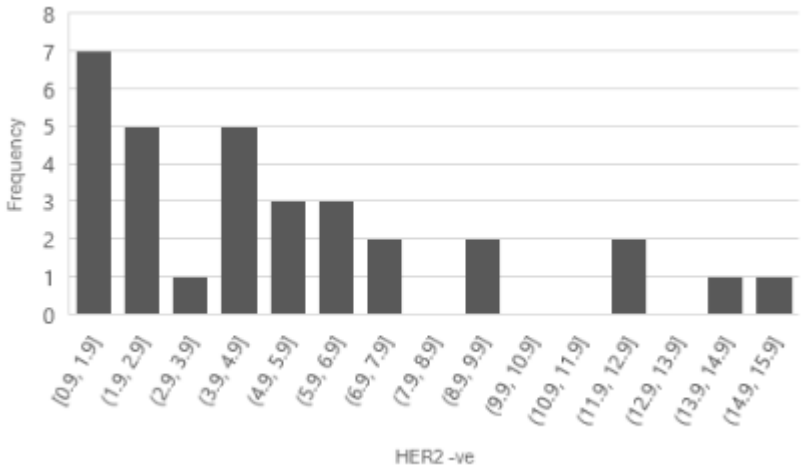

Figure 7. The frequency of the *HER2* negative cases as detected by qPCR.

DISCUSSION

The samples investigated in this study were all from higher pathological grade and more aggressive features such as *HER2* positivity. Which was noticed in the GCC countries [20]. This study was performed to investigate *HER2* gene quantity by using real-time PCR in grades I, II and III breast cancer archival tissue which were previously categorized by IHC into *HER2* negative, *HER2* equivocal and *HER2* positive (3+). The relative fold values obtained in this study were elevated in most of the samples with no significant association with the grade of the tumor. The molecular biology screening for the *HER2* gene is essential. Some patients who showed a negative *HER2* status by IHC, presented high qPCR values. In addition, *HER2* equivocal samples which were negative by FISH presented an elevated qPCR relative value. Notably the sample with an equivocal evaluation by IHC and -ve *HER2* status by FISH which belonged to a grade III patient. Quantitative reverse transcriptase real time PCR (qRT-PCR) may outperform FISH in identifying patients overexpressing *HER2* protein [24]. FISH analysis depends on the *HER2* positive cells observed with respect to

the amount of tissue analysed. Quantitative PCR analysis in this sense, avoids the possible misinterpretation by sensitive measurement of the gene quantity ratio that is independent from tissue amount. Accordingly, molecular techniques such as RT-qPCR might be added to the methods for screening *HER2* status in breast cancer patients.

Many laboratories have adopted IHC, as a screening method, and FISH as a conformation test for *HER2* equivocal cases, taking into consideration higher failure rate, longer procedure time and higher reagent cost of FISH, compared to that of IHC. Nevertheless, the IHC and FISH might not be as sensitive as qPCR especially in the higher grades of breast cancer who might get more accurate evaluation and might benefit from better treatment options. False negative or positive *HER2* evaluations may result in inappropriate treatment administration. Regarding the samples that were evaluated negative for *HER2* by IHC and presented elevated qPCR relative values, further assessment would be recommended. The differences between *HER2* 2+ or equivocal by IHC with respect to FISH and qPCR were previously reported [24,25]. There are factors such as tissue quality, fixation time and type of antibody used that might contribute to the variability in the results obtained for *HER2* 2+ by IHC [26,27]. Furthermore, considerations must be taken to the alterations of *HER2* status which might occur after neo-adjuvant chemotherapy or during metastatic progression, due to biologic or methodologic issues [28]. Studies have shown that *HER2* amplification-positive patients had a higher mutation frequency than the *HER2* amplification negative patients [29]. Another important factor in the determination of the *HER2* status would be the screening for the gene mutation. Hence, *HER2*- mutated non-amplified breast cancer may benefit from pyrotinib treatment as well.

Some researchers have suggested that qPCR cut-off should be greater than 2.7 to be considered as *HER2* amplification [30,31,26]. In our study 12 samples out of 32 in the *HER2* negative group showed lower relative qPCR fold values (≤ 2.3). On the other hand, 20 samples out of 32 at the *HER2* negative group showed high qPCR values (>3.6). These results necessitate the revaluation of the *HER2* testing methods at the hospital and adopting supporting tests such as molecular quantification methods. A study found out that a better correlation of clinicopathologic features and oncotype DX recurrence score was found between ER-positive, *HER2*-negative breast cancers by quantifying *HER2* mRNA levels than *HER2* IHC [14]. Thus, introducing molecular techniques such as qPCR in the routine evaluation for *HER2* status will support accurate quantification and interpretation.

In this study at the *HER2* equivocal group, 2 out of 6 cases analysed showed low qPCR relative value (≤1.6) and 4 presented higher values (5.1-28). These samples were negative by FISH. A study showed that qRT-PCR may outperform FISH in identifying patients overexpressing *HER2* protein, suggesting that qRT-PCR might be more accurate than qPCR and FISH [32]. Measuring the protein level is quite crucial together with that of the gene level. DNA copy number amplifications are one of the most important mechanisms leading to deregulated gene expression in cancer [15]. In this study, among the *HER2* positive group by IHC one case presented a qPCR relative value equal to 2.2 and 5 presented higher values (3.1-97). The two highest qPCR values in the *HER2* positive group were a grade three patients.

CONCLUSION

As observed in the amplified qPCR values of the cases that were negative in *HER2* protein status by IHC or FISH, a molecular technique such as qPCR or RT-qPCR might be added to the methods for screening HER2 status in breast cancer patients. The routinely used methods in breast cancer evaluation IHC and FISH might not be as sensitive as qPCR especially in the higher grades of breast cancer who might get more accurate evaluation and might benefit from better treatment options. Inaccurate *HER2* evaluations may result in inappropriate treatment administration. Future studies that involve the screening for *HER2* mutations alongside the conformational evaluation by qPCR in *HER2* negative compared to *HER2* positive are important. Finally, hormone receptors and *HER2* as molecular markers are of prime therapeutic importance and have the capability to take part in future drug development techniques.

REFERENCES

1. Tzahar E, Waterman H, Chen X, Levkowtiz G, et al. (1996) A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol. Cell. Biol*. 16: 5276-5287.
2. Moasser MM (2007) The oncogene *HER2*: Its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene*. 26: 6469-6487.
3. Ursini-Siegel J, Schade B, Cardiff RD and Muller WJ (2007) Insights from transgenic mouse models of ERBB2-induced breast cancer. *Nat. Rev. Cancer*. 7: 389-397.
4. Gutierrez C and Schiff R (2011) *HER2*: Biology, detection, and clinical implications. *Arch. Pathol. Lab. Med*. 135: 55-62.
5. Seol H, Lee HJ, Choi Y, Lee HE, et al. (2012) Intratumoral heterogeneity of *HER2* gene amplification in breast cancer: its clinicopathological significance. *Mod. Pathol*. 25: 938-948.
6. Mohanty SS, Sahoo CR and Padhy RN. (2020) Role of hormone receptors and *HER2* as prospective molecular markers for breast cancer: An update. *Genes. Dis*. 9: 648-658.

7. Bergstein I (1999) Molecular Alterations in Breast Cancer. In: Breast Cancer. Contemporary Cancer Research (Bowcock AM, eds.). Humana Press, Totowa, NJ.
8. Yamashita H, Ishida N, Hatanaka Y and Hagio K (2020) *HER2* Gene Amplification in ER-positive *HER2* Immunohistochemistry 0 or 1+ Breast Cancer With Early Recurrence. *Anticancer. Res*. 40: 645-652.
9. Ellsworth RE, Ellsworth DL, Patney HL and Deyarmin B (2008). Amplification of *HER2* is a marker for global genomic instability. *BMC. Cancer*. 8: 297.
10. Sestak I and Cuzick J (2015) Markers for the identification of late breast cancer recurrence. *Breast. Cancer. Res*. 17: 10.
11. Sestak I, Dowsett M, Zabaglo L and Lopez-Knowles E (2013). Factors predicting late recurrence for estrogen receptor-positive breast cancer. *J. Natl. Cancer. Inst*. 105: 1504-1511.
12. Yamashita H, Ogiya A, Shien T, Horimoto Y, et al. (2016) Clinicopathological factors predicting early and late distant recurrence in estrogen receptor-positive, *HER2*-negative breast cancer. *Breast. Cancer*. 23: 830-843.
13. Davies C, Godwin J, Gray R, Clarke M, et al. (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: Patient-level meta-analysis of randomised trials. *Lancet*. 378: 771-784.
14. Tyburski H, Karakas C, Finkelman BS, Turner BM, et al. (2023) In ER-positive, *HER2*-negative breast cancers, HER2 mrna levels correlate better with clinicopathologic features and oncotype DX recurrence score than HER2 immunohistochemistry. *Lab. Invest*. 104: 100309.
15. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, et al. (2007) American Society of Clinical Oncology/ College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Arch. Pathol. Lab. Med*. 131: 18-43.
16. Sauter G, Lee J, Bartlett JM, Slamon DJ, Press MF. (2009) Guidelines for human epidermal growth factor receptor 2 testing: biologic and methodologic considerations. *J. Clin. Oncol.* 27: 1323-1333.
17. Mitas M, Mikhitarian K, Walters C, Baron PL, et al. (2001) Quantitative real-time RT-PCR detection of breast cancer micrometastasis using a multigene marker panel. *Int. J. Cancer*. 93: 162-171.
18. Bernard PS, Wittwer CT (2002) Real-time PCR technology for cancer diagnostics. *Clin. Chem*. 48: 1178-1185.
19. Alawadhi E, Al-Awadi A, Elbasmi A, Coleman MP, et al. (2019) Cancer survival by stage at diagnosis in Kuwait: A population-based study. *J. Oncol*. 2019: 8463195.
20. Al-Shamsi HO, Abdelwahed N, Abyad A, Abu-Gheida I, et al. (2023) Breast Cancer in the Arabian Gulf Countries. *Cancers (Basel)*. 15: 5398.
21. Sadia H, Bhinder MA, Irshad A, Zahid B, et al. (2020) Determination of expression profile of p53 gene in different grades of breast cancer tissues by real time PCR. *Afr. Health. Sci*. 20: 1273-1282.
22. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2- ΔΔCT method. *Methods*. 25: 402-408.
23. Dunn OJ, Clark VA (1986) Applied statistics: analysis of variance and regression. 3rd Edition. John Wiley & Sons, Inc.
24. Benöhr P, Henkel V, Speer R, Vogel U, et al. (2005). *HER-2*/neu expression in breast cancer-A comparison of different diagnostic methods. *Anticancer. Res*. 25: 1895-1900.
25. Kulka J, Tôkés AM, Kaposi-Novák P, Udvarhelyi N, et al. (2006) Detection of *HER-2*/neu gene amplification in breast carcinomas using quantitative real-time PCR - a comparison with immunohistochemical and FISH results. *Pathol. Oncol. Res*. 12: 197-204.
26. Tsuda H. (2006) *HER-2* (c-erbB-2) test update: Present status and problems. *Breast. Cancer*. 13: 236-248.
27. Dobson L, Conway C, Hanley A, Johnson A, et al. (2010) Image analysis as an adjunct to manual *HER-2* immunohistochemical review: A diagnostic tool to standardize interpretation. Histopathology. 57: 27-38.
28. Ahn S, Woo JW, Lee K, Park SY (2020) HER2 status in breast cancer: Changes in guidelines and complicating factors for interpretation. *J. Pathol. Transl. Med*. 54: 34-44.
29. Yi Z, Rong G, Guan Y, Li J, et al. (2020) Molecular landscape and efficacy of HER2-targeted therapy in patients with *HER2*-mutated metastatic breast cancer. *NPJ. Breast. Cancer*. 6: 59.
30. Owens MA, Horten BC, Da Silva MM (2004) *HER2* amplification ratios by fluorescence in situ hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues. *Clin. Breast. Cancer*. 5: 63-69.
31. Murthy SK, Magliocco AM, Demetrick DJ (2005) Copy number analysis of c-erb-B2 (*HER-2*/neu) and topoisomerase IIalpha genes in breast carcinoma by quantitative real-time polymerase chain reaction using hybridization probes and fluorescence in situ hybridization. *Arch. Pathol. Lab. Med*. 129: 39-46.

32. Zoppoli G, Garuti A, Cirmena G, di Cantogno LV, et al. (2017) *HER2* assessment using quantitative reverse transcriptase polymerase chain reaction reliably identifies *HER2* overexpression without amplification in breast cancer cases. *J. Transl. Med*. 15: 91.