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Detection of Topoisomerase IIα Gene Amplification in Archival Breast Cancer Specimens by Quantitative Real-Time Polymerase Chain Reaction

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Abstract
Topoisomerase IIα is an important nuclear enzyme involved in cell division. The inhibition of the DNA topoisomerase IIα is achieved by using anthracyclines which are powerful drugs for the treatment of breast cancer. The sensitivity of topo II inhibitors such as anthracyclines is dependent on the expression level of topo IIα in target cancer cells. Accordingly accurate determination of the amplification level of topo IIα in breast cancer patient is crucial to identify the patients who will benefit from the treatment. The aim of this study focusses on the detection of topo IIα gene amplification status in invasive breast cancer compared to the normal values and its association with the HER2 gene status.

Forty-four (44) FFPE tissue samples were examined for topo IIα and HER2 genes quantity by qPCR. Statistical analysis using one sample t-test to determine the significant difference between the mean of the relative fold values of each gene from the normal sample value. Pearson correlation test was performed using Excel to determine the relation between the topo IIα Ct values and the HER2 Ct values.

Out of 44 patients 21 showed down regulation of the topo IIα gene (48%) and 23 showed up-regulation (52%). Only 2 samples out of 44 showed down regulation of the HER2 gene (0.05%) and 42 showed up-regulation (95%%). The statistical t-test performed on the relative values of the topo IIα gene and the HER2 gene separately showed a significant difference from the normal sample relative fold value. A positive correlation was found between the topo IIα gene and the HER2 gene relative values (r = 0.61522262 and p-value = 0.00000838321).

This study confirmed the association of the topo IIα gene abnormal fold values with HER2 gene amplification and its association with invasive histological types of breast cancer. Cut-off values of the amplification of the key genes such as HER2 and topo IIα should be established. The results of this study may add to the information required to understand the functional roles of these genes in the diagnosis of breast cancer and the use of target therapy.

Keywords: Breast cancer, HER2 gene, Topo IIα gene amplification

1. Introduction
Breast cancer is the most frequently diagnosed life-threatening cancer in women worldwide [1]. It is a heterogenous disease with several biological subtypes that have a well-defined characteristics and responses to therapy [2]. 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 patients ages were 15-99 years. Across the Gulf Cooperation Countries (GCC) breast cancer stands as the prevailing malignancy. 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 [3].

Cancer cells carry amplified oncogenes that contributes to tumorigenesis. Amplification quantities of oncogenes correlate in different tumors with their aggressive potential and proliferative activity [4-7]. Breast cancer is a heterogenous disease where many genes identified contribute to the development of the disease. Amplifications and deletions are the most common mechanisms leading to gene deregulation [2]. The significance of each of these oncogenes must be established in terms of the gene contribution to

the histological subtype of breast cancer and hence prognosis and therapy plans.

Topoisomerase IIα (topo IIα) is an important nuclear enzyme involved in cell division. It releases torsional stress in double-stranded DNA by inducing transient breaks that are then subsequently released [8]. Catalytic activity of topo II in mammalian cells is mediated by two isoforms topo IIα and topo IIβ. Anthracyclines are powerful drugs for the treatment of breast cancer used in adjuvant setting. The mechanism of action of Anthracyclines is related to the inhibition of the nuclear enzyme DNA topoisomerase II [9] together with other antitumor mechanisms. Studies have suggested that sensitivity of topo II inhibitors is dependent on the expression level of topo IIα in target cancer cells [10-13]. Thus, the abnormality of topo IIα plays a critical role in chromosome instability and tumorigenesis. Cells with low concentration of topo IIα protein are less sensitive to topo II-inhibiting drugs compared to cells with high concentration of topo IIα protein. Although anthracyclines are powerful agents, they are associated with severe adverse effects, such as cardiac toxicity [14], bone marrow dysfunction, including acute leukemia and myelodysplasia [15]. Accordingly, there is a need to identify the patients who will benefit from these drugs.

Topo IIα gene is located at chromosome 17 and is up regulated by the proliferating cells [16]. The epidermal growth factor receptor-2 (HER2) gene amplification is observed in breast cancer patients [17] which is an adverse prognostic factor but predictive for the response to anti-HER2 therapy. Gene amplification at chromosome 17q (locus 17q12-21) involves the HER2 gene, the co-amplification of topo IIα and other gene in this locus [18]. The genomic proximity of the genes at 17q12-21 and their frequent co-amplification in breast cancer provides prognostic potential for these genes as predictive tools.

The current study focusses on the detection of topo IIα gene amplification status in invasive breast cancer compared to the normal values and its association with the HER2 gene status using the t-test and Pearson correlation test. The results of this study may add to the information required to understand the functional roles of these genes in the diagnosis of breast cancer and the use of target therapy.

2. Materials and Methods

Ethics committee approval was taken and forty-four (44) formalin fixed paraffin embedded (FFPE) breast cancer tissue samples from Royal Hayat hospital in Kuwait were examined for topo IIα and HER2 gene quantity by qPCR. According to the pathology consult report obtained from Royal Hayat Hospital, 8 samples were grade I, 21 samples grade II and 15 samples grade III. The TNM grading system was used for samples grading. The archival tumour tissues were 33 Invasive Ductal Carcinoma ,10 Invasive lobular carcinoma and 1 was diagnosed with undifferentiated pleomorphic sarcoma.
DNA isolation from FFPE samples was performed using the NucleoSpin®DNA FFPE XS kit. Quantitation of the two genes was done by real time PCR. Samples with no DNA and with normal DNA were included. β actin gene was used as a reference gene for HER2 and topoIIα genes. The primer sequences used for the three genes are as described before [19,20] (Table 1).

Name	Forward primer	Reverse primer
HER2	5′AGC CTC TGC ATT TAG GGA TTC TC 3′	5′CTA GCG CCG GGA CGC 3′
Topo IIα	5′GCC AGA ATC TGT TCG CTT CAA C 3′	5′AGG AAA CTG AGT GCC GGC TT 3′
β actin	5′ACT CCT ATG TGG GCA ACG AG 3′	5′AGG TGT GGT GCC AGA TCT TC 3′

Table 1: Forward and reverse primer sequences for HER2, topo IIα and β actin genes.

For real time PCR TaqMan™ Fast advanced Master Mix (Appliedbiosystems, Thermo Fisher Scientific Baltics UAB) was used. The 7500 Fast Real-time PCR system (Appliedbiosystems) was used. Amplification conditions were 50ºC for 2 min, 95ºC for 10 min, 40 PCR cycles at 95ºC for 15 s, and 60ºC for 1 min. The qPCR for the two studied genes and the reference gene was setup individually 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 the gene probe, and 6.5 µL of molecular water.

For the calculation of the relative fold values of the HER2 and topo IIα genes the 2^ΔΔCt method was used [21]. Averages for the Ct values were calculated for the studied genes. The HER2 and topo IIα genes Ct values were separately 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.

Statistical analysis was done on the data collected by applying one sample t-test to determine the significant difference between the mean of the relative fold values of each gene from the normal sample value. Pearson correlation test was performed using Excel to determine the relation between the topo IIα Ct values and the

HER2 Ct values.

3. Results

The qPCR values for the topo IIα gene, HER2 gene and the β actin gene were obtained. Amplification plots for the topo IIα gene and the HER2 gene Ct values using the 7500 fast system SDS software are shown in figures 1 and 2 respectively. The Ct values for each breast tumor sample were normalized with β actin gene Ct values. The results were compared with the normal tissue sample. The normal sample 2^ΔΔCt value was 1, accordingly any value below that will be considered as a down-regulation and any value higher than that will be considered as an up-regulation.

All the samples showed abnormal relative gene values for the topo IIα gene (Figure 3). The results were compared with the normal sample that had a 1-fold change relative value. Accordingly, any value below 1 was considered a down-regulation and any above 1 was considered an up-regulation. Out of 44 patients 21 showed down regulation of the topo II α gene (48%) and 23 showed up-regulation (52%). The highest relative fold value of the topo IIα gene was 5.710828 and the lowest relative fold value was 0.285606.

All the samples showed abnormal relative gene values for the HER2 gene Figure 3. The results were compared with the normal sample that had a 1-fold change relative value. Accordingly, any value below 1 was considered a down-regulation and any above 1 was considered an up-regulation. Out of 44 patients only 2 showed down regulation of the HER2 gene (0.05%) and 42 showed up-regulation (95%%). The highest relative fold value of the HER2 gene was 26.31475 and the lowest relative fold value was 0.889891. The statistical t-test performed on the relative values of the topo IIα gene and the HER2 gene relative values separately showed a significant difference from the normal sample relative fold value that was equal to 1 (Tables 2 & 3 respectively). A significant linear relationship between the relative fold values of the topo IIα gene and the HER2 gene was found with an ANOVA F value of 0.00000883 (< 0.05) (Table 3). The intercept values showed the significant relationship between the relative values of the topo IIα and HER2 gene from the calculation intercept values and a p-vlaue of <0.05 (0.005046 and 0.0000083 respectively). The line fit plot shows the relationship between the two genes relative values. Figure 4. A positive correlation was found between the topo IIα gene relative values and the HER2 gene relative values (r = 0.61522262 and p-value = 0.00000838321).

The The range of patients age was from 30 to 82 years of age. The highest age frequency was observed in the 50 to 60 years old age group (Figure 5).

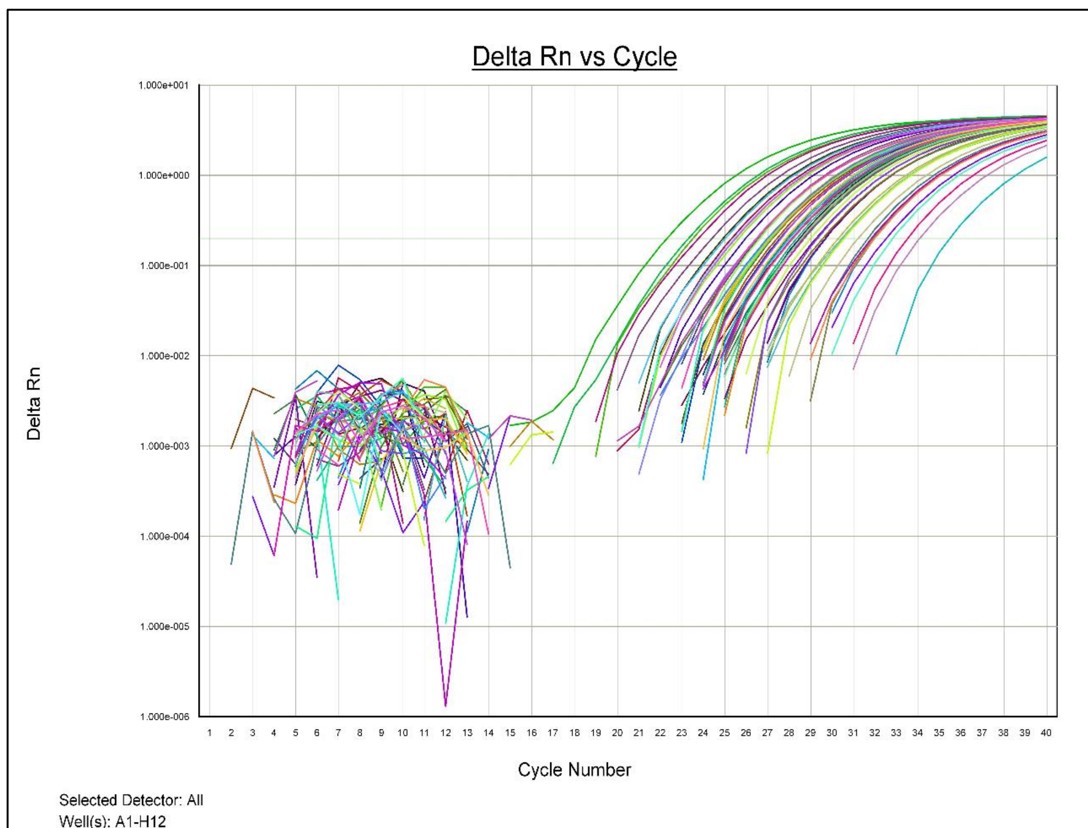

Figure 1: Amplification plot for the topo IIα gene Ct values using 7500 fast system SDS software. The magnitude of the fluorescence signal (ΔRn) (Y axis) vs PCR cycles number (X axis).

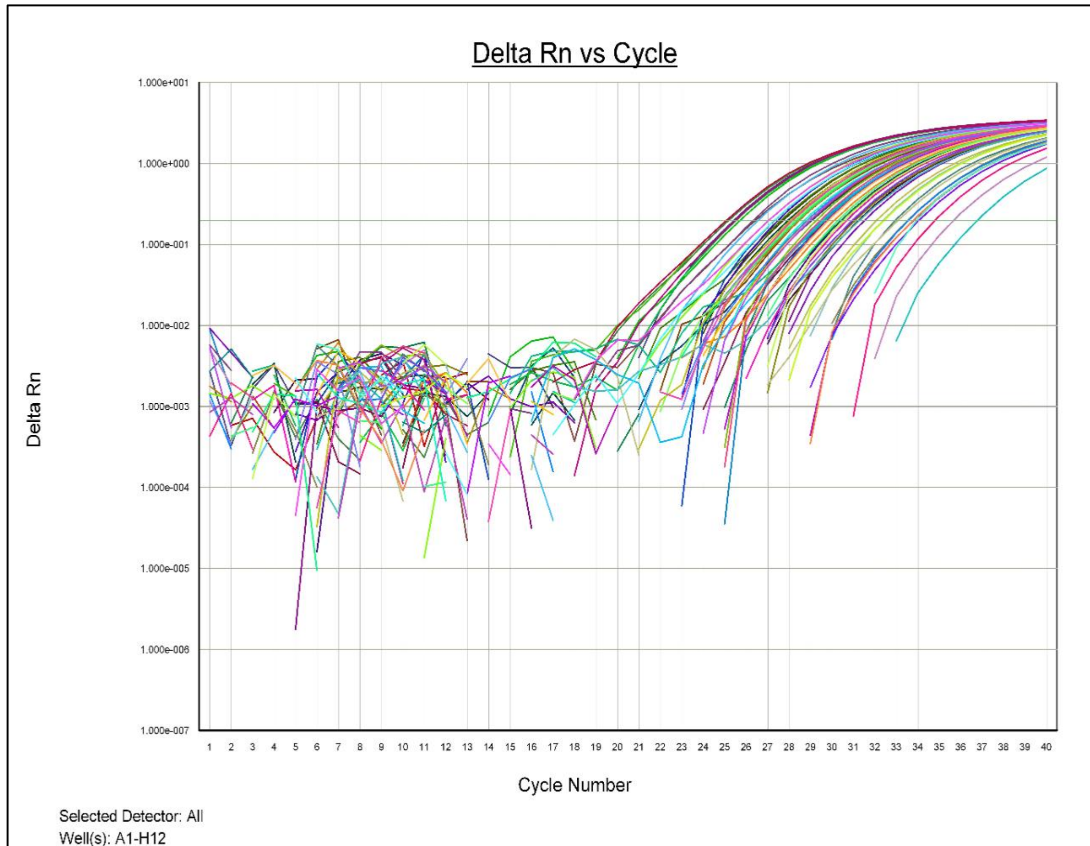

Figure 2: Amplification plot of the HER2 gene Ct values using 7500 fast system SDS software. The magnitude of the fluorescence signal (ΔRn) (Y axis) and PCR cycles number (X axis).

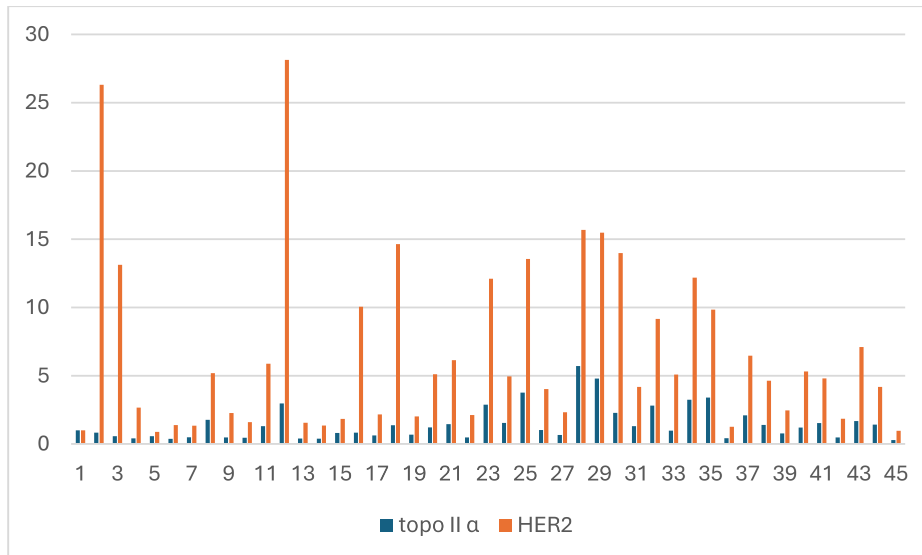

Figure 3: Topo IIα and HER2 genes relative fold values

Table 1: One sample t-test results for the topo IIα gene relative fold values

MEAN	1.444258
STANDARD DEVIATION	1.233011
COUNT	44
STANDARD EROR OF MEAN	0.185883
DEGREES OF FREEDOM	43
HYPOTHESIZED MEAN	1
t-statistics	2.389982
p-value	0.021303

Table 2: One sample t-test results for the HER2 gene relative fold values

MEAN	6.75924201
STANDARD DEVIATION	6.383041377
COUNT	43
STANDARD EROR OF MEAN	0.973404684
DEGREES OF FREEDOM	42
HYPOTHESIZED MEAN	1
t-statistics	5.916595741
p-value	0.000000523565

Table 3: Pearson correlation test results

Coefficient (r)	0.61522262
N	44
T statistic	5.05750103
DF	43
p-value	0.00000838321

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	25.16489	25.16489	25.57832	0.00000883
Residual	42	41.32115	0.983837		
Total	43	66.48604			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.648519	0.219126	2.95957	0.005046	0.206305	1.090734	0.206305	1.090734
HER2	0.119849	0.023697	5.057501	8.83E-06	0.072026	0.167673	0.072026	0.167673

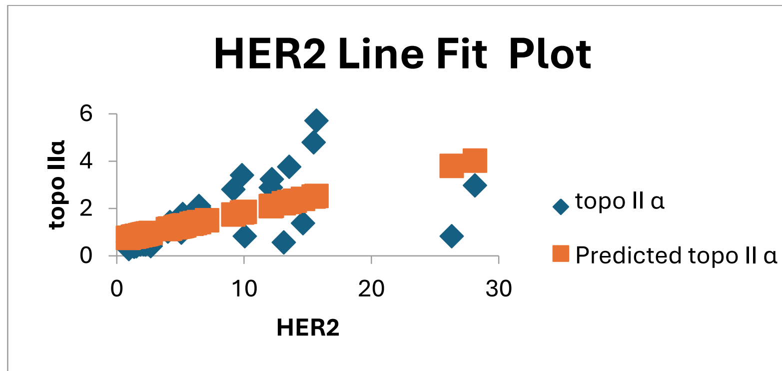

Figure 4: Fit plot for the correlation between topo IIα gene relative values and HER2 relative values.

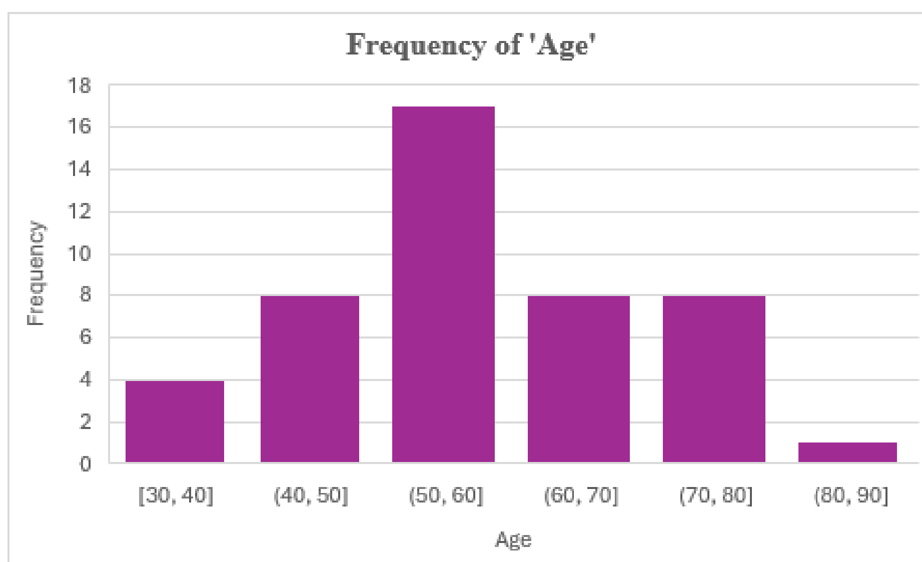

Figure 5: The frequency of age groups for the patients studied.

4. Discussion

Gene copy number alterations is one of the most important mechanisms leading to deregulation of gene expression and neoplastic transformation. Many studies correlate copy numbers of certain loci with disease progression and response to therapy [22]. Among those loci are the Chr17 q12-21 that houses the HER2 and topo IIα genes which play a crucial role in the pathology, prognosis and treatability of breast cancer. The results of this study showed that there is a significant difference of the topo IIα gene relative value and the HER2 relative value from the normal sample. The histological types of the archival samples studied were 75% Invasive Ductal Carcinoma and 23% Invasive Lobular Carcinoma. These histological types showed a shared significant linear relationship between the relative fold values of the topo IIα gene and the HER2 gene which emphasized the related alterations of the topo IIα gene with the HER2 gene. The ANOVA test showed no significant association between the topo IIα and HER2 fold values with any of the grades of the breast cancer.

The key role of topo IIα was found to be in DNA replication and accordingly it is a target for chemotherapeutic drugs such as Anthracycline. Topo IIα over expression in breast cancer has been linked to cell proliferation and HER2 protein overexpression [23]. This study confirms the association of the topo IIα gene abnormal fold values with HER2 gene amplification and its association with invasive ductal and lobular carcinoma histological types of breast cancer. In this study 48% of the samples showed down regulation of the topo IIα gene and 52% showed up-regulation. The highest relative fold value of the topo IIα gene was 5.710828 it was for a 51 years old IDC patient with pT2N0M0 pathology and an elevated HER2 relative value of 15.68703. This may indicate the role of gene amplification in the tumor prior to metastasis. The lowest relative fold value for the topo IIα gene was 0.285606, HER2= 0.962394, for a 54 grade II IDC pT1CN0 patient. The highest relative fold value for the HER2 gene was 26.31475 with topo IIα value of 0.828458, for an IDC pT2 N2a Mx, 53 years old grade III patient. Amplification of the HER2 is observed in 18-20% of breast cancers and is used as a selection criterion for HER2

targeted therapy [24].

In this study, the amplification status of the topo IIα gene was detected and correlated to the status of the HER2 gene in invasive breast cancer archival samples using qPCR. The analysis of the qPCR relative values of the topo IIα gene in the studied samples revealed that the gene has abnormal relative values. Abnormal gene relative values of the HER 2 gene were also detected in the studied samples. Copy number abnormal changes of HER2 correlated to topo IIα were determined by previous studies using FISH. The study by [25], found that the amplification of the two genes may be caused by different mechanisms, leading to higher level of amplification for HER2 compared to topo IIα and that simultaneous aberration of the two genes is not explained by simple co-amplification. In this study the higher amplification of the HER2 gene over the topo IIα gene relative values was observed (figure 3). The significance of each of these oncogenes must be established in terms of the gene contribution to the histological subtype of breast cancer and hence prognosis and therapy plans.
In the studied samples 52% showed co-amplification of the topo IIα and HER2 genes. The genomic proximity of the genes at 17q12-21 and their frequent co-amplification in breast cancer provides prognostic potential for these genes as predictive tools. In this study All the samples showed abnormal relative gene values for the topo IIα gene and HER2 gene. HER2 amplification could be the cause of the genomic instability along Chr17q leading to different patterns of gene amplification [26].

Different studies used different methods for the evaluation of topo IIα HER2 and other important markers in breast cancer such as qPCR, gene expression, microarray, immunohistochemistry and fluorescence in situ hybridization. Further studies are required to find the most relevant method for the quantification of these genes and the cut-off values to evaluate the gene.

In the GCC region breast cancer was recorded before the age of 50 [3]. In our study, the highest frequency of patients fell in the 50-60 age group. A limitation of this study was the group size. A lager group shall give a clearer picture to the age group with the higher risk. However younger patients were also included in the study. The youngest patient was 35 years old IDC with pT2N1aMx, Topo IIα = 0.805748 and HER2= 1.842417. The relative fold values of the youngest patient are close to the normal values. The patient was a grade II. It is crucial to establish cut-off values for the qPCR results taking into consideration the level of both genes where relative drugs can be effective.

5. Conclusion

The results of this study confirmed the difference of the topo IIα gene relative value and the HER2 relative value from the normal sample. This study confirmed the association of the topo IIα gene abnormal fold values with HER2 gene amplification and its association with invasive histological types of breast cancer. 48% of the samples showed down regulation of the topo IIα gene, 52% showed up-regulation and 52% showed co-amplification of the topo IIα and HER2 genes. Quantitative PCR is a powerful tool that can be used for the quantification of oncogenes. The determination of the amplification values of these key genes plays a significant role in the treatment plans that could benefit the patient. However, cut-off values of the amplification of the key genes such as HER2 and topo IIα should be established.

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