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Research Article

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Detection of p53 Gene Status in Different Grades of Archival Breast Cancer Specimens by Quantitative Real-Time PCR

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Abstract

Background: Breast cancer is the most diagnosed cancer globally and contributes to the top five leading causes of cancer mortality. In Kuwait, breast cancer contributes for 49.4% from all types of cancers. P53 gene normal function is crucial in controlling cell proliferation and preventing the development of malignant cells. The aim of this study was to evaluate the p53 status in formalin fixed paraffin embedded (FFBE) samples of different grades of breast cancer using qPCR. The method of detecting p53 status affects the determination of its predictive value and the gene status present in the invasive forms of breast cancer. The aim of this study was to evaluate the p53 status in FFPE of different grades of breast cancer samples compared to the normal tissue by using qPCR. The method of detecting p53 status affects the determination of its predictive value and the gene status present in the invasive forms of breast cancer.

Methods: *In this study 44 breast cancer tissue samples were used. DNA isolation was followed by quantitation of the HER2 gene which was done by real time PCR. The calculation of the relative fold values of the p53 gene was performed using the 2^ΔΔCt and ΔCt methods were the p53 gene was normalized with β actin gene. A one-way analysis of variance (ANOVA) test was used to determine whether the three groups of patients; grade I, II and III p53 qPCR relative values are statistically different.*

Results: *The ΔCt values for Grade I, Grade II and Grade III groups showed a noticeable down-regulation of the gene copy number values. However, the one-way ANOVA test P value for each of the 2^-ΔΔCt and ΔCt values were above the significance threshold 0.05. Accordingly, no significant differences among the three patient groups were found.*

Conclusion: *The down regulation of the gene observed in the invasive ducal and lobular carcinomas presented in the studied samples forms the start to investigate the p53 gene status in a larger group of patients. Determination of the p53 mutations is necessary and further studies are required to develop safe and efficient treatment to target p53 mutations in breast cancer.*

Keywords: P53, Breast cancer, qPCR

1. Introduction

Breast cancer is the most diagnosed cancer globally and contributes to the top five leading causes of cancer mortality [1]. Breast cancer 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 [3]. 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].

Chemotherapy, hormone therapy and radiotherapy are currently used for patients with metastatic disease. However, 20-40 % of the patients exhibit primary resistance to medical treatment [4-6]. This suggests that a better knowledge of the predictive factors of response to treatment is required. Studies on p53 status indicates its strong prognosis impact which could be useful in choosing the best treatment for breast cancer [7]. Mutated p53 is associated with poor response to chemotherapy, hormone therapy or radiotherapy. The method of detecting p53 status affects the determination of its

predictive value and hence response to treatment [8]. Quantitative Real-time PCR is one of the effective molecular techniques for clinical trials and, eventually, routine use [9]. Accurate determination of the p53 mutations is necessary and further studies are required to develop safe and efficient treatment to target p53 mutations in breast cancer.

P53 gene normal function is crucial in controlling cell proliferation and preventing the development of malignant cells. The alterations in the p53 gene can result in a lack of function, allowing cells to divide out of control and may trigger the development of cancer [10]. As a tumor suppressor gene p53 gene plays a significant role in maintaining genome stability. This is achieved by the p53 function in allowing time for the DNA repair machinery to eliminate damaged lesions before cell proliferation resumes [11]. Another function of the p53 is in the cell cycle regulation. In the presence of DNA damage, p53 is activated to regulate the G1-S and G2-M phase restriction points. When activated, p53 transcriptionally regulates p21, 14-3σ, Cdc25c, and GADD45 to promote cell cycle arrest [12]. Multiple studies have demonstrated that p53 plays a major role in triggering apoptosis [13-14]. P53 mediates both major signalling pathways that propagate apoptosis: extrinsic pathways and intrinsic pathways [15]. An additional role of p53 is exhibited in senescence which is an irreversible cell cycle arrest that can be induced by different types of cellular stresses. The p53 levels, kinetics, and threshold of stressor intensity can dictate if a cell decides to undergo senescence or apoptosis [16].
The most frequently mutated gene in breast cancer is p53, accounting for 30% of all breast cancer cases [17]. The different mutations in the p53 gene are highly linked to tumor subtypes and are associated with poor survival in breast cancer patients [18-19]. Basal-like tumors have the highest frequency of p53 mutation frequency (88%) compared to the luminal-like, while about half of HER2-amplified tumors (53%) harbour p53 mutations [20]. Significantly poor prognosis of breast cancer was associated with the presence of p53 mutation together with HER2 overexpression [21].

The aim of this study was to evaluate the p53 status in FFPE of different grades of breast cancer samples compared to the normal tissue by using qPCR. The method of detecting p53 status affects the determination of its predictive value and the gene status present in the invasive forms of breast cancer.

2. Materials and Methods

In this study Forty-four 44 FFPE breast cancer tissue samples from Royal Hayat Hospital in Kuwait were studies. Project number RN 01/06 was approved by the ethical committee of the faculty of medicine, Kuwait university which ensures that the conducted research complied with both national and international ethical norms, regulations. According to the pathology consult report obtained from Royal Hayat Hospital, 8 samples were grade I, 21 samples grade II and 15 samples were 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 that was diagnosed with undifferentiated pleomorphic sarcoma.
DNA isolation from FFPE samples was performed using the NucleoSpin®DNA FFPE XS kit. Quantitation of the p53 gene was done by real time PCR. Samples with no DNA and with normal DNA were included. The primes used for the p53 gene were as follows: Forward primer for p53 gene, 5′CCC CTC CAT CCT TTC TTC TC 3′, reverse primer for the p53 gene, 5′ATG AGC CAG ATC AGG GAC TG 3′. For the quantitation of the β actin gene the following forward and reverse primer sequences were used respectively: 5′ACTCCTATGTGGGCAACGAG3′, and 5′AGGTGTGGTGCCAGATCTTC3′9 [22].

For real time PCR SYBR®Green Master Mix (Appliedbiosystems, Thermo Fisher Scientific Baltics UAB) was used. The beta actin gene was used as a reference gene to the p53 gene investigated. 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 95 ºC for 15 s, and 60 ºC for 1 min.

The qPCR for the p53 gene was set up in 96-well plates, in a final volume of 20µL containing of up to 1µL of isolated DNA, 10µL of SYBR Green Master Mix, 1µL of forward gene primer, 1µL of reverse gene primer, and 8µL of molecular water. For the β actin gene qPCR reaction the following mixture was used: 1µL of isolated DNA, 10µL of PowerUpTM SYBR®Green Master Mix, 1µL of forward gene primer, 1µL of reverse gene primer and 7µL of free molecular water.

3. Calculation of the Relative Fold Values of the p53 Gene

Averages for the Ct values were calculated for the p53 gene. The p53 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].

4. 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 p53 qPCR relative values are statistically different by calculating the means of the three groups. The ANOVA test was performed on the 2^ΔΔCt and ΔCt values separately. 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 [24]. The breast cancer is considered as the independent variable. The qPCR values in the three groups were considered as the

dependent variables. The null (HO) 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 0.05. The P value was interpreted to be significant if it is ≤ 0.05.

5. Results

The qPCR values for the p53 gene were obtained. Amplification plot for the p53 gene and the β actin gene Ct values using the 7500 fast system SDS software are shown in figures 1 and 2. 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 ΔCt value was 6.6854, 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. Abnormal ΔCt values were obtained from the three groups of patients. The ΔCt values for Grade I, Grade II and Grade III groups showed a noticeable down-regulation of the gene copy number values as shown in figure 3. However, the one-way Anova test P value for each of the 2^-ΔΔCt and ΔCt values were above the significance threshold 0.05 (Tables 1 and 2 respectively). Accordingly, the null hypothesis was accepted which indicates that there were no significant differences among the three patient groups.

The lowest ΔCt value was 0.4117 while the highest value was 5.5103, and were for two grade II patients. The patient with the lowest ΔCt which was 0.4117 was 71 years old diagnosed with ILC. The patient with the 5.5103 was 75 years old diagnosed with IDC.

It was observed that grade II p53 relative fold values were highly correlated with grade III gene relative values as shown in figure 5. The range of the ΔCt value

The patients ages were from 30 to 82 years of age. The highest age frequency was observed in the 50 to 60 years old age group (Figure 6).

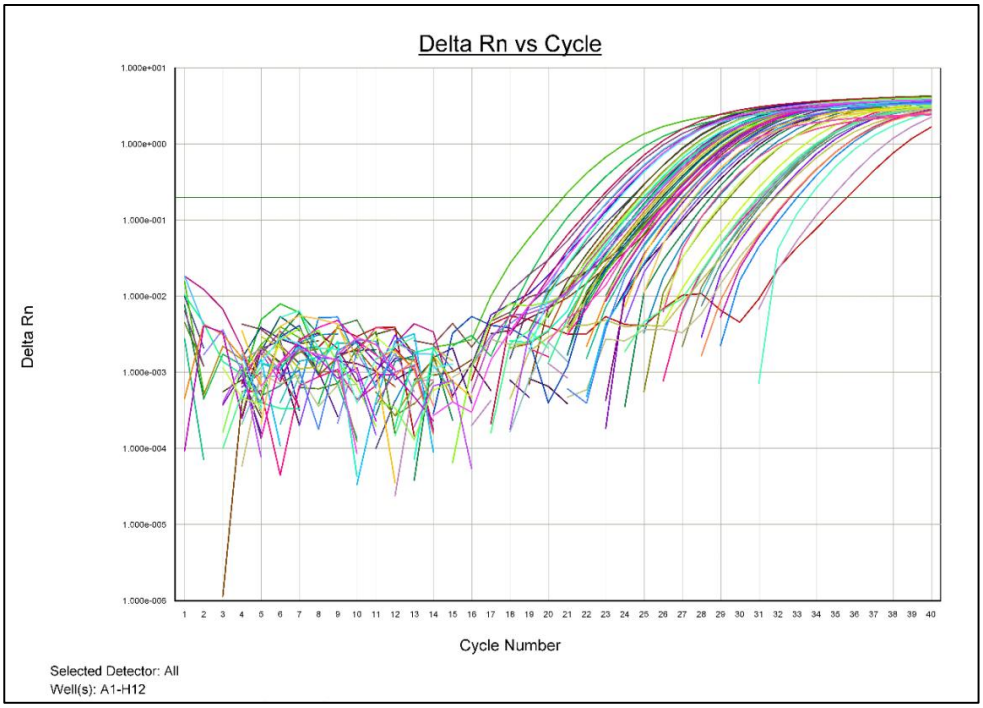

Figure 1: Amplification plot for the p53 gene Ct values using the 7500 fast system SDS software. The magnitude of the fluorescence signal (ΔRn) is in the Y axis and PCR cycle numbers are shown in the X axis.

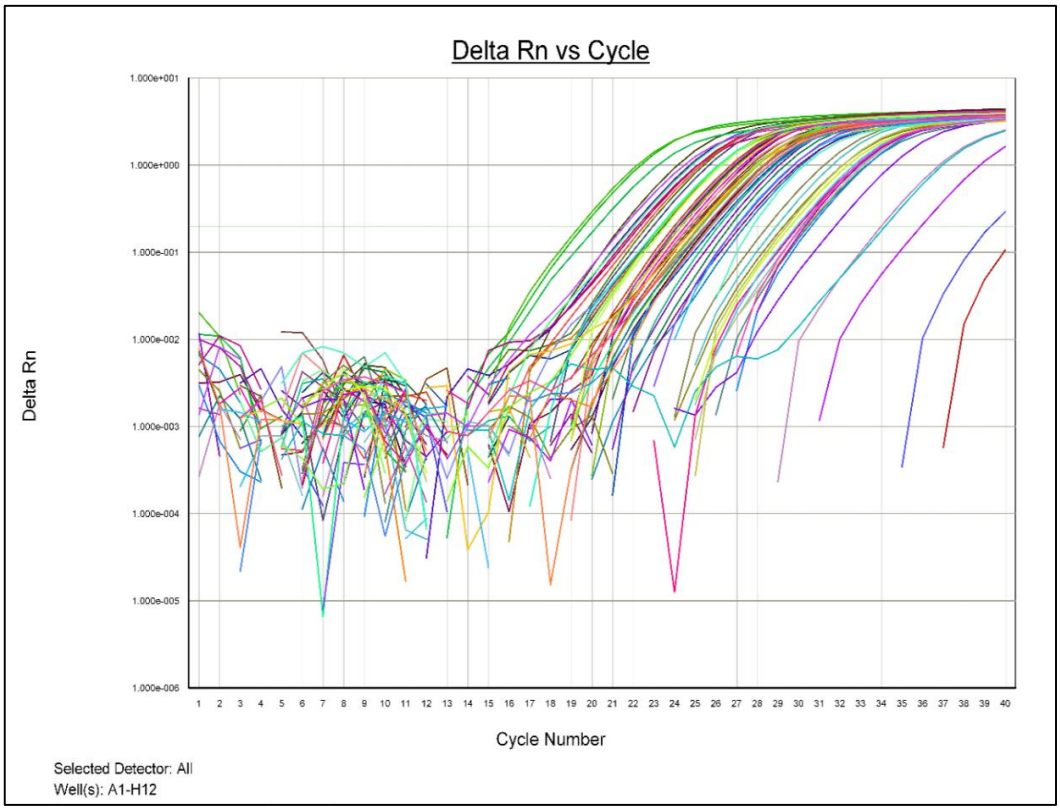

Figure 2: Amplification plot for the β actin gene Ct values using 7500 fast system SDS software. The magnitude of the fluorescence signal (ΔRn) is in the Y axis and PCR cycle numbers are shown in the X axis.

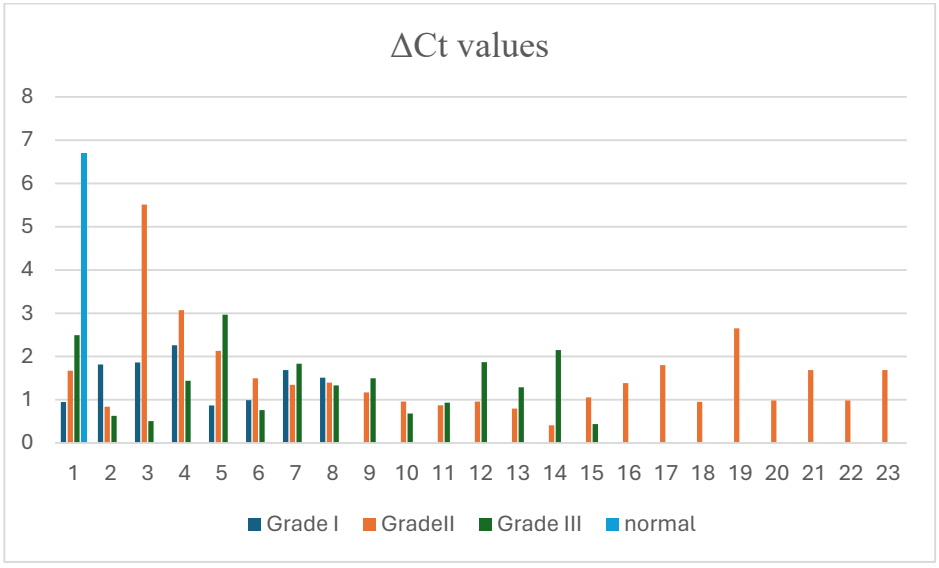

Figure 3: p53 gene ΔCt values in grade I, II, and III compared to the normal value.

SUMMARY						
Groups	***Count***	***Sum***	***Average***	***Variance***		
Column 1	8	308.5816	38.5727	177.3678		
Column 2	23	79518.53	3457.327	1.79E+08		
Column 3	15	672.7299	44.84866	395.1082		
ANOVA						
Source of Variation	***SS***	***Df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	1.34E+08	2	67044604	0.731394	0.487128	3.21448
Within Groups	3.94E+09	43	91666863			
Total	4.08E+09	45	---			

Table 1: ANOVA: Single factor for 2^-ΔΔCt values

SUMMARY						
Groups	***Count***	***Sum***	***Average***	***Variance***		
Grade I	8	11.944	1.493	0.257805		
GradeII	23	16.628	0.722957	9.223627		
Grade III	15	20.8191	1.38794	0.577778		
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	5.717184	2	2.858592	0.577593	0.565542	3.21448
Within Groups	212.8133	43	4.949147			
Total	218.5305	45	---			

Table 2: ANOVA: Single factor for ΔCt vlaues

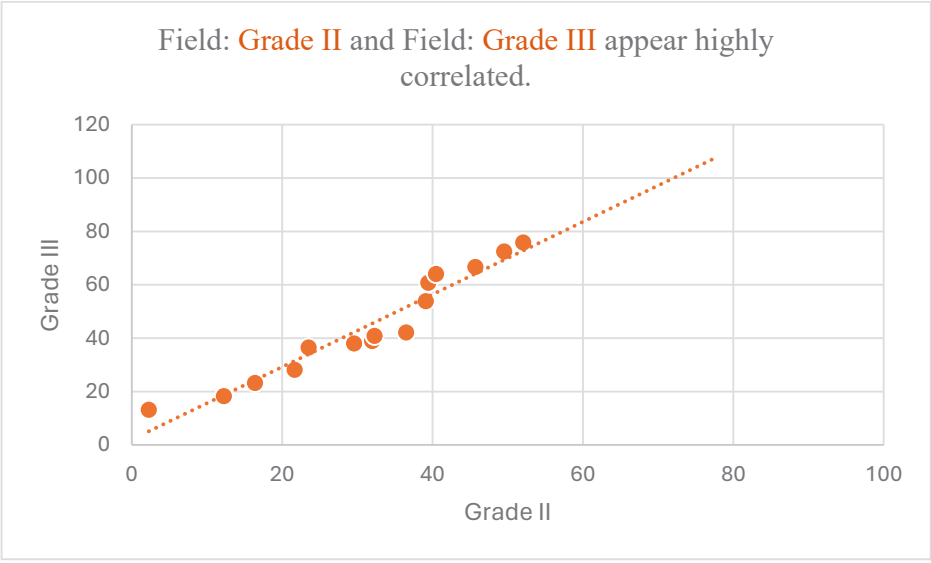

Figure 4: It was observed that grade II p53 relative fold values were highly correlated with grade III gene relative values.

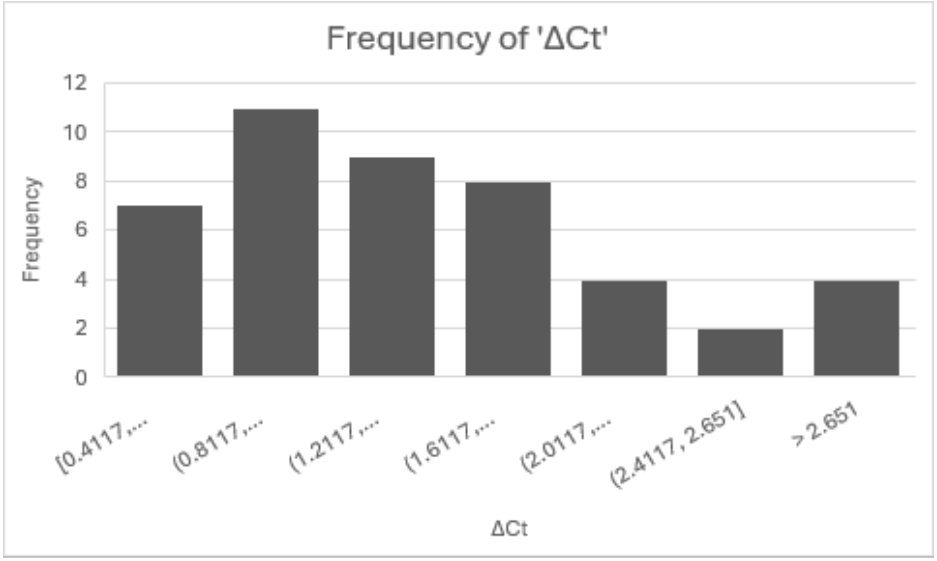

Figure 5: Frequency of ΔCt values for Grade I, II and III combined

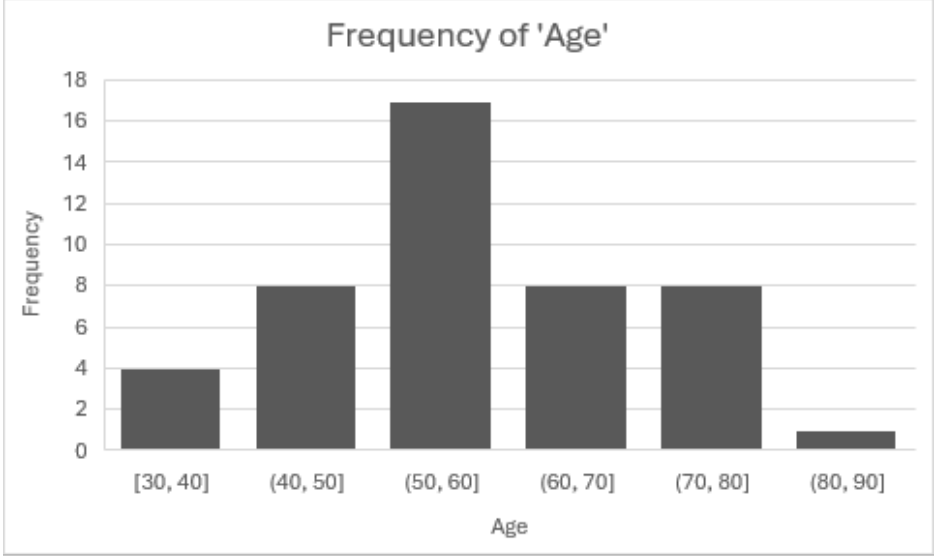

Figure 6: The frequency of age groups for the patients studied.

6. Discussion

Wild-type p53 is crucial for the regulation of cell growth and apoptosis. The p53 gene was found mutated or deleted in 60-70% of human cancers. Dysregulation of p53 in breast cancer due to various factors, such as gene mutation, signalling pathways alterations, and hormones, can alter p53 function and cause breast cancer [24]. Detecting p53 dysregulations in breast cancer can help in the development of promising strategies for individualized cancer treatment. This study was performed to investigate the status of the p53 gene in different grades of breast cancer by using qPCR on archival specimens.

The 2^-ΔΔCt values were used in the correlation plot. Converting the data to linear form by calculating the 2^ΔΔCt values depicts the individual variation among the replicate reactions [23]. However, the ΔCt value were used to detect the amplifications and deletions of the p53 gene by relating the test samples to both the normal sample Ct value and the reference gene Ct value. The correlation obtained between grade II and III p53 values may relate to the advance stages of the disease. Abnormal ΔCt values were obtained from the three groups of patients. The ΔCt values for Grade I, Grade II and Grade III groups showed a noticeable down-regulation of the gene copy number values. The lowest ΔCt value was 0.4117 while the highest value was 5.5103, and were for two grade II patients. The patient with the lowest ΔCt which was 0.4117 was 71 years old diagnosed with ILC. The patient with the 5.5103 was 75 years old diagnosed with IDC. The highest age frequency was observed in the 50 to 60 years old age group. The American cancer society stated that breast cancer mainly occurs in middle-aged and older women. The median age at the time of

breast cancer diagnosis is 62. This means half of the women who developed breast cancer are 62 years of age or younger when they are diagnosed. A very small number of women diagnosed with breast cancer are younger than 45 [26].

Many studies showed the association between p53 mutations and breast cancer severity. The polymorphism of p53 codon 72 was linked to breast cancer development among Saudi women [27]. A study by Zheng et al 2004 [28] showed that a decrease in the amount of wild-type p53 protein is due to the overexpression of HER2 which is a proto-oncogen. The over expression of HER2 was linked to the decrease in the amount of wild-type p53 protein via activating PI3K pathway and inducing MDM2 nuclear translocation in MCF7 human breast cancer cells. HER2 quantification by qPCR to the studied samples have shown up-regulation of 66% of the HER2 gene [29]. Previous Immunohistochemical detection of p53 protein expression in breast cancer in young Kuwaiti women (≤ 30 years old) found that p53 was positive in 57.32% and 63.7% of these patients displayed positive HER2 by IHC. In the young women study for the p53 overexpression by IHC no correlation was found with the tumor grade or tumor size [30]. This agrees with the current study which showed no significant association between p53 down-regulation and any of the three grades. Worse prognosis of breast cancer is significantly associated with the presence of p53, HER2 overexpression and shorter overall survival in metastatic breast cancer subtypes [21,31]. HER2 positive cancers frequently involved mutations in the p53 tumor suppressor gene, which provokes the unfavourable prognosis. The mechanism of RTK-specific drugs (receptor tyrosine-protein kinase HER2) which is a member of the ERBB family was described by Fedorova et al 2020 [32] as well as new perspectives of combinational treatment of HER2-postive cancers through inhibition of the mutant form of p53.

It has also been shown that inflammatory breast cancer (50%) has a higher frequency of p53 mutations than non-inflammatory breast cancer (20-30%) [33]. Higher frequences of p53 mutations are found in patients with advanced stage and high-grade tumours. Moreover, p53 mutations have been associated with aggressive behaviour such as triple negative breast cancer or HER2-amplified BC [34]. The acquisition of genetic and epigenetic changes plays a role in the development of cancer due to p53 dysregulation. A study by Yu Liu et al 2016 [35] showed that that somatic heterozygous deletion of mouse chromosome 11B3, a 4-megabase region syntenic to human 17p13.1, produces a greater effect on lymphoma and leukaemia development than p53 deletion. Mechanistically, the effect of 11B3 loss on tumorigenesis involves co-deleted genes such as Eif5a and Alox15b (also known as Alox8), the suppression of which cooperates with p53 loss to produce more aggressive disease. These findings indicates that the selective advantage produced by human chromosome 17p deletion reflects the combined impact of TP53 loss and the reduced dosage of linked tumour suppressor genes.

A previous study demonstrated that p53 mutation or deletion promotes immunosuppressive cells differentiation [36]. Patients with p53 mutations display non-T cell infiltrated phenotype where the number of cytogentic T-cells, helper T cells, as well as NK cells was significantly reduced compared with an expansion of immunosuppressive regulatory T cells [37], and M2 macrophages in cases with p53 mutations [38]. These studies were followed by the development of new targeted p53 strategies for immunotherapy such as ICIs, cancer vaccination and adoptive cell therapy.

7. Conclusion

The findings of the current study gave an insight into the status of p53 in grades I, II and III of Kuwaiti women. The current study showed no significant association between p53 down-regulation and any of the three grades. A limitation of this study was the size of the studied groups and lack of information on the survival status of the patients. A study on a larger group will provide better evaluation. In addition, studies that investigate combined impact of p53 loss and the involvement of other genes are necessary. Studies that investigate the mutations of the p53 present in breast cancer women in Kuwait will provide the basics for tailored treatment plans development.

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