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EVALUATION OF TP53 GENE EXPRESSION IN FFPE OF BREAST CANCER PATIENTS

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ABSTRACT

Background

Breast cancer is a malignant tumor arising from the terminal duct lobular unit of the breast tissues. It is most prevalent malignancy in women and its incidence is increasing globally and the lifetime risk of developing breast cancer between 5-10%. Tumor suppressor geneTP53 is essential for preventing inappropriate cell proliferation and maintaining genome stability and integrity following genotoxic stress. TP53 gene is the most commonly mutated gene in cancer and approximately 20% of breast cancer. Thus, gene expression was investigated.

Patients and Methods

One hundred formalin fixed paraffin embedded (FFPE) tissues samples of malignant breast tumor as well as 100 FFPE samples of normal breast tissues samples obtained from same patients were investigated. The blocks of breast cancer patients were collected from Al Sadder Medical City, Najaf, Iraq. Ages of patients were expressed as Mean ± S.D 48.4 ± 10.90 year with a minimum to maximum of 20 -75 years. Nucleic acid (RNA) isolated and evaluated by picodrop spectrophotometer for the expression of TP53 gene by qRT-PCR. cDNA was prepared using the High Capacity RNA-to-cDNA (ABI). For real-time quantitative PCR (RTQ-PCR), 100 ng cDNA was added to SYBR Green Master Mixture (Applied Biosystems) and run in Corbett Real-time PCR System.

Results

The current study involved investigated of TP53 gene expression in FFPE tissues of breast cancer tissues. TP53 gene expression was indicated to be 44 fold ($P<0.001$) relative to housekeeing gene (18S rRNA).

Conclusions

TP53 gene is a major tumor suppressor that is altered by mutations in 50% of human cancers and functionally inactivated in other 50%.

KEYWORDS: TP53, Breast Cancer, Tumor Suppresser Gene

INTRODUCTION

Tumor suppressor protein (TP53) considered first identified in 1979 as a transformation-related protein [(1)]. TP53 gene is a 20 Kb gene located on the short arm of chromosome 17p13.1 locus, contains 11 exons. It considered the most commonly somatic mutated gene in human cancers and occurring in about 50% of all human cancers [(2)]. Approximately 90% of the TP53 gene mutations are localized between domains encoding 5 - 8 exons [(3)].

Usually functions of TP53 as a tumor suppressor protein by regulating transcription, cell cycle, and programmed cell death. Primarily point mutations of P53 that lead to loss of function of wild type p53 and over expression of mutant p53 in malignant cells are detected in breast cancer [(4)]. The bad prognosis of breast cancer are involved in the alterations of TP53 gene products. Up-expression of TP53 in follicular lymphoma was observed in high grade and oversized tumors and correlated with poor prognosis and also in a subset of ductal carcinomas in situ, with no expression observed in atypical lesions [(5)]. In the different cancer cells various tumor suppressor genes are mutated in addition to overexpressed of oncogenes [(6)]. TP53, p21, and p27 tumor suppressor genes, c-erb-b2, an epidermal growth factor, BRCA1 and BRCA2 genes are known to be more prone to mutated in breast cancer patients [(7)].

The second most common malignancy in women worldwide is breast cancer and accounting about 23 percentage of all cancers [(8, 9)]. In Iraq, breast cancer is the commonest type of female malignancy, and represent approximately one-third of the registered female cancers according to the latest Iraqi Cancer Registry [(10)].

Some studies and reviews have demonstrated that mammography may reduces mortality, and several authors have controversy that early detection and screening represent best way to decrease breast cancer mortality [(11, 12)].

PATIENTS AND METHODS

Step 1: Cellular Specimens

One hundred human breast carcinoma tissues of formalin-fixed paraffin embedded tissues (FFPE), further that to normal breast tissues of same patients stored between 1and 2 years were obtained from Al Sadder Medical City in Najaf / Iraq. The baseline information of all the patients with breast cancer enrolled in this study is presented in Table 1. For Nucleic acid extraction, four 5-µm-thick sections of archival FFPE blocks sliced by using microtome. The slices placed in sterile eppendorf tube and deparaffinized by adding 1ml of xylene and following the protocol given in the pack insert of the miRNeasy FFPE Kit qiagen (Qiagen, Valencia, CA, Cat#217504). The quantity and quality of the total RNA extracts were determined by measuring the absorbance at 260 nm and 280 nm by using picodrop spectrophotometer (United kingdome).

Step 2: Reverse Transcription of the Isolated Total RNA

Reverse transcription reactions were performed according to procedure in the pack insert of the High Capacity RNA-to-cDNA Kit obtained from applied biosystem (Cat# 2387951). Quantitatively converting up to 2 µg (20-µL of total volume reaction) of total RNA to cDNA.

Step 3: CDNA Quality Check

In order to check the quality of cDNA generated from FFPE material, a qPCR using SYBR-Green I detection format was performed on the Corbett RG-6000 6 plex instrument. The following protocol was applied: GoTaq® qPCR Master Mixture contained 10 µl 2x SYBR Green I, 10 pmole of each primer and 5 µl of cDNA. The cycling conditions included initial incubation step at 95°C for 15 minutes followed by a 50 cycles of amplification with 25 seconds at 95°C, 30 seconds at 58°C, and 20 seconds at 72°C (single acquisition). The melting curve analysis consisted of 95°C for 30 seconds followed by cooling to 65°C for 30 seconds before the temperature was raised to 95°C at a rate of 0.1°C/s with continuous fluorescence acquisition. The final cooling step was 40°C for 30 seconds. Two concentrations of reference gene, was used to check cDNA from FFPE material.

Step 4: qRT-PCR Analysis

A qPCR using the SYBR-Green I detection format was performed on Corbett RG-6000 6 plex instrument to quantify gene expression of FFPE blocks. Two sets of genes, including one gene of interest and one reference gene, were quantified.

The transcripts of the 18S rRNA and TP53 genes were detected by the following pair of primers: 5'- CCT GCG GCT TAA TTT GAC TCA-3' (forward) and 5'- AGC TAT CAA TCT GTC AAT CCT GTC C-3' (reverse); 5'- TCA ACA AGA TGT TTT GCC AAC TG -3' (forward) and 5'- ATG TGC TGT GAC TGC TTG TAG ATG -3' (reverse), respectively.

The PCR protocol is as follows: The real-time PCR mixture contained 10 µl of 2x GoTaq® qPCR Master Mixture contained 10 µl 2x SYBR Green I, 10 µmol/L of each primer and 5 µl of cDNA. The cycling conditions included initial incubation step at 95°C for 15 minutes followed by a 45 cycles of amplification with 25 seconds at 95°C, 30 seconds at 58°C, and 20 seconds at 72°C (single acquisition). The cycling conditions included initial incubation step at 95°C for 10 minutes, followed by a 50 cycles of amplification with 10 seconds at 95°C, 30 seconds at 60°C (single acquisition), and 1 seconds at 72°C. The final cooling step was 40°C for 30 seconds. Housekeeping/target gene sets, for FFPE material were measured.

Table 1: Characteristics of all Breast Cancer Patients in this Study

Characteristics	Number
Age	
≤40 year	29
>40 year	71
Histopathological Type	
IDC	91
ILC	7
Rare type	2
ER Status	
Positive	44
Negative	53
Unknown	3
PR Status	
Positive	50
Negative	47
Unknown	3
Her 2 Status	
Positive	16
Negative	76
Unknown	8
Laterality	
Right	53
Left	47

IDC, Invasive ductal carcinomas; ILC,Invasive lobular carcinoma

Statistical Analysis

Calculations were based on the comparison of the distinct cycle determined by threshold values (Ct) at a constant level of fluorescence [(13)]. Kinetic PCR efficiency correction was considered suitable and appropriate model to allow

determination of single transcription difference between one control and one sample, assayed in triplicates (n = 1/3). The relative expression ratio of a target gene is computed, based on its real-time PCR efficiencies (E) or a static efficiency of 2, and the threshold (Ct) difference (Δ) of one unknown sample (treated) versus one control (ΔCt control - treated) [14]. Using relative expression software tool (REST) and REST-XL, the relative calculation procedure is based on the mean of Ct for each sample. Only Ct values < 34 were used for calculation of the PCR efficiency from the given slope in Corbett Rotor-Gene 1.7.61 software according to the following equation of PCR efficiency:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct,\ \text{target (cal. – test)}}}{(E_{\text{ref}})^{\Delta Ct,\ \text{ref (cal. - test)}}}$$

E: Efficiency; ref: Reference; cal: Calibrator; Ct: Threshold

The efficiency for each gene was calculated from the data of standard curve as below.

PCR **Efficiency** = $(10^{(-1/\text{slope})} - 1)$ X100

RESULTS AND DISCUSSIONS

Tell now challengeable treated with uses of FFPE material for molecular analysis, and the amount of nucleic acids and quality depend on the isolation method and FFPE block long storage [15].

Total RNA was extracted from FFPE specimens prepared from malignant breast tumors tissues, Nucleic acid extraction, carried out by taken three 5-µm-thick sections of archival FFPE blocks were cut using a microtome. The slice was deparaffinized by adding 1000 µl Xylene and following the protocol given in the pack insert of the miRNeasy FFPE Kit qiagen (Qiagen, Valencia, CA, Cat#217504). The quantity and quality of extracted RNA determined by Biophotometer spectrophotometer (Eppendorf, Germany) through measuring the absorbance at 260 nm and 280 nm.

The concentration of extracted RNA was expressed as (Mean ± SD) exhibited a level of 55.5 ± 13.61 µg/ml. The purity of the extracted RNA was estimated by measuring the ratio of A_{260}/A_{280}. It was found to be 1.93 ± 0.04 suggesting an appropriate purity.

Validation of TP53 and 18s rRNA Genes Primer Specificity

Extracted RNA from breast cancer tissues was converted to cDNA. The products were amplified with the use of two levels (5 and 10 pmole) of the designed primers. Both levels were observed to amplify the cDNA successfully as demonstrated in Figure 1-A. A level of 10 pmole of the primer was considered to be the best . The specificity of the designed primer was confirmed by monitoring the melting curve obtained (Figure 1-B). The appearance of a single peak suggests a specific primer.

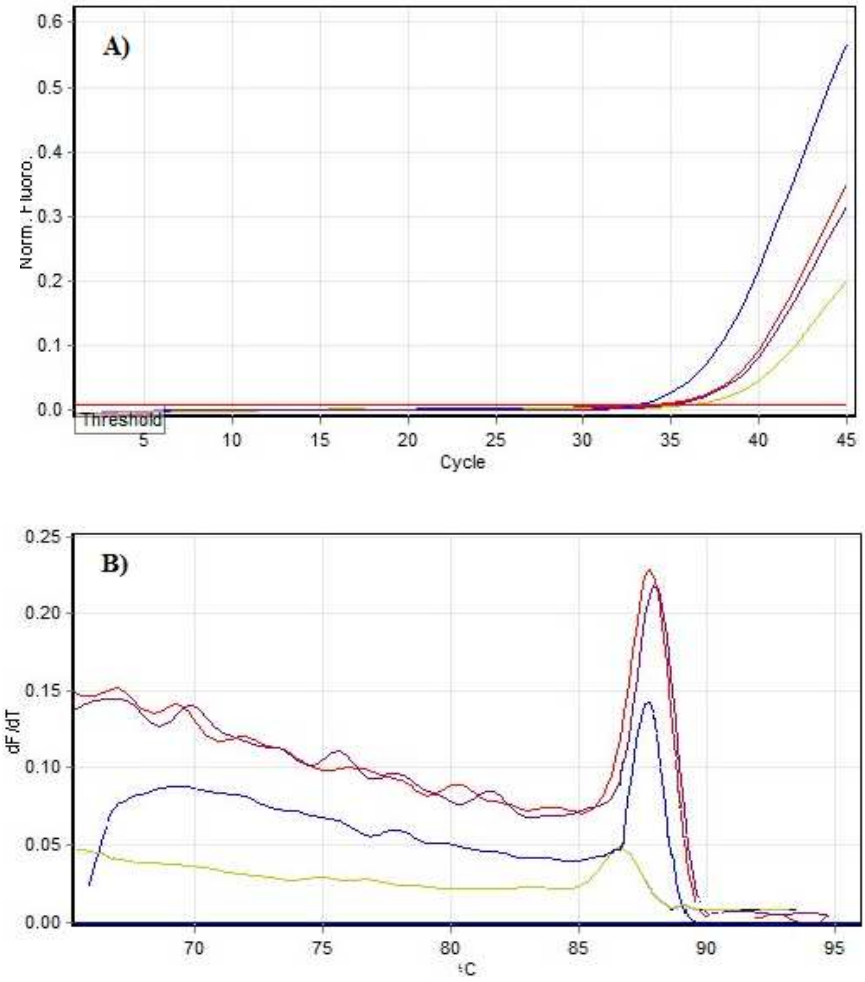

Figure 1: A) Validation of the Concentration of Specific Primer for TP53 Gene; B) Melting Curve Measured as -ΔF/ΔT versus Temperature (°C) of TP53 Gene, with two Different Concentrations 5, 10 pmol, C) Melting Curve Measured as Florescence (F) versus Temperature (°C)

No.	Colour	Name	Type	Ct
1		10pmol	Unknown	33.68
2		5pmol	Unknown	35.84
3		5pmol	Unknown	32.84
4		10pmol	Unknown	34.82

The real-time PCR efficiency was evaluated from the construction of a standard curve of Ct verses concentrations. A serial dilution ($1/10^3$, $1/10^4$, $1/10^5$, $1/10^6$, and $1/10^8$) of one amplified cDNA sample was carried out. Diluted samples were re-amplified and successful amplification was achieved as pointed out in Figure 1-A. The melting curve of the amplification product was shown in figure 1-B. Only Ct values < 34 were used for calculation of the PCR efficiency from the given slope in Corbett Rotor-Gene 1.7.61 software according to the equation of PCR efficiency. Therefore a standard curve was plotted (Figure 1.C), from which the efficiency was found to equal to 78% and 96% for TP53 and 18S rRNA respectively.

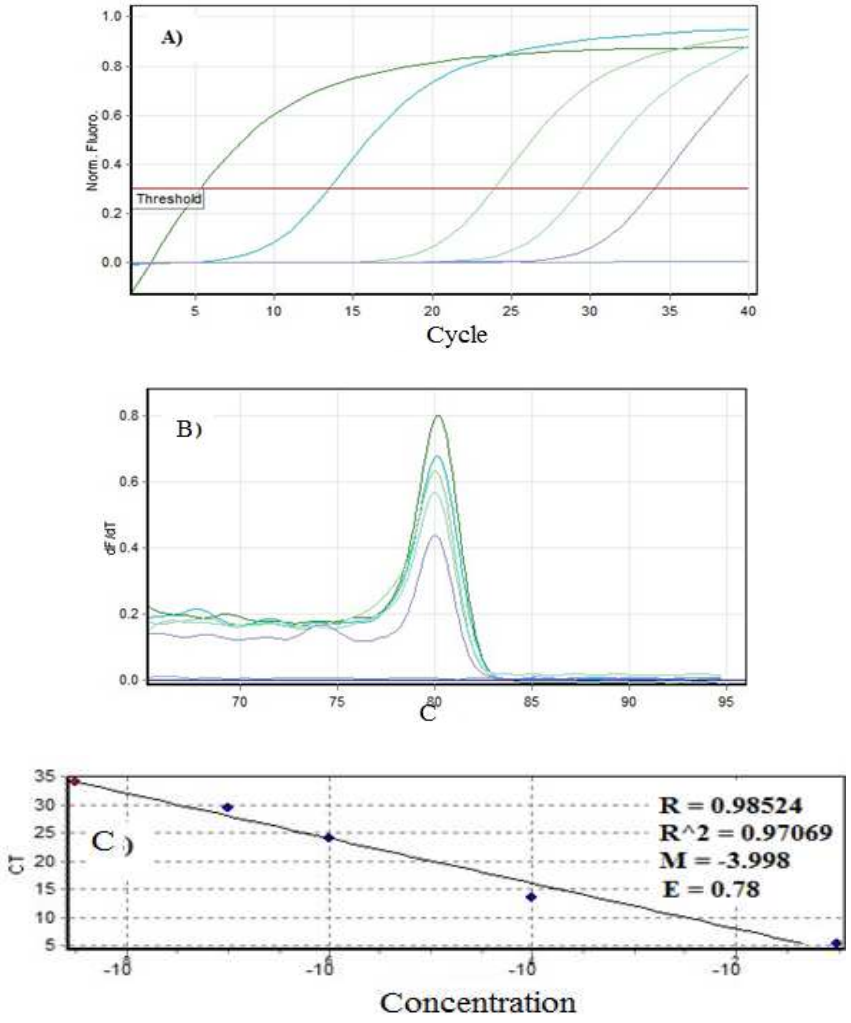

Figure 2: Generating a Standard Curve to Assess Reaction Optimization for TP53 Gene by Using the Corbett® Real-Time System. A) Amplification Curves of the Dilution Series. B) Melting Curve Measured as -ΔF /ΔT Versus Temperature (°C) for Standard Curve. C) Standard Curve with the CT Plotted against the Log of the Starting Quantity of Template for Each Dilution, The Calculated Amplification Efficiency was 78%

Validation of Housekeeping (18s rRNA) Gene Primer Specificity

The real-time PCR efficiency was evaluated from the construction of a standard curve of Ct verses concentrations. A serial dilution ($1/10^3$, $1/10^4$, $1/10^5$, $1/10^6$, and $1/10^8$) of one amplified cDNA sample was carried out. Diluted samples were re-amplified and successful amplification was achieved as pointed out in (Figure 2-A). The melting curve of amplification product was shown in (Figure 2-B). Only Ct values <34 were used for calculation of the PCR efficiency from the given slope in Corbett Rotor-Gene 1.7.61 software according to the following equation of PCR efficiency. Therefore a standard curve was plotted (Figure 2-), from which the efficiency was found to equal to 96%.

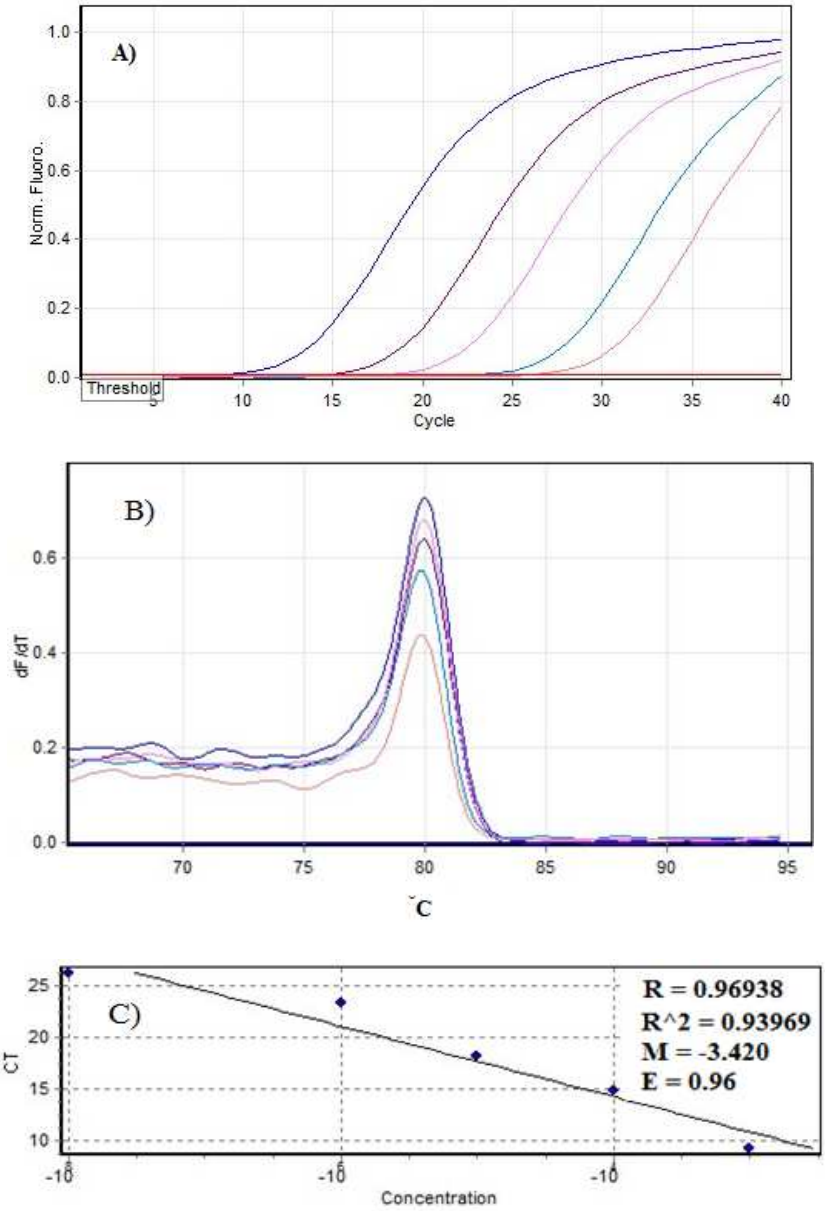

Figure 2: Generating a Standard Curve to Assess Reaction Optimization for 18S rRNA Gene by Using the Corbett® Real-Time System. A) Amplification Curves of the Serial Dilution. B) Melting Curve for Standard Curve. C) Standard Curve with the CT Plotted Against the Log of the Starting Quantity of Template for Each Dilution. The Calculated Amplification Efficiency was 96%

TP53 Gene Expression in Malignant Breast Tissues

The expression of TP53 genes in malignant breast tumors was estimated in the 100 specimens of cDNA prepared from RNA of malignant breast tissues. The TP53 cDNA was amplified successfully with 78% efficiency. As well as 18S rRNA cDNA was amplified successfully with 96% efficiency.

A melting curve was examined to confirm the amplification of each intended gene. When the temperature is raised, a double stranded DNA (dsDNA) intercalated with the dye molecules is dissociated or "melts", into single –stranded DNA (ssDNA). Thus, changes of the fluorescence of the dye is plotted against changes of temperature (F vs T and -ΔF/ΔT vs T) for both TP53 and 18S rRNA genes respectively. Results indicated a decline of the fluorescence as the temperature

raised (Figures 3 &4 respectively). Differential evaluation of the melting dynamics revealed a single peak, illustrating a single amplicone of the intended gene with an appropriate specificity.

The relative quantification of TP53 gene expression in malignant breast tumors was achieved through the calibration against the expression of the same gene in normal tissues (calibrators). A normalized gene (18S rRNA) was used as a control for the experimental variability in this quantification. Thus, the expression folds of TP53 gene was calculated with respect to the internal control gene (housekeeping gene), i.e.,18SrRNA. TP53 gene expressions was found to be significantly ($p<0.0001$) raised in malignant breast tumors with 44 folds (Table 1).

Table 1: Relative Expression Results of TP53 Gene Malignant Breast Tissues

Gene	Type	Efficiency	Expression	P value
18srRNA	REF	0.96	1.000	
TP53	TRG	0.78	44.000	0.0001

REF: reference; **TRG:** Target

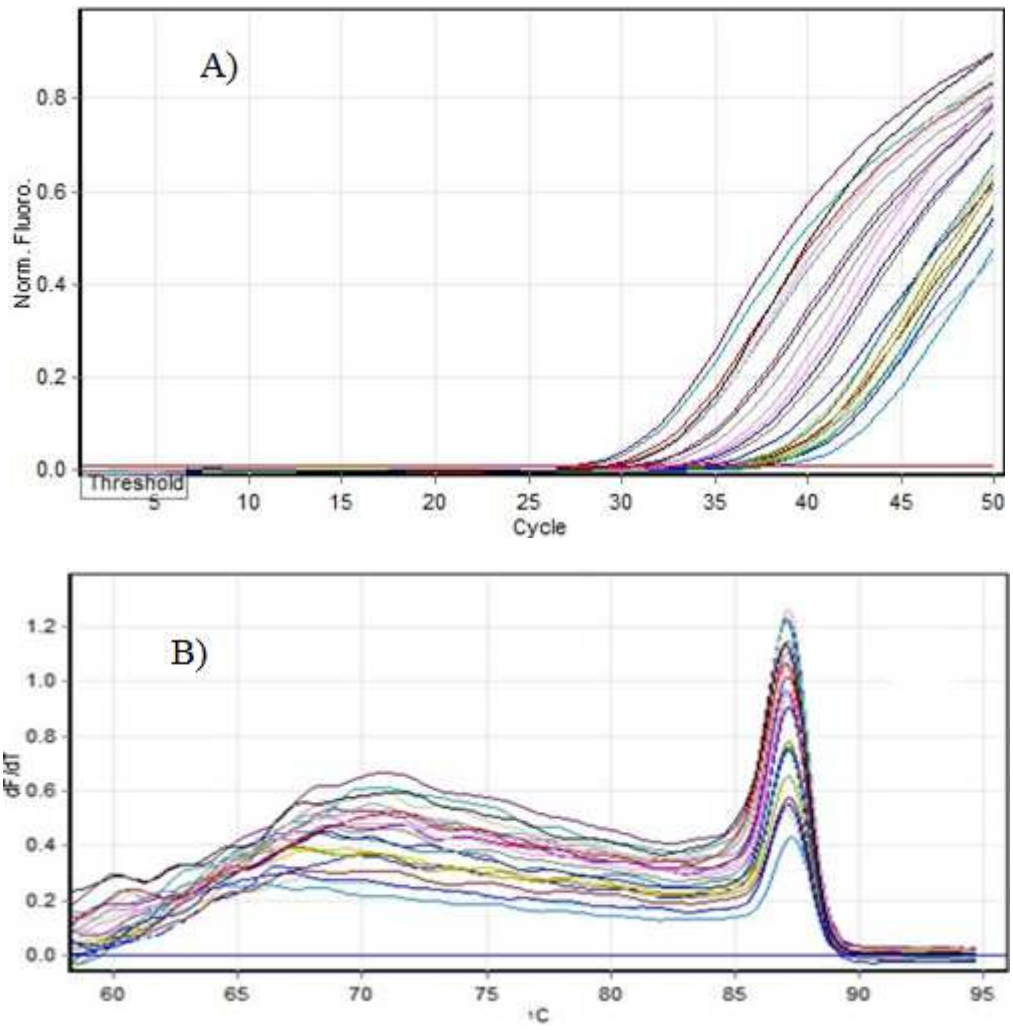

Figure 3: A) Amplification Curve of the Quantification Data of Cycling of Different Samples Analyzed for TP53 Gene Amplification. (B) Melting Curve Measured as -ΔF/ΔT versus Temperature (°C)

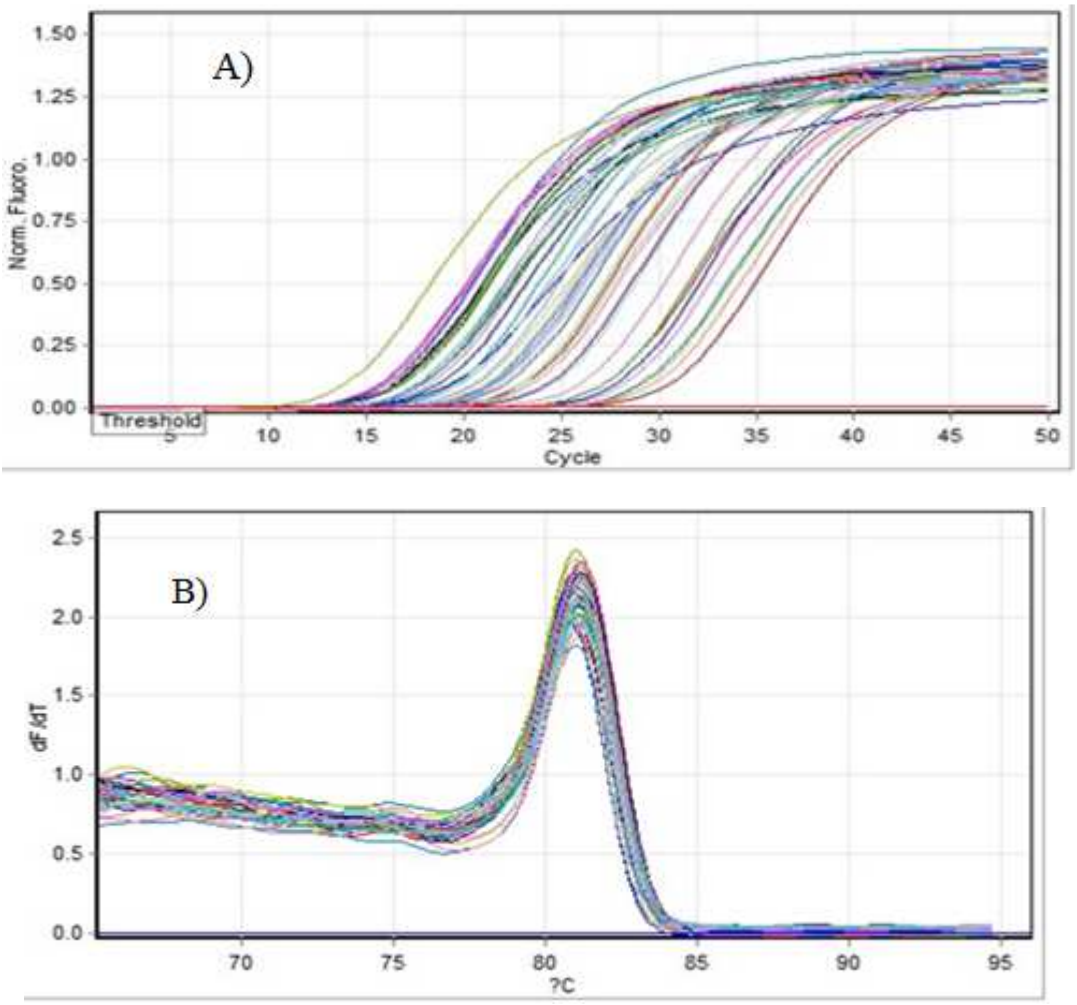

Figure 4: A) Amplification Curve of the Quantification Data of Cycling of Different Samples Analyzed for 18S rRNA Gene Amplification. (B)Melting Curve Measured as -ΔF/ΔT vs Temperature (˚C)

In the current investigation, RNAs of TP53 and 18S rRNA genes were extracted from 100 FFPE tissues, converted to cDNA and successfully amplified with appropriate efficiencies. To our knowledge, this is the first study dealt with such extraction and amplification carried out in Iraq. Moreover, the comparison with other studies carried out abroad highlights a remarkable notice which is the high number of samples investigated relative to those enrolled abroad.

TP53 was evident to be expressed extensively in malignant breast tumors with 44 folds relative to normal tissues. Such results suggested the up regulation of the expression of both genes during carcinogenesis.

Tumor suppressor gene mutation or activation of oncogenes can lead to breast cancer development spontaneously, but may occur by germline mutations in tumor suppressor genes that result from familial predisposition to breast and ovarian cancer. A multiple proteins involving TP53 have been found to be specifically over expressed in approximately more than half of human cancers due to mutations involving breast cancer [(16)].

Normally TP53 protein prevents and suppresses tumor formation, while the mutant TP53 can cause cancer through inactivation of the TP53 signaling pathway either by increasing the number of TP53 inhibitors or decreasing the number of cooperators. So, in the absence of TP53, tumor can develop easily due to the genetic alterations and defective cells accumulations [(17)].

The major cause of TP53 over expression may be related to high proliferation rate (S-phase fraction), in addition to mutation or changes in the TP53 gene and the resulting protein different from the wild type protein [(18)]. Since most TP53 mutations result in increased protein stability, over expression of TP53 has been used as a surrogate of TP53 dysfunction. However, some mutations result in the absence of protein. Abnormalities of TP53 expression have been associated with worse prognosis in cases of breast cancer [(19)], while TP53 expression was associated with tumor aggressiveness [(20)]. Usually TP53 function is tumor suppressor through regulating transcription, cell cycle, and apoptosis. Mutations of TP53 in breast cancers are primarily point mutations that often lead to loss of function of the wild type TP53 and over-expression in malignant cells [(21)]. DNA methylation is the common feature of all cancer types and responsible for the genomic instability. Such mutations frequently occur in the tumor suppressor genes involving TP53 [(22)].

In normal conditions, wild-type TP53 protein is rapidly degraded but in carcinomas, the acquisition of a mutant genotype is thought to increase the half-life of the mutant protein, which leads to accumulation within the cells causing over expression [(23)]. In breast carcinoma the mutations or alterations in the TP53 gene are common. These mutations are associated with poor prognosis, thought to be due to proliferative advantage and poor response to chemotherapy associated with loss of TP53 function [(24)]. The expression of mutant TP53 was demonstrated in malignant breast cell lines [(25)]. A common event in primary carcinoma of breast is the Loss of heterozygosity (LOH) in the TP53 gene and this is accompanied by mutation of the remaining allele in some cases [(26)]. To clarify the relevance of histopathological type with the expression of TP53 gene, the gene expression results were analyzed in regards to the type of malignant tumors. There were 91 ductal carcinoma, 7 lobular carcinoma and 2 rare cases. Significant ($P<0.001$) elevation of TP53 gene expression was evident in ductal carcinoma with respect to the of lobular carcinoma (Table 2).

Table 2: TP53 Gene Expression in ductal and Lobular Breast Cancer Tumors

Gene	Type	Type	Efficiency	Expression	P Value
18S rRNA	REF	Ductal Lobular	0.96	1.000 1.000	
TP53	TRG	Ductal Lobular	0.78	45.6 27.2	0.001

REF: reference; TRG: Target

Over expression of TP53 gene was evident in the investigated breast cancer patients. Disruption of TP53 functions due to alteration or mutations appears to play an important role in carcinogenesis[(27)]. Mutations may lead to excessive expression of this gene. Such conditions were frequently associated with poorer disease-free and overall survival [(28)]. It has been reported that over-expression of TP53 gene in tumor tissues and accumulation of their protein have poor prognosis [(29)]. Some authors have mentioned a relatively good prognosis for HER2 positive breast cancer patients when TP53 was over expressed [(31)]. On the other hand it has been found that TP53 expression may correlate with the hereditary mutations in genetic breast cancer [(32)].

CONCLUSIONS

TP53 gene is a major tumor suppressor that is altered by mutations in 50% of human cancers and functionally inactivated in other 50%. In our study we find increased their expression in FFPE of breast cancer patients relative to reference gene. It can be used with TP53 gene mutation assessment as

survival markers of breast cancer. Markers may provide prognostic information that complements clinical variables.

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