Introduction:

Breast cancer is the most common malignancy among women in worldwide (1) and it ranks second (after lung cancer) as a cause of cancer mortality (2). Over the last several decades the incidence of breast cancer has increased globally (2-5), the greatest increase has been in Asian countries too (6-7).

In Iran, due to increasing its incidence rate, breast cancer is one of the most frequent malignancies during last four decades comprising 21.4% of all cancers among females (9). Based on the statically reports, the mortality rate of breast cancer was 5.8 per 100,000 women in Tehran in 1998 (10), 2.5 per 100,000 for the female population, and 7762 years life lost in the 18 provinces of Iran in 2001 (11).

Breast cancer is a collection of biologically distinct disease subtypes characterized by unique gene expression profiles, molecular or protein markers (12). The researches indicated that six hormones controlled the tissue growth and development in the mature stage. In women, estrogen regulated these changes more than others. Estrogen produced in ovary and then entered to blood to control the growth in childhood until adult period (13). Evidence suggests that during breast cancer development, estrogen signaling pathways including estrogen receptor α (ER-α) and estrogen receptor β (ER-β) were changed (12). Previous studies showed that estrogen mainly estrogen receptor α (ER-α) (7-8) plays essential role in the development, growth control and differentiation of the normal mammary gland. Role of estrogen receptors and its modulators in breast cancer have been widely studied in western population (14). The overexpression of estrogen receptor α (ERα) is frequently observed in the early stage of breast cancer (15-17). Ali and Coombes in 2000 reported ER alpha was overexpressed in half of all breast cancers and around 70% of these respond to anti-estrogen (for example tamoxifen) therapy (15). In the other study, the expression of several candidate genes in breast cancer tissues including the estrogen receptors were analyzed by real-time RT-PCR and by immunohistochemical techniques. This study was confirmed the previous result too (17). Recently, there were few studies have been reported recently on the expression of estrogen receptors in the Iranian population (12, 18). Totally, many risk factors are involved in breast cancer and most of them are linked to estrogens. By early menarche, late menopause, obesity in postmenopausal women, and high concentrations of human estrogens, risk of breast cancer can be increased (19). Therefore, it is crucial to develop a gene and hormone therapy treatment for breast cancer due to the limited success of conventional treatment (20). The analysis of estrogen receptor is as a suitable tool for diagnose and predict to hormone therapy response in breast cancer (21, 22). In order to directly determine how ER expression may change and to gain insight into expression patterns of ERα in individual cases of breast cancer is an important question. So, objective of this study was to quantify of expression of ERα gene by Real-time PCR and study of the association between ERα expression, age and menopause of Iranian women with breast cancer.

Material & Methods:

Sample collection

31 specimens, embedded in paraffin wax were collected in coordination with a pathologist at Moayyed laboratory, Mashhad, Iran for this study. The samples were composed of 21 women with malignant breast tumors and 10 normal (non-cancerous). Histological analysis was performed, by the same pathologist for the evaluation of grade, estrogen receptor (ER) status and tumor size. The mean age of the patients with breast ductal carcinoma and normal samples was 50 (range, 38 to 75) and 34 (range, 19 to 44), respectively. Most patients were married and non-menopause (85% and 70%, respectively). The characteristics of samples is displayed in table 1.

Total RNA extraction and cDNA synthesis

Twelve micro sections were cut for each selected block and total RNA was either extracted. RNA was extracted with FFPE Kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. The isolated RNA samples were stored in RNAse-free distilled water at –80ºC. Total RNA concentration and quality were determined nanodrop ND-2000 spectrophotometer (Thermo, Wilmington, USA) at 260 and 280 nm, while RNA integrity was evaluated using agarose gel electrophoresis.

Table 1. The characteristic of samples

Factors	Class of factors	Total number	Percentage	P-value
Age	**≤ 45**	21	67.64%	<0.0299
	> 45	10	32.36%	
Material status	**Married**	14	45.16%	<0.401
	Single	17	54.84%	
Menopausal status	**Menopause**	9	29%	<0.13
	Non menopause	22	71%	

Table 2. The thermal protocol of Real-time PCR

Cycles	Segment	Target	Hold time
		Temperature	
1		**Pre-Incubation**	
		95^0c	10 min
		Amplification	
	Denaturation	95^0c	30 s
	Annealing	60^0c	30 s
45	Extension	72^0c	30 s
		Melting curve	
	Denaturation	95^0c	15 s
	Annealing	60^0c	1 min
1	Extension	95^0c	15 s
	Melting	60^0c	15 s

Reverse transcription of the mRNA molecules into first-strand cDNA, was carried out using 1µg of total RNA from each tissue specimen, RevertAid™ H Minus Reverse Transcriptase kite (Fermentas, Burlington, USA) and an Oligo (dT) oligonoucleotide as reverse transcription primer. The reaction mix was incubated at 42ºC for 60 min, and the reverse transcriptase enzyme was inactivated by incubation at 70ºC for 5 min. All of cDNAs were stored at –80ºC until used for measurement. Purity of cDNA was obtained by spectrophotometer. Each cDNA sample was diluted to 100 ng/µL by DNase free diluted water to provide uniformly concentrated sample for real-time PCR.

Primers

Primers were designed with the assistance of the computer program Primer premier software, version 5 (23) for the target gene – ERα and a housekeeping gene - GAPDH. GAPDH primer set was located in different exons of the gene in order to avoid amplification of contaminating genomic DNA. We also conducted BLASTN searches (24) and Primer Data Base such as RT (25) to confirm the total gene specificity of the nucleotide sequences chosen for the primers, and the structure of primers. The primers were produced through Bioneer Company of southern Korea. Designed primers ERα (F: 5'TGGTCAGTGCCTTGTTGGATG3', R: 5'TGTCTTGCCAGGTTGGTCAGTAAG3') and GAPDH (F: 5'GAAGGCTGGGGCTCATTTGA3', R: 5'GCTGATGATCTTGAGGCTGTTGT3') amplify 111 bp products and 127 bp products, respectively.

Real-time PCR amplification

A SYBR-Green fluorescence-based quantitative Real-Time PCR assay (qPCR) was used for the determination of ERα gene expression levels in the breast specimens on an ABI Prism 7300 Thermal Cycler (Applied Biosystems, Foster city, USA). The final 25 µL reaction volume includes 10 µL SYBRضGreen PCR Master Mix (Applied Biosystems), 0.15 µL of each primer (5pmol), 2 µL of template cDNA (100ng/µL), 1.2 µL of Mgcl2 (50 mM) and 11.4 µL of diethylpyrocarbonate (DEPC) treated water. The thermal protocol of Real-time PCR was performed based on the table 2. Reactions without reverse transcriptase or without template served as controls for ERα genomic DNA contamination. To ensure that the correct product was amplified in the reaction, all PCR products were separated on 1.5 % agarose gel electrophoresis (Invitrogen, Carlsbad, USA).

PCR efficiency and data analysis

A standard curve was constructed from a known concentration of cDNA sample for determination of PCR efficiency for ERα and GAPDH genes. A series of seven 1/10 dilutions, starting from a concentration of 100 ng/µL were used. Delta Ct for ERα and GAPDH was determined. Normal probability plot for genes were made for measuring PCR efficiency. The delta Ct slope of the line was calculated from a plot with delta Ct on the Y-axis and the logarithm of total cDNA on the X-axis. Efficiency for ERα and GAPDH genes was found to be comparable according to the absolute value of the slope of the lines. Data was obtained as Ct values according to the manufacturer's guidelines and expressed as a ΔCt value (ΔCt = Ct of the target gene – Ct of housekeeping gene) (26). Analysis was also performed by normalizing the Ct ERα to the Ct GAPDH. The fold change in the target gene for the results of quantitative amplification was calculated for each sample using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCt = ΔCt of target cancer _ ΔCt of target normal (26).

All the data was entered into a computer database. Statistical analysis was performed using the SDS v1.4.1 software (27). Fisher's exact test was used for categorical variables. Student t-test procedure was performed in SAS v9.1 software (28) and excels Microsoft to determine statistical significance. Significance level of the tests was taken at $P < 0.05$.

Results:

Quantity and quality of results of extracted RNA and synthesis cDNA

The spectrophotometer results of extracted RNA and synthesis cDNA showed all RNAs were with high purity and without any EDTA and phenol contamination. Furthermore, the results demonstrated that quality and quantity of cDNAs was suitable for real-time PCR too.

PCR optimization

For quantification of cDNAs by real-time PCR and achieve accurate results, we accomplished qualitative PCR before. PCR reaction was accomplished to obtain to optimum annealing temperature and concentration of primers and determination of $Mgcl_2$ content too. Annealing temperature was obtained 60^{OC} for all primers and concentration of primers and $Mgcl_2$ was 0.3 and 1.2 µl, respectively. The expected size of PCR products (111bp and 127bp for ERα and GAPDH, respectively)after run on agarose gel was indicative specificity of PCR products (see Figure 1).

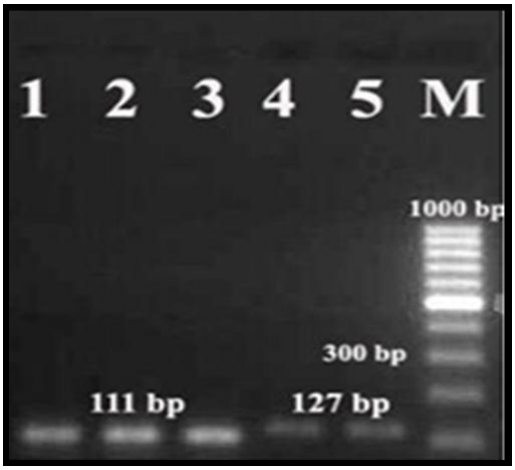

Figure 1. The results of PCR reaction on 1.5% agarose gel. Lane 1, Sample 1 (ERα); lane 2, Sample 14(ERα); Lane 21, Sample 1 (ERα); lane 4, Sample 1(GAPDH); Lane 5, Sample 14 (GAPDH); M: 100-bp ladder (100-1000 bp)

Real-time PCR assay

Quantitative Real-time PCR (qRT-PCR) approach based on SYBR Green were used to measure relative expression levels of ERα gene in non-malignant and malignant specimens. The results and amplification curve of ERα as target gene and GAPDH as reference gene by real-time PCR showed that genes replication was done very well and without any noise. SYBR green florescence dye doesn't act specific in real-time PCR reaction, so melting curve was plotted to be sure from specific segments production, the absence nonspecific band, second structures and dimer primer. The results of melting plot indicated that each two pairs of primer acted specifically and there were any nonspecific segments and second structures. Standard curve design is a reliable and simple alternative to the relative real time PCR-efficiency evaluation. The standard curve method has been compiled and validated. It simplifies calculations and avoids practical and theoretical problems currently associated with PCR efficiency assessment. A standard curve was derived from the serial dilutions and is graphically represented as a semi-log regression line plot of CT value vs. log of input nucleic acid. To produce the standard curve of sufficient quality we should have sufficient number and range of standard dilutions and proper laboratory

practice (29). A standard curve slope of −3.32 indicates a PCR reaction with 100% efficiency. Slopes more negative than −3.32 indicate reactions that are less than 100% efficient. Slopes more positive than −3.32 may indicate sample quality or pipetting problems (29). ERα standard curve by real-time PCR was plotted serial dilution of cDNA vs. ct. The slop of this curve was −3.1 and it was in the expected range. PCR efficacy was 95% for ERα gene (Figure 2).

Statistical analysis

Real-time PCR data as ct (threshold cycle) value were analyzed by SDS software v1.4. The determination of ERα expression was obtained using ct value and Pfaffl formulation (30). The obtained Ct and ΔCt values were analyzed through Excel and SAS software. ANOVA technique and student t tests procedure were performed in SAS v9.1 software to ascertain statistical significance difference ($\alpha = 0.05$). The results showed that there was a significant difference between cancerous and normal tissue for ERα expression amount. The fold change in the target gene (ERα) for the results of quantitative amplification was calculated using the $2^{-\Delta\Delta Ct}$ method. Fold change was calculated about 15 is indicative that ERα expression in cases with breast cancer was over expressed ($P<0.01$). Over expression of ERα in breast cancer led to increase the ratio of ERα/ERβ in adenoma carcinoma tissue compared with normal one.

ERα mRNA expression in breast tumor tissues

The expression content was evaluated based on obtained ct value from real-time PCR. A reverse relationship between ct value and expression mount. NERα values (calculated as described in Materials and methods) ranged from 2.34 to 14.53 (mean 8.53+standard deviation 0.98) for the breast tumor RNA samples tested, whereas NERα values ranged from 7.39 to 14.53 (mean 12.47 +standard deviation 0.81) for normal samples.

Study of relation between ERα expression and age of patient

We used SAS software to determination association between ERα expression and age of patient. Data and plot of SAS software showed there was a significant nonlinear relation between ERα expression and age of patient and relation between ct of ERα (ERα expression) and age was analyzed using a quadratic equation. The results indicated that ct value variation have reduction process at age 37. The minimum of ct value was at 18 to 19 ages and after that the ct value has rising trend and it seems that the maximum of ct value was seen at older (see Figure 3).

Study of relation between ERα expression and menopause of patient

We have to use logistic regression for determination of relation between ct value and menopause status. H0: the logistic regression fits well. With a P-value of 0.4726, we cannot reject this null hypothesis. So it's fine to use the logistic regression model. The graph show that with increasing ct number, the estimated probability of menopause decrease. We could find the intercept and regression slope of the model which calculated by maximum likelihood method (see Figure 5).

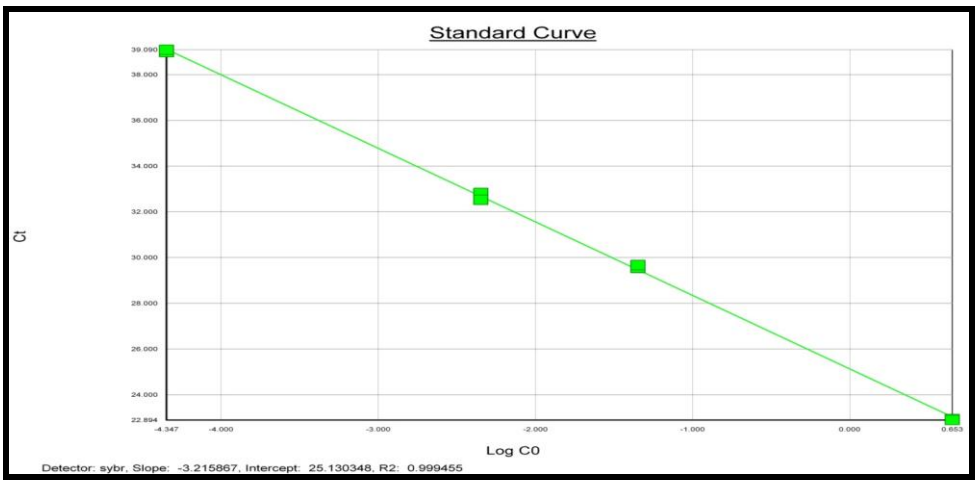

Figure 2. Standard curve of ERα expression. The slop of this curve was –3.1

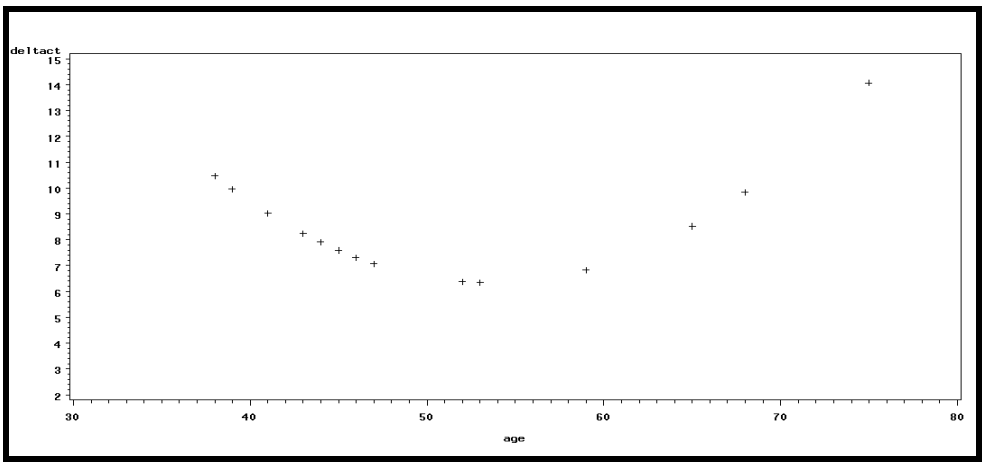

Figure 4. Nonlinear curve of ERα expression and age of patient

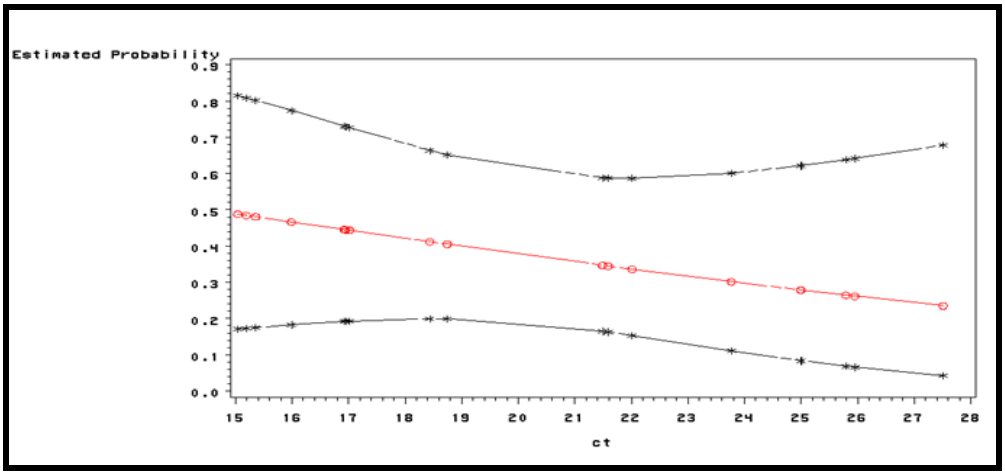

Figure 5. Curve of ct value and menopause status. Y is a binary response variable here. Estimated odds ratio = 0.913, and 95% Confidence interval for odds ratio is (0.725 1.150). PROC PLOT gives a crude plot of the estimated logistic regression curve and the 95% Confidence interval are also plotted

Conclusion:

Human breast cancer is considered a heterogeneous disease according to estrogen receptor, tumor grade and age (31). Over expression of ERα is one of the main factors of malignant tumor growth, human breast proliferation and led to promote and progress breast cancer in human. It is still widely accepted that one of the basic predictive indicators of hormonal therapy success is evaluation of estrogen and progesterone receptors (ER, PR) in breast cancer tissue (32). So, we can diagnose tumors rather and enterprise to treat them. In this study, we validated a real-time PCR method for quantification of ERα expression in human breast tumors. The method is based on real-time analysis of PCR amplification and SYBR green. This recent approach to nucleic acid quantification is suited to the development of target gene assays, having a high degree of inter-laboratory standardization and also yielding statistical confidence values (33-34). The primary goal of our study was to measurement of ERα gene expression content in breast carcinomas and studying of relation between ERα, age and menopause of women with breast cancer. Finding showed that ERα express in both tumor and normal tissue but expression content of this gene is different. The statically significant difference test between gene expressions in malignant cases in comparison with non-malignant samples showed that ERα gene expression increased in 100 percent of malignant cases. Also the amount of ERα mRNA increased with age and was consequently higher in postmenopausal patients' tumors ($P<0.01$). So, this study results indicated that this gene can be used as biomarker for screening and diagnosis patients against normal humans even, at the beginning stages of breast cancer. Furthermore, this method can be help to patient selection for anti-estrogen therapy and monitoring response to therapy. Ivan BieÁche and et al (2001) investigated Quantification of estrogen receptor α and β expression in sporadic breast cancer using real-time PCR and they showed that ERα mRNA expression in most breast tumor tissues increased (33). De cremoux and et al in 2002 obtained to similar results using real-time reverse transcription-polymerase chain reaction correlation with protein assay for quantitation of estradiol receptor alpha and beta and progesterone receptors in human breast tumor (35).

In 2004, Martin C Abba worked on Gene expression signature of estrogen receptor α status in breast cancer and the real-time PCR statistical analysis of this paper revealed that genes differentially expressed between ERα (+) and ERα (-) primary breast carcinomas (36).

In summary, we have identified changes in the expression of ERα in progression of breast neoplasm. In breast adenocarcinomas, ERα amplification was often noted. The difference in expression of ERα between normal and cancerous tissue suggests that ERα expression may be a useful surrogate molecular marker to adenocarcinoma. Further studies into this gene should be performed to identify the function of this novel gene and its role in cancer development. These results indicate that levels of estrogen receptor alpha expression are related to age and menopausal status in women with mammary carcinoma.

Conflict:

Authors declare no conflict of interest.

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