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Gene Expression Analyses of HER-2/neu and ESR1 in Patients with Breast Cancer

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ABSTRACT

Background: Her-2 and ESR1 genes, that interact in the cell signaling pathway, are the most important molecular markers of breast cancer, which have been amplified or overexpressed in 30% and 70%, respectively. This study was performed to evaluate the gene expression levels of Her-2 and ESR1 genes in tumor cells and its adjacent normal tissue of breast cancer patients and compared them whit clinical-pathological features.

Methods: In total, 80 tissue specimens from 40 patients, with an average age of 48.47 years, were examined by Real-time PCR technique, and ultimately evaluated the expression level of Her-2 and ESR1genes. The data were analyzed by REST 2009 V2.0.13 statistical software.

Results: HER2 and ESR1 overexpression was identified in 19 (48%) and 12 (30%) of 40 patients respectively, which was higher and lower than that recorded in international statistics, respectively. ESR1 overexpression was associated with Stage 3A and lymph node involvement 2 (N2) (P = 0.04 and P = 0.047, respectively). No significant correlation was observed between the expression of HER2 and ESR1 and other clinical-pathological features, however, the relative differences were identified in the expression levels of genes between main group and groups that were classified according to the clinical-pathological features and age.

Conclusions: Overexpression of Her-2 and ESR1 genes in the patients of our study are higher and lower than international statistics, respectively, indicating the differences in genetic, environmental and ethnic factors that involved in the developing of breast cancer.

Key Words: Breast Cancer, Her-2/neu, ESR1, overexpression, molecular marker, real-time PCR

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INTRODUCTION

Breast cancer is one of the most common cancers in women around the world, and the main causes of death in middle-aged women (1-6). About 15-10% of women during his lifetime, to be diagnosed with breast cancer (2, 4, 5, 7). Breast cancer is one of the three most common cancer in Iranian population and the highest rate of cancers diagnosed among Iranian women. Although the incidence rate of breast cancer in Iranian women is almost six times lower than Caucasian women in the United States (23.65 per 100,000 to 140.8 per 100,000), but the number of newly diagnosed cases is rising rapidly (8-10). Breast cancer is very poor prognosis and there are limited therapeutic options for patients with advanced and metastatic forms. Breast cancer patients with the same prognosis and clinical diagnostic, Reveals different clinical results. This difference is concerned to the current classification of breast cancers, because the diseases with distinct molecular markers are classified as clinical classes, mainly based on the morphology (11), indicating the need to find new treatment methods and therapeutic agents, and identify breast tumor markers for monitoring progression and treatment of the disease. Although breast cancer is diagnosed by using of classical methods such as mammography, but using of the highly sensitive and accurate molecular methods for early diagnosis and treatments, can be useful (3). By measuring the expression levels, some of specific genes can be attributed to early diagnosis and choice the proper treatment method applied (1, 12). Human epidermal growth factor receptor 2 (Her-2 neu) and estrogen receptor 1(ESR1) are two important biomarkers that, separate the breast cancer subtypes in terms of biological diversity, effective and purposeful treatments (1, 3, 12-17). Her-2 neu gene, is also known as EGFR2 and erbB2 names which is located on the long arm of chromosome 17(17q21.1). Her-2 an oncogene, that is known as a molecular marker and used commonly in the study of breast cancer. This oncogene is overexpressed and amplified in 20-30% of cases of invasive breast cancers (13, 17-23), and encodes for a 185-kDa protein that is a cytoplasmic trans-membrane receptor and a member of the receptor tyrosine kinase family (13, 17, 24-27). Her-2 overexpression and amplification is associated with poor prognosis, DFS, OS, aggressive phenotype (17, 21-23). In addition, it is related with estrogen receptor and progesterone receptor negativity, leads to increased histologic grade, proliferation index, and an elevated rate of lymph node metastases (17, 21, 28, 29). Her/2-positive breast cancers respond to treatment with selective monoclonal antibody (trastuzumab) therapy (16). Recent studies have revealed the role of a strong crosstalk between the ER and HER family signaling pathways as a substantial factor to the acquired resistance to endocrine therapies (tamoxifen) against the ER pathways (30). ER (inclusive; ESR1 and ESR2) expressed in about 80% of breast cancers. There are two different forms of the nuclear estrogen receptor which are ligand-dependent transcription factors encoded by two different genes on the sixth and fourteenth chromosome (6q25.1 and 14q23.2) called; ESR1 and ESR2, respectively. These genes demonstrate high homology in their sequence. Estrogen and receptors plays an important role in the development and malignant progression of breast cancer, and it should be mentioned that ESR1 is one of important genes to this process. ESR1 receptor activated by ligand (estrogen) and then bind to DNA, and regulate the expression of target genes that promoters contain estrogen-responsive element (ERE). ESR1 expression in tumor is a favorable prognosis to respond in endocrine (tamoxifen) treatment and tend to have a lower recurrence rate (14, 31, 32). Thus, accurate measuring of Her-2 and ESR1 expression levels is very important in the prognosis and treatment of breast cancer (14, 17). Two common techniques for measuring the expression levels of these genes are

immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). The first on is not sufficiently accurate and the second is costly and time-consuming, but the use of molecular diagnostic techniques, such as real-time polymerase chain reaction (PCR), compared to the mentioned methods, is cheaper, more accessible, more accurate and faster (13, 18, 33-38) . The main goal in this study is to measure and compare the expression levels of Her-2 and ESR1 genes in tumor cells and its adjacent normal tissue of breast cancer and evaluate the association between the expression of these genes and breast tumorigenesis and also clinical-pathological features of breast tumor.

MATERIALS AND METHODS

Study design:

A descriptive study design was carried out under collaboration between the Noor-E-Nejat Hospital and Tabriz Genetic Analysis Center of Tabriz University of Medical Sciences. The protocol of the study was approved by the Ethical Committee of Tabriz University of Medical Sciences, which was in compliance with the Helsinki Declaration. Written informed consent was obtained from all participants.

Study population:

A total of 40 breast tumor samples and its adjacent tumor free margin samples were obtained during surgery, from 40 women. Based on pathological results, all tumors were ductal carcinoma, and all margins were free of tumor cells. All patients underwent appropriate surgery at Noor-E-Nejat Hospital of Tabriz in 2014. All patients were clearly identified as having breast cancer based on the clinic-pathological findings. The median age of patients with breast cancer was 48.47 ± 12.29 years (range 28-80 years). 80 samples were separated into two groups including; 40 tumors and its 40 tumor marginal sample controls.

Sample preparation, RNA Extraction and cDNA Synthesis:

All samples were obtained immediately after surgery and fresh tumor tissues were snap frozen in liquid nitrogen, and then were carried out to -70˚C freezer. All samples were subjected to total RNA extraction using TRIZOL (Invitrogen), as recommended by the manufacturer. The quality and quantity of extracted RNA was analyzed by agarose gel electrophoresis and nanodrop (nd1000). For removing DNA contamination probability, 2μg of extracted RNA was treated by DNAase1 (Fermentase). Then first-strand cDNA (complementary DNA) was synthesized using random hexamer primers and Reverse Transcriptase (Fermentase). For determining of cDNA synthesis PCR were performed by using house-keeping gene(GAPDH) primers. For showing nonentity of genomic DNA contamination RT minus control PCR are done; until all samples that are used for quantitative real time PCR been without genomic DNA contamination.

Standards as gene-specific quantity marker:

A dilution series of human genomic standards for absolute quantification was generated using human genomic DNA (hgDNA). HgDNA (Promega, USA) was homogenized by sonication and serially diluted in Tris–EDTA buffer to produce standards in which the number of DNA molecules ranged from 1/10 to 1/100,000. Each standard was then placed into aliquots of 1 ml and stored at -20˚C.

Real-time quantitative PCR:

Primers for SYBR green real-time PCR were designed for each gene (Table 1). For each gene, SYBR Green real-time PCR was performed in 15µl target volume using 4µl of cDNA from each sample, 7.5µl SYBR Green master mix (Takara, Japan) and 3.5µl of each gene specific forward and reverse primers. All real time PCRs were performed using the Roto gene 6000 system, with the following settings: 2 min at 95℃, followed by 40 cycles of 95℃ for 10 second, 60℃ for 60 second. All samples, as well as the five serial human genomic standards, were measured in each gene. Negative controls (NTC) were prepared each time. Heat separation (PCR product melting curve) analysis at the end of the PCR was performed for confirmation of single product amplification in each micro-tube.

Table.1. Primers used for PCR

Gene	Reverse primer	Forward primer	Primer length
Her-2 neu	CATAACTCCACACATCACTCTG	GTCAAGAGTCCCAACCATGTC	22 / 21
ESR1	AGTGGCTTTGGTCCGTCTC	TCCTCATCCTCTCCCACATC	19 / 20
GAPDH	GGTTGAGCACAGGGTACTTTA	CATGGCCTCCAAGGAGTAAG	21 /20

Normalization

Normalization using the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used in order to identify the expression levels of the Her-2 neu and ESR1 genes. The expression levels of the housekeeping and the Her-2 neu and ESR1 genes were measured by real-time quantitative PCR. The mean cycle threshold (Ct) values and the copy number of each gene were determined. The mean copy number of the housekeeping gene was calculated and then divided by the highest housekeeping gene in all samples, resulting in a normalization correction factor. Following real-time PCR amplification and quantification of the selected genes, this factor was used for the normalization of the expression levels for the Her-2 neu and ESR1 genes measured. The standard curves, which were plotted, demonstrated the ability of the method to accurately measure the expression levels of Her-2 neu and ESR1 genes.

Statistical analysis

Initial analysis indicated that the data were not normally distributed; therefore, nonparametric tests were used. Spearman correlation coefficient was used. moreover, statistical analysis was performed in each of the diagnostic groups using Mann–Whitney U with $p \leq 0.05$ deemed as statistically significant. All analyzes were performed using REST 2009 V2.0.13 and SPSS v.19 Softwares.

RESULTS

All 80 samples were separated into two groups; tumor, margin (40 tumor cells and 40 marginal cells) then were analyzed for her-2 and ESR1expression levels. For these genes and GAPDH gene (housekeeping gene), real-time PCR was applied using specific primers. Negative template controls (NTC) and human genomic standards were prepared each time. Standard curves were plotted using Ct values of the fivefold serial dilutions of the human genomic DNA for the optimization and efficiency of real-time PCR reactions. Heat dissociation analysis at the end of the

PCR confirmed amplification of single product in each micro-tube and it was shown that without interfering dimers and unspecific products in the reaction.

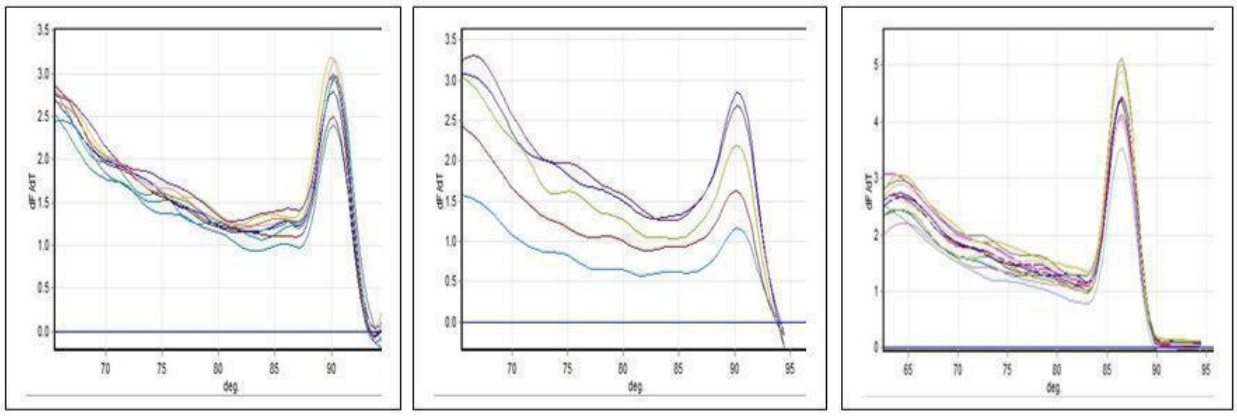

Fig.1. Melt curve analysis of real time PCR (Curves from left to right: Her-2, ESR1, GAPDH)

According to the results; in 27 of 40 patients (67.5%) her-2 expression level in tumor cells was higher than marginal cells. In addition, it's; 47.5%, for ESR1 gene (in 19 of 40 patients). In 9 of 40 patients (22%), Her-2 and ESR1 expression levels in tumor cells was lower than marginal cells, but the 15 patients (37.5%), the expression levels of both genes in tumor cells was higher than marginal cells and also; in the 16 patients (40%), expression level in tumor cells, was higher than marginal cells, only for one of these genes. To neglect any small increases of the gene expression levels, ratio ≥2 was chosen as the gene overexpression or gene amplification. Based on this criteria, 19 patients (47%) showed overexpression for Her-2 gene and 12 patients (30%) showed overexpression for ESRI gene, that in both groups the difference in gene expression level between tumor cells and marginal tumor cells (P=0.003, P=0.002 respectively for her-2 and ESR1 genes). Moreover, 5 patients (12.5%), Showed overexpression in both genes.

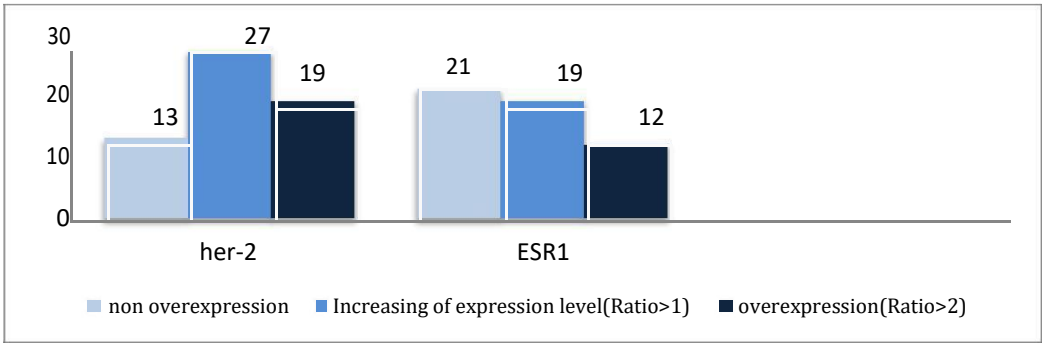

Fig.2. Expression of genes status in 40 patients.

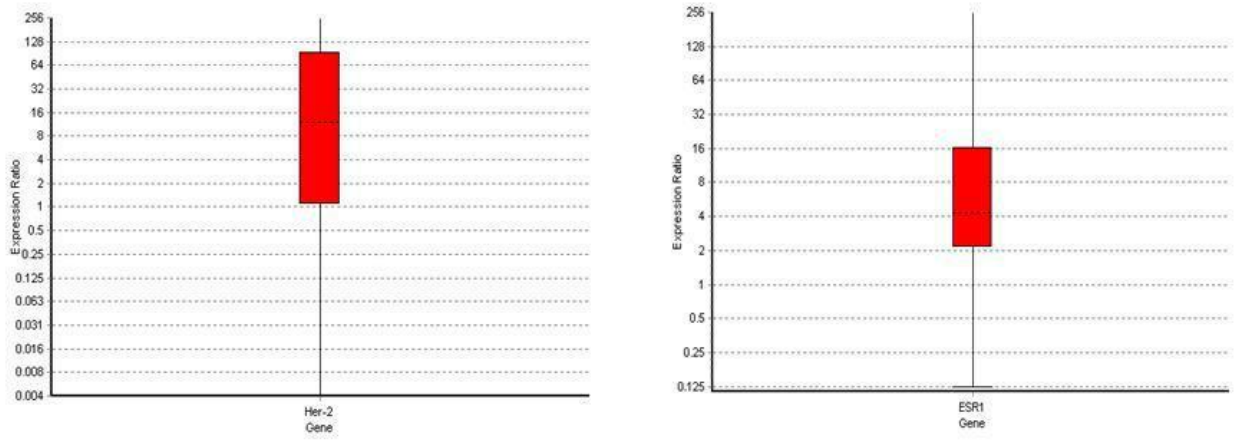

Fig.3. Relative expression of Her-2 and ESR1 genes in patients that was Ratio≥0, using relative expression software tool (REST 2009). Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Table.2. Relative expression of Her-2 and ESR1 genes in patients that was Ratio≥0, using relative expression software tool (REST 2009).

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
GAPDH	REF	1.0	1.000				
Her-2	TRG	1.0	10.365	0.435 - 229.958	0.027 - 2,486.671	0.003	UP
ESR1	TRG	1.0	5.281	1.128 - 30.967	0.229 - 82.139	0.002	UP

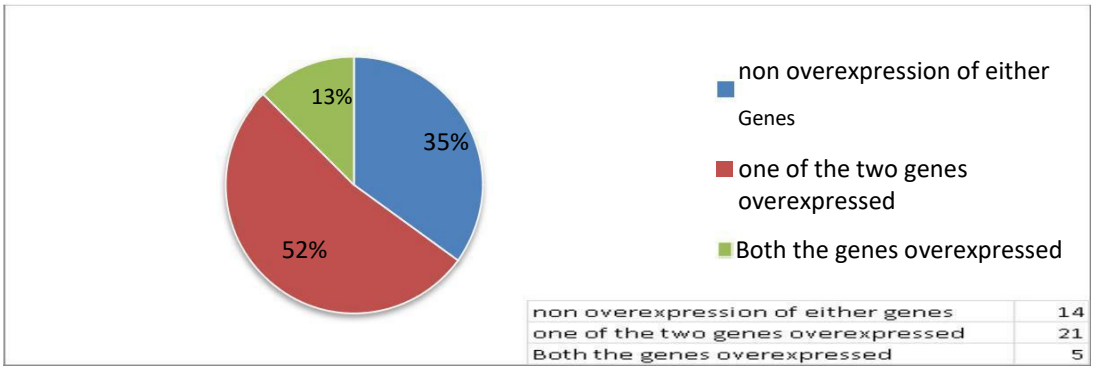

Fig.4. percentage of overexpression of genes that's Ratio≥2.

The patients were classified according to expression increase or non-increase of each of the genes, then the expression status of second gene was evaluated. the results are as follow:

Table.3. expression levels of second gene compared to different expression modes of other gene.

	Members of group	age average of group	Percentage (Members) with an increase of second gene expression level		Percentage (Members) with overexpression of second gene		Relative expression levels of second gene in tumor cell compared to margin cell	P-value
			group	Main group	group	Main group		
Samples with an increase of ESR1 expression	19	49.47	79% (15)	67.5%	47.4% (9)	47.5%	2.697	0.131

level (Ratio>1)								
Samples without an increase of ESR1 expression level (Ratio<1)	21	47.35	57.1% (12)	67.5%	47.6% (10)	47.5%	1.684	0.490
Samples with overexpression of ESR1 (Ratio≥2)	12	50.42	75% (9)	67.5%	41.7% (5)	47.5%	3.356	0.123
Samples without overexpression of ESR1 (Ratio≤2)	28	47.5	57.1% (16)	67.5%	50% (14)	47.5%	1.725	0.391
Samples with an increase of Her-2 expression level (Ratio>1)	27	46.3	55.6% (15)	47.5%	37% (10)	30%	0.927	0.886
Samples without an increase of Her-2 expression level (Ratio<1)	13	52.4	30.8% (4)	47.5%	15.4% (2)	30%	0.321	0.135
Samples with overexpression of Her-2 (Ratio≥2)	19	44.13	47.4% (9)	47.5%	26.3% (5)	30%	0.608	0.386
Samples without overexpression of Her2 (Ratio≤2)	21	51.57	47.6% (10)	47.5%	33.3% (7)	30%	0.705	0.508

The patients classified based on clinical and pathological features of the patients and were studied individually. The cases classified according to their ages into two groups; less than 50 years (17 members) and equal or more than 50 years (19 members). Also 6 patients whose age and some pathological and clinical features were not available. The number “50 years” which is the based on division of the two group ages was attained according to different factors; the median age of population number, menopause age and hormonal changes, the age of onset for breast cancer risk in Iranian women and very close to the average age of our study population. According to the results; in the” ≥50” group, 5 and 7 Of the 19 patients showed overexpression for Her-2 and ESR1 genes respectively. The proportion of patients with overexpression, that compared to the main group, for Her-2 gene was less (26.3% versus 48%) and for ESR1 gene was more (36.8% versus 30%). 3 and 10 of 19 patients Showed overexpression and non- overexpression of both genes, respectively. On the contrary, in other group (<50) the number of patients that had overexpression for HER-2 and ESR1 genes were 10 and 5 of 17 patients, respectively, which in comparison to the main group shows a higher ratio for HER-2 gene but this ratio was fixed for ERS1 gene (58.8% versus 48% and 29.4% versus

30%). In this group 2 patients overexpressed in both genes and 4 patients showed non-overexpression of both genes. Even though Mann–Whitney U test showed no statistical significance for none of cases mentioned above. In relation to clinical-pathological features of patients with gene expression, the patients in group N2 (lymph node involvement 2) showed an increasing of expression levels of both her-2 and ESR1 genes. In regard to ESR1 gene, a statistically significant was found (p=0.047). 5 out of 7 members (71%) showed gene overexpression for ESR1 gene. The ratio of people with gene overexpression in this group for her-2 and ESR1 genes were 42.9% and 71% that in comparison to the main group (48% and 30 %) showed a decrease and increase, respectively. The average age of this group was 48.29 years, that are equal to the average age of the main group (48.47 years).

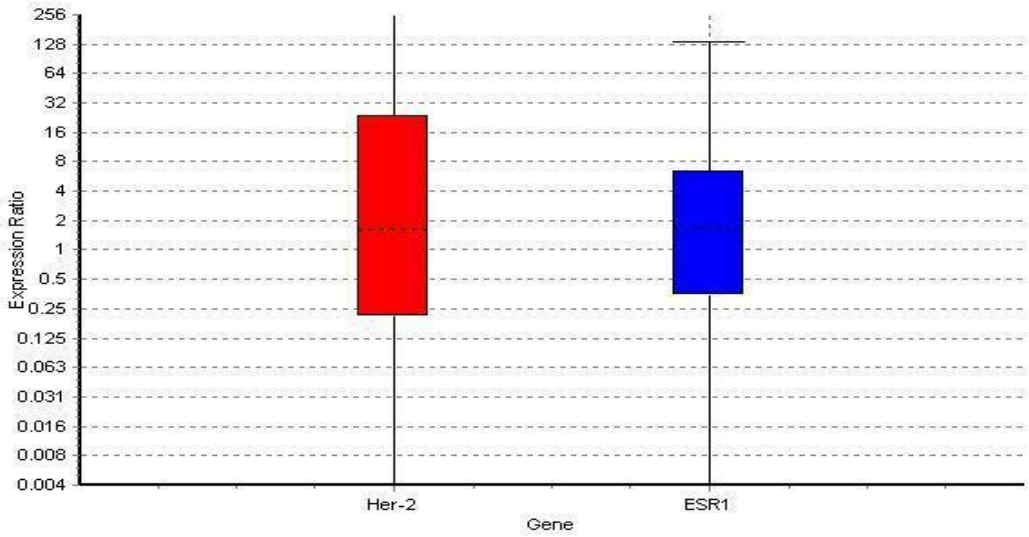

Fig.5. Relative expression of Her-2 and ESR1 genes of the patients in group N2 (lymph node involvement 2). Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Table.4. Relative expression of Her-2 and ESR1 genes of the patients in group N2 (lymph node involvement 2).

Gene	Type	Reaction Expression	Std. Error 95%	C.I. P(H1) Result	Efficiency		
GAPDH-N2	REF	1.0	1.000				
HER-2	TRG	1.0	3.071	0.115 - 76.890	0.015 - 1,497.820	0.426	
ESR1	TRG	1.0	2.917	0.815 - 8.189	0.538 - 28.636	0.047	UP

Also patients of subgroup stage3A, for both genes showed an increasing of expression levels of both genes, That for her-2 gene a statistically significant was not found but for ESR1 gene showed statistical significance (p= 0.04).

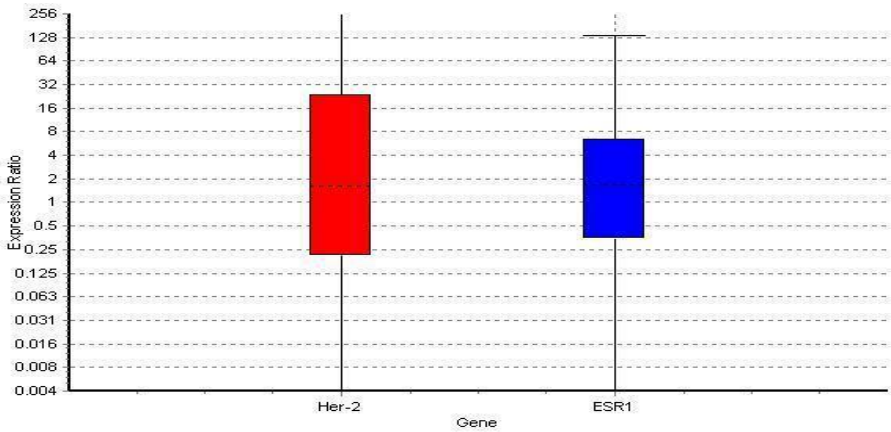

Fig.6. Relative expression of Her-2 and ESR1 genes of the patients in group STAGE-3A. software tool (REST 2009). Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Table.5. Relative expression of Her-2 and ESR1 genes of the patients in group STAGE-3A.

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
GAPDH-STG 3A	REF	1.0	1.000				
HER-2	TRG	1.0	3.417	0.115 - 100.453	0.010 - 1,708.673	0.387	
ESR1	TRG	1.0	2.623	0.815 - 6.940	0.595 - 19.650	0.040	UP

These genes overexpressed among 4 out of 7 cases (57.1%). Although the occasional significant differences in the proportion of patients with overexpression were found in other classified groups. Compared with the main group, a statistically significant was not found among gene expression levels and other pathological-clinical features.

DISCUSSION

Our results from comparison of her-2 and ESR1 expression levels between tumor and margin samples showed 48% of cases overexpressed for Her-2 neu and 30% of them overexpressed for ESR1 gene. Moreover, 65% of the cases overexpressed for one of the two above mentioned gene, that confirms the former studies focusing the direct role of these genes in the development of breast cancer. But the ratios collected from this studies were not compatible with the former studies results and there has been a double increase in overexpression of Her-2 neu gene. According to previous studies, this gene overexpressed in 20% - 30% of women with breast cancer (13, 17-23). This high rate in patients with overexpression in Her-2 can be attributed to difference in racial, life style, environmental factors and other genetic and non-genetic factors. Breast cancer is a heterogenetic disease and its development is affected by many factors and also the rate of occurrence and its types is different among countries and nations. In addition, genetic and hormone factors, and the age at which the disease has started in eastern countries is significantly different with that of the western ones (8, 39). Moreover, the other reason for this difference can be due to techniques used in previous studies and our study where according to the previous statistics most of them were accumulated through IHC technique which is not a high-precision technique, while using the real-time technique PCR can detect

the least of changes in gene expression level (13, 17). In contrast to Her-2 gene, the ratio of patients with ESR-1 overexpression is less in our study, in comparison to the ratio of 60 to 70 % that mentioned in international statistics. These differences can be due to following reasons; the average age of beginning of breast cancer in Asian and Iranian women, about 10 years earlier than the ones in western countries. Also the lower risk of developing postmenopausal breast cancer and its correlation of estrogen receptor negative cancer in Asian women compared to the western women (39). In addition to high rate of patients with Her -2 overexpression and low rate of patients with ESR1 overexpression, the average young age of the patients in this study (47.48 years old) and also the difference in gene expression profiles of these genes in two age groups (<50 and ≥50), demonstrating the existence of the differences in factors that are affecting the development of breast cancer in Iranian women. Even tough in <50 group, the disease was in advanced stage, in average, but we can`t attribute it to difference in gene expression because the cancer in advanced stages is diagnosed in our country (39, 40, 41). Other noteworthy is the correlation between the overexpression/non-overexpression of these genes and patients average age. In patients whom showed an overexpression in ESR1 gene possess the average age of 50.42 but the average age of the opposite group (non-overexpression ESR1) was 47.5 years. Also, the average age of patients with overexpression Her-2 gene was 44.13 and the average age (non- overexpression her-2) was 51.57, that showed a relative correlation between gene amplification of Her-2 with low age and gene amplification ESR-1 with high age, and the necessity for evaluate expression level and determining Her-2 status in breast cancer patients, especially younger ones. A relative correlation is established between the expression level of these genes and the cancers grade and other clinical-pathological features. The overexpression of Her-2 gene and non-overexpression of ER genes accompanies a poor prognosis and The development of breast cancer. A high proportion of the patients with grade 1 (55.6% versus 30% in main group) had overexpression of ESR1 gene. In patients with grade 2 the rates had not a significant difference with the main group. In three patients with grade 3, two patients had overexpression of Her-2 gene and the third one did not show overexpression for ESR1 gene. These relative correlations can be generalized to cancer stages, tumor size and lymph node involvement with the expression level of these genes. However, this correlation is relative and statistically significant was not found. An interesting point from the results of this study is the relative correlation between the Her-2 and ESR-1 genes expression levels. Although the coordination in expression level changes of these genes was not statistically significant, but the group of patients with an increase expression level of Her-2 gene(Ratio>1) had an increase expression level of ESR1 gene compared to the main group and in patients that Her-2 ratio was less than 1, the rate of patients with an increase expression level of ESR1 was significantly lower compared to the former group and to the main group, and vice versa. Previous studies demonstrated a strong interaction between ER and Her-2 signaling pathways and its important role in ER physiological functions and acquiring a new resistance to endocrine therapy (30). Given that the nuclear estrogen receptors can be phosphorylated and activated by intracellular kinases, Her-2 can play a role in activating ER and activate tyrosine receptor kinase, leading to activation of cellular signaling pathways and consequently, the results in cell division and cell proliferation. An increase in both Her-2 and ESR-1 receptors after the amplification of these genes can activate different signaling pathways and subsequently elevate cell proliferation rate. 13 of 15 patients who had the higher expression levels of both genes in tumor cells than marginal cells (Ratio>1) were in the advanced stages of cancer (Stage1: 2 patients, Stage2: 4 patients, Stage3: 9 patients) and 4 of 5 patients who had higher overexpression of both genes (Ratio>2) were in the advanced stage of cancer

(Stage3). The progression of breast cancer is affected by different genetic, environmental, nutritious and life style factors and gene profile in each patient is different from the other. In addition, different signaling pathways involved in the process of breast cancer.

CONCLUSION

In this study, the percentage of patients with amplification or overexpression of Her-2 gene was higher than western country statistics. In contrast this percentage for ESR1 is less than the amount stipulated in the mentioned statistics which can be due to differences in genetic, environmental and racial factors. Further studies with larger sample size could lead to more comprehensive and precise results. Additionally, real-time PCR technique can be an accurate complement to the primary test, IHC and paves the way for target therapy.

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