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Original Article

Study of Fibroblast Growth Factor 3 (fgf-3) Gene Expression in Breast Cancer Tissues Infected with Mouse Mammary Tumor Virus (MMTV)

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Abstract

Background and Aims: Breast cancer (BC) is the second most common cancer among women worldwide. Several factors can influence the occurrence of BC, including the presence of murine mammary tumor virus (MMTV). In this study, we aimed to perform the epidemiology of MMTV and investigation of human fgf-3 gene expression in cancerous and noncancerous individuals infected with MMTV

Materials and Methods: A total of 85 BC tissue samples and 85 tumor-free margin tissue samples (normal tissue) were collected from women undergoing breast surgery at general hospitals in the Qom Province, Iran. The presence of the MMTV env gene was determined using the nested PCR method. Additionally, the correlation between the fgf-3 gene expression and the MMTV-env gene conducted by Real-time PCR for the first time in Iran.

Results: The MMTV env gene was detected in 16 (18.8%) out of 85 BC tissue samples, but in only 2 (2.35%) samples without cancer. In the MMTV-positive samples, the expression of the fgf-3 gene was increased compared to MMTV-negative samples (P=0.012). Furthermore, there was no significant difference in fgf-3 gene expression between grade I and II BC samples. However, a significant increase in fgf-3 gene expression was observed in grade III and TNBC cases. (P< 0.05).

Conclusion: The mouse mammary tumor virus represents a significant risk factor for BC. Therefore, implementing preventive measures, including timely MMTV infection detection and control, can be effective in BC prevention programs in Iran and globally.

Keywords: MMTV, Breast cancer, fgf-3 gene, Real-time PCR method.

Introduction

Breast cancer (BC) is the leading cause of cancer-related death in women worldwide. Numerous risk factors including diet, obesity, pregnancy, hormones, longer life expectancy, and family life have been identified for the development of this tumor (1). However, the etiology and molecular mechanism of breast carcinogenesis remain unclear despite decades of research (2).

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Previous studies suggest that viral infections may play a key role in the development of BC (3, 4). As early as 1936, John Bittner discovered the "mouse milk factor", which can increase the incidence of BC in mice (5). Later studies demonstrated that the "causative agent" was mouse mammary tumor virus (MMTV) (6). It is a beta retrovirus and has been a major suspect as a cause of some human breast cancers for more than 50 years (7).

MMTV-like antigens and virus particles were detected in human milk and cancer cells in the 1970s and 1980s (8). The prevalence of MMTV-like gene sequences is 15-fold greater in human BC tissue than in normal human breast tissue controls and is present in up to

40% of human BCs (9). Recently, improved molecular sequencing techniques have allowed the identification of MMTV-like sequences using highly specific primer pairs that recognize MMTV envelope gene sequences (env) with low homology to HERV. The env gene sequence of MMTV virus has been detected in 30-40% of BC samples (10).

In addition, Env is involved in breast tumorigenesis and it has an immuno-tyrosine-based activation motif (ITAM) that is normally found in hematopoietic cell receptors. Cultured expression of Env in normal mouse or human mammary epithelial cells results in morphological changes in culture (11).

Moreover, integration of the MMTV viral sequence using its terminal repeat sequence (LTR) leads to the activation of cellular oncogenes, the most important of which are several common integration sites (CIS) that play essential roles in BC including fibroblast growth factor 3 (fgf-3) gene (12, 13).

The products of fgf-3 gene are secretory glycoproteins involved in various cellular processes, such as cycle regulation. FGF signa-ling plays a role in the control of proliferation, differentiation, migration, survival, polarity, and angiogenesis. FGFR alterations have been reported in approximately 7.1% of cancers (most commonly in urothelial and BC).

Increasing evidence suggests that the genetic mutation-driven activation of FGF signaling is the key factor in breast cancer metastasis (13, 14).

Considering that two or more viral genomes are integrated in or near the target genes and that the target genes expression is increased in these tumors compared with other tumors that do not have viral integration at this site or with normal breast tissue. The expression level of the fgf gene is also different, and thus the severity of the disease is distinct (15). A large percentage of mammary tumors isolated from MMTV-infected wild-type mice have "knock-outs" in the fgf-3 gene. Therefore, it is possible that additional genes are activated in these tumors. Also, the generation of double transgenic mice, such as MMTV-Fgf3, accelerates the development of mammary tumors (16).

In this study, the env gene epidemiology of the MMTV was investigated and a quantitative comparison of the expression of the fgf-3 gene in a noninvasive manner in cancerous and non-cancerous individuals infected with MMTV using real-time PCR method was analysed.

Methods and Materials

Patient

A total of 85 BC tissue (women undergoing breast surgery for mastectomy) and 85 tumor-free margin tissue (normal tissue) samples were collected from women (between the ages of 33 and 71 years) referred to two general hospitals in the Qom Province, Iran. All the tissue samples were formalin fixed. The WHO Criteria were followed in the histopathological diagnosis of each cancer tissue (17). Based on the modified Scarff-Bloom-Richardson SBR system, the invasive ductal carcinomas were classified into three grades (18). The peripheral tissue of the tumor was considered as a control specimen.

Additionally, the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) in malignant mammary tumors in samples was studied by immunohistochemical assays in pathology laboratory of Valiasr hospital.

Preparation of Samples

According to FavorPrep^{TM} kit (Taiwan), 25 mg of sample was placed in 2 ml tubes and 1 ml of PBS buffer was added to each tube. Then, the samples were ground with sterile and disposable pestles under liquid nitrogen.

DNA and RNA Extraction

To test our hypothesis, both DNA and RNA were isolated for this study. A commercial kit (FavorPrep^{TM}, Taiwan. Cat No.: FATGK001-1) and a kit (FavorPrep^{TM}, Taiwan. Cat No.: FATGK000-Mini) was used for DNA and RNA extraction, from all tumor, and normal samples.

Quantitative testing by NanoDrop spectrophotometer and qualitative testing by gel electrophoresis were performed for each sample to

confirm the extracted DNA and RNA. Subsequently, all the samples were stored at -20°C until PCR reaction.

Synthesis of the First-Strand cDNA

Two different protocols were used to generate cDNA according to EpiNext™ kit (Cat No.: P-9004-20) instructions: one using oligo dT primers and the other using random primers (Promega). Real-time PCR was then performed for expression of human GAPDH and fgf-3 genes transcripts according to the mentioned protocols.

Screening for MMTV env sequences

The nested PCR method had 100% sensitivity, 91% specificity and simplicity was used for the env gene detection of MMTV (19). Primers for MMTV env gene amplification were designed according to the sequences of a MMTV prototype in GenBank (accession number AF228 551.1). Approximately 200 ng of DNA template from normal and neoplastic mammary gland tissues were used for the first PCR amplification run with the outer primers. The 2 µl of resulting DNA products (647 bp) were used as a template for the second round of amplification with the inner primer.

Thermocycler conditions for PCR with the external primers (env-1F and env-1R) for MMTV env sequences began with denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30s, 55 °C for 30s, and 72 °C for 45s, terminated at 72 °C for 7 min. The second PCR run with internal primers (env-2F and env-2R) was performed under the same conditions as the first run. At the same time, conventional PCR was performed to detect GAPDH (glyceraldehyde phosphate dehydrogenase) gene as an internal control (Table 1). The amplification of GAPDH gene was done by initial denaturation at 94 °C for 8 min; subsequent denaturation at 94 °C for 1 min; annealing at 64 °C for 1 min; extension at 72 °C for 1 min and final extension at 72 °C for 7 min.

A synthetic oligonucleotide of MMTV and the serum sample without DNA template were used as positive and negative controls, respectively. For evaluation, 5 µl of each reaction mixture was run on a 1% agarose gel by safe stain (GelRed®, Biotium Company, California. Cat No.: 41003) visualization using a UV transilluminator.

Gene Expression

The expression level of fgf-3 gene in env-positive and env-negative samples was determined by real-time PCR using Real Q Plus 2x Master Mix Green Cat No.: A323402 (AMPLIQON - Denmark) and Roche Light Cycler® 96. The total volume for the qPCR reaction was 20 µl, including 10 µl SYBR Green PCR Master Mix, 0.5 µl forward and 0.5 µl reverse primer, 3 µl cDNA templates, and 6 µl ddH2O.

The Real-time PCR program for the reaction was based on a holding step at 95 °C for 10 min, followed by 45 cycles of initiation at 95 °C for 30s, annealing at 58 °C for 30 s, and a single final step at 58 °C for 90s. Values for mRNA expression were normalized by using GAPDH gene (as housekeeping gene). Finally, the Real-time instrument data were analyzed and the melting curve and threshold cycle (CT) were calculated. The relative fold-change (FC) of gene expression (fgf-3 and GAPDH) in MMTV-positive samples was calculated using the $2^{-\Delta\Delta ct}$ standard method.

Statistical Analysis

For descriptive statistics, N (percent) was reported for qualitative variables, whereas the mean (SD) was reported for quantitative variables. The t-test for independent samples was used to compare a variable with two independent groups and a quantitative variable. A one-tailed ANOVA was used to compare a variable with more than two independent groups and a quantitative variable. Also, SPSS (IBM Corp, Armonk, NY) was used for statistical analysis. The statistical significance level of this study was considered as $p < 0.05$.

Ethics approval and consent to participate

This research was performed based on the ethical principles and national standards for medical research in Iran (Approval ID: IR.UT. 1401.151513).

Table 1. The oligonucleotide sequences of used for detection of MMTV

Gene	Sequence	Location	Accession No	Product Size
env	Outer primer: Forward: 5'- TTCCTTCTCCTTTTCTACCCC-3' Reverse: 3'- GCAGATATGCCCAGGATAATG-5' Inner primer: Forward: 5'- TCCTCACTGCCAGATCGCCT-3' Reverse: 3'- CTAGGCGAGGGAAGGGAGAA-5'	5846-5866 6763-6746 6047-6066 6205-6186	AF033807.1 AF033807.1	918 bp 160 bp
GAPDH	F: 5′- CCACTCCTCCACCTTTGACG -3′ R: 3′- CCACCACCCTGTTGCTGTAG -5′	7450-7431 7671-7690	NG_007073.2	120bp
fgf-3	F: 5′- TCCATCTCCTGGCTGAAGAACG -3′ R: 3′- TGTTCTCCACGACGCAGGTGTA -5′	828-849 972-951	XM_054349265.1	145 bp
Oligonucleotide of MMTV	5′-TCCCTATAAAAAAGAAGTTGCCCCCCA AATATCCTCACTGCCAGATCGCCTTTAAGAAGG ACGCCTTCTGGGAGGGAGACGAGTCTGCTCCTC CACGGTGGTTGCCTTGCGCCTTCCCTGACCAGGG GGTGAGTTTTTCTCCAAAAGGGGCCCTTGGGTTA CTTTGGGATTTCTCCCTTCCCTCGCCTAATATAG ATCAGTCAGATCAGATTAAAAACAAAAAGGATC TATATGGAAATTATACTCCCCCTGTCAATAAAG AGGTTCATCGATGGTATGAAGCAGGATGGGTAG AACCTACTTGGTTCTGGGAGAATTCTCCTAAGG ATC-3′	6016-6041 6345-6317	NC_001503.1	335 bp

Results

Patient

In this study, 85 tissue samples were collected from patients aged between of 33 and 71 years. All patients were undergoing breast surgery for mastectomy. The distribution of tumors was as follows: 78 invasive ductal carcinomas, 6 invasive lobular carcinomas, and 1 mucinous adenocarcinoma. Patient characteristics and tumor descriptors are summarized in Table 2.

In addition, Figure 1 shows appearance of cells in human BC and MMTV-positive cancerous breast tissue.

Table 2. Clinicopathological characteristics of patients with BC (n=85).

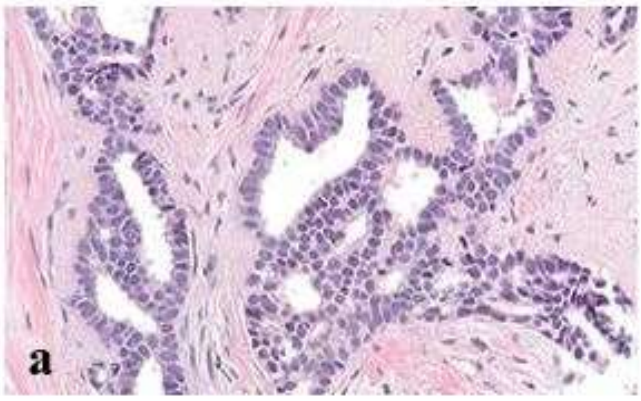
Parameters		Number (%)
Age (years)	X ≤50 X >50	29 (34.1) 56 (65.8)
Tumor size	X ≤50 X >50	26 (30.5) 59 (69.4)
Histological grade (Nattingham score)	Grade I Grade II Grade III	3 (3.5) 64 (75.3) 18 (21.1)
Estrogen receptor (ER)	Positive ER Negative ER	54 (63.5) 31 (36.5)
Progesterone receptor (PR)	Positive PR Negative PR	38 (44.7) 47 (55.3)
HER-2 receptors	3^{+++}	21 (24.7)
Triple negative BC (TNBC)*	-	11 (12.94)

*Triple-negative breast cancer (TNBC) is the most aggressive subtype in BC that does not have any of the receptors that are commonly found in breast cancer.

Results of Nested PCR Method

The expected size of the product for MMTV-positive samples (based on the env gene of MMTV) according to the nested PCR method was 160 bp. To ensure the extraction of DNA and cDNA synthesis of RNA, amplification of human GAPDH sequences of 120 bp was observed in all samples, confirming the presence of this gene in the positive samples (Figure 2).

Based on the nested PCR technique, the env gene of MMTV was detected in 16 (18.8%) and 2 (2.35%) ($P < 0.05$) out of 85 BC tissue



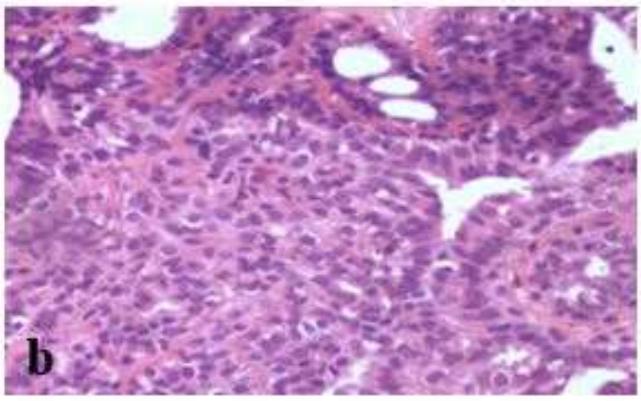


Fig. 1. Mammary epithelial cells appearance in: **a.** Human breast cancer; **b.** MMTV-positive human breast cancer. The scale bar is 50 microns.

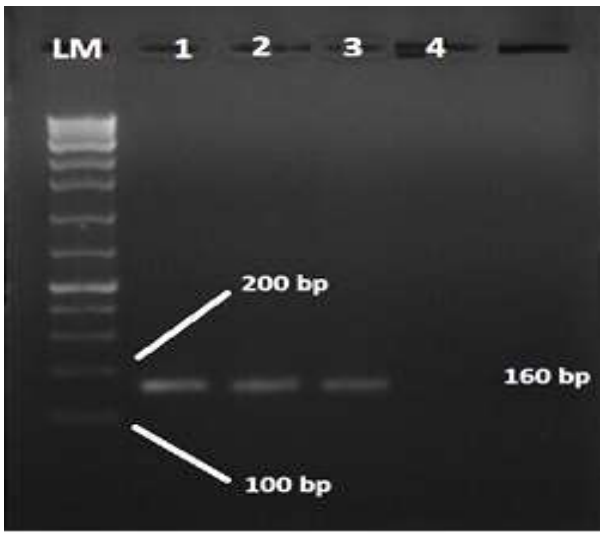


Fig. 2. Amplification of the env gene in some samples. LM: 100 bp DNA ladder; Lane 1, 2: 160 bp *env* gene; Lane 3: positive control; Lane 4: negative control.

and without cancer samples, respectively.
Most malignant samples of MMTV-positive were II grade. env gene prevalence in BC tissue samples showed that none of the grade I samples was positive for env gene target sequences. The env gene of MMTV detected in 11 (61%), 5 (28%) and 2 (11%) of the grad II, III and TNBC samples, respectively.

Results of the Real-Time PCR Method

The expression level of the fgf-3 and GAPDH genes in the MMTV-env positive samples are showed as the normal real-time amplification curve to verify the specificity of the primers and to ensure the specific amplification of the studied genes in the real-time PCR product (Figure 3).

The quantitative analysis results of fgf-3 gene expression in env-positive samples were normalized using Rest PCR software compared with the reference gene human (GAPDH gene).

The data on the expression of the fgf-3 gene in two groups of tumor tissue compared to that in healthy marginal tissue showed a normal distribution (Figure 4). The results of the statistical comparison of the mean of these data using the statistical t-test for independent samples, assuming unequal variance of the samples, showed a significant difference between the two groups studied. The expression value of this gene was about 6 times higher than that of the marginal tissue and this increase is statistically significant (P= 0.012) (Figure 5).

Discussion

Breast cancer is the most often diagnosed cancer and the one cause of death in women (20). In the late 19th century, the role and mechanism of viruses in the development of cancer were proposed, so many studies have been conducted on the association between MMTV and human breast carcinogenesis. The findings showed that they detected MMTV-like antigens or viral particles in human breast or BC cells. Furthermore, immune responses to MMTV antigens were also detected in women with BC (10, 21).

The nested PCR method is considered as a gold standard for the diagnosis of viruses (22). In fact, it confirmed the presence of MMTV in

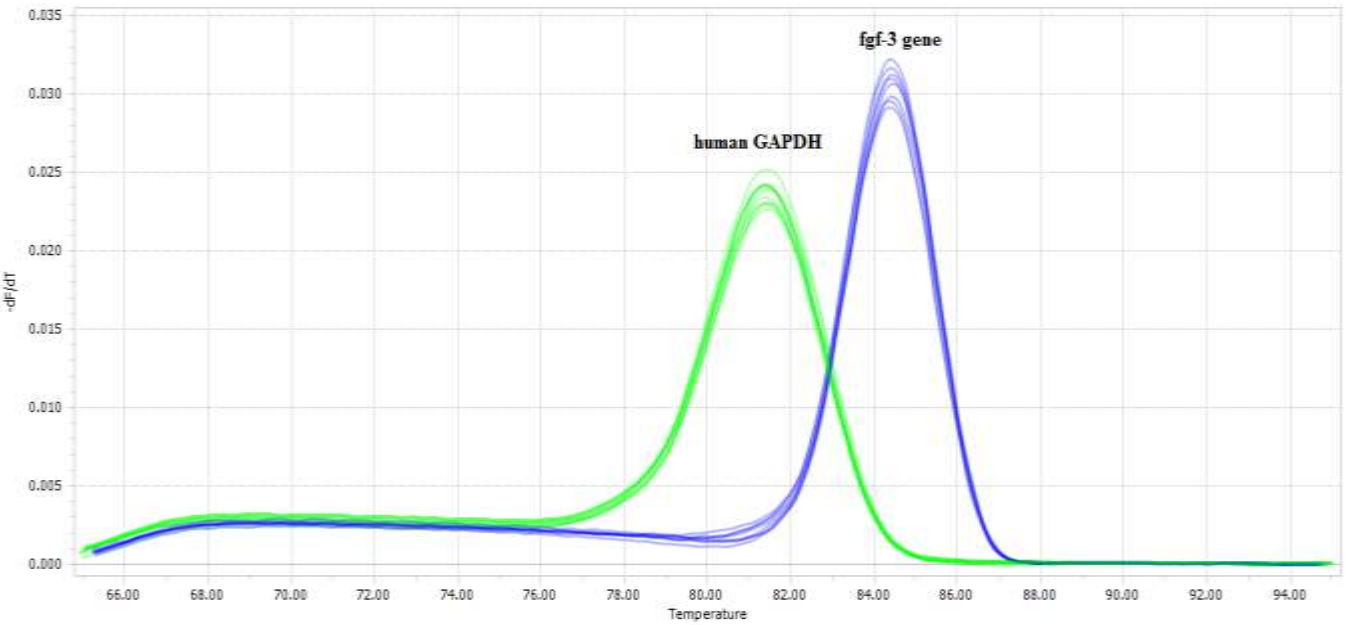


Fig. 3. The melting curve of human GAPDH and fgf-3 genes.

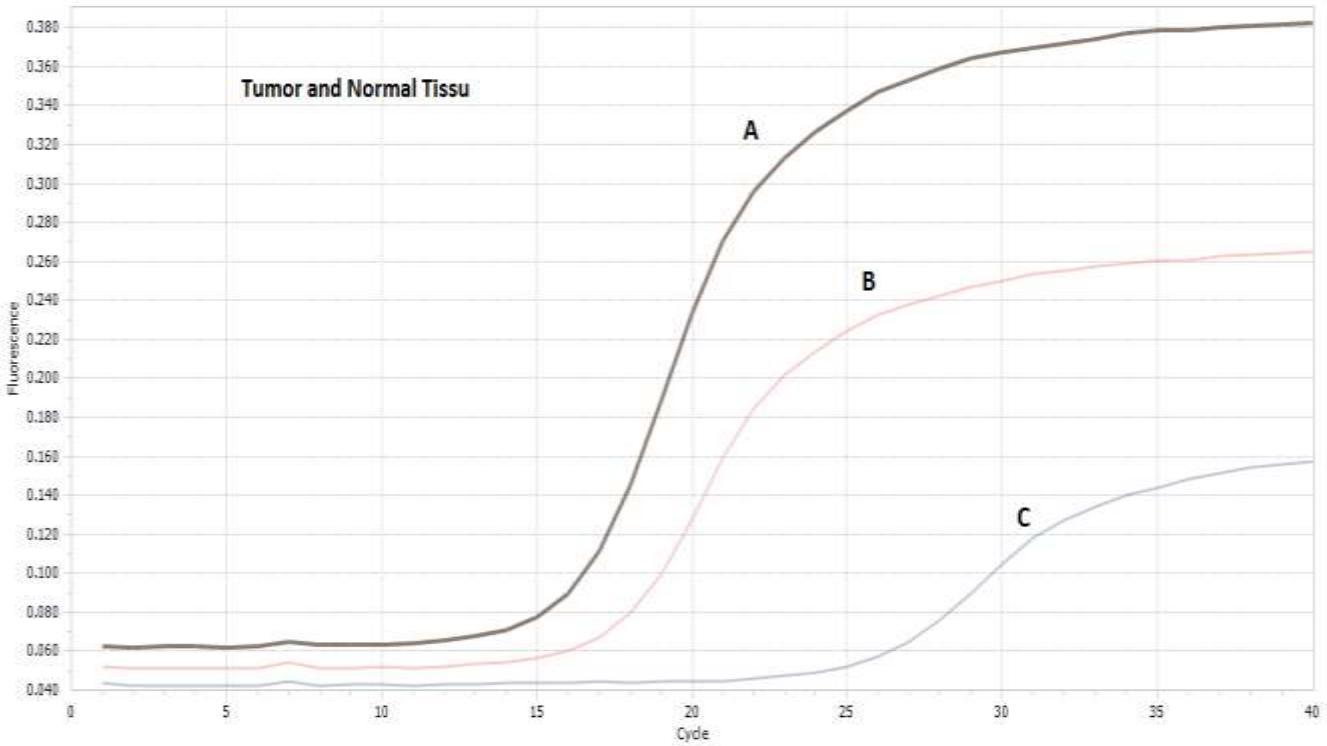


Fig. 4. The sigmoidal shape cycles. (A): fgf-3 gene in tumor tissue; (B): GAPDH gene; (C): fgf-3 gene in normal tissue.

human breast tissue epithelial cells. The frequency of MMTV DNA based on the env gene in women with BC was 16/85 (18.8%). Other studies as follows: USA, Pakistan, Iran, Argentina, Italy, Tunisia, Morocco, Saudi Arabia, Korea, Iraq, China and Australia with the reported MMTV prevalence of 30-38.5% (23-26), 20% (7), 32.2% (27), 31% (28), 33% (29), 14% (30), 57% (31), 5.9% (32), 9.4% (33), 18.18% (20), 17.65% (34) and 36-78% (21, 35, 36), respectively. All these results are evidence that env gene detection is variable and it is actually widespread in most countries of the world.

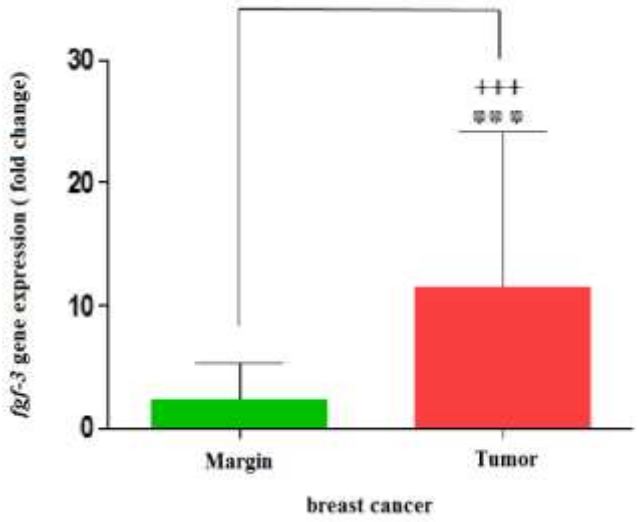


Fig. 5. Diagram of the relative expression of the env gene in the group of women with breast cancer, tumor tissue and confidence margin

env gene of MMTV was presented in 2/85 (2.85%) of normal breast tissues. This have a high frequency unlike Wang et al. (1995), Pogo et al. (2010), and Melana et al. (2001) reports 1-1.8% in USA.

In the event that Ahangar et al. (Iran, Tabriz), Seo et al. (Korea), Al Dossary et al. (Saudi Arabia), Lawson et al. (Australia), Hachana et al. (Tunisia), Etkind et al. (USA), and Zammarchi et al. (Italy) did not detect MMTV sequences in human normal breast tissue (25, 29, 30, 32, 33, 35, 37). On the other hand, Shariatpanahi et al., in Iran (5%) (27), Melana et al., in Argentina (10%) (28), Glenn et al. in Australia (33%) (36), Wang et al. in China (4%) (34) and Slaoui et al. in Morocco (33%) (31) reported high percent comparing to our results. There are vast differences in results that they are attributed to number of samples, identification method, the geographical location of the study and other inherent difficulties such as scarcity of virus genome in the samples, the quality of the DNA used and so on.

Fibroblast growth factor 3 (FGF-3) regulates several developmental processes including brain patterning, branching morphogenesis, and organ growth and activating cell surface FGF receptors (FGFRs). Expression and associated amplification of FGF3 and FGFRs have been identified in breast cancer, they are associated with advanced stage and high grade tumors as well as decreased patient survival time which may contribute to resistance to chemotherapy (3, 14).

Real-time PCR is a major development of PCR technology that enables reliable detection and measurement of products generated during each cycle of PCR process. This technique became possible after introduction of an oligonucleotide probe which was designed to hybridize within the target sequence. The correlation between the fgf-3 gene expression and the MMTV-env gene conducted by Real-time PCR for the first time in Iran. It can be helpful in determining the role of virus in proliferation, metastasis and invasiveness of breast cancer. Similar to the study by Mirali-malek et al. (38), There was no significant difference in env gene expression in grade I and II breast cancer samples, on the contrary, a significant increase in env gene expression was observed in grade III and TNBC ($P<0.05$). It seems that with the progression of human breast cancer, FGF-3 gene expression increases in breast cancer tissues infected with MMTV, which may be due to the loss of cellular physiology and disruption of cellular balance (9).

In the current study, relationship between fgf-3 gene (human) expression and env gene (MMTV) in human breast cancer tissues were evaluated for the first time in the world. Based on fold change analysis, fgf-3 gene expression in env-positive samples comparing to env-negative was increased 6-fold.

MMTV is also transmitted via the mother's milk to newborn mice. The virus transverses the gut via M cells and then infects B cells in the Peyer’s patches. The transfer of retro-viruses from one species to another is a highly contentious issue (39). On one hand, Lawson et al. shown localization of the MMTV env sequences to the nuclei of breast cancer cells that indicates integration of the provirus particularly near the FGF family genes in human breast cancer (35). MMTV is present in human saliva and salivary glands of the general population and it's transmission by human milk and saliva is possible (35, 40). Therefore, it is

transmitted from mouse and/or human to human.

Conclusions

MMTV can be one of the important risk factors for breast cancer and authors believe that preventive measures considering MMTV infection control and timely treatment would be quite effective in BC preventive program in Iran and other countries.

Acknowledgment

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Conflict of Interest

No conflict of interest is declared.

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Ethics Approval and Consent to Participate

Approval ID: IR.UT.1401.151513

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