Original Article

Evaluation of Neurofibromatosis Gene Expression in Non-Hereditary Breast Cancer

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Abstract

Background: Breast cancer is the most common cause of death in women. Studies have shown that changes in neurofibromatosis gene expression can cause breast cancer. The aim of this study was to investigate the change of neurofibromatosis type 1 gene expression in non-hereditary breast cancer using real time PCR.

Materials and Methods: In this study, 160 tissue samples were collected from patients following ethical principles. After lysis of tissues, extraction of RNA and synthesis of cDNA was performed. The amount of gene expression changes was investigated.

Results: The results showed that the level of *NF1* gene expression was dependent on the stages of the disease and as the stages progress, the level of expression of this gene showed a significant decrease.

Conclusion: The use of gene biomarkers can help to diagnose and treat diseases faster. Along with examining other candidate genes, using *NF1* gene expression analysis in breast cancer patients can be a suitable option for diagnosing the stages of disease progression.

Keywords: Breast cancer, Gene expression, Neurofibromatosis gene

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Introduction

Breast cancer is the most common cause of death for women between the ages of 35 to 55[1]. According to the Iranian Cancer Registry, breast cancer with a relative incidence of 24.8% was the most commonly diagnosed cancer in women (11.3% of all cancers in 2008). The peak age of this cancer in Iranian women was reported in the fourth and fifth decades of life, one decade below global statistics[2]. Studies have shown that the incidence of breast cancer in Iran is lower than in developed countries. However, it has been reported as the most common cancer in Iranian women and the available information indicates an increase in the prevalence of this malignancy in Iran over the past two decades[3]. According to the latest statistics from the Cancer Research Center in Iran, about 8500 new cases of breast cancer are reported annually, and 1,400 die for this reason. Currently, about 40,000 people are living with this disease in the country[4]. According to numerous studies, family and pregnancy history are two important and influential factors in Iranian patients[5]. Factors such as age, hormone replacement therapy, breastfeeding for longer than one year, menstrual history, family history, personal history of cancer and oral contraceptive use are considered as risk factors for breast cancer. The result of 25,000 women showed that pregnancy history and the lower age of the first

pregnancy, the risk of breast cancer is significantly reduced. In some studies, oral contraceptive use was unaffected and in others; they clearly have reduced the risk of cancer. Family history, smoking, eating unhealthy foods and being overweight raise the risk of cancer[6]. Recently, common genetic variations with low penetration, at least in 120 candidate genes are identified. The study was performed on breast cancer cases and genomic association studies that study genetic variations (single nucleotide polymorphism) in the development of hereditary breast cancer. Among the candidate genes, the main effective factors that associated with the risk of breast cancer are the effective genes in cell cycle control, metabolism of steroid hormones, and cellular signaling pathways. By increasing the expression of *LSP*, *MAP3K1*, *TNC9*, *FGFR2*, *TGFβ1*, *CASP8* genes, the incidence of breast cancer increases significantly. Studies have shown that mutations in candidate and unknown genes make up about 53% of breast cancer cases[7]. *NF1* is one of the candidate genes that produces a protein called neurofibromin that is actually a tumor suppressor. Problems in its production, predisposing a person to develop a variety of tumors and molecular changes in the gene, causes neurofibromatosis[6]. In one study, neurofibromatosis and breast cancer in a mother and daughter were investigated, that mother had breast cancer. In this study, 84 genes were studied, including the neurofibromatosis gene. The results showed that the expression of the genes were similar in both cases and the expression of the *DDB2*, *MGMT*, *MLH1*, *POLB UNG*, and *XPA* genes were high in both persons. Therefore, due to the potential for breast cancer, the girl should be under the supervision of a doctor[8]. In another study, mutations of several genes were investigated in a mother and young child with breast cancer. Hereditary mutations of *NF1*, *BRCA1* or *BRCA2* were not seen in the peripheral blood of her daughter. In MDA-MP-231 human neoplasms, the neurofibromin encoded by the *NF1* gene was almost absent. Neurofibromin is similar to the active protein P120GAP, GTPase (GAP), which inhibits RAS activity. LOH in the tumor tissue supports the role of *NF1* gene in breast cancer[9]. Nearly 3 to 5% of people with neurofibromatosis develop cancerous tumors. The origin of these tumors is usually neurofibromatosis subcutaneous or retinoid neurofibromatosis. In these people, the risk is also high for other types of cancer such as breast cancer, leukemia, brain tumors, and some types of soft tissue cancers. As a result, type 1 neurofibromatosis is one of the factors contributing to the increased risk of developing breast cancer factors contributing to the increased risk of developing breast cancer[10]. Due to the high prevalence of breast cancer in Iran, the ascending slope of this malignancy over the past two decades, high mortality rate, lack of comprehensive report on the effect of neurofibromatosis in breast cancer, the aim of this study was to investigate the effect of gene expression in breast cancer using Real Time PCR technique. The results of this study can improve the recognition of the effect of type 1 neurofibromatosis on the formation of breast cancer cells. The innovative aspect of this study is to investigate the effect of type 1 neurofibromatosis genetic disease on non-heritable breast cancer and achieve the effect of gene expression on the incidence rate of breast cancer.

Methods

Sampling: The type of study is descriptive and laboratory. Partial mastectomy was performed on 160 breast cancer patients between January 2017 and January 2018 at Farmanieh Hospital and Sina oncologic hospital (both from Tehran, Iran). Healthy tissues were excluded from the cancerous tissue. All participants signed a written consent form to participate in this study. A questionnaire was prepared based on the risk factors that patients responded. All procedures performed in the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study has an ethics code with an ID IR.IAU.SRB.REC.1400.016. The stages of breast cancer progression were determined by the hospital pathologist. All tissues were transferred to the laboratory by the N-portable tanks.

Molecular examination

***RNA extraction and cDNA synthesis*:** In the molecular study, the expression of *NF1* in the samples of both groups was quantitatively evaluated. First of all, extraction of RNA was done using the RNX-Plus method. BIOZOL Total RNA Extraction Reagent Kit (Bioer technology Co. Ltd) was used to extract RNA. Purity and concentration of extracted RNA were

Table 1: Primer sequence

Primer sequence	Length (Nt)	Amplicon (Pb)	Tm (°C)	Gene
F: CTGCCACCCAGAAGACTGTG	20	154	60.60	*GAPDH*
R: AAGGCCATGCCAGTGAGCT	19		61.92	
F: TAAAGCTGTTGGAAGACGAC	20	140	55.72	*NF1*
R: TAGTCATAAATTCCTCAAAC	20		47.96	

investigated by NanoDrop 2000c Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the wavelength of 260 to 280 nm for all specimens varied from 1.9 to 1.7. The quality of RNA was examined by agarose gel 1.5%. The presence of 18S and 28S ribosomal RNA bands represents healthy and intact RNA. High quality specimens showed at least a smear on the top, between, and bottom of the bands and the intensity of the 28S band was roughly twice the intensity of the 18S band. The lack of clear bands of 18S and 28S indicates a breakdown of the RNA sample by the RNase enzyme, especially if the band smearing is limited to the lower half of the gel. The synthesis of the cDNA was done using the Revert Aid First Stand cDNA Synthesis Kit of Thermo Scientific Company. Materials for the synthesis of cDNA, mixed prepared with random hexamer (40µM) 1µl, Oligo dt (100 µM) 1 µl, and dNTP (10 mM) with 1 µl volumes. 10 µl of RNA were added to each tube and placed at 65 ° C for 5 minutes and then immediately placed on ice. After about 2 minutes, the reaction mixture was mixed with nuclease free water to a volume of 4.5 µl, M-MuLV 10X buffer 2 µl (µl M-MuLV) (200 µl / 0.5 µl) and for each tube 7 µl of this mixture was added. Therefore, the final volume of the reaction eventually turned out to be 20 µl. The reaction was made at 42°C for 60 min.

***Design the primer and do Real Time PCR*:** Primers of this study were designed using Gene runner and Allele ID software. The gene sequences were obtained from the National Center for Biotechnology (NCBI) and the correct connection of primers to the corresponding sequence was investigated and then by the Blast program at NCBI, the connection of primers to other sequences was investigated. It should be noted that the primers were ordered to Macrogen Company after being tested by this software. Primers with sterile distilled water reached the desired volume (100 pmol/µl). A dilution of one tenth of each primer was prepared and stored at -20°C. The sequence of primers examined presented in Table 1.

The PCR test was performed for each replication in patient samples and controls separately for the evaluation of *NF1* and *GAPDH* genes. To did this, first of all dilutions 1, 1 to 5, 1 to 25 and 1 to 125 were prepared from the cDNAs (75, 15, 3 and 0.6 ng/µl). Then, the real time PCR reaction was repeated twice, for these dilutions, along with each of the primers individually. At the end, the standard curve for each primer was plotted based on the values of Ct obtained versus the dilutions used. The reaction efficiency was calculated for each primer by using the curve line gradient and the following equation.

$E= 10^{(-1/slope)} – 1$ E= Reaction efficiency Slope= Curve line gradient

After adding the necessary quantities to each microtubes, genes multiplied and determination of relative quantity in it was done by measuring the amount of fluorescence light increase caused by cyber green connection by ABI 7500 real time PCR system (Applied Biosystems Inc., Foster City, CA, USA). Then, measurement of the target gene expression (*miR-21*) to the control gene in each group was performed with ΔΔct method and, the gene expression chart was drawn by the REST software. Statistical analysis was performed by SPSS version 20. *NF1* expression of microRNAs was obtained in two groups of control and patient via software.

All participants signed a written consent form to participate in this study. A questionnaire was prepared based on the risk factors that patients responded. All procedures performed in the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Results

The information of questionnaire completed by the

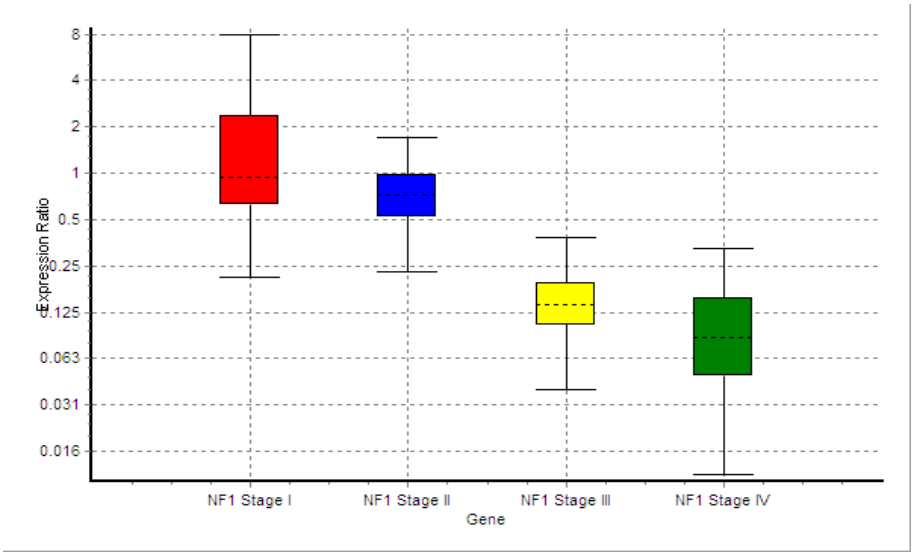

Figure 1. *NF1* expression in different stages of breast cancer

patients, statistical analysis was performed by SPSS program version 23 (Table 2). The results of quantitative variables were presented in the form of Mean± standard error (SE) and the results of qualitative variables in the form of number and frequency.

The Kolmograph-Smirnov test was used to examine the normal distribution of data. The two-way Anova statistical test was used to evaluate the variables that had normal distribution. To assess risk factors, the logistic regression test was used to measure odds ratio (OR) and the 95% confidence interval (95% CI). All were two-tailed P values, and confidence intervals were set at 95%. There was no significant relationship between the disease and oral contraceptive use ($P<0.05$). There was a significant relationship between other demographic characteristics and disease.

The quality and quantity of RNA samples was studied by agarose gel electrophoresis and spectrophotometry respectively, to make sure non-degradation of extracted RNA. In the agarose gel electrophoresis, two bands of 18S and 28S RNAs were clearly observed, indicating a lack of RNA degradation. In spectrophotometric study, the ratio of absorption of 260 to 280 nm was obtained 380 ng/µl, which indicates high purity of the RNA and its low protein binding with genomic DNA. In general,

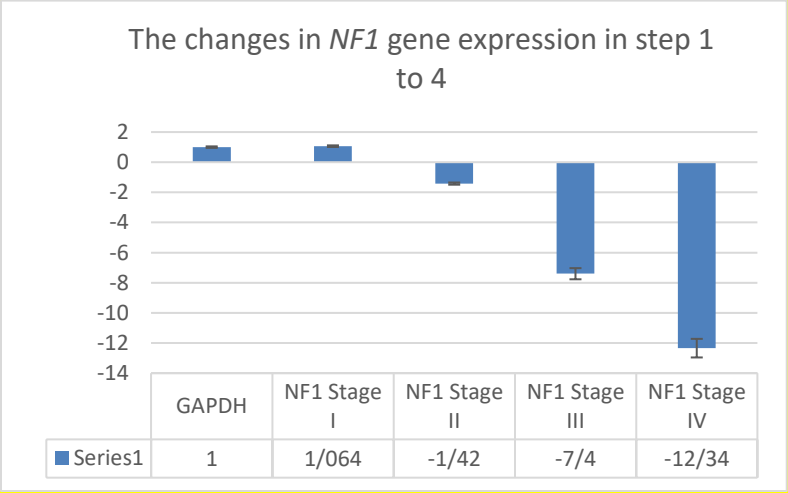

Figure 2. Analysis of the expression of *NF1* in different stages of breast cancer

Table 2: The relationship between demographic information and disease generation

Risk Factors	N=160 n (%)	P Value	OR	95%CI
Age	88(55%)	0.029	0.264	0.011-2.977
Menstrual History	90 (56.25%)	0.016	0.467	0.112-5.629
Family history	91 (56.87%)	0.014	0.549	0.295-5.221
Personal History of Cancer	100 (62.5%)	0.031	0.146	0.034-2.238
Breastfeeding for longer than one year	85 (53.12%)	0.024	0.278	0.013-3.374
Oral contraceptive use	72 (60%)	**0.057**	0.207	0.019-1.097
Pregnancy history (first child after age 30)	79 (49.37%)	0.038	0.136	0.044-5.727
Hormone Replacement Therapy	89 (55.62%)	0.019	0.527	0.141-6.474
Being overweight	68 (42.5%)	0.041	0.079	0.037-3.583
Eating unhealthy foods	44 (27.5%)	0.033	0.088	0.015-4.588
Smoking	50 (31.25%)	0.038	0.085	0.019-4.128
Personal History of Cancer	99(61.87%)	0.030	0.144	0.034-3.344

the result was that extracted RNA can be used with confidence in the next stages of the study. Prior to Real Time PCR, design primers should be evaluated at NCBI and the primer-blast software to verify their performance. By study the melting curves of the genes, there can be no contamination in the replicated samples. Finally, the results were analyzed using the software REST 2009 V2.0.13. The expression levels of *NF1* and *GAPDH* have been compared in Figures 1, 2 and Table 3.

In this study, the expression of genes was studied in four stages of breast cancer. According to Figure 2, it can be concluded that the rate of expression of *NF1* gene in the first to fourth stages of breast cancer is 1.064, 1.42, 7.4 and 12.34, respectively. Changes in the expression of *NF1* in the third and fourth stages are significant. The results show that the expression of *NF1* gene compared to *GAPDH* gene decreased in some stages of breast cancer.

Discussion

Few studies with controversial results have been made on the expression of *NF1*, most of which have been done on breast cancer cell lines. In the present study, the expression of this gene is investigated in human cancerous and normal tissues. The result of the study showed that the expression of this gene is significantly dependent on the progression of the disease and gradually decreases significantly. In research on genes involved in the development of cancer, impairment of *BIF-1* gene expression in cancer cells compared with adjacent healthy tissues in many types of cancer, including colorectal cancer, prostate cancer, pancreatic cancer, bladder and stomach cancer. The results showed that *BIF-1* gene significantly decreased expression in 68% of mammary tumor samples compared with normal breast tissue. Low expression in the tumor tissue confirms the suppression of the *BIF-1* gene relative to the normal tissue around the tumor[11]. The result of the

Table 3: The result of changes in gene expression

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
GAPDH	REF	0.96	1.000				
NF1 Stage I	TRG	0.98	1.064	0.348 - 2.582	0.245 - 7.536	0.963	
NF1 Stage II	TRG	0.98	0.700	0.433 - 1.102	0.292 - 1.514	0.186	
NF1 Stage III	TRG	0.98	0.135	0.089 - 0.229	0.045 - 0.318	0.005	DOWN
NF1 Stage IV	TRG	0.98	0.081	0.036 - 0.195	0.017 - 0.321	0.007	DOWN

BACH2 gene in breast cancer revealed that expression of this gene was significantly reduced in comparison to the non-tumor tissue adjacent to the tumor. Also, there was a significant reduction in expression of the gene in metastatic samples in the lymph nodes than in non-metastatic samples. Reducing the amount of gene expression in the metastasis can be due to the lack of regulation in cell proliferation and transplantation and apoptosis violation[12, 13]. The rate of *P53* expression in both T47-d and MCF-7 breast cancer cell lines has been investigated in a study. The results showed that the gene expression level increased in the T47-d cell line and decreased in the MCF-7 cell line[14]. It can be inferred that the pattern of expression of genes in each cell line is almost unique. Studying and measuring the expression level of each gene in different cell lines can help identify genes and identify appropriate drugs[14]. Type 1 neurofibromatosis gene (*NF1*) produces a tumor suppressor protein called neurofibromin, which in adults is expressed primarily in neurons, Schwann cells, and medulla adrenal cells. The loss of *NF1* gene activity in patients with neurofibrosarcomas and in patients with malignant melanoma and neuroblastomas is reported. In a study, it was shown that neurofibromin does not occur in a group of patients with *NF1*. This can confirm the idea that neurofibromin may be an essential regulator for growth in these cells[15]. By performing cloning in another study, type 1 neurofibromatosis gene (*NF1*) was introduced as the tumor suppressor gene, which is most expressed in the brain. Type II neurofibromatosis *NF1* or isoform type II has an additional 21 amino acids in a protein region that is involved in the regulation of p21-ras. To determine the role of *NF1* gene in mammalian growth, the expression of *NF1* gene and neurofibromin formation were evaluated during the development of mouse embryos. From the birth to the 10th day, type II isoform was associated with the formation of *NF1* mRNA and the production of neurofibromin-dominant type. However, after the tenth day and throughout the growth period of the mice, Type I isoform was identified by the creation of *NF1* mRNA and the production of neurofibromin as the dominant type. Therefore, these experiments showed that the dominance of the two-mode change to the type of a neurofibromin isoform during embryogenesis may have significant consequences. [16]

Studies have shown that children with type 1 neurofibromatosis (*NF1*) have a high risk of developing gliomas in the optic nerve. Several evidences suggest that the *NF1* gene acts as a tumor suppressor in pilocytic astrocytoma. Therefore, it is assumed that the *NF1* gene plays a similar role in dispersed pilocytic astrocytoma. In one study, the mechanism of inactivation of *NF1* gene in pilocytic astrocytoma was investigated. Minimizing the protein size (PTT) did not affect the mutation in ten examined tumors. The use of real time PCR showed that the total levels of *NF1* transcripts in pilocytic astrocytoma are high and the ratio of expression of type I and type II *NF1* in pilocytic astrocytoma is comparable in normal tissue of the brain and grade one glioma. As a result, it has been proven that there is an increase in the expression of *NF1* gene in pediatric tumors of pilocytic astrocytoma[17].

Conclusion

Results of the study on clinical samples of breast cancer showed that the expression of *NF1* gene was dependent on the stages of the disease and the expression of this gene showed a significant decrease with progression of the disease stages. The use of gene biomarkers can be useful in early diagnosis and treatment more effectively. In patients with breast cancer, the use of *NF1* gene expression, along with other candidate genes, can be a good way to diagnose the progression of the disease. Also, *NF1* gene expression can be described as a possible cancer-related biomarker.

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