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Differential Expression Analysis of Dopamine Receptor Genes DRD2, DRD3 and DRD4 in the Tumoral and Tumor Margin Samples of Breast Cancer Patients

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ABSTRACT

Cancer is one of the major causes of mortality in the world, and breast cancer is one of the most common types of cancer affecting adult women. Various neurotransmitters may performed to tumor development through stimulation, migration, or metastasis. One of these neurotransmitters is dopamine, also involved in cell proliferation. Some neurotransmitters, dopamine in particular, can cause T-cells to proliferate and secrete cytokines. In this study, the expression levels of D2-like dopamine receptors were assessed in breast cancer tissues. Total mRNA was extracted from breast cancer tissues and tumor margin samples and reverse-transcribed into complementary DNA (cDNA). Internal control samples were also included prior to real-time PCR, and the results were statistically analyzed. The expression levels of the three genes DRD2, DRD3 and DRD4 were significantly increased in breast cancer tissue compared to those of the normal tissue, in the order of DRD2, DRD4 and DRD3. The correlation between dopamine receptors and breast cancer is discussed.

Keywords: Breast cancer, Dopamine receptors, Gene expression

INTRODUCTION

Cancer is a multifactorial disease and reported as the third leading cause of death in Iran after cardiovascular disorders and accidents. Because the molecular mechanisms involved in carcinogenesis are different in the different cell types, and the pattern of cell proliferation varies from the primary site, the treatment protocols are not the same. The main source of breast cancer is usually squamous cell lining of the lobules and mammary ducts [1]. Breast cancer has been reported as the prevalent type of cancer in which the mortality rate is very high. This cancer, despite extensive research on the management and treatment, continues to be the leading cause of death in women [2].

Neurotransmitters are chemical messengers. Recently, evidence indicated the role of neurotransmitters in immune action and regulating the migration of lymphocytes and tumor cells. Dopamine is one of the most critical neurotransmitters in the nervous system, and many neurological and mental disorders have been ascribed to malfunctioning of secretion of dopamine [3]. Dopamine is a catecholamine, while serotonin is an indole amine. Dopamine has at least five types of receptors by which it exerts its biological effects [4]. The family of dopamine receptors belongs to G-coupled proteins possessing seven alpha-helix transmembrane domains. Dopamine binds to the receptor binding site on extracellular proteins, activating G-coupled proteins, which in turn transforms an external signal to the internal secondary messenger [5]. In summary, five types of dopamine receptors have been recognized, including D1-D5. These receptors are divided into two categories. The D1-like class contains D1 and D5, while the D2-like group comprises D2, D3 and D4 [6]. The D1-like family is a stimulatory receptor that stimulates the function of adenylate cyclase. In contrast, the D2-like family are inhibitory receptors that prevent the function of adenylate cyclase. Dopamine receptors are also present on peripheral blood lymphocytes, with specific functions in these cells. A genomic study on dopamine receptors indicated that they have two gene families that are divergent [7]. To date, most of the pharmacological functions of D1 and D2 receptors have been identified. These two receptors have been characterized in the primary efferent neurons of the striatum, limbic cortex, prefrontal, and other areas of the

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cortex. The dopamine receptors are expressed on cell bodies and at the ends of axons. Both D1- and D2-like dopamine receptors are activated by G-proteins [6]. Despite recent scientific advances, cancer is yet considered as the second case of death worldwide. Although radiation and chemotherapy are effective in the treatment of malignancies, their use as therapeutic strategies have harmful effects on tumor margin tissues and normal cells [3]. According to the epidemiological studies, approximately 6160 new cases are annually diagnosed in Iran, of which 17.26% result in death. Such a mortality rate causes irreparable damages to society, since women are the mainstay of families [8].

The nervous system and the immune system are closely related. The interactions of these systems and the agents of the environment and genetic agents on the immune system can lead to various autoimmune diseases and cancers. A dopaminergic system produces dopamine, which acts as a neurotransmitter in the nervous system and mediates the nervous system and the immune system [9]. As dopamine receptors are located on lymphocytes, It is therefore, reasonable to assume that neurotransmitters such as dopamine can mediate between the nervous system and the immune system, and play a role in pathological pathways [10]. The association of dopamine with breast cancer has been established in peripheral mononuclear blood cells, but no studies have yet been performed on the tissue of patients with breast cancer [11]. Given that 70% of the disease is caused by several risk factors, especially in women, can be a key issue in this disease. Therefore, a molecular approach seems to be a very useful tool for research in this field, and on the other hand, the analysis of neuroimmunoendocrine super-system may provide further insight into cancer treatment [8].

The main goal of this study is to reveal the expression profiles of dopamine receptors DRD2, DRD3 and DRD4 in breast cancer tissues with the hope of using them as potential markers for therapeutic purposes.

MATERIALS AND METHODS

Sampling

The breast cancer tissue samples and their normal adjacent tissues were obtained from patients with breast cancer after obtaining written consent, and the protocol of sampling was in accordance with the method of our previous study performed on the expression change of Caveolin [11-16]. The experimental procedures were confirmed by the Ethics Committee of Shahid Beheshti University with the ethical code of IR.SBMU.RETECH.REC.1397.562.

The expression of DRD2, DRD3 and DRD4 genes was analyzed in cancerous tissues of patients with breast cancer, and their normal adjacent tissues were used as the control samples

RNA Extraction and cDNA Synthesis

The mRNA contents of cancerous and healthy or tumor margin tissues were extracted using High Pure mRNA Isolation Kit (Roche, Mannheim, Germany) based on the producer's instructions. Total RNA concentration of all specimens was measured using a nanodrop instrument, and the concentrations were adjusted to 70 ng ml^{-1} using the deposition buffer of the kit. The total amounts of the extracted mRNA were calculated based on the following formula: C2V2 = C1V1. Agarose gel was used for qualitative evaluations of the extracted mRNA. Then, the extracted mRNA was converted into cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

PCR Procedure

To ensure the precision of previous steps and the intactness of the synthesized cDNA, beta-actin, as a housekeeping gene was used as the internal control. Following confirmation, PCR was carried out for each gene using a positive control sample. The PCR reactions were performed in a reaction mixture at a volume of 20 µl containing 2 µl of 1 X Buffer, 2 µl of forward and reverse primers both at a concentration of 1 µM, 0.5 µl of 2.5 mM $MgCl_2$, 11.8 µl of ddH_2O, 0.5 µl of dNTPs, and 0.2 µl of 0.5 U Taq polymerase enzyme. Primers were designed, using the software program OLIGO™ version 7. The sequence of primers and their characteristics are shown in Table 1. The thermocycling program was set as follows: initiatial denaturation at 95 °C for 3 min, 25 cycles of denaturation at 95 °C for 10 s, annealing (60 °C for DRD2 gene; 62 °C for DRD2 and beta-actin genes; 58 °C for

Table 1. Sequence of the Designed Primers Designed and their Characteristics

Primer name	Primer sequence	Accession number
B-actin-forward	5'-AGACGCAGGATGGCATGGG-3'	NM_001101.3
B-actin-reverse	5'-GAGACCTTCAACACCCCAGCC-3'	
DRD2-forward	5'-TGTACAATACGCGCTACAGCTCCA-3'	NM_016574.3
DRD2-reverse	5'-ATGCACTCGTTCTGGTCTGCGTTA-3'	
DRD3-forward	5'-TCTGTGCCATCAGCATAGACAGGT-3'	NM_000796.3
DRD3-reverse	5'-TAAAGCCAAACAGAAGAGGGCAGG-3'	
DRD4-forward	5'-TCTTCGTCTACTCCGAGGTCCA-3'	NM_000797.3
DRD4-reverse	5'-TGATGGCGCACAGGTTGAAGAT-3'	

DRD4 gene) for 10 s, 35 cycles of extension at 72 °C for 30 seconds, followed by a final extension at 72 °C for 300 s.

Real-time PCR and Statistical Analysis

To investigate the expression levels of the three genes, real-time PCR reactions containing 5 × HOT FIREPol® EvaGreen® HRM Mix (ROX) (Solis BioDyne, Germany) were performed using the primers listed in Table 1 on a MIC instrument. The temperature and time adjustment programs for beta-actin, DRD2, DRD3 and DRD4 genes were the same as the one mentioned for the PCR reaction.

After determining the cycle threshold for each sample, the target and Ct values of the reference gene in both cancerous and tumor margin tissues were examined with LinReg software using the fluorescent analysis read for each sample and the efficiency of each sample. Calculations for differential expression were performed as following:

$$R = \frac{(E_{t\,\mathrm{arg}\,et})^{\Delta CP_{t\,\mathrm{arg}\,et}\,(mean\ control\ -\ mean\ sample)}}{(E_{ref})^{\Delta CP_{ref}\,(mean\ control\ -\ mean\ sample)}}$$

this way, for the target and reference genes, the degree of gene expression is calculated as absolute and finally, the calculated concentration of target genes is divided by the calculated concentration of the reference gene. Finally, the relative changes in the expression of target genes were measured using the $2^{-\Delta\Delta CT}$ method. Beta-actin housekeeping gene was used to normalize the data. The statistical significance of changes was assessed using Independent-Samples T test with a p-value ≤ 0.01 considered as significant**.**

RESULTS

RNA Extraction

Following preparation of a 1.5% agarose gel, the extracted RNA was loaded on the electrophoresis gel, and the results after gel visualization are shown in Fig. 1. The bands related to 28S RNA and 18S RNAs indicate and confirm the quality of the extracted RNA.

PCR Analysis

After the RNA extraction, cDNA was synthesized and for confirming the accuracy of the synthesized cDNA, PCR reactions were performed based on materials and programs set for the DRD2-DRD4 genes as well as the beta-actin

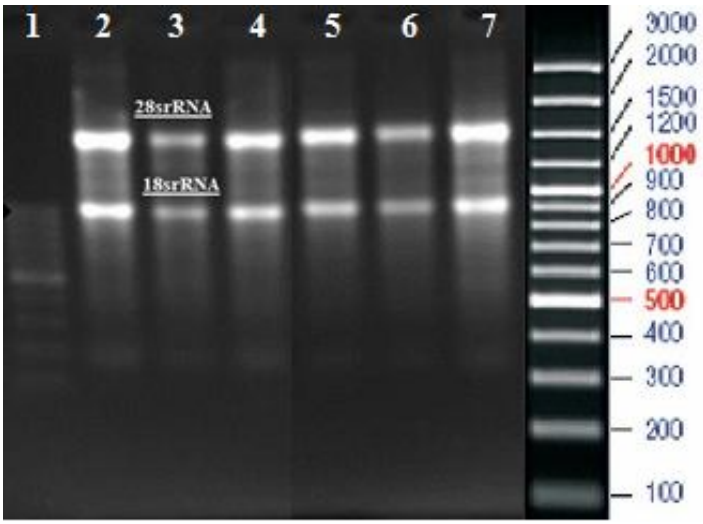

Fig. 1. RNA extracted from six patient samples on 1.5% agarose gel. The bands related to 28S RNA and 18S RNAs. Well 1: The 100-pair ladder related to products produced by Fermentase Company, Germany. Wells 2-7: RNA extracted from breast tissues samples of six patients.

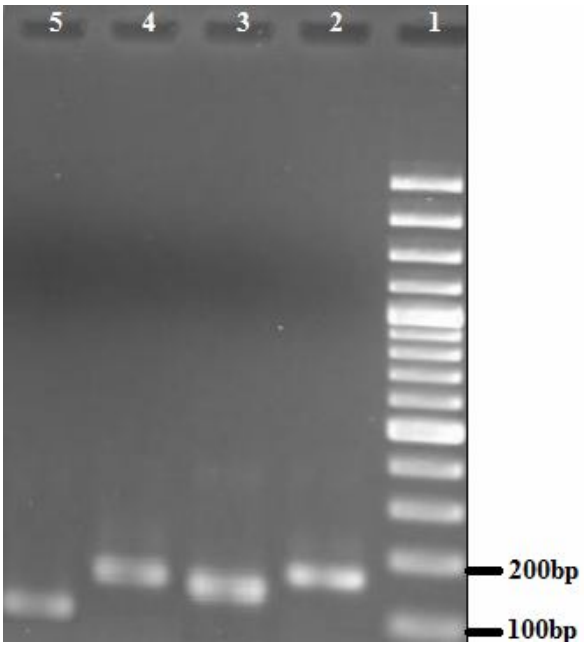

Fig. 2. Expression of dopamine and beta-actin genes in tissue samples were appeared on agarose gel. Well 1: The 100-bp ladder prepared by the Fermentase Company. Well 2: The PCR product related to the beta-actin housekeeping gene (161 bp). Well 3: The PCR product related to the DRD2 gene (127 bp). Well 4: The PCR product related to the DRD3 gene (155 bp). Well 5: The PCR product related to the DRD4 gene (110 bp).

housekeeping gene using the designed primers. The reaction products were loaded onto the electrophoresis gel. The results showed the precision of the experiments and the PCR products related to the DRD2 gene (127 bp), DRD3 gene (155 bp), DRD4 gene (110 bp) and the beta-actin housekeeping gene (161 bp) were appeared on agarose gel (Fig. 2).

Real-time PCR and Statistical Analysis

Real-Time PCR was carried out to assess the changes in the expression levels of dopamine receptors as well as the beta-actin housekeeping genes.

The results of real-time PCR showed that the expression levels of DRD2, DRD3 and DRD4 genes were significantly increased in breast cancerous tissues compared with the margin adjacent tissues (Table 2). Table 2 shows the extent of changes in the expression of each gene (alone, not compared to other genes). A statistical graph of the expression changes in the three genes of the dopamine receptors in breast cancerous tissues compared to margin adjacent tissues was provided (Fig. 3). Figure 3 shows an increase in the expression of the three genes with DRD2 having the greatest upregulation followed by DRD4 and DRD3.

DISCUSSION

Cancer drugs are expensive and despite costs imposed on patients or their family, the treatment is not definitive and may not have considerable effects on recovery of patients and the same is true for other therapeutic protocols used for the treatment of other types of tumors [17]. In addition to radiation and chemotherapy, the high cost of the treatment courses and the emergence of side effects caused by them, there the cure is not definitive [17]. More than 90% of cancer-related deaths in women, following lung cancer, is due to breast cancer, necessitating a greater attention needed to be paid to the disease [18,19]. Therefore, gene therapy and treatments based on genetics can be helpful. This is one of the objectives of this study and in this regard, considering the relationship between dopamine receptors and breast cancer, the present study aimed at monitoring changes in the expression levels of three D2-like receptor genes.

The neurotransmitters have a regulatory effect on various cells in the body, including different glands, which control the release of hormones. The impact of neurotransmitters on different cells of the body is through the neurotransmitter and dopaminergic systems which have various receptors on the cells. Neurotransmitter systems are critical in the nervous system that release and control other neurotransmitters such as dopamine, serotonin, GABA and epinephrine as well as some hormones such as oxytocin and prolactin. They play a role in pathogenesis or treatment of diseases through interactions with other systems [6]. Also, dopamine receptors, as parts of these systems are involved in the transmission of neural messages, and have numerous roles in various disorders. Dopamine receptors are classified into two groups; the first group is named D1-like dopamine receptors with stimulatory effects on cells, while the second group, named D2-like dopamine receptors, have inhibitory effects on cells. D1-Like receptors stimulate cAMP production and result in potential changes and message transmission within the cells, while D2-like receptors inhibit the synthesis of cAMP. These two groups of dopamine receptors make a balance in the nervous and immune systems [6]. Any disruption in the balance of these systems could lead to the development of various disorders. The analysis of dopamine receptors shows that there are many differences and similarities between them. It has been demonstrated that D1 and D2 receptors have about 30% similarity in the sequence of their transmembrane domains, and this percentage for D2, D3 and D4 is approximately 50%. [4,20-22]. The transcribed regions of D2, D3 and D4 contain 6, 5 and 4 introns, respectively. The position of introns is the same for D2, D3 and D4 receptors but their difference stems from the number of introns as the D3 receptor losses the fourth intron of the D2 receptor, while the D4 receptor losses the third and fourth introns of the D2 receptor. It is interesting that the third intron of the D4 receptor has an unusual connection between the exon and the intron [3]. The effect of dopamine on lowering the number of viable cells in salivary gland tumors the overexpression of its genes in patients with diabetic foot ulcers, and its effect on the intracellular accumulation of cAMP in T lymphocytes in preventing their proliferation, thus reducing the body's resistance to cancer have been reported by various studies (23, 15, 16). In general, there are

Table 2. Changes in the Gene Expression of Dopamine Receptors in the Breast Cancerous Tissues Compared with Healthy Adjacent Tissues

Genes	P-value	Rate of change	Standard error	Level of changes
DRD2	0.000[a]	6.574782	±1.603605252	Increase
DRD3	0.000[a]	6.832121	±1.666370995	Increase
DRD4	0.001[a]	5.950661	±1.451380768	Increase

[a]The asterisk denotes statistically significant difference ($p < 001$).

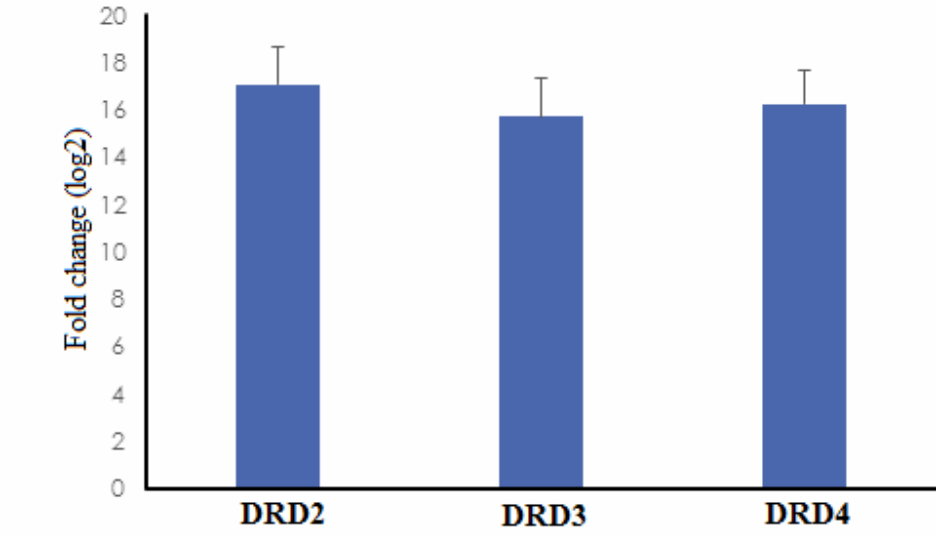

Fig. 3. Statistical graph of the expression changes in the three genes of the dopamine receptors in breast cancerous tissues compared to margin adjacent tissues. Graph shows the changes in the gene expression of D2-like receptors compared to each other based on the results obtained from real-time PCR.

several signaling pathways affected by dopamine and its receptors, including the PI3 kinase and MAPK [24]. The expression pattern of dopamine receptor genes in patients with chronic stress is altered (source). Several investigations have aimed at identifying the roles played by dopamine receptors in the pathogenesis of a number of disorders, such as schizophrenia, lupus erythematosus, psoriasis and lung cancer [25]. S have also reported the changes in the gene expression of dopamine receptors in patients with Parkinson's disease [26]. A significant increase in expression has been reported in the levels of D2, D3 and D4 receptors in lung cancer. In previous studies, it has been shown that in patients with breast cancer, the expression of dopamine receptors is increased in peripheral blood mononuclear cells (PBMCs) compared to health subjects, and that such a change resulted in a reduced number of T cells in breast cancer patients. These studies concluded that the upregulation of the of D2-like dopamine receptors in breast cancer patients may have led to a weaker immune system, in addition to increased proliferation and growth of cancer cells [11].

Several lines of evidence have suggested an association between the expression levels of D2 receptor gene family and the stage of breast cancer when analyzed in PBMCs of patients [25].

CONCLUSIONS

In this study, the expression levels of dopamine D2-like receptors (D2, D3 and D4) were monitored in cancerous tissues in breast cancer patients. As a control group, tumor margin breast tissues were also included. It was shown for the first time that the expression of the three genes was significantly increased in cancerous tissues. Neurotransmitters, dopamine in particular, play an inhibitory role in the growth and proliferation of malignant cells through their receptors (usually D2-Like receptors), and previous studies have shown that dopamine and its agonists can cause apoptosis in cancer cells. Given that the expression levels of D2-like receptors were higher in breast cancerous tissues than in the normal ones, this could provide further insight into cancer biology. As dopamine is known to induce apoptosis in tumor cells, and considering our results on the expression levels of DRD2, DRD3, DRD4 receptors in breast tumor tissue cells, it is suggested that dopamine agonists have the potential to be used as drugs. In addition, since dopamine has negative effects on tumor control, causing cancer cells to grow and metastasize, it is therefore suggested that use agonists as drugs, in addition to affecting only dopamine receptors in breast tissue tumor cells, has a higher adhesion strength than dopamine and outperform binding to dopamine receptors.

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DECLARATIONS

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Conflicts of Interest/Competing Interests

The authors declare no competing interests.

Availability of Data and Material

Supplemental data are provided.

Authors' Contributions

Both authors contributed to the study conception and design. Material preparation performed by Garshasb Rigi. The biological experiments and data collection were done by Amirhossain Mirzaghasab as a MSc student in Genetics. The first draft of the manuscript was written by Amirhossain Mirzaghasab and all authors commented on previous versions of the manuscript. Garshasb Rigi as a supervisor of the project and corresponding author, designed the whole experiments, supported the project, analyzed data, and wrote the final manuscript. All authors read and approved the final manuscript.

Consent for Publication

Not applicable

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