RESEARCH ARTICLE

Dopamine Receptor Gene (DRD1-DRD5) Expression Changes as Stress Factors Associated with Breast Cancer

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Abstract

Breast cancer is the most common cancer among females worldwide and a most prevalent malignancy in Iranian women. Chronic stress may make an important contribution to cancer, especially in the breast. Numerous studies showed roles of neurotransmitters in the occurrence and progression of cancers which are mediated by their various types of receptors. This study was conducted to evaluate alterations in the expression profile of dopamine receptor genes in peripheral blood mononuclear cells (PBMC) as stress factors in breast cancer patients and the human breast cancer cell line (MCF-7). Peripheral blood samples were obtained from 30 patients and 30 healthy individuals. Total mRNA was extracted from PBMC and MCF-7 cells and RT-PCR was performed to confirm the presence of five dopamine receptors (DRD1-DRD5). Expression changes of dopamine receptor genes were evaluated by real time PCR. We observed that DRD2-DRD4 in PBMCs of breast cancer patients were increased compared to healthy individuals. In addition, all dopamine receptor subtypes but DRD1 were expressed in MCF-7 cells. Therefore, alterations of these receptors as stress factorsshould be assessed for selecting appropriate drugs such as D2-like agonists for treatment of breast cancer after performing complimentary tests. Determining the expression profile of dopamine receptor genes thus seems promising.

Keywords: Stress - breast cancer - dopamine receptors - gene expression - neurotransmitters - peripheral blood cells

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Introduction

In the last three decades, the incidence of breast cancer has increased and now it is one of the most common cancers in females worldwide, particularly in Iran (Brennan and Houssami, 2011). It is one of the main reasons of cancer-related mortality in women and its rate in developing countries is annually 70% of which will occur (Haghighat S, 2012). Researchers believe that mental and physiological conditions of individuals are important in the promotion and development of cancers and changes in patient's life style could be effective in incidence of stress (Elenkov et al., 2000; Cohen et al., 2007; Barik et al., 2013). Improvement the quality of life can be effective in reduction of breast cancer patients' distress and the risk of breast cancer (Yavuzsen, 2012).

In addition, the occurrence of different types of cancers including breast cancer could be associated with chronic stress (Marsh et al., 2010). Chronic stress has an important role in evidence of cancers specially breast cancer and cause to secretion of neurotransmitters. Numerous studies showed the role of neurotransmitters in the occurrence and progression of cancers which is mediated by their various types of receptors (Elenkov et al., 2000; Marsh et al., 2010; Mancino et al., 2011). In general, the nerve fibers are interconnected with lymphatic and blood vessels (Elenkov et al., 2000). Recently, several studies have shown that different mediators of the nervous system like neurotransmitters have an important role in immune system functions, lymphocyte migration and tumor angiogenesis, lymphogenesis and progression. Several neurotransmitters may lead to tumor progression via stimulating the migration and dissemination of tumor cells to distant sites (Mancino et al., 2011; Hejazi et al., 2014).

One of these neurotransmitters is dopamine which has proliferative effects in non-transformed cells (Mancino et al., 2011). Whereas, some of neurotransmitters particularly dopamine can inhibit the T cell receptor (TCR)-induced cell proliferation and stimulation of T cells to secretion of cytokines and consequently leads immune system depletion (Basu et al., 2010). Dopamine affects different types of cells via its various receptors. These receptors are from G-protein family containing D1and D2 family

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receptors. Both D1 and D5 receptors are members of D1-like family that play a role as stimulator and induce increases intracellular cyclic AMP (cAMP). In contrast, D2 and D4 receptors, members of D2-like receptor family, inhibit intracellular cAMP on stimulation (Beaulieu and Gainetdinov, 2011).

Different studies showed that these receptor genes expression profiles were changed associated with chronic stress condition in various types of diseases including lupus erythematosus, schizophrenia and non-small lung cancer (Kwak et al., 2001; Shaikhpoor et al., 2012; Jafari et al., 2013). Whereas, regarding to the importance of dopamine receptors gene expression changes in pathogenesis of diseases related to chronic stress, in breast cancer patients has not been reported yet. It is assumed that the stress can be conducted to alterations in expression of dopamine receptor genes in some diseases which could be attributed to development of the disease. Therefore, we hypothesized that change in the expression level of dopamine receptor genes (DRD1-DRD5) in the PBMCs as well as a human breast cancer could be a stress factors in breast cancer.

Materials and Methods

Patient's sample

The naive patients group consisted of 30 breast cancer patients with the mean age of 34 years (ranged from 20 to 40 years) who were referred to Shohada hospitals, Tehran, Iran, were enrolled. All of the cases had a chronic stress or a life event in their background. The control group included 30 individuals and the average of 35years old (ranged from 20 to 40 years). Patient consent for all of samples was obtained according to the Declaration of Helsinki principles. The pathological information of all patients was obtained from Pathology Department of this academic Hospital. Also, thirty healthy individuals were included as controls. Exclusion and inclusion criteria were determined according to the revised criteria for classification of breast cancer patients, published by cancer research center of Iran, and demographic data of the studied patients, were collected from cancer department of Shohada hospital. This project was approved by the National Institute for Genetic Engineering and Biotechnology (NIGEB) and written informed consent was obtained from all participants of this study.

Cell isolation

Peripheral blood samples (5ml) were obtained from the cubical vein and were collected in cell preparation tubes containing an anticoagulant (0.05 M EDTA). Blood samples were then diluted with an equal volume of PBS. Peripheral blood mononuclear cells (PBMC) were isolated from 4ml of each blood sample by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. Cell density and osmolity were 1.077±0.001 g/ml (20˚C) and 290±15 moms, respectively. Horizontal swing-out centrifuge was used for cell isolation in 2500 rpm, for 20 minutes and 1.0 speed regulation. The Buffy coat (lymphocyte layer) was collected and centrifuged in 1600 RPM, for 10 minutes and 2.0 Speed regulations. Finally, the resulting pellet was washed in Phosphate buffer saline (PBS) (Hejazi et al., 2014).

Cell culture

Media, serum and MCF-7 cell line were provided from Gibco (Germany) and Pasture institute of Iran. The cells were cultured in DMEM supplemented with 10 percent fetal bovine serum. To evaluate gene expression of dopamine receptors, 1×10^6 of MCF-7 cells were used for RNA extraction.

RT-PCR

The total mRNA was isolated from PBMC by High pure RNA isolation Kit (Roche, Germany), according to the manufacturer's instructions. The RNA extracted samples read with Nanodrop to measuring the RNA concentration in samples and in this stage we synchronize all other samples. The RNA (1µg) from each sample was used to synthesize first-strand cDNA and cDNA synthesis was carried out by cDNA synthesis kit (Fermentase, Germany). Similarly, cDNA synthesis was performed based on manufacture's protocols. Primer design was carried out using oligo5 software. Primer specificity was theoretically checked by BLAST database search against nucleotide reference NCBI database and experimentally verified by the positive control amplification. To confirm the presence of dopamine receptor genes in blood and MCF-7 cells, a common PCR technique was carried out for all subtypes (DRD1-DRD5). Reaction mixtures contained 2-2.5 mM $MgCl_2$, 0.5 mM each of the dNTPs, 0.8 -1mM primers, 2.5μLTaq DNA polymerase (Sinagene), and 1 μL of the cDNA was used as template in each RT-PCR reaction. In order to amplify the DRD1-DRD5 and b-actin genes, PCR was initiated at 95°C for 5min and amplified during 35 cycles at 95°C for 1min, 56, 60, 62, 58, 58 and 62°C for40 s and 72°C for 1 min and followed by a final extension step at 72 for 10 min. Finally, the PCR products were visualized by gel electrophoresis on a 2% agarose gel. The primers for DRD1-DRD5 receptor genes, and b-actin gene as housekeeping gene were designed based on GenBank sequences (Table 1).

Real time PCR

Reactions were also carried out in a Real Time-PCR (Corbett, Germany) with a Cyber green flourgenic nucleotide to monitor cDNA amplification by (Roche kit, Germany) measuring the increase in Fluorescence intensity and using primer pairs specific for five dopamine receptors (DRD1-DRD5) mRNAs and β-actin as the internal control. The PCR was performed in 10 μL of solution, consisting of 2 μL of Fast Start Master solution and 0.3 μM of each primer. A total of 9 μL of this reaction mix was placed into 0.1 vials, and 1 μL of cDNA was added as a template. Thermal cycling consisted of an initial denaturation step 95°C for min followed by an amplification program (primer annealing, amplification and quantification) repeated for 45 cycles. The amplification program was 95°C for 10 sec, 56, 60, 62, 58, 58 and 62°C for 10 sec, respectively for DRD1 to DRD5 and 72°C for 10 sec with a single fluorescence acquisition at the end of the elongation step. The third

Table 1. Primer sequences used in RT-PCR and Real time -PCR

Locus	Primers	Accession number(Gene Bank)	Size
β-actin- F	5'-AGACGCAGGATGGCATGGG-3'		
β-actin -R	5'-GAGACCTTCAACACCCCAGCC-3'	NM_001101.3	161bp
DRD1-F	5'- CTTCCTCAACGTTTCGGAGCC-3'		
DRD1-R	5'- AGCTCTCCAAACGCCTTGCCTT-3'	NM_000794.3	115bp
DRD2-F	5'- TGTACAATACGCGCTACAGCTCCA-3'		
DRD2-R	5'- ATGCACTCGTTCTGGTCTGCGTTA-3'	NM_016574.3	127bp
DRD3-F	5'- TCTGTGCCATCAGCATAGACAGGT-3'		
DRD3-R	5'-TAAAGCCAAACAGAAGAGGGCAGG-3'	NM_000796.3	156bp
DRD4-F	5'- TCTTCGTCTACTCCGAGGTCCA-3'		
DRD4-R	5'- TGATGGCGCACAGGTTGAAGAT-3'	NM_000797.3	100bp
DRD5-F	5'-TCATCTATGCCTTCAACGCCGACT-3'		
DRD5-R	5'- AGCTGCGATTTCCTTGTGGAAGAC-3'	NM_000798.4	155bp

segment consisted of a melting curve program performed by default program of the real time-PCR instrument. Melting curve analysis showed only one peak for each reaction and this also confirmed by electrophoresis of PCR products that showed only one band of the expected size.

Sequencing

Receptors and β-actin fragments were sequenced by DNA sequencer ABI 3700 capillary system (Applied Bio System, USA) to confirm amplified sequences.

Statistical analysis

The number of samples was determined by Minitab 16.1 software and efficiency of each reaction was precisely evaluated by Linreg software. Real time PCR data were analyzed by Rest 2005 and 2009 software. The correlation between the changes in dopamine receptor gene expression and the items such as age of patients, stages of the disease, Progesterone receptor (PR),human epidermal growth factor Receptor (HER-2) and estrogen receptor (ER) gene expression was assessed by SPSS software (version 16.0). In the current study, the p-value less than0.05 ($P < 0.05$) was considered statistically significant.

Results

Pathologic analysis

Pathology examinations were showed that 30, 23, 37 and 10 percent of the patients respectively were related to stages of I, II, III, and IV. Almost 75 percent of patients expressed ER and 67 percent of them expressed PR. Also, 40 percent of patients were HER-2 positive. Six percent of patients had a mutation in P53 protein. In this study, expression of different types of dopamine receptors genes were evaluated in PBMC breast cancer patients and healthy individuals PBMC and also in human breast cancer cell line (MCF-7).

PBMC expresses in breast cancer

The results of RT-PCR showed that all types of dopamine receptors were expressed in PBMC of breast cancer and healthy individuals (Figure1).We also evaluated gene expression of dopamine receptors in human breast cancer cells (MCF7).The results of RT-PCR revealed that all dopamine receptor subtypes (DRD2-DRD5), expressed on MCF-7 cell line. However, dopamine receptor gene

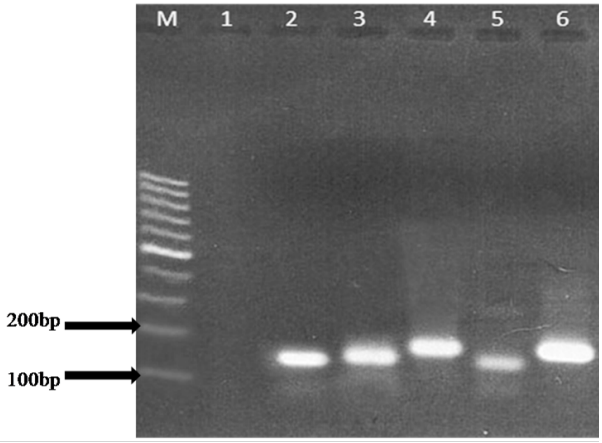

Figure 1. Show All Types of Dopamine Receptors have been Expressed in PBMCs Cells. Lane M: Molecular size marker 100bp (Fermentase, Germany), Lane1: control negative of DRD1-115bp, Lane2:DRD1-115bp, lane3: DRD2-127bp, Lane4: DRD3-156bp, Lane5: DRD4-100bp and Lane6: DRD5-155bp

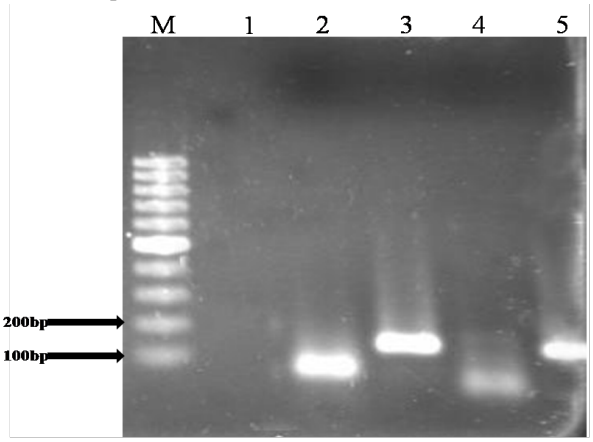

Figure 2. Show all types of dopamine receptors have been expressed in MCF-7 cells except DRD1. Lane M: Molecular size marker 100bp (Fermentase, Germany), Lane1: DRD1-115bp, Lane2: DRD2-127bp, Lane3: DRD3-156bp, Lane4: DRD4-100bp and Lane5: DRD5-155bp

(DRD1) was not detected on electrophoresis gel (Figure2).

Expression analysis

Analysis of Real time-PCR revealed there were considerable differences in gene expression rates between patients and healthy controls. DRD1, DRD2, DRD3 and DRD4 genes in PBMC of breast cancer cases showed a

Table 2. Compared to healthy persons' PBMC DRD1-DRD4 genes expression in breast cancer patients PBMCs were increased significantly

Gene	P-value	Rate of change	Standard error	Changes
DRD1	0.001*	5.854	±0.64	UP
DRD2	0.001*	6.488	±0.28	UP
DRD3	0.001*	4.863	±0.45	UP
DRD4	0.001*	6.791	±0.41	UP
DRD5	0.965^{ns}	0.046	±0.57	No significant

ns: no significant

* Significant increasing at P≤0.001 level (up regulation)

significant over expression compared to their counterparts in healthy individuals. (Table 2). Furthermore, all sequenced fragments were checked by BLAST database against nucleotide reference NCBI database and confirmed amplicon sequences. Investigation of the association of dopamine receptor gene expression with factors such as patient's age, stage of the disease, and the expression of ER, PR and HER2 genes showed a significantly higher expression of DRD2 gene in all stages of breast cancer. One way Anova analysis confirmed the significant association between over expression of DRD2 gene with the progression of the disease (P-value≤ 0.004). Over expressions of dopamine receptor genes were not associated with patient's age or expression of ER, PR and HER-2 genes.

Discussion

Based on our hypothesize, the results show difference of dopamine gene expression profile in PBMCs as well as a human breast cancer cell line (MCF-7) as stress factors in breast cancer patients. Previous study demonstrated that mental and physiological conditions of individuals are important in the promotion and development of cancers and changes in patient's life style could be effective in incidence of stress (Cohen et al., 2007; Mancino et al., 2011; Barik et al., 2013). Cancer cells and nerve fibers have cross-talks and thus could influence on each other. Cancer cells cover up to 33% of the surrounding space of perineural cells and secret soluble factors which lead to perineural cell invasion (Sarkar et al., 2008; De Leeuw van Weenen et al., 2011). Moreover, different signaling pathways are shared by cancer cells whose presence is important for successful axon growth and elongation towards its target region. For example, serine/threonine specific protein kinase (Akt) and phosphoglycogen synthase kinase 3 (Gsk3) pathways are important for neuronal polarity and microtubule stability (Tonge et al., 1998). Several neurotransmitters have been found to be related to tumor progression via activation of signaling pathways linked to cell proliferation and survival, including Phosphatidylinositide 3-kinases (PI3K), Mitogen-activated protein kinases (MAPK) and Akt pathways (Beaulieu and Gainetdinov, 2011). These types of events occur due to secretion of catecholamines (stress-mediators) which increases in response to stressful events (Ganguly, 2010; Beaulieu and Gainetdinov, 2011). Most of catecholamines including epinephrine and norepinephrine are related to carcinogenesis and tumor progression (Mancino et al., 2011). In addition, there is a close relationship between immune and nervous systems. They interact with each other through cytokines, neurotransmitters, hormones and neuropeptides. Neurotransmitters are mediators that have traditionally been regarded as signal substance. Various studies have shown that neurotransmitters, including dopamine and serotonin affected immune cells in a number of diseases through their receptors (Reiche et al., 2004; Ganguly S, 2010). Dopamine receptors activity is mediated by G-proteins. There are five different subtypes for dopamine receptors (DRD1-DRD5) which are classified into two families including D1-like family (stimulatory receptors) and D2-like family (inhibitory receptors) (Kirillova et al., 2008; Beaulieu and Gainetdinov, 2011). Prior studies reported that in different diseases the gene expression of dopamine receptors have been altered in immune and nervous systems (Kwak et al., 2001; Jafari et al., 2013). For instance, significant decreasing in D2-D4 were reported in PBMCs of non-small cell long cancer (Shaikhpoor et al., 2012). Kwak et al (2001) were reported significant elevation in the expression level of D2 and D3 genes in schizophrenia (Kwak et al., 2001). In arthritis rheumatoid, the expression of D2 was decreased and D4 was elevated, respectively. Jafari et al (2013) indicated an over expression of D4 in lupus erythromatosus (Jafari et al., 2013). In the current study, expression rates of DRD2-DRD4 genes have been observed to be significantly higher in PBMC of breast cancer patients than healthy controls and also the expression of DRD2 increased with promoting the stages of disease. These alterations could lead to changes in the amount of T-cells which play a key role in both cellular and hemoral immune systems (Elenkov et al., 2000). T-cells control the natural tumor cells via secretion of cytokines TNF-α (Jiang et al., 2007). Thus, the coordinated over expression of DRD2 family receptor genes enhances each other (additive effect) and leads to reduction of T-cells amount. Therefore, it may be a result for decreasing of anti-tumor cell cytokines and subsequently tumor progression may be occurred. We also observed the expression of DRD2 to DRD5 genes in MCF-7 cells. However, DRD1 gene expression was not detected in this cell line. It might be as a result of the inhibitory effect of dopamine in breast cancer cells. As Sarker et al (2008) had previously suggested that dopamine have an inhibitory effect in the cells of breast cancer bearing- mice (Sarkar et al., 2008). Other studies had also disclosed an inhibitory role for dopamine, D2-agonists and antagonists in several types of cancers including bone marrow (HL-60), gastric and colon cancers cells (Inayat-Hussain et al., 2000; Chakroborty et al., 2004; Sarkar et al., 2008).

In conclusion, recent investigations give an idea about DRD2 gene expression changes as stress factor along with other diagnostic markers could be used as a reliable marker attributed to development of breast cancer. However, further investigations using D2-agonist as a new therapeutic agent are recommended.

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