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Original Article

Analysis of the Expression of Dopamine Receptors, DRD1 and DRD5, in Cancerous Tissue of Patients with Breast Cancer and the Impact of Quercetin on the Expression of These Receptors in Hs578t and HDF Cell Lines

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Abstract

Background & Objectives: The expression of neurotransmitters during cancer progression is one of the factors to consider. This study aimed to evaluate the possible expression changes in dopamine receptors (DRD1 and DRD5), in breast tumor tissue compared to healthy adjacent samples, and the effect of quercetin on the expression of these receptors.
Materials & Methods: Expression levels of DRD1 and DRD5 were evaluated in 20 breast tumors and healthy adjacent samples using qPCR. The RNA was extracted from all samples, followed by cDNA synthesis and real-time PCR. *In vitro* experiment was accomplished on breast cancer cell line, Hs578t. Cells were treated with quercetin and evaluated cell viability by MTT assay. DRD1 and DRD5 gene expression was performed in treatment cells compared to untreated control. Statistical analysis was performed to determine the significance.
Results: The results showed that the DRD1 and DRD5 were unregulated in breast cancer tissue ($p<0.01$). For cellular experiment, MTT assays revealed that the quercetin induced a significant decrease in cell viability and proliferation in dose and time-dependent but it was not seen in a normal cell line (HDF). Hs578t cells showed a significant reduction of DRD5 in response to quercetin. DRD1 gene downregulation was indicated significant in 72h treatment.
Conclusions: The effect of quercetin on the expression of genes encoding DRD1 and DRD5 showed that this substance reduced the expression of these two genes in treated Hs578t cells compared to untreated cells in the same cell line.

Keywords: Dopamine receptor, Breast cancer, Quercetin, Gene expression, Hs578t cell line, HDF cell line

Introduction

Breast cancer is the most common cancer among women worldwide and among women in Iran, and it is currently one of the leading causes of cancer-related death in the world (1).

Numerous studies have shown the role of neurotransmitters in the occurrence and progression of cancer (2-5). The results of several recent studies indicate that various neural mediators, such as neurotransmitters, play an essential role in the immunity of the human body (3, 4). Multiple neurotransmitters have been reported to participate in tumor growth, stimulating tumor migration and proliferation. Among these neurotransmitters, dopamine, effectively contributes to the proliferation of cancer cells. To date, five receptors have been identified that are bound to dopamine, namely DRD1-DRD5.

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These receptors are part of the G-Protein family (3, 6). DRD1 is a protein receptor and cells expressing its receptor constitute about half of neurons in the brain and spinal cord. Upon dopamine binding to the DRD1 receptor, this receptor induces apoptosis in tumor cells by activating the cGMP-PKG-D1R pathways (2, 7). The DRD1 receptor is the most abundant type of dopamine receptor in the central nervous system. This receptor contains 446 amino acids and stimulates the adenylate cyclase enzyme. It has also been shown that it is capable of producing ATP-dependent kinases. The D1 receptor gene is located on chromosome 5 and lacks any introns (8), while the D5 receptor gene is positioned on chromosome 4, lacking any introns, and has 2062 bp (9).

The D1-like family (DRD1 and DRD5) increases cell cycle activity as a stimulus and inducer of AMP to cAMP conversion (3, 10). DRD5 can inhibit the growth of pituitary tumors. Also, it plays an essential role in the inhibition of the mTOR pathway and the increase in the generation of reactive oxygen species (ROS) (11). According to the literature, the involvement of DRD5 in the progression of some types of human cancer, including, colon, glioblastoma, and gastric cancer, has also been addressed (12).

Quercetin is a plant flavonoid found in fruits and vegetables and sold as a dietary supplement in the form of quercetin aglycone. Quercetin inhibits tumor proliferation by inducing cell cycle arrest and is a known factor in treating autophagy-mediated cancer (13). Flavonoids have anti-inflammatory and antioxidant activities (14). In further searches of up-to-date databases, it was found that articles on the effect of quercetin on DRD2 have been reported (13), but the impact of this plant flavonoid on DRD1 and DRD5 has not been studied, albeit it has been on a different cell line from the present study.

This study aimed to evaluate the possible expression changes in dopamine receptors (DRD1 and DRD5), in breast tumor tissue compared to healthy adjacent samples, and the impact of quercetin on the expression of these receptors in Hs578t and HDF cell lines. From this perspective, no study has been done so far. However, in previous analyses, from a different perspective, studies were performed on the peripheral blood mononuclear cell of patients with breast cancer and not on the tissue of these patients (2, 3).

Materials & Methods

Sample collection

Before the initiation of the experiment, written consent information was obtained from all patients diagnosed with breast cancer. In the process of sampling, cancerous tissues, along with adjacent healthy tissues at the proximity of breast tumors, were taken from 20 breast cancer patients.

Extraction of RNA and synthesis of cDNA

To extract the RNA contents of breast cancer tissue and its adjacent healthy tissue, the high-pure RNA isolation kit (Roche) was used following the manufacturer's instructions. Firstly, the concentration of the extracted RNA was determined by a nanodrop instrument with the help of a sediment buffer, and the concentration of mRNAs was reported to be 72 ng/ml. Employing the following formula; C1V1 = C2V2, the total amount of extracted mRNA was calculated. Finally, the Revert aid first-strand cDNA synthesis kit (Thermo Scientific, USA) was utilized to reverse-transcribe the extracted RNA into complementary DNA (cDNA).

Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out to examine whether the integrity of cDNA was preserved in previous steps. As an internal control, the β-actin gene, known as a house-keeping gene, was applied. The amplification of synthesized cDNA was conducted in a 20 µl reaction mixture. The reaction mixture consisted of 0.5µl of 2.5 mM $MgCl_2$, 1 µl of forward primer, 1 µl of reverse primer, 11.8 µl of ddH_2O, 2 µl of the 1X buffer, 0.5 µl of dNTPs, and 0.2 µl of 0.5 U of Taq polymerase. The sequences of the designed primers are depicted in Table 1.

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Table 1. Sequences of specific primers for beta-actin and target genes (DR1-DR5)

Locus	primer	Accession number	Product Size(bp)
β-actin forward	5′-AGACGCAGGATGGCATGGG-3′	001101.3	161
β-actin reverse	5′-GAGACCTTCAACACCCCAGCC-3′	001101.3	
DRD1 forward	5′-CTTCCTCAACGTTTCGGAGCC-3′	000794.3	115
DRD1 reverse	5′-AGCTCTCCAAACGCCTTGCCTT-3′	000794.3	
DRD5 forward	5′-TCATCTATGCCTTCAACGCCGACT-3′	000798.4	155
DRD5 reverse	5′-AGCTGCGATTTCCTTGTGGAAGAC-3′	000798.4	

The amplification of thermocycling included the initial denaturation at 94 °C for 3 minutes, followed by 25 cycles of denaturation at 95 °C for 10 seconds, and annealing at 62 °C, 56 °C, 58 °C for 30 seconds for β-actin, DR1, and DR5 genes, respectively. The final extension was conducted at 72 °C for 4 minutes.

qPCR reaction

The qPCR technique was employed to evaluate the level of DRD1 and DRD5 expression. The cycling reactions were performed on a 7500 real-time PCR system (Applied Biosystems) in a reaction mixture, containing the designed primers (Table 1) and 5x HOT FIREPol EvaGreen HRM Mix (ROX) (Solis BioDyne Co, Germany). Temperature and time adjustment programs for DRD1 and DRD5 genes were performed according to the previous PCR temperature and time settings. Following the determination of the threshold cycle for each sample, CT values of target genes, along with the β-actin gene, were obtained for both cancerous and healthy adjacent tissues using the LinReg software (4). In this way, the absolute expression of all genes (target and control genes) was calculated. The relative expression of target genes was analyzed against the β-actin gene using the ΔΔCT method. The difference in the relative expression of genes between cancerous and healthy adjacent tissues was investigated by the independent T-test. The level of the statistical significance was set at $p<0.05$.

Cell culture

The breast cancer cell line Hs578t and skin fibroblast cells (HDF) (purchased from the National Center for Genetic Resources of Iran) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in T-25 flasks. They were incubated in a 5% CO_2 incubator at 37 ° C. HDF as a normal cell was used to evaluate the toxicity of quercetin on healthy cells.

MTT assay

The MTT assay was performed to determine the IC50 concentration of plant quercetin (Sigma, Q4951). In this assay, 104 cells were seeded onto each well of a 96-well microplate containing 102µl of the culture medium. The microplate was placed in a 5% CO_2 incubator at 37 ° C for 24 hours, and then quercetin was prepared at concentrations of 25 µM to 175 µM was added to wells containing Hs578t and HDF cells. After

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incubation, the culture media were removed, the cells were washed with PBS buffer, and the fresh culture medium supplemented with 5% FBS was replaced and incubated again. After 24, 48, and 72 hours, the cells were washed with PBS buffer, and 1.5 µl of the MTT solution was added. After 3-4 hours of incubation, DMSO was added to the wells, and in the next step, the optical density of the wells was measured by a plate reader at a wavelength of 570 nm, in triplicate. The IC50 values are defined as the concentration of quercetin, causing cell death in half of the cells compared with control cells.

Analysis of gene expression

To treat cells with quercetin plant flavonoids and analyze the impact of this substance on the expression of genes encoding dopamine receptors (DRD1 and DRD5), cells at a density of 10^6 Hs578t cells were seeded onto a 6-well plate and cultured in 2 ml of culture medium. Cells were treated with 40 µM and 25 µM quercetin for 48 and 72 hours, respectively. The procedures of RNA extraction and cDNA synthesis were similar to those mentioned earlier. In order to assess the expression of genes encoding DRD1 and DRD5 in Hs578t cells treated with quercetin, the qPCR technique was used based on the method previously described in the "qPCR reaction" section.

Statistical analysis

Statistical analysis of the data was accomplished using SPSS software version 20 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software, San Diego, CA). Kruskal-Wallis and Dunn's tests were used to evaluate the statistical analysis between the quercetin treated and the control groups for MTT assay. The 2-ΔΔct method was used to analyze the real-time PCR data. Data were then presented as mean [± standard error of the mean (SEM)]. P-values of less than 0.05 were considered statistically significant.

Results

qPCR reaction for tissue samples

The results obtained from the qPCR method demonstrated that expression rates of DRD1 and DRD5 genes were significantly higher in breast cancer tissue than in healthy adjacent tissue. The degree of change in the expression of these genes is shown in Table 2.

Table 2. The alteration in the expression rate of DRD1 and DRD5 genes in breast cancer tissue and healthy adjacent tissue of the same individuals diagnosed with breast cancer

Gene	p-value	Rate of change	Standard error	changes
DRD1	0.5** (<0.0001)	6.278	±1.54	up
DRD5	0.5** (<0.0001)	4.192	±1	up

*The asterisk denotes a statistically significant change at a significance level of 0.05
In chart 1, the bar graph of the qPCR analysis demonstrates that the gene encoding the DRD5 receptor was significantly increased in cancerous tissue compared with healthy adjacent tissue ($p<0.001$).

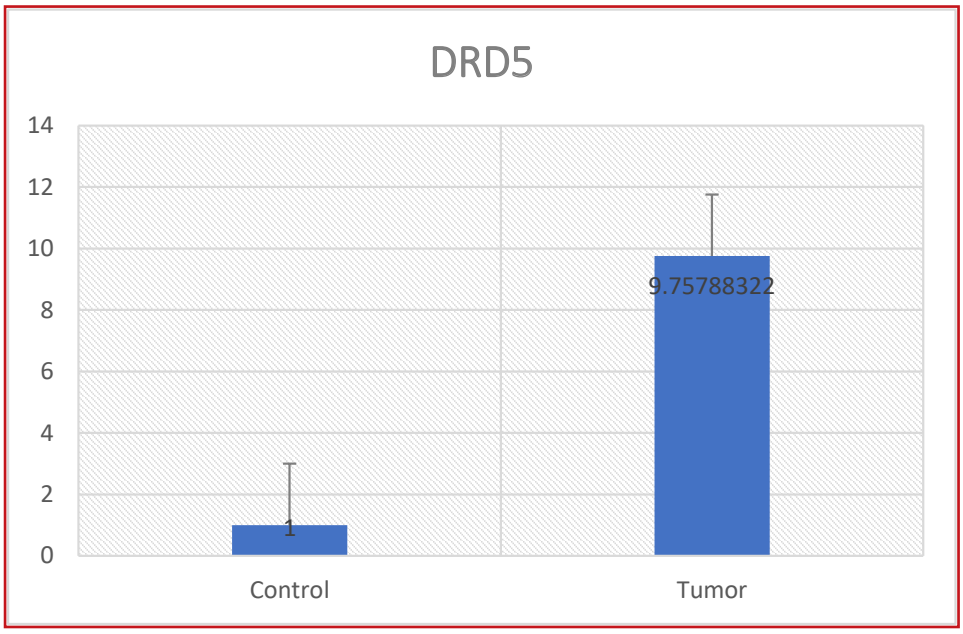

Chart 1. The bar graph for depicting the alteration in gene expression of DRD5 in cancerous tissue compared with healthy adjacent tissue

Chart 2 also displays the bar graph of alterations in DRD1 gene expression in cancerous and healthy adjacent tissues. According to the obtained results, the expression of the DRD1 gene was significantly higher in cancerous tissues than that of the healthy adjoining tissue ($p< 0.001$).

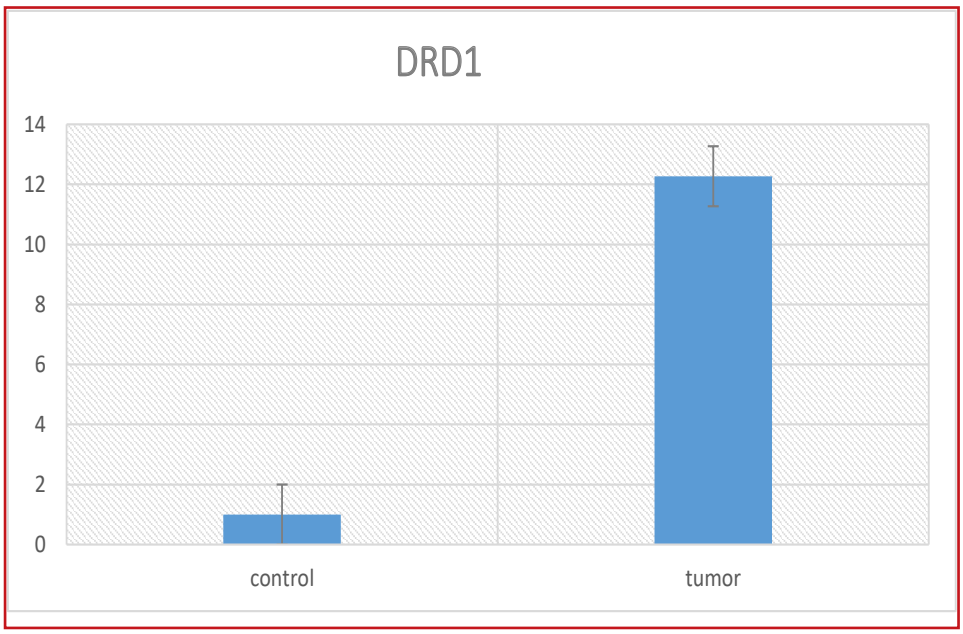

Chart 2. The bar graph of changes in DRD1 gene expression in cancerous tissue compared with healthy adjacent tissue

It was found that the rate of increment in the expression of DRD1 was pronounced in cancerous tissue than the expression of DRD5 in the same tissue (Chart 3).

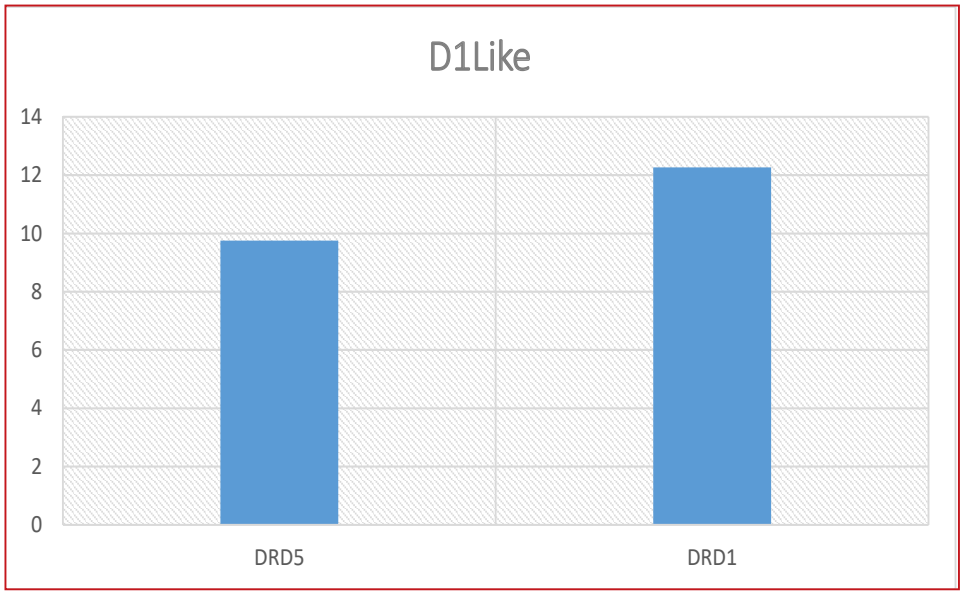

Chart 3. The diagram of changes in DRD1 gene expression versus the expression of DRD5 in cancerous tissue

Cell experimental results

MTT Assay analysis

The obtained data were expressed as dose-response for each quercetin concentration. According to the results of the MTT assay in which the effect of quercetin plant flavonoid was investigated on the Hs578t cancer cell line (Chart 4a), it was found that quercetin causes death in cancerous tissue in a dose-dependent manner. At a 24-hour incubation time, the IC50 value of quercetin was calculated to be 121.454 µM, while at 48- and 72-hour treatment incubation periods, such values were 73.635 µM and 64.309 µM, respectively. The MTT assay was also conducted against the human skin fibroblast cell line to compare the results (Chart 4b). Quercetin in 24, 48, and 72 h has no detrimental effect on HDF cells at specified doses (up to 175 µM). According to figure 4, quercetin selectively targets cancer cells and has no cytotoxicity effect on normal cells. These findings confirm that quercetin can cause death in cancer cells in a dose- and time-dependent manner.

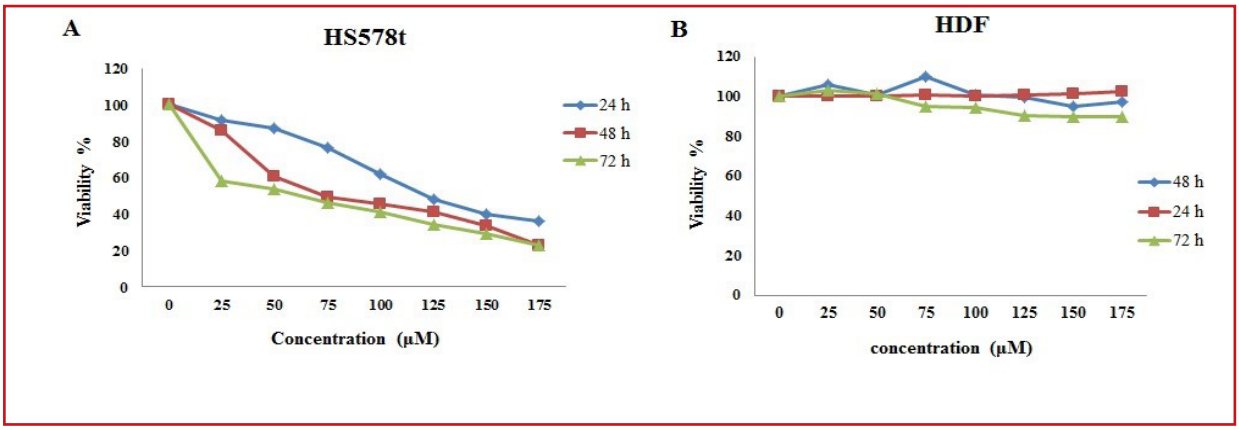

Chart 4. The MTT assay results on the Hs578t cancer cell line (A) and HDF (B) treated with quercetin and incubated at 24, 48, and 72 hours

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Analysis of gene expression following quercetin treatment

Changes in the expression of genes encoding DRD1 and DRD5 in the Hs578t breast cancer cell line following treatment with quercetin revealed that this compound is able to inhibit the expression of DRD1 and DRD5 genes. Chart 5 depicts the results of gene expression analysis after 24, 48, and 72 hours of quercetin treatment in cancer cells showing a significant decrease in the expression of the DRD5 gene. According to chart 5, the expression of DRD5 was significantly reduced in cells treated with quercetin after 24 ($p<0.002$), 48 ($p<0.01$), and 72 ($p<0.001$) hours.

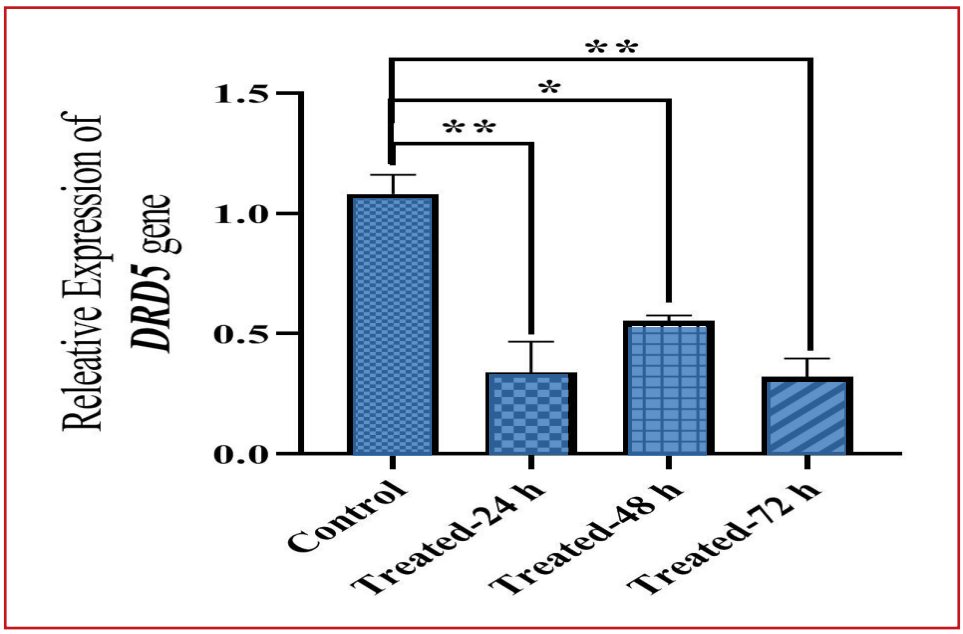

Chart 5. The comparison of changes in the expression of DRD5 receptor between quercetin-treated Hs578t cells and untreated Hs578t cells

However, the expression of the DRD1 gene after 72 hours of treatment with quercetin showed a significant decrease ($p<0.03$), as shown in chart 6. Moreover, a slight decrease in DRD1 expression was seen at 24 and 48 hours, but this decrease was not statistically significant.

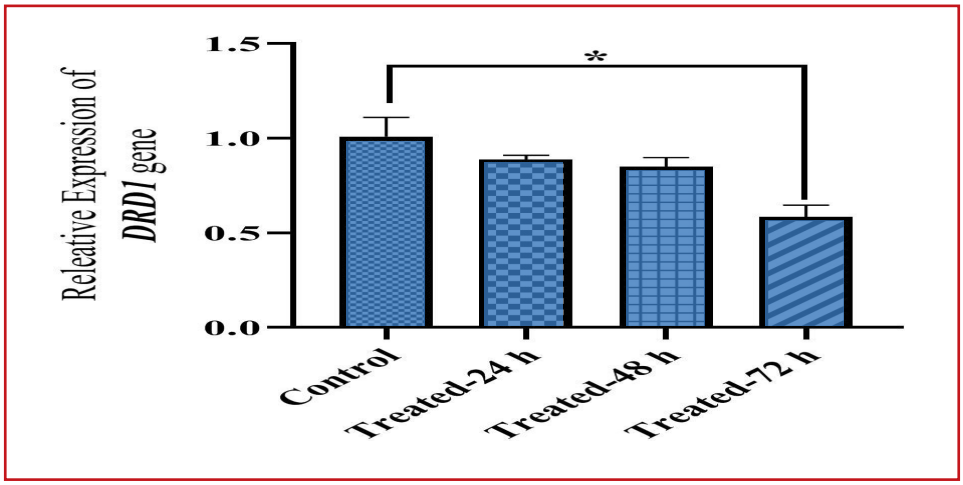

Chart 6. The comparison of changes in the expression of DRD1 receptor between quercetin-treated Hs578t cells and untreated Hs578t cells

Discussion

The drugs available for cancer treatment are costly, and the treatment outcome is not always definitive. Also, significant side effects have been reported for chemotherapeutic agents. Chemotherapy drugs are spread throughout the body. The side effects of chemotherapy may be more severe during treatment. Most side effects are temporary and go away after treatment. In some cases, chemotherapy can have long-term or permanent effects (4). Some chemotherapy drugs can affect the nerves in the hands and feet, leading to numbness, pain, tingling, sensitivity to cold and heat, or weakness in the limbs. These side effects usually go away after treatment or one year after chemotherapy. In some cases, they may be long-lasting (6). To further explain, the remedies currently used for patients with breast cancer are limited to a few treatment options; radiotherapy, hormone therapy, and adjuvant chemotherapy for these patients after surgery have severe side effects. Therefore, finding new therapeutic agents with low toxicity is essential for treating the patients (4, 6, 15), due to the relationship between dopamine and its receptors with various types of cancer, including breast cancer, the current research aimed to evaluate the alterations in the expression of DRD1 and DRD5 genes. Neurotransmitters have different effects on the cells of various body tissues and endocrine cells, such as regulatory effects. It is now known that several neurotransmitters, including serotonin, dopamine, and epinephrine, as well as other types of hormones such as oxytocin and prolactin, effectively contribute to the treatment or pathogenesis of some diseases by interacting with other systems. There are two types of dopamine receptors, namely D1-like and D2-like receptors. D1-like receptors exert stimulatory effects on cells and include DRD1 and DRD5, whereas D2-like receptors have inhibitory roles and include DRD2, DRD3, and DRD4. It has been shown that D1-like receptors can activate the adenylate cyclase pathway, leading to the altered expression of some genes and transmission of molecular messages within the cells. Any imbalance between these two groups of receptors can lead to different diseases (16).

Several lines of evidence indicate that dopamine and its cognate receptors can influence multiple signaling pathways such as PI3K and MAPK (17). Also, the role of dopamine receptors in the pathogenesis of some systemic diseases, such as lung cancer, psoriasis, and schizophrenia has been addressed (18). Previous research has found that the expression of dopamine receptors in peripheral blood mononuclear cells (PBMC) of breast cancer patients was higher than in healthy subjects (3). In this study, significant changes were detected in the expression of genes encoding dopamine receptors (DRD1 and DRD5) in cancerous tissues compared with healthy tissues at the proximity of breast tumors. Quercetin is a plant flavonoid with anti-inflammatory and antioxidant activity (14), preventing tumor proliferation by inducing cell cycle arrest. It is also a known factor in the treatment of cancer mediated by autophagy (13).

In-vivo and in-vitro experiments have demonstrated that quercetin can have antitumor properties by altering cell cycle trends, inhibiting cell proliferation, inducing apoptosis, suppressing angiogenesis, and metastasis. Quercetin can induce apoptosis and activate autophagy-related pathways in cancer cells by modulating the catenin, mTOR / Wnt / Akt / PI3K / ERK1,2 / MAPK pathways. Quercetin has two-phase and dose-dependent effects, as it acts as an antioxidant in low concentrations, whereas as a pro-oxidant in high concentrations. On the other hand, this plant flavonoid halts the cell cycle at the G1 stage and prevents the proliferation of breast cancer cell lines MDA-MB-453 and SK-BR in a dose-dependent manner. Quercetin also increases the induction of apoptosis by an increase in the expression of caspase-3. At the same time, it reduces metastasis by reducing the secretion of vascular endothelial growth factor (VEGF) and matrix metalloproteinase protein (MMP) levels. Besides, it also exerts its metabolic effect on cancer cells and inhibits glycolysis enzymes by interfering

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with PI3K / Akt and mTOR pathways (19). Our findings showed that quercetin could promote cell death in the Hs578t cell line in a time- and dose-dependent manner. It also reduces the expression of genes encoding DRD1 and DRD5 in quercetin-treated Hs578t cells compared with untreated Hs578t cells.

Conclusion

In the current research, for the first time, the expression of D1-like receptors was assessed in cancerous tissue and healthy adjacent tissue of breast cancer patients. It was found that the expression of DRD1 and DRD5 genes was markedly higher in cancerous tissue than in healthy adjacent tissue.

The inhibitory effects of a neurotransmitter in the proliferation and migration of cancer cells through their cognate receptors have been well documented. According to the literature, dopamine and its agonists can incite programmed cell death in tumors. Regarding the expression status of DRD1 and DRD5 genes in the Hs578t cell line, it would be plausible that the administration of dopamine agonists could attenuate the disease course in patients with breast cancer. The flavonoid quercetin induced apoptosis and cell death in the Hs578t breast cancer cell line, indicating that quercetin could reduce the expression of D1-like encoding genes.

Data Availability Statement

All data related to this research have been included in the manuscript.

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The Ethics Committee of Shahid Beheshti University of Medical Sciences approved the experimental procedures of the present research (IR.SBMU.RETECH.REC.1397.562)

Conflict of Interest

The authors have no conflicts of interest to declare.

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