

## RESEARCH ARTICLE

## Evaluation of Gene Expression Changes of Serotonin Receptors, 5-HT3AR and 5-HT2AR as Main Stress Factors in Breast Cancer Patients

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### Abstract

Breast cancer is a serious and potentially lethal multi-factor disease among 40-50 aged women in both developed and developing countries. Also, various studies have pointed to roles of neurotransmitters like serotonin in development of cancers, through action on various types of receptors. This study was conducted to evaluate serotonin receptor (5HT2AR and 5HT3AR) genes expression in peripheral blood mononuclear cells (PBMCs) of breast cancer patients in comparison with the healthy people and in the MCF7 cell line. Peripheral blood samples were obtained from 30 patients and 30 healthy individuals. Total RNA was extracted from PBMCs and MCF-7 cells. and 5HT2AR and 5HT3AR were detected by RT-PCR techniques. Finally, serotonin receptor gene expression variation in breast cancer patients and MCF-7 cells were determined by real time-PCR. This latter indicated significant promotion in expression of 5HT3AR and 5HT2AR in PBMCs in breast cancer patients but expression of 5HT2AR in the MCF-7 cell line was significantly decreased. In conclusion, after performing complimentary tests, determine of gene expression changes in serotonin receptors (5HT2AR and 5HT3AR) may be useful as a new approach in treatment of breast cancer based on use of antagonists.

**Keywords:** Breast cancer - serotonin receptors - gene expression - real time-PCR

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### Introduction

Breast cancer is a serious and potentially lethal disease among 40-50 aged women and its incident rate is one out of eight in the United States and one out of thirty five people in Asia. Incidence of breast carcinoma is 10 per 100,000 of population with 7000 new cases annually in Iran. The age specific rate in females is 23.16/100,000. Every year 1200 women die due to breast carcinoma with burden of 0/31 DALY/1000, which is the third in ranking of burden of disease (BOD) among women and seventh rank in both sexes. It is expected that the number of new cases will rise up to 20,000,000 by 2015 globally and 12,000,000 deaths annually 70% of which will occur in developing countries (Akbari et al., 2011; Haghigat et al., 2012). It is a multi-factor disease, genetically mutation and environmental condition plays important role in the breast cancer (Madigan et al., 1995). Only 5-10 percent of the breast cancer patients have a known mutation and about 70% of breast cancer patient are not related to genetically mutation. The breast cancer is

commonplace disease between women but 1-2 percent of patients were men and because of their late diagnosis, men have a higher mortality rate (Donegan and Redlich, 1996; Haghigat et al., 2012). Whereas the greatest risk factor for breast cancer are female, ageing parity, and breastfeeding inheritance, but environmental factors are critically important in initiation of disease (Helmrich et al., 1983; Akbari et al., 2011). Environmental factors like stress, can lead to the disruption of the balance of central nervous system and immune system. Relationship between stress and breast cancer development remain controversial (Pinquart and Duberstein, 2010; Antonova et al., 2011). Many researchers believed that mental and psychological conditions have a fundamental role in progression of cancers (Reiche et al., 2004). Interaction and balance of nervous and immune systems are essential for maintaining of homeostasis. There are strong evidence that the CNS, endocrine and immune system talk to each other and this process mediated with neurotransmitters (serotonin, norepinephrine and dopamine), neurohormones (growth hormone and prolactin) and cytokines (interleukin1,

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TNF $\alpha$ , interferon  $\alpha$  and  $\gamma$ ) (Reiche et al., 2004). Serotonin is one of the most ancient neurotransmitter and its receptor is found in divers' organism from planaria to human. The serotonin has several roles in normal brain including mood state, memory, anxiety, emotion, sex, sleep and many others (Webster et al., 2002). Serotonin or 5-hydroxytryptamine (5-HT) receptor includes seven classes from 5HTR1 to 5HTR7 and many subtypes. The 5HT2A class is subdivided into 5HT2a, 5HT2b and 5HT2c which are coupled to G-Protein. 5HT3A receptor, an inotropic receptor, affects the potassium voltage gated channel, permit actions sensitive to Na $^+$  and play important role in regulation of Na $^+$ /K $^+$  movement in some immune cells (Kopparapu et al., 2013). Furthermore, some studies showed that serotonin can promote transition T cells from S-phase to G2/M in the cell cycle via serotonin receptors such as 5HT2A and 5HT3A receptors. On the other hand, some studies revealed that stressful conditions have an inhibitory role in immune system cells including T-lymphocyte mitogenesis, natural killer cell (NKC) activity and production of interleukin 2 and interferon  $\gamma$  (Reiche et al., 2004). That is one of the most important mechanisms against development of tumors cells. So, the study of serotonin receptors gene expression changes in the progression of breast cancer patient seems unavoidable. The major aim of this study is to evaluate the relationship between increasing incidence of breast cancer and psychosocial stress factor at molecular level.

## Materials and Methods

We studied the thirty patients suffering from breast cancer, admitted to the CRC and demographic data of Shohada Hospital of Tehran, Iran, were enrolled in this study. Five mL of peripheral blood samples was taken from each patient, among which there were 1 male and 29 female. The patient population had a mean age of 34 years and ranged from 20 to 40 years. Samples were also taken from 30 healthy subjects, of which there were 15 male and 15 female. The healthy group had a mean age of 35 years, where ages ranged from 20 to 40 years. Healthy participants were excluded provided that they were diagnosed with cigarette smoking, alcohol drinking or mental illness.

All clinical procedures were approved by the local ethical committee of CRC Shohada Hospital. All participants gave their informed consent prior to their inclusion in the study. PBMCs from collected samples were isolated using density gradient centrifuge by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation whose density was 1.077 $\pm$ 0.001 g/ml (20°C) and its osmolity 290 $\pm$ 15 mosm. Horizontal swing out centrifuge was used for cells isolation with 2500 rpm, at 20 minute and 1.0 speed regulation. The buffy coat (lymphocyte layer) was collected and centrifuged with 1600 rpm, at 10 minute and 2.0 gulation. Finally the resulting pellet was washed three times in Phosphate buffer saline (PBS), resulting in a final count of 10 $^6$  to 3 $\times$ 10 $^6$  cells for total RNA extraction. MCF7 breast cancer cell line provided from Gibco (Germany) and Pasteur institute of Iran. The cells were cultured in DMEM supplemented with 10

percent fetal bovine serum. To evaluate gene expression of serotonin receptors, 1 $\times$ 10 $^6$  of MCF-7 cells were used for RNA extraction

Total RNA was extracted using High pure RNA isolation kit (Roche, Germany) according to the manufacturer's instructions. To confirm existing total RNA in the samples and to verify previous steps, we continued by conventional PCR and gel electrophoresis. The RNA extracted samples read with Nano drop to measuring the RNA concentration in samples and in this stage we synchronize all other samples. The RNA (1 $\mu$ g) from each sample was used to synthesize first-strand cDNA by using cDNA Synthesis Kit Fermentase, USA. PCR cycles used Techne Flexigen, Minneapolis, MN, USA. PCR products were separated using 2% agarose gel electrophoresis supplemented with Ethidium bromide for DNA product visualization under UV light. After verifying RNA extraction, product was amplified using RT-PCR with primer sets specific for 5HT3AR and 5HT2AR. Also Beta-actin was used as an internal control (Table I). Beta-actin, 5HT2AR and 5-HT3AR transcripts were quantified in samples using real time PCR (Rotor gene, Corbett, Germany) and light cycler fast start DNA master plus SYBR green I kit (Roche, Germany) with specific primer (Table 1). PCR products of serotonin receptor Beta-actin, 5HTR2A and 5-HT3A were sequenced by DNA sequencer ABI 3700 capillary system (Applied Biosystem, USA) to confirm amplicon sequences. The number of samples was determined by Minitab 16.1 software. Efficiency of each reaction was determined precisely by Linreg software. Real time PCR data were analyzed by Rest 2005 and 2009 software. The relationship between changes in serotonin receptor gene expression data obtained from case patients on age, disease stage, HER2 and ER gene expression was evaluated by SPSS 16.0 software. Also, the relationship Significant value in this study was less than 0.05 ( $p<0.05$ ).

## Results

We examined the change in expression of serotonin receptor subtype 5HT3AR and 5HT2AR genes were evaluated in breast cancer patients PBMC, healthy individuals PBMC and human breast cancer cell line (MCF-7) by a highly sensitive method of real-time PCR. The amplicon sequences and size were corroborated using gel electrophoresis and sequencing. The results of RT-PCR showed that serotonin receptor subtype such as 5HT3AR and 5HT2AR were expressed in PBMC of breast cancer patient , healthy individuals and human breast cancer cell- line (MCF-7) as previously published (Sonier et al., 2006; Shariati et al., 2009).

But there were differences between the genes expression rates. Compared to healthy persons' PBMC, 5HT3AR and 5HT2AR receptor genes expression rates were increased significantly in patient (Table 2). We also measured gene expression of serotonin receptors in MCF7 cell line and compared them with patients and healthy persons. So, we found that 5HT3AR was increased in MCF-7 cells significantly compared with the healthy persons' profile and mRNA rates of 5HTR2A receptor gene were significantly reduced as well. Also, it was

**Table 1. Primer Sequences used in RT-PCR and Real Time PCR**

Locus	Primers	Accession number	Amplicon size
B-actin-forward	5'- AGACGCAGGATGGCATGGG-3'	NM_001101.3	161bp
B-actin-reverse	5'-GAGACCTTCAACACCCCAGCC-3'		
5HT2AR -forward	5'- TTGGGCTACAGGACGATT -3'	NM_001165947.2	383bp
5HT2AR -reverse	5'- GAAGAAAGGGCACCATC -3'		
5HT3AR-forward	5'- CTATGTGGTCATCCGCCGG -3'	NM_001161772.2	457bp
5HT3AR- reverse	5'- CTATGTGGTCATCCGCCGG -3'		

**Table 2. Compared to Healthy Persons' PBMC 5HT2AR & 5HT3AR Genes Expression in Breast Cancer Patients PBMCs were Increased Significantly**

Gene	p value	Rate of changes	Changes
5HT2AR	0.027	2.769±2.4	UP
5HT3AR	0.001	3.6±2.8	UP
5HT2AR*	0.045	5.9±0.00097	DOWN
5HT3AR*	0.002	6.4±3.9	UP

\* In human breast cancer cells (MCF-7), 5HT3AR had significant promotion in comparison to healthy individuals' PBMCs, but 5HT2AR was decreased

**Table 3. Relationship between Changes in 5HT3AR Gene Expression and Disease Stages**

5HT3AR	ANOVA				
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	114.216	3	38.072	4.780	.012
Within Groups	151.327	27	7.965		
Total	265.543	30			

the first time that two types of receptors with different structure were evaluated simultaneously in breast cancer. Furthermore, 5HT2AR was decreased significantly in MCF-7 cell line in comparison to breast cancer patients' PBMC (Table 2). Also, sequencing analysis by capillary sequencer did not show any structural changes in genes in breast 5HT2AR and 5HT3AR cancer patients, compared to sequences available through NCBI. Investigate the relationship between serotonin receptor gene expression with age, disease stage, HER2 and ER expression showed that 5HT3AR gene expression was significantly increased in all stages of breast cancer (Table 3). However, such a significant relationship between increased expressions of 5HT2AR receptor with different stages of disease was not observed. There were also no significant association between serotonin receptor gene expression increases with age of the patient, human estrogen Receptor (HER2) and estrogen receptor (ER) genes expression.

## Discussion

Serotonin is an ancient neurotransmitter with a long evolutionary history and serotonin receptor virtually exist in peripheral blood cells and all of region of central nervous system and play different roles in sleep, memory, mood, aggression, appetite and sex. (Prelusky, 1996). It has been demonstrated that distress among breast cancer patients can be reduced via group therapy for women especially those with high initial distress (Yavuzsen et al., 2012). So far, the relationship between depression and breast cancer remain controversial. There are different reports for promoting and descending the risk of breast

cancer through depression conditions (Persky et al., 1987; Schoevers et al., 2000; Aro et al., 2005; Nazlican et al., 2012). Many studies indicated that some cytokine (IL-1b, IF-a, IF- c and TNF- $\alpha$ ) whether directly or indirectly can modulate metabolism of serotonin via peripheral depletion of tryptophan. Also, some cytokines can stimulate HPA axis and induce depression (Leonard, 2010). Depression has been shown that attenuate of the immune system as lower NKC activity and T cell subpopulations are occurred which are needed to defense against cancer disease. Also, the level of tryptophan as a precursor of serotonin is low in depression mood and tryptophan-free amino acid diet has a negative effect on therapeutic anti depression drug (Herbert and Cohen, 1993). Some anti-depression drugs like serotonin specific reuptake inhibitor (SSRI) can inhibit reuptake of serotonin and increase the serotonin level in synaptic clefts (Moorman et al., 2003; Wang et al., 2001). Some clinical studies demonstrated that 5HT3A receptor antagonists have anxiolytic effects in humans. The presence of serotonin receptors on the PBMC cells and nervous system indicates the role of serotonin in relationship between CNS and immune system. Thus, disruption in this relation can lead to relieve the symptoms of cancer. In a dose-dependent manner mitogenic, the effect of serotonin on B-lymphocyte proliferation and hepatocytes has been observed (Iken et al., 1995). Phospholipase D (PLD) dependent protein kinase C (PKC) pathway can be activated in T-cells via induction of 5HT3AR (Khan and Hichami, 1999). Serotonin also has a mitogen effect on hepatocytes and promotes liver regeneration (Soll et al., 2009). Serotonin cooperates as a growth factor in several type of cancer and non-cancer cells (Siddiqui et al., 2005). This data was in line with our results which showing increases level of 5HT3AR expression not only in PBMC on breast cancer patient but also in MCF7 cell line compared to normal individual PBMC.

Also, 5HT2AR, a member of G-protein-coupled receptors, is expressed in several tissues and has variety of functions (Hannon and Hoyer, 2008) including: arterial fibroblast proliferation via mitogen activated protein kinase (MAPK) pathway (Welsh et al., 2004) and migration of aortic muscle cell by activation of PLC and PKC pathways (Cogolludo et al., 2006). Another researchers reported that increase level of 5HT2AR and activity of serotonin lead to apoptosis and activity of 5HT1AR decreased apoptotic signaling via caspase3 suppression (Adayev et al., 2003).

Moreover, different expression pattern of serotonin receptors (5-HTRs) was observed in patient with breast cancer and this diversity can be applied as a prominent

index for understanding the distribution and function of each subtype in the pathogenesis of breast cancer (Kopparapu et al., 2013). Modification of 5HT2AR can promote the risk of breast cancer. Also, the results of this study indicated that expression of 5HTR2a was increased in breast cancer patients PBMC compare to healthy individuals. While, expression of this receptor has descending effects on MCF-7 cells significantly. In conclusion, simultaneity analysis of 5HT2AR and 5HT3AR showed that 5HT3AR increases and 5HT2AR descending expression in MCF-7 cell line can promote the risk of breast cancer.

At least, regarding to these studies observations, performing of additional studies using 5HT3AR antagonists and 5HT2AR agonists as new therapeutic strategy and definition of serotonin receptors gene expression profile for the diagnosis and curmeant of breast cancer patients seems hopeful.

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