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Significant Association between Catechol Amine O-Methyl Transferase (COMT) Gene Expression Changes and Breast Cancer Pathogenesis

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Abstract

Objective: The most prevalent cancer among Iranian women and worldwide is breast cancer. Catechol amine-o-methyltransfrase (COMT) methylates some neurotransmitters such as dopamine which is secreted in chronic stress conditions and controls the growth of cancer cells. This study was conducted to evaluate the role of alterations in the gene expression of COMT in peripheral blood mononuclear cells (PBMCs) and its specific enzyme activity in blood samples of breast cancer patients as a stress factors suppressor.

Methods: Peripheral blood samples were obtained from 40 patients and 40 healthy individuals. Total mRNA was extracted from PBMCs and their plasma was stored to evaluate specific enzyme activity changes. RT-PCR was performed to confirm the presence of COMT gene expression in PBMCs. Expression changes of COMT gene were evaluated by real time PCR technique. Finally, specific enzyme activity of COMT was investigated.

Results: We observed that COMT gene expression in PBMCs of breast cancer patients was increased compared to healthy individuals. In addition, the enzyme activity of COMT was elevated in breast cancer patients compared to healthy individuals.

Conclusion: Increasing in COMT gene expression in PBMCs lead to the further amount of dopamine methylation and promotion of breast cancer. Therefore, assessment of alterations in COMT as metabolizers of dopamine (a risk factor) in diagnosis of breast cancer seems to be necessary and using selected appropriate drugs such as COMT inhibitors after performing complimentary tests may be promising perspective in breast cancer therapy.

Keywords: Breast cancer; Catechol amine-o-methyltransfrase; Methylation; Peripheral blood mononuclear cells; Real time-PCR; Risk factor; Specific enzyme activity

Abbreviations

COMT: Catechol amine-O-methyltransfrase; PBMCs: Peripheral Blood Mononuclear Cells; CNS: Central Nervous System; cAMP: cyclic Adenosine Mono Phosphate; SAM, S-adenosyl-L-methionine; PBS: Phosphate Buffered Saline; DHAP: 3,4-Dihydroxyacetophenone; DTT: Dithiothreitol; PR: Progesterone Receptor; HER: Human Epidermal Growth Factor Receptor 2; Er: Estrogen Receptor; Vpf/Vegf: Vascular Permeability Factor/Vascular Endothelial Growth Factor; MAP: Mitogen Activated Protein

Introduction

Breast cancer as the most common and frequent cancer-related death is prevalent in Iranian and world women. The main reason and mechanism for incidence of breast cancer are still ambiguous [1]. Among hereditary genetic markers, occurrence of mutations in BRCA1 and BRCA2 are associated with increased risk of the disease [2]. However, they were only observed in about five percent of breast cancer patients and the low percentages of genetic markers explain the weak penetrance of these factors in disease cases [2]. Several studies have reported that a high number of life events and stressful conditions can increase the risk of breast cancer among women. Health and homeostasis of the body are preserved via interaction and balance of nervous and immune systems [3-5]. Central nervous system (CNS), endocrine and immune system interaction are mediated by neurotransmitters (serotonin, norepinephrine and dopamine), neurohormons (growth hormone and prolactin) and cytokines (interleukin, TNFα, interferon α and γ). Neurotransmitters were recognized as cell mediators of nervous system for a long time [6]; however, they have effects on the cells in other tissues and persuade them to show different behavior via their special receptors. For instance, catechol amines especially dopamine and serotonin influence the immune system functions or these mediators can affect tumor cells [3]. Neurotransmitters affect cells through their special receptors which have been changed in stressful conditions [3,6]. One of the neurotransmitters, dopamine, which increases in chronic stress also the expression of its receptors are changed. Dopamine affects different types of cells via its various receptors. These receptors are from G-protein family containing D1and D2 family receptors. Both D1 and D5 receptors are members of D1-like family that play a role as a stimulator and induce increased intracellular cyclic adenosine mono phosphate (cAMP). In contrast, D2-D4 receptors, members of D2-like

receptor family, inhibit intracellular cAMP on stimulation [7]. Different studies showed that these receptor gene expression profiles were associated with chronic stress condition in various types of diseases including lupus erythematosus, schizophrenia, non-small lung cancer and breast cancer) [8-10]. Although stressful conditions can increase the amount of dopamine and inhibit the tumor growth and attachment [4], it needs to be noted that the body has a regulatory system for descending of dopamine rate and whole of these variations could be feckless, if dopamine was methylated and metabolized [11].

COMT plays a key role in the metabolism of dopamine and catechol estrogens [12]. COMT catalyses the transfer of a methyl group from S-adenosyl-L-methionine to one of the two hydroxyl groups of catecholic compounds, including L-dopa, catechol estrogens, endogenous and exogenous catecholamines as well as their hydroxylated metabolites [12]. The relation between COMT val158met polymorphism and incidence of different diseases such as neural pain, schizophrenia, obsessive-compulsive disorder and breast cancer has been reported [13-16]. However, over expression and excessive activity of COMT can lead to depression and it can be harmful [12]. There were no explanations about the distinctive character of COMT in incidence of diseases especially breast cancer and most of studies indicated the relation between susceptibility of patients to COMT haplotype [14,17].

Regarding to the importance of COMT, chronic stress and incidence of breast cancer, gene expression changes of COMT in pathogenesis of diseases related to chronic stress in breast cancer patients have not been reported yet. To the best of our knowledge, it is assumed that the stress can lead to alterations in expression of COMT gene and this could be attributed to the development of the disease. Therefore, evaluation of expression level changes of COMT gene and its specific enzyme activity associated with chronic stress in breast cancer patients seem to be necessary.

Materials and Methods

This study consists of two parts, including the study of gene expression and evaluation of specific enzyme activity.

Samples

The naive breast cancer patient group included 40 patients with the average of 35 years old (ranged from 20 to 55 years) who referred to Imam Khomaini Hospital, Tehran Medical Sciences University, Tehran, Iran. All of the cases suffered from chronic stress and they had a life event in their background. The control group consists of 40 healthy individuals with the average of 32 years old (ranged from 20 to 50 years). Patient consent for all of samples was obtained according to the Declaration of Helsinki principles.

All pathological information related to patients was collected from the Pathology Department of Hospital (Table1). Exclusion and inclusion criteria were determined according to the revised criteria for classification of breast cancer patients. 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 (5 ml) were obtained from the cubical vein and were collected in cell preparation tubes containing an anticoagulant (Heparin). First, blood samples were centrifuged in 300 g for 5 min and plasma of samples was preserved for enzyme assay (as substrate). Then, blood samples were diluted with an equal volume of phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMC) were isolated from 4 ml 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 850 g for 20 minutes and 1.0 speed regulation. The Buffy coat (lymphocyte layer) was collected and centrifuged in 300 g for 10 minutes and 2.0 Speed regulations. Finally, the resulting pellet was washed in PBS.

Demographic data	specimen number	Age(range)	Sex
Stage I	8	31-54	Female
Stage II	17	20-55	Female
Stage III	12	27-55	Female
Stage IV	3	40-55	Female
ER-positive	31	27-55	Female
PR- positive	28	27-55	Female
HER2- Positive	14	33-55	Female
p53	4	20-53	Female

Table 1: Pathological information related to patients.

RT-PCR technique

The total mRNA was isolated from PBMC by High pure RNA isolation Kit (Roche, Germany), according to the manufacturer's instructions. Concentration of extracted RNA samples read with Nanodrop to synchronize all other samples. The RNA (1µg) from each sample was used to synthesize first-strand cDNA by cDNA synthesis kit (Fermentase, Germany). Similarly, cDNA synthesis was carried out based on manufacture's protocols. There were primers designed using oligo5 software (WWW.oligo.net) for COMT and b-actin genes as housekeeping gene based on GenBank sequences and their specificity theoretically checked by BLAST database search against nucleotide reference NCBI database (Table2).

To confirm the presence of COMT gene in PBMC cells, a common PCR technique was carried out for all samples in a final volume of 20 µl with 1 Unit of Taq DNA polymerase (Sinagene, Iran). Reaction mixtures contained 2–2.5 mM MgCl, 0.5 mM each of the dNTPs, 0.8–1pM primers, 2.5 µL Taq DNA polymerase (Sinagene, Iran), and 1 µL of the cDNA was used as a template in each RT-PCR reaction. In order to amplify the COMT and b-actin genes, PCR was initiated at 95°C for 5 min and amplified during 35 cycles at 95°C for 1 min, 54 and 62°C for 40 s and 72°C for 1 min and followed by a final extension step at 72°C for 10 min. Finally, the PCR products were visualized by gel electrophoresis on a 2% agarose gel. Moreover, positive control amplification was used experimentally to verify of primers.

Real time PCR

Real time PCR was also carried out by 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 COMT mRNA and β-actin as the internal control in a Real Time-PCR instrument (Corbett, Germany).

Locus	Primers	Accession number (Gene Bank)	Size
β-actin-F	5′-AGACGCAGGATGGCATGGG-3′	NM_001101.3	161bp
β-actin-R	5-GAGACCTTCAACACCCCAGCC-3′		
COMT-F	5′-CTGGAGGCCATTGACACCTA -3′	NM_000754.3	202bp
COMT-R	5′-GGTTGATCTCGATGGTGATGAG -3′		

Table 2: Primer sequences used in RT-PCR and Real time-PCR.

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 10 min followed by an amplification program (primer annealing, amplification and quantification) repeated for 45 cycles. The amplification program was 95°C for 10 sec, 54 and 62°C for 10 sec, respectively for COMT to β-actin and 72°C for 10 sec with a single fluorescence acquisition at the end of the elongation step. The third 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 was also confirmed by electrophoresis of PCR products that showed only one band of the expected size.

Sequencing

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

Enzyme assay

For each dilution test, following materials were pipetted (in 250 μl) in each well. 50 μl of each 3,4 Dihydroxyacetophenone (DHAP) (sigma, Germany), 5mM S-adenosyl-L-methionine(SAM) (Sigma, Germany), 6 mM Magnesium Chloride and 20 mM Dithiothreitol (DTT) (Sigma, Germany) mixed by swirling and equilibrate to 37°C. Then, 50 μl of plasma from each samples as COMT solution were added in different wells. Then, the dilutions mixed by swirling and incubated at 37°C for 60 min or preparation of blank 50 μl water used instead of substrate in respective blank well. Finally, absorbance was measured at 340 nm by an ELISA plate reader (ELX800TM, USA). This test was performed in triplicate form [18]. Finally, specific enzyme activity was calculated for normalization of each sample. The specific COMT enzyme activity is evaluated for enzyme units per milligram of total protein (expressed in μmol/min/mg) [18].

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 COMT gene expression and the items such as age of patients, Progesterone receptor (PR), D2 receptors family, human epidermal growth factor Receptor 2 (HER-2) and estrogen receptor(ER) gene expression was assessed by SPSS software (version 16.0). One way Anova test was performed to investigate of the relationship between the changes in COMT gene expression and the stages of the disease. Furthermore, relative COMT specific enzyme activity changes were evaluated by independent t-test. In the current study, the p-value less than 0.05 ($P<0.05$) was considered statistically significant.

Results

Pathologic analysis

Pathology examination results showed that 20, 30, 42 and 8 percent of the patients were respectively related to stages of I, II, III, and IV. Almost 77 percent of patients expressed the estrogen receptor ER and 70 percent of them expressed PR. Also, 35 percent of patients were HER2 positive. 10 percent of patients had a mutation in P53 protein.

PBMC expresses COMET in breast cancer

In this study, expression of the COMT gene was evaluated in PBMC of breast cancer patients and healthy individuals PBMC. The results of RT-PCR showed that all types of dopamine receptors were expressed in PBMC of breast cancer and healthy individuals (Figure1).

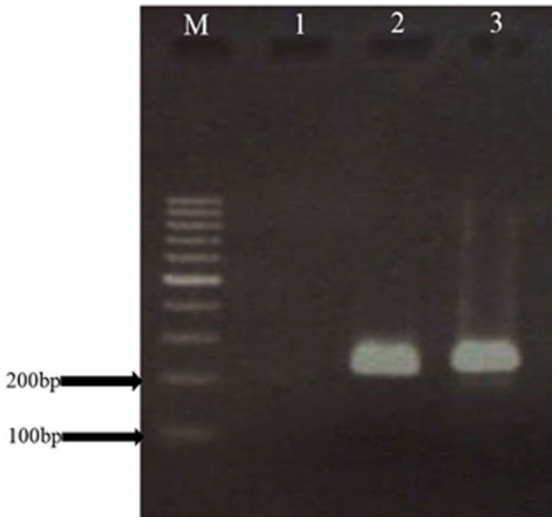

Figure 1: Show COMT gene expression in PBMCs cells. Lane M: Molecular size marker 100bp (Fermentase, Germany), Lane1: control negative, Lane2: control positive of COMT-202bp, Lane3: COMT-202bp

There was a considerable difference in gene expression rate between patients and healthy controls, COMT enzyme gene in PBMC of breast cancer cases that showed a significant over expression compared to their counterparts in healthy individuals. Furthermore, all sequenced fragments were checked by BLAST database against the nucleotide reference NCBI database and confirmed amplicon sequences.

Expression analysis

Statistical analysis described a significant correlation between COMT specific activity and over expression of the enzyme. Investigation of the association of COMT 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 COMT gene in all stages of breast cancer (Table 3). One way anova analysis confirmed the significant association between the elevating expressions of COMT and the progression (stages) of the disease (P-value<0.001). In addition, there was a significant correlation between COMT and D2- receptor of dopamine (DRD2) at P-value<0.039. Over expressions of COMT gene was not associated with patients' age or expression of ER, PR and HER-2 genes.

Gene	P-value	Rate of change	Standard error	Changes
COMT	0.001***	4.51	0.249	UP
***Significant increasing at P ≤ 0.001 level (up regulation)				

Table 3: The expression of COMT gene in breast cancer patients PBMCs were significantly higher than in healthy individuals.

Specific enzyme activity analysis

Under physiological temperature 37°C and pH 7.6, COMT specific activity in breast cancerpatient's plasma elevated compare to individual healthy people at P-value <0.001 (Figure2). Moreover, the results related to enzyme activity showed a linear relationship between COMT specific activity and over expression of COMT gene expression. In breast cancer patients in comparison with healthy individual people, COMT specific activity elevated almost 4.3 fold and over expression of COMT gene expression increased 4.5 fold. So, it seems that elevation of the COMT level in breast cancer patients is related to pathology of breast cancer.

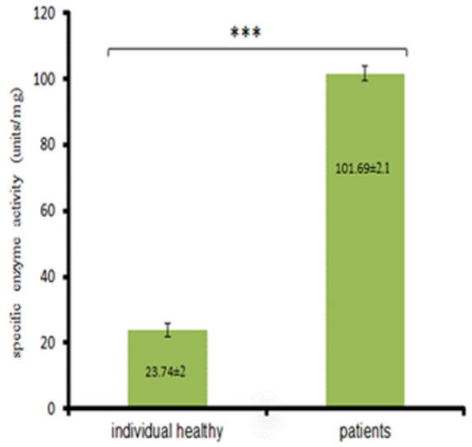

Figure 2: Show COMT specific enzyme activity changes in breast cancer patients compare to healthy individual. ***Significant increasing at P<0.001 level.

Discussion

Based on our hypothesis, the gene expression and COMT activity in PBMCs of breast cancer patients was different from healthy individual as a stress factor inhibitor. Previous studies revealed that patient's lifestyle can be effective in incidence of stress and their mental and physiological conditions which can play a key role in promotion and development of cancers [4,19,20]. Naturally, there were crosstalk between Cancer cells and nerve fibers and they can influence each other. Up to 33% of the surrounding space of perineural cells covered by cancer cells and they cause perineural cell invasion via secretion of soluble factors [4]. Axon growth and their elongation occurred in the presence of cancer cells [4,21]. Moreover, cancer cells stability is influenced by some neuronsthrough which the overgrowth of cancer cells can be limited via secretion of some mediators such as dopamine and serotonin [4]. Dopamine inhibits the cell proliferation via their inhibitory receptors which suppressed the cell growth and apoptosis induction via increasing p38mitogen activated protein (MAP) kinase activity [4,7]; however, Sarker et al. showed that dopamine cannot suppress breast cancer cells and it can increase only the efficacy of anticancer drugs via suppression of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) pathway instead of increasing p38 MAP kinase activity [22]. In spite of expression of the dopamine inhibitory receptors on breast cancer cells, dopamine could not suppress their development directly [22].

COMT, dopamine metabolize enzymes, neutralized dopamine effects via methylation. Various studies implied significant changes in COMT enzyme and they claimed that it has an important role in the incidence of breast cancer [23,24]. RT-PCR results associated to this study confirmed the expression of COMT in both PBMCs. Other studies emphasize on the relationship between COMT val158met Polymorphism and incidence of breast cancer, but they had conflict in this case [23,24]. Bergman-Jungeström et al. reported that frequency of different alleles with Val→Met polymorphism in young women does not have any differences in breast cancer pathogenesis [25]. Moreover, the presence of this polymorphism has various effects on breast cancer incidence in the patients with different physiological conditions and ages [23]. Therefore, this controversial result cannot be reliable for diagnosis of breast cancer and it cannot be a vigorous reason for breast cancer incidence [17]. Gene expression analysis and its relation with stage showed that the rate of COMT gene expression level in breast cancer patients PBMCs are higher than individual healthy people and it is associated with progression of disease. This increasing is along with elevation of specific enzyme activity of COMT in breast cancer patients compare to healthy individuals. Also, Wen et al. revealed that the gene expression of COMT decreased in tumoral tissue compare to the adjacent non tumor tissue of both benign and malignant breast cancer patients. This finding does not controvert our studies results because they measured COMT gene expression in tumoral and adjacent non-tumor tissues but we had performed in PBMCs. Due to production and secretion of dopamine in blood via other tissues especially CNS in chronic stress condition, elevation in COMT rates in PBMCs can cause metabolizing of further amount of dopamine and it makes a disruption in control of cancer cells growth by dopamine. Furthermore, decreasing of COMT gene expression in tumoral tissue can lead to accumulation of catechol estrogens which increase breast cancer risk [26]. In conclusion, the risk of breast cancer was elevated via continuous reduction of dopamine level in PBMCs and accumulation of catechol estrogens in tumoral tissue.

Some other studies disclosed that COMT inhibitors (Tolcapone and Amphetamine) had improvement effects in some diseases such as Parkinson and some neural disorders depend on COMT genotypes [24]. However, according to our knowledge, there are not any preclinical or clinical studies about the effect of COMT inhibitors related to growth of cancer cells.

Conclusion

Based on the results obtained in this study, after performing some complementary studies, COMT gene expression changes as stress factor inhibitors and risk factor along with other diagnostic markers may be used as a reliable marker attributed to the development of breast cancer. However, further investigations using COMT-inhibitors after definition COMT genotypes as a new therapeutic agent are recommended. Also, based on estrogen and catechol estrogens roles in inflammatory diseases[27] and COMT expression changes' importance associated to its roles in estrogen and catechol estrogens metabolism, further studies about such changes along with the diseases are suggested.

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MP and HB were PhD students obtained samples and performed RT-PCR, Real time-PCR and SA (PhD of Biochemistry) performed enzyme assays. HRA was psychiatric consultant. GA designed, analyzed and wrote the paper. We also thank patients who donated blood.

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