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Significant Changes of 5-Hydroxytriptamine 3A Receptor Gene Expression in Peripheral Blood Mononuclear Cells of Allergic Asthmatic Patients

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ABSTRACT

Asthma is a chronic inflammatory disorder of the airways. The stress is a factor for asthma which indicates a disorder in the function of communicational mediators of nervous and immunological systems such as neurotransmitters. A study indicated that blood serotonin concentration increases in asthmatic patients. Other study indicates that one kind of the serotonin receptors, named $5HT_{3A}$, on PBMCs causes secretion of series of pro-inflammatory cytokines which play important roles in allergic asthma disease. Thus, we evaluated the ratio expression level of $5HT_{3A}$ subtype receptors in asthma.

The Peripheral Blood Mononuclear Cells were separated from whole blood of 30 allergic asthmatic patients and 30 normal controls by a gradient density centrifugation technique, then the total cellular RNA was extracted and the cDNA was synthesized. This process was followed by real-time PCR using primer pairs specific for 5-hydroxytryptamine 3A subtype receptor mRNA and beta-actin as internal control.

Results revealed that relative gene expression of 5-hydroxytryptamine 3A subtype receptor increased significantly in Peripheral Blood Mononuclear Cells of patients with asthma in comparison with normal individuals.

To conclude, considering 5-hydroxytryptamine 3A subtype receptor role in accomplishment of asthma symptoms, this increase in its expression may exacerbate the seriousness of asthma disease.

Keywords: Asthma; Gene expression; $5HT_{3A}$; Real-Time PCR

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways.[1] In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough particularly at night and/or in the early morning. Asthma remains one of the most common disorders encountered in clinical medicine in both children and adults. The main cause of asthma disease has not been clearly understood but it is noticed as an inflammatory disorder which is correlated with

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increased expression and releasing of pro-inflammatory cytokines from some Peripheral Blood Mononuclear Cells (PBMCs). One of the inducers of these cytokines released from PBMCs is serotonin.[2,3] Serotonin, 5-hydroxytryptamine (5-HT), is one class of monoamine neurotransmitters, which does multiple physiological regulative roles in body.[4] Furthermore, it is demonstrated that the levels of free serotonin in plasma of symptomatic asthmatic patients increased[5] and its pathological role in asthma was obviously confirmed.[2,3,6]

5-HT has been recognized to attach to 7 types of receptors, 5-HT_{1-7}. Each group of these receptors is also divided into subgroups. All 5-HT receptors are G protein-coupled receptors except 5-HT_3 receptor, which is a ligand-gated cation channel belonging to the superfamily of Cys-loop receptors and has 9 exons. This receptor has 3 subunits named 5-HT_{3A}, 5-HT_{3B}, and 5-HT_{3c}. 5-HT_{3A} subunit which is necessary for functional 5-HT_3 receptor, so the amount of this subunit in the cells reflect the amount of 5-HT_3 receptor.[7,8] 5-HT_3 receptors are known to be expressed and presented in/on a variety of neurons and immune cells such as T and B cells, monocytes, macrophages, and dendritic cells (DC).[2,3,9-11] In response to serotonin, the 5-HT_3 receptors on some cells of PBMC like monocytes and DC proceed a cascade of signal transduction in the cells which results in releasing cytokines such as IL-6, IL-1β, and IL-8/CXCL8.[2,3] It is demonstrated that all of these cytokines have an important role in inflammatory response in the asthmatic patients.[12-14]

Thus we hypothesized that whether human PBMC could contribute to the functional mechanism of 5–HT_3 receptors in allergic asthmatic patients. Based on this model we tried to analyze the 5-HT_{3A} receptor mRNA expression changes in PBMC of asthmatic patients compared with healthy individuals.

MATERIALS AND METHODS

Study Population

Thirty allergic asthmatic patients in the clinic department of Imam Khomeini hospital and thirty healthy individuals took part in this study.

The diagnosis of asthma was based upon an appropriate clinical history and characteristic findings from a series of pulmonary function tests (PFT) including bronchodilator responses, lung volumes, and the diffusing capacity. The symptoms of patients with asthma had been controlled with appropriate treatments for years. The patients showed some specific symptoms such as coughing, wheezing, shortness of breath, chest tightness, eczema and urticaria especially in spring.

Inclusion and Exclusion Criteria

The patients (aged 23-70 years) with different severity of symptoms for at least 5 years were included and they did not necessarily have severe asthma but all of them had typical asthma with above mentioned symptoms. The participants who smoked, or had other current inflammatory or infectious diseases were excluded from the study.

The members of control group were healthy individuals and they had no other allergic diseases.

PBMC Isolation

We collected 4 ml of peripheral blood samples from the cubital vein of two study groups of normal controls and patients in EDTA-containig tubes. Then, PBMCs were separated from total blood samples based on gradient density centrifugation technique by Ficoll-hypaque (Pharmacia, Uppsula, Sweden).[15] Then, we examined the viability of the cells by trypan blue staining and the percentage of viable cells was >99%.

RNA Isolation and cDNA Synthesis

The total cellular RNA was extracted by High Pure RNA Isolation Kit (Roche, Germany) on the basis of its instruction. To normalize RNA concentration, we assigned its absorbance by using Nano Drop 2000 instrument (Wilmington, USA) in 260 nm and then for each reaction of cDNA synthesis, we used a constant concentration RNA (70ng/µl). To obtain cDNA, total mRNA was reverse-transcribed into first-strand cDNA at 42°C for 1 hour using Oligo (dT) primer and Revert Aid First Strand cDNA Synthesis Kit (Fermentas, USA). Then for inactivation of Reverse transcriptase (RT), cDNA products were maintained at 70°C for 5 minutes. Ultimately, the cDNA samples were kept at -20°C until we used them for PCR.

PCR and Real-Time PCR Analyses

Primers for 5-HT_{3A} and housekeeping gene β-actin were designed using primer express software to exclude amplification of genomic DNA and pseudo genes and confirming validity of these primers by blasting these primers in:

http://www.ncbi.nlm.nih.gov/tools/primer-blast/.

We designed 4 primers for 5-HT_{3A} receptor gene that each of them amplified specific exons of 5-HT_{3A} receptor gene. Primer sequences are depicted in table 1:

In order to be certain that the changes in level of mRNA 5-HT_{3A} were not for mutation in coding region sequence of 5-HT_{3A} gene, we performed PCR with four primers of S_1, S_2, S_3 and S_4 , then loaded them on gel 2% (Figure 1) and ultimately sequenced these products of PCR (ABI 3500 Applied Biosystem, USA).

Real-time-PCR was performed with SYBR® Green fluorescent dye (Light Cycler Fast Start DNA Master Plus SYBR Green I, Roche, Germany) to scan cDNA amplification by binding only to double-stranded DNA and its fluorescent intensity identified by Rotor gene (Termocicler Rotor-Gene™6000 Corbett Research/ Australia). Real-time PCRs were performed in 0.1 ml 4-Strip Rotor-Gene® Style Tubes (Rotor Gene Q, STARLAB, Germany), in a final amount of 10 µL including 1 µL cDNA template, 0.4 µL pairs of primer (200 nM, β-actin, S_2) and 2 µL of the SYBR® Green I Master Mix. The protocol for real-time PCR for each primer pairs are presented in table 2.

As it is shown in table 1, at the end of the real-time PCR, a melting curve was drawn by gently increasing the temperature from 72 to 95°C. To avoid general error in a real-time PCR, normalization performed. Normalization contained 1. Number of cells 2. Total amount of RNA (70ng/µl) 3. PCR dependent reference (we used β-actin as a reference gene) 4. Scale of threshold in real-time PCR and amplification efficiencies for the genes in each reaction (we used LinReg PCR software).[16]

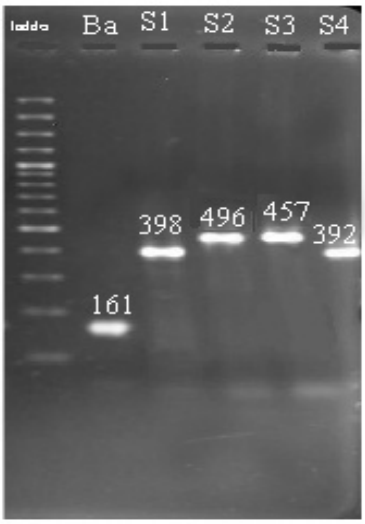

Figure 1. Gel electrophoresis of RT-PCR products for S_1-, S_2-, S_3-, and S_4- 5-HT_{3A} fragment on 2% gel.

Statistical Analysis

Data output from LinReg PCR software were imported to Relative Expression Software Tool 2009 version (REST 2009). Then the data output normalized and analyzed in statistic REST. This program uses the following formula:

Table 1. Primer sequence of β-actin, and four primer sequences of S_1, S_2, S_3 and S_4 for 5-HT_{3A} gene.

Genes	Sense	Anti-Sense	Size (bp)
β-actin	5'AGACGCAGGATGGCATGGG -3'	5'-GAGACCTTCAACACCCCAGCC -3'	161
S_1(1,2 and 3exons)	5'-CAGAAGGTGTGAGCAGTGG -3'	5'-GTACTGCCGGTACCAGATGTAG-3'	398
S_2(4,5 and 6 exons)	5'-GGTACCGGCAGTACTGGACT -3'	5'-CGGCGGATGACCACATAG -3'	496
S_3(7 and 8 exons)	5'-GAAGTTCTATGTGGTCATCCG -3'	5'-GTGGTTTCCCATGGCTGAG -3'	457
S_4(9 exon)	5'-GATGACTGCTCAGCCATGG -3'	5'-GGTCCTGAGGGGCCTAAG- 3'	392

Table 2. Statistic results for 5-HT_{3A} gene expression variation.

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
β-actin	REF	0.9115	1.000				
5-HT_{3A}	TRG	0.8868	3.777	0.193-86.072	0.016-1,709.185	0.021	UP

Legend: P (H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance.

TRG: Target, REF: Reference, Std. Error: Standard Error, C.I.: Confidence Interval

$$R = \frac{E_{target}^{\Delta CPtarget(MEAN\ control - MEAN\ sample)}}{E_{ref}^{\Delta CPref(MEAN\ control - MEAN\ sample)}}$$

R is relative expression ratio of a target gene in asthmatic patients in comparison with control (normal) cases. Calculation of relative expression ratio is based on its real-time PCR efficiencies (E) , and the crossing point (CP) difference (Δ) of one unknown sample (asthmatic patients) against one control (ΔCP control – asthmatic patients).[17-19] *P* value is calculated by REST software and if it is less than 0.05, it was considered significant.

RESULTS

cDNA Validation

We used cDNA for RT-PCR amplification in a final volume of 25 µl with 0.8 unit of Taq DNA polymerase (Roche, Germany) by β-actin primers and then loaded the PCR products on 2% agarose gel (Figure 2).

Real-time PCR Outputs

At the end of real-time PCR, melting and quantification curves for each gene of β-actin and 5-HT_{3A} were drawn by rotor gene. The results showed that human PBMCs express 5-HT_{3A} receptor in asthmatic patients. By this quantification curve and LinReg software, the C_p(crossing point) and efficiency for each reaction were determined.

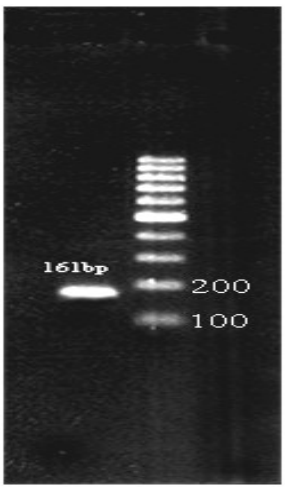

Figure 2. Gel electrophoresis of RT-PCR products for gene β-actin (161bp) on 2% gel.

For verifying the true product of real-time PCR, we loaded 5 µL of PCR products on 2% agarose gel and stained the gel in ethidium bromide (Figure 3). Figure 4 shows Electrophorogram of real-time PCR product 5-HT_{3A} receptor gene by S2 Primer (496bp) which confirmed sequence of this product.

5-HT_{3A} mRNA Expression

We estimated 5-HT_{3A} receptor gene expression alterations in the PBMC of asthmatic patients compared with normal cases. Expression of the 5-HT_{3A} receptor gene was sought by analyzing total mRNA extracted from the PBMC samples of two groups of normal and asthmatic patients. Thus, real-time PCR was performed to detect serotonin gene receptor expression on RNA level. A significant change, in up regulated manner, in 5-HT_{3A} receptor RNA level in PBMC was observed between the asthmatic patients and normal group with mean factor of 3.777 (p=0.021) (Table 2).

The median gene expression of 5-HT_{3A} receptor is shown in figure 5.

Sequencing of Coding Region of 5-HT_{3A}

As we mentioned before, to confirm that the up regulation in level of 5-HT_{3A} mRNA was not due to mutation in coding region sequence of 5-HT_{3A} gene, we sequenced PCR products which were performed with four primers of S_1, S_2, S_3 and S_4. These PCR products were then loaded on 2% gel (Figure 1).

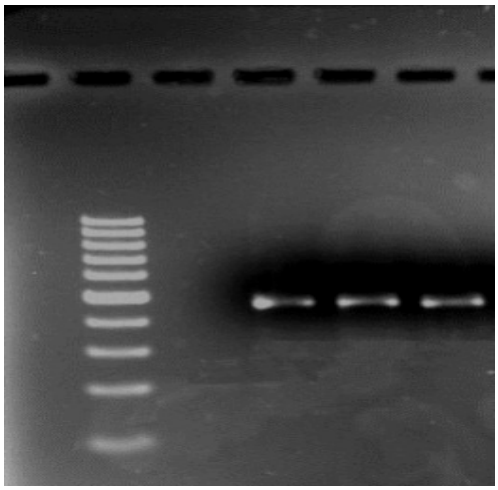

Figure 3. Gel electrophoresis of real-time PCR product of cDNA samples for 5-HT_{3A} receptor gene by S2 Primer (496bp), the first lane after ladder is empty.

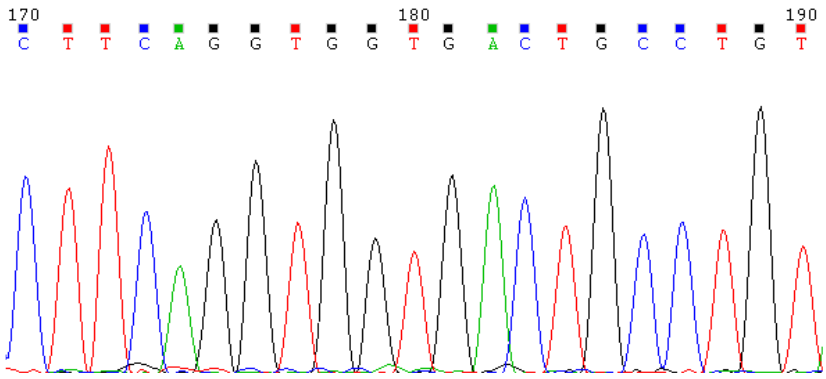

Figure 4. Electrophorogram of real-time PCR product 5-HT$_{3A}$ receptor gene by S2 Primer (496bp) which confirmed sequence of this product.

DISCUSSION

Asthma is a chronic inflammatory disease.[1] Disorders in endocrine, immune, and nervous systems are involved in asthma pathogenesis. A stimulation which affects one system (causing stress) can stimulate and change the function of the other two systems by mediators.[20] Serotonin is one example of these mediators. In this case, serotonin can link nervous and immune systems by its receptors on the cells of both systems. When some environmental stress influences the nervous system, it can convey to immune system by means of the some neuro-immunological factors like serotonin, and lead or help to proceed to an inflammatory disease[21] like asthma. Some related studies depict the relationship between stress and asthma.[22] Moreover, it is demonstrated that the levels of free serotonin in plasma of symptomatic asthmatic patients increased.[23] This increase in the level of serotonin can be as a result of some environmental stress.

Serotonin is present in some PBMCs such as platelets, basophils, and lymphocytes but the main source of serotonin is platelet. PBMCs other than basophils and lymphocytes uptake serotonin from platelet and then release it in inflammatory response.[5,8,9,24,25] When allergens or other stimuli affect the airways of asthmatic patients, ultimately IgE antibodies are produced by B cells. These antibodies activate classical pathway of complement system which subsequently induces platelet activating factors. These factors trigger resealing of serotonin from platelets in inflammatory sites.[9] In response to serotonin, the 5-HT$_3$ receptors on some cells of PBMC such as monocytes, macrophages and T and B cells which are recruited to inflammatory sites, cause influx of Ca^{2+} into these cells. This bivalent cation ion eventually results in some pro-inflammatory mediators such as IL-6, IL-1β, and IL-8/CXCL8.[2,3] Significant increase in CXCL8/IL-8 and IL-6 concentrations in bronchoalveolar lavage fluids of asthmatic patients compared with normal individuals confirms this situation. [26] Furthermore, it is demonstrated that IL-6, IL-8 and IL-1β have important roles in inflammatory response in the asthma disease by triggering Th2-dominated reaction.[12,13,27,28] IL-8 is also a chemokine which leads to an enhancement of neutrophils and eosinophils.[28] IL-6 also causes mucin genes expression and "airway remodeling", which is another aspect of the asthmatic lung symptoms. Thus, increase in secretion due to IL-6 may then exacerbate the asthmatic symptoms by hyper secretion of mucus.[29] In another study, it is shown that 5-HT$_3$ receptor modulated the function of human monocyte–derived dendritic cells and monocytes, which leads to provoke the Th2 immune response in allergic asthmatic patients.[3] All these findings show the action of 5-HT$_{3A}$ receptor on PBMC and verify our finding of up regulating of this receptor.

Our study was performed for the first time on 5-HT$_3$ receptor and shows consistent 5-HT$_{3A}$ receptor gene expression up regulated in PBMC of asthmatic patients compared with control cases. This result supports the suggestion that PBMC may be useful in investigating the action mechanism of some antagonists of 5-HT$_{3A}$ receptor like as tropisetron in clinical treatment of some chronic inflammatory diseases[30] and we have used tropisetron or the other antagonists of 5-HT$_{3A}$ receptor in asthma treatment. Furthermore, it has been denoted that "Tianeptine" which is a serotonin-uptake accelerator causes a clinical alleviation of asthmatic

symptoms.[31,32]

Further studies by investigation of effect 5-HT_{3A} antagonist on asthmatic patients might help to establish new clinical treatment strategy for allergic asthma diseases in future.

As we mentioned in results, the changes in 5-HT_3 receptor expression was not due to changes in its mRNA sequence. This finding shows that some other factors other than mutation cause this up-regulated change in 5-HT_3 receptor expression. Some of these factors could have been environmental pollution and allergens, stress, hormones and other factors which can ultimately induce the expression of 5-HT_3 receptor in PBMCs of patients with asthma.

On the basis of our finding, this significant change (up regulated change) in serotonin receptor gene expression could exasperate the influence of serotonin which is increased in plasma of asthmatic patients.

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