Evaluation of Serotonin Receptor (5HT2RA) Gene Expression Changes in Peripheral Blood Mononuclear Cells of Asthma Allergic Patients

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Abstract: Asthma is an inflammatory airway disorder which many immune cells and cellular elements play a role in it. The airway inflammation in asthma is accompanied by bronchial smooth muscle spasm that causes airway obstruction. Increasing prevalence of asthma all over the world, and lack of any definite treatments makes this disease a global concern. Therefore discovering cellular changes in asthma can be useful.

Although the role of serotonin in asthma has been controversial, there are some studies showed high concentration of free serotonin in plasma is related to asthma exacerbation and severity. According to the studies on 5-hydroxytryptamine (5-HT) receptor family, it is showed that 5-HT2A receptors are essential for mediating many physiological processes. So it is important to evaluate HTR2A gene expression in asthma allergic subjects in comparison with healthy individuals.

Mononuclear cells were separated from peripheral blood of 30 asthma patients and 30 normal people. Total mRNAs were extracted and first strand cDNAs were synthesized. Real-time PCR was used to amplify HTR2A and beta-actin (as a housekeeping gene) with their designed pairs of primers. Then analysis was performed to determine the 5-HT2A mRNA level. The expression ratio was calculated in both asthmatic and normal groups.

The results show that HTR2A gene expression is significantly increased in peripheral blood mononuclear cells of asthma patients in comparison with normal group (P = 0.001).

Considering the result of this study, it seems that 5-HT2A receptors can be used as one of the diagnostic biomarkers of asthma. In addition, the potential role of this receptor in bronchoconstriction can lead us to use its antagonists as a new treatment in asthma.

Keywords: Allergic asthma, 5-HT2A receptor, Real-time PCR, Serotonin, Serotonin receptor 2A gene.

INTRODUCTION

Asthma is a chronic and heterogeneous airway disorder that lots of inflammatory cells and elements such as eosinophils, mast cells, neutrophils, T lymphocytes and epithelial cells play a crucial role in it [1]. The airway inflammation in asthma is accompanied by bronchoconstriction, mucus hyper secretion, thickened and inflamed airway and remodeling of cell walls that all leads to airflow obstruction in asthmatic patients [1, 2]. Asthma is a complex genetic disorder that 118 genes have been associated with it [3-8]. In the last 4 decades, the incidence and prevalence of asthma has increased all over the world, due to factors like global warming, modern lifestyle and becoming urbanized [9, 10].

Serotonin or 5-Hydroxytryptamine (5-HT), is a monoamine neurotransmitter which can be found in pineal gland, digestive tract, central nervous system and blood platelets; However about 80% of 5-HT in mammals is produced by enterochromaffin cells in gut and the rest is synthesized in central nervous system [11-13]. This hormone is well-known because of its extremely important functions in some physiological conditions and behaviors like anxiety, depression, sleep, learning and memory [14], although its role in asthma has been very controversial and there are some facts indicating its potential effects on asthma.

Serotonin as a neurotransmitter is in need of its receptors to have its effects on the cells. Recent molecular biology studies have confirmed 15 subtypes of serotonin receptors [15-17]. These receptors are divided in 7 groups and all of them are G-protein coupled receptors, except 5-HT3 which is a ligand dependent ionic channel [16, 18-20]. 5-HT2 receptor is one of the most studied serotonin receptor and has three subtypes: 5-HT2A, 5-HT2Band 5-HT2C [19, 21, 22]. Plenty of studies have been done on 5-HT2A on account of its great role in some pathophysiological conditions.
conditions like suicide, schizophrenia, anxiety, depression and alcoholism [23-26]. It is encoded by HTR2A gene in 13q14-q21 locus. This receptor can be seen in different tissues and cells particularly brain cells, kidney, skeletal, smooth muscles and even platelets [25-27].

Although the role of serotonin in causing and exacerbation of asthma has not been clearly found, there are some studies that directly or indirectly related to asthma and serotonin or its receptors. Some studies focus on relationship between amount of free plasma serotonin in asthmatic patients [28-31]; or study serotonin as a molecule that triggers allergic cascade in asthma [32]; Also there are some other studies which discuss the role of 5-HT receptors in airway smooth muscles [33-34].

In regard to the economic costs that asthma imposes on patients and society and since any definite treatments have not been found yet; it seems essential to study more about the intricate cellular and molecular changes in asthma. This research will be classified in neuro-immunogenetic science which studies the relationship between nervous and immune system by means of mediators that present in both systems, like serotonin and its receptors. It also studies the effect of nervous stimulating factors (such as stress) on immune diseases.

According to other findings we hypothesized the potential role of 5-HT2A as a cause of asthma attack. So it can be helpful to evaluate the expression changes of 5-HTR2A gene in allergic asthma in comparison with healthy individuals.

MATERIAL AND METHODS

Sample Collection

Thirty allergic asthmatic patients and thirty healthy individuals were included in this study. The group of patients was consisted of 30 allergic asthmatic patients equally from both men and women between ages 18 to 60. Individuals who were challenging with asthma at least for five years were included and they also did not have any other diseases and health conditions that would influence the study. Participants were selected from patients who came to pulmonary division of Imam Khomeini hospital and were diagnosed with atopic asthma based on the spirometry tests and clinical symptoms; the samples were taken with respect to hospital laws and each patient has filled a consent form, stating that the sampling process was fully voluntary and safe. The control group was thirty healthy individuals who had not been a smoker, without any lung or allergic diseases.

For each individual 4 mL of peripheral blood from cubital vein was obtained and transferred to blood sample tubes containing 0.5 mM Ethylene Diamine Tetra-acetic Acid (EDTA). Tubes were transferred to the lab in flask.

PBMCs Isolation

The density gradient centrifuge was used by Ficoll Hypaque (Pharmacia, Uppsula, Sweden) in order to isolate peripheral blood mononuclear cells of the collected samples. Then the cells were washed with phosphate-buffered saline (PBS) and centrifuged. Viability test by Trypan blue staining was confirmed and their concentration was normalized (7×10⁶ cells/mL). The percentage of viable cells was >%99. Plaques were suspended in PBS and placed in a 1.5 mL tube for further use [35-36].

Total RNA Extraction

PBMCs total RNA were extracted using High pure RNA isolation kit (Roche, Germany) according to the manufacturer’s instructions. The cDNA Purity and concentration was determined by nanodrop 2000 instrument (Wilmington, USA) at 260/280nm.

cDNA Synthesis and PCR

Total mRNA was reverse-transcribed into first-strand cDNA using Oligo (dT) primer by Revert Aid First Strand cDNA Synthesis Kit (Revert AidTM, Fermentase, USA) according to the manufacturer’s instructions. Primer pairs for both target genes and β-actin were designed by using primer express software. The designed primers were blasted (http://www.ncbi.nlm.nih.gov/tools/primer-blast); they just adhered to appropriate loci in mentioned genes (Table 1).

These first strand cDNAs were used for PCR amplification of HTR2A and beta-actin (as a housekeeping gene) with their designed pairs of primers. PCR products were determined by gel electrophoresis on a 2% agarose geland sharp appropriate bands acknowledged the accuracy of the exact segments amplification of HTR2A...
This step also confirmed the expression of 5-HT$_{2A}$ mRNA in PBMCs.

Figure 1: Gel electrophoresis of Real-time PCR product.

Quantitative Real-Time PCR

Quantitative real-time PCR were performed with fast start DNA master plus SYBR green I kit (Light Cycler Fast Start DNA Master Plus SYBR Green I, Roche, Germany) to quantify beta actin and 5-HT$_{2A}$ receptor transcripts in samples using the Corbett Rotor gene 6000 (Termocycler Rotor-Gene™6000, QIAGEN, USA).

Differences in the total RNA volume of each reaction were synchronized by using beta-actin as endogenous control.

Table 2: Schedules for Real-Time qPCR

<table>
<thead>
<tr>
<th>Amplified gene</th>
<th>Annealing temperature</th>
<th>Time for annealing</th>
<th>Cycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR2A</td>
<td>54°C</td>
<td>5</td>
<td>45</td>
</tr>
<tr>
<td>beta-actin</td>
<td>61°C</td>
<td>10</td>
<td>40</td>
</tr>
</tbody>
</table>

Figure 2 shows melting curve of 5-HT$_{2A}$ receptor gene and beta-actin expression.

STATISTICAL ANALYSIS

Real-Time PCR data were inserted to LinReg software in order to calculate mean and individual
efficiency and cycle threshold (Ct) of each sample. Gene expression ratios and p-value analysis of HTR2A was performed by REST 2006 (Relative Expression Software Tool 2006) program. P-value up to 0.05 is accepted by the software as significant.

RESULTS

The mRNA expressions of serotonin receptors detection were examined in PBMC with highly sensitive methods. In this study, we performed real-time PCR on cDNA synthetized from mRNA extraction of PBMCs of individuals who had been challenging with asthma by using primers specific for HTR2A. Relative mRNA expression was calculated by REST 2006 program. The results show that HTR2A expression is significantly increased in PBMCs of asthmatic patients (in comparison with normal group) by a mean factor of 3.996.

(P value = 0.001) Table 3 shows gene expression changes of HTR2A in asthmatic patients compared to healthy individuals.

Sequencing of Coding Region of Target Genes (Mutations in HTR2A cDNA)

Sequencing results acknowledged accuracy sequence of HTR2A in PBMCs of allergic asthmatic patients and showed no changes in their sequences.

CONCLUSION AND DISCUSSION

Asthma is a complicated and chronic disease that its increasing prevalence between different races and ages makes it a global concern. The role of 5-HT in asthma has been controversial, but several studies done by Lechin et al. [28-30] shows that high concentration of free serotonin in plasma is related to asthma severity [28] and they reported that Tianeptin (a drug that lowers plasma serotonin by re-up taking it by platelets and serotonergic neurons) enhanced lung function of asthmatic children [29]. So this studies suggested that serotonin can involve in pathophysiology of asthma [34]. Therefore it seems 5-HT receptors can involve in asthma. In this study, mRNA expression of HTR2A has evaluated with Real Time PCR. The results show that HTR2A expression is significantly increased in PBMCs of asthmatic patients.

When airway epithelial cells are exposed to an allergen, dendritic cells and other probable antigen presenting cells like macrophage [40, 41] present it to T-helper lymphocytes [38]. Dendritic cells release chemokines which attract T-helper type 2 cells (Th2). Also T-lymphocytes release a distinct pattern of cytokines, especially Interleukine-4 (IL-4), IL-5 & IL-13 that make imbalanceratio of Th2 cells [37, 38]. These cytokines trigger an inflammatory response and gather immune cells including eosinophils, mast cells, macrophages and neutrophils [37, 38]. In this inflammatory response, it seems IgE (released by B-lymphocytes) causes platelet aggregations [39] and serotonin stored within platelets is released [33]. Also free 5-HT increment can be caused by decreasing in its absorption by platelets [33]; in fact if releasing 5-HT from enterochromaffin cells is more than absorbing it by platelets, concentration of free plasma serotonin will rise [39]. Another probable source of serotonin in airway system is releasing from nervous terminals [34], which can be due to environmental stimulating factors like stress. In cells expressing 5-HT2A, serotonin activates phospholipase C (PLC) via 5-HT2A; this activation leads to accumulation of IP3 and increase in intracellular Ca2+ [40-42]. These changes activate ligand Ca2+ channels and induce Protein kinase C (PKC) [40]. In some other cells, 5-HT2A can lead to increasing of cAMP [17]. These cascades eventually cause pathophysiology symptoms of asthma, including smooth muscle constriction.

The exact role of 5-HT2A in asthma has not been found yet. Our previous study had shown significant increase in gene expression of 5-HT3A (HTR3A), in people who have been exposed to air pollution and allergic asthmatic patients [44, 45]; But there are not any further publications about association of 5-HT2A gene expression with asthma. However some studies have shown the effect of this receptor in airway smooth

Table 3: Expression Ratio of HTR2A in Allergic Asthmatic Patients

<table>
<thead>
<tr>
<th>Gene Type</th>
<th>Control Individuals</th>
<th>Allergic Asthmatic Patients</th>
<th>P values</th>
<th>PCR Efficiencies</th>
<th>Expression Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>standard error</td>
<td>Mean</td>
<td>standard error</td>
<td></td>
</tr>
<tr>
<td>HTR2A</td>
<td>15.82</td>
<td>0.36</td>
<td>17.90</td>
<td>0.48</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.908697369</td>
<td>3.996</td>
</tr>
</tbody>
</table>

Expression of HTR2A gene was calculated relative to that of β-actin.
muscle contraction. Cazzola & Matera et al. reported that four groups of 5-HT receptors are important in controlling airway functions: 5-HT1a, 5-HT2a, 5-HT3a, 5-HT7; and serotonin directly has its contractile effect through 5-HT2A located on smooth muscles [33, 34]. Other studies showed that blocking 5-HT2A by its antagonists (Ketanserin & Droperidol) can induce a weak anti-asthmatic effect [34]. Moreover Prezant et al. could treat two patients (with status asthmatics) by Droperidol [46]. These findings can confirm our results, because up-regulating of 5-HT2A gene in patients can indicate possible effect of this receptor in exacerbation of asthma. However there are different findings about the effect of serotonin and 5-HT2A on constriction or relaxation of airway smooth muscles [31, 47, 48]. Thus it seems essential to do more studies about serotonin receptors’ effects on human pulmonary cells.

In this research we measured mRNA expression of HTR2A gene in both groups’ PBMCs. The reason why we used PBMCs is that some studies have revealed that serotonin concentration in pulmonary tissue is proportional to its concentration in plasma, because its main source is platelet [31, 33, 34]. Shin et al used PBMCs instead of lung tissue cells or Broncho alveolar lavage (BAL) [56]. According to Yang et al. 5-HT1a, 5-HT1b, 5-HT1e, 5-HT2A, 5-HT3, 5-HT4 and 5-HT6 are expressed in human and monkey PBMCs [50]. Rollins et al declared PBMCs can be used in evaluation of brain cellular mRNA expression, both in vivo and in vitro [51]. Similar studies by Sabery Anvar et al, and Shariati et al. have demonstrated serotonin receptors are expressed in PBMCs [52, 53] and related studies showed 5-HT receptors can be synthesized and functioned in nervous system cells and lymphocytes too [53, 54]. Our research demonstrated the same result that 5-HT2A mRNA is expressed in human mononuclear blood cells.

Considering the result of this study, it seems that 5-HT2A receptors can be used as one of the diagnostic biomarkers of asthma. In addition, the potential role of this receptor in bronchoconstriction can lead us to use its antagonists as a new treatment of asthma. Therefore our results can be quite beneficial in order to find a new way of diagnosis and treatment of allergic asthma. Moreover our finding can demonstrate the important role of 5-HT2A, as a mediator of immune and nervous system. This molecule and other similar neurotransmitter receptors can be the connection ring that transfers the environmental stimulating factors (like stress) from nervous system to immune system.

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