Analysis of 5HT3Ra gene expression by real time PCR in Systemic Lupus Erythematosus (SLE) patients

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ABSTRACT

Systemic lupus erythemathosus is an autoimmune disease that affected many various types of tissues in 10% of world population and over 30 genes has associated with it. Nouroimmunoendocrynology concepts have shown that immune system could be affected by neuron system and vice versa, 5-hydroxytryptamine receptor a (5HT3Ra) was studied as a main receptor in these relations.
In this study, peripheral blood sample were collected from (SLE) patient and normal individuals. The total cellular RNAs were extracted and the cDNAs were synthesized. This process was followed by real-time PCR using specific primers for 5HT3Ra gene and beta-actin gene as internal control. Eventually PCR products have been sequenced.
Results of this study suggested that this special receptor expressed in polymorpho-nuclear cells. We found over expression of 5HT3Ra in patients in comparison with healthy individuals group. Interestingly, some nucleotide changes have been found in 5HT3Ra gene in patients but not found sequential nucleotide changes in healthy individuals group.
This study supposed that over expression of 5HT3Ra gene in SLE patients lead to over activation of immune cells that derived from over stimulation of them from serotonin blood serum that finally lead to autoimmune reactions that terminated in SLE.

Keywords: Systemic Lupus Erythematosus (SLE), real time quantitative PCR, 5HT3Ra receptor, expression

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is an autoimmune disease that affected various types of tissues of 10% of world population. Many genes has been shown to be associated with this complex disease[1],[2]. SLE usually affected young and pregnant women (approximately %90 of SLE patient)[3]. In recent studies have been illustrated that most of diseases such autoimmune made from disruption of homeostasis between neurological system, immune system and endocrine system[4].

Neuroimmunoendocrinology is a multidisciplinary science that studied this network. This network is consisted of neurotransmitters, cytokines and endocrine glands hormones and related receptors [5, 6]. These connections, environmental effects and genetic factors in healthy conditions prepares an appropriate homeostasis but any alteration in this network can terminate to numerous disease such as autoimmune disease including SLE[5]. Serotonin receptors are the critical factors in this relation and consist of seven members that controlled other neurotransmitters (e.g. dopamine,

GABA, acetylcholine and etc) and hormones (prolactin, cortisol and etc). These receptors are distributed in two main groups: 5HT3 (5-hydroxytryptamine) group and others. This particular receptor group belongs to ligand-gate ion channel receptors super family against the other groups that belong to G-proteins coupled receptors[7]. First and most important isotype of serotonin receptor 3 (5HT3Ra) was recognized in 1987 by Maricq[8]. At first thought that these receptors expressed only in nervous system but in 1997 Meyniel et al shown that this receptors also expressed on trout lymphocyte [9]. 5HT3 is a pentameric receptor that is in two heteropentameric and homopentameric forms with different functions[10], [11],[12]. Considering published data, it seems that 5HT3Ra expression has been changed in SLE patients. In this study, we therefore performed real time reverse transcriptase quantitative PCR by SYBR Green to investigate 5HT3Ra gene expression in SLE patient in comparison with normal group.

MATERIAL AND METHODS
Sample Collection
Healthy individuals and affected cases have been selected base on SLE patient criteria from American college of rheumatology [13]. We obtained 5ml peripheral blood sample from healthy and SLE patients.
Sample Preparation
PBMCs isolation
For Human blood PBMCs isolation from collected samples from Healthy individuals and patient cases, the density gradient centrifuge was used by Ficoll Hypaque (lymphoperp[TM], Oslo, Norway). Buffy coat layer removed and washed twice with sterile phosphate buffer saline (PBSs), then counted and finally cells were isolated 105-106 for total RNA extraction.
Total RNA extraction and real time reverse transcriptase quantitative PCR
PBMCs total RNA were extracted using High pure RNA isolation kit (Roche, Germany) according to the manufacturer's instructions. Extracted samples were saved on -70° refrigerator.

To confirm of RNAs existing in samples, 11µl of total RNA was used as template and Revert AID TM first strand SL end cDNA synthesis (M-MLV) (Fermentase, USA) with oligo dT primer was used to convert total mRNA to cDNA according to the manufacturer's instructions.

Beta actin and 5HT3Ra primer sequences designed for this study [14]. The cycling conditions were set up for each primer sets. For PCR cycling 1µl of cDNA was used as template and cycling did for 26 cycle with 95°C for 10 sec, 59°C for 10 sec and 72°C for 30 sec. All of these cycles were accomplished using TECHNE Flexigene (Techne Flexigen, Minneapolis, MN, USA).

PCR products were run in 2% agarose gel for electrophoresis and plunge in ethidium bromide pool and finally visualized and imaged under UV light.

Quantitative real-time PCR assay
After confirmation of existing of total RNAs in samples, beta actin and 5HT3Ra transcripts were quantified in samples using lightcycler ver. 2 (Roche, Germany) and light cycler fast start DNA master plus SYBR green I kit (Roche, Germany) with relative method and Livac analysis, standard curve was depicted in Figure1. Primer sequences of beta actin and 5HT3Ra were the same as previously reported [14], PCR products recapped and were sent to sequencing.

Statistical Analysis
To analyze the data of SLE patients in comparison of healthy individuals and t test SPSS 16.0 (SPSS, Inc, Chicago, IL) was used. Relative mRNA expression was calculated by the δδCt method. The significant value in this study was less than 0.05 (P≤ 0.05).

Table1. Mean of 5HT3Ra gene expression in each patient and healthy individuals groups.

	number	means	Standard deviation	Standard error of mean
Normal	32	0.15178	0.255713	0.045204
SLE patient	31	0.002706	0.011812	0.0021215

Table.2. Levene's test on equality of variances used to examine the effect of SLE disease on variance of 5HT3Ra expression levels.

	F.	Sig.
Equal variance assumed	290191	0.000

RESULTS

The mRNA expressions of serotonin receptors detection where examined in PBMC with highly sensitive methods. focus was only on one of subtype of serotonin receptors (5HT3Ra) because based on latest research only this receptor is functional related some diseases. We divided nine exons into four segments and then expression of the different serotonin receptor gene segments studied by analyzing total RNA extracted from the samples. Results revealed that all segments of serotonin receptor gene expressed on PBMC of healthy and patients individuals. In order to detect serotonin gene receptors expression on RNA level, Real time PCR was performed for the regions of different serotonin receptor segments. The specificities of the obtained PCR products for the respective serotonin receptor fragments were confirmed by capillary sequenced analysis ABI 3700 machine [14]. However, firstly, receptor gene fragments expression of SLE patients and matched normal individuals were analyzed by Real time PCR and normalized to the housekeeping gene β-actin for each sample using the δδCt method.

Table3. 2-tail t-test has illustrated significant expression changes in patient group

	t	df	Sig.(2 tail)	Mean difference
Equal variance assumed	-3.242	61	0.002	-0.14907
Equal variance not assumed	-3.294	31.137	0.002	-0.14907

Table 1 showed mean of 5HT3Ra gene expression in each patient and healthy individuals group. Levene's test on equality of variances was used to examine the effect of SLE disease on variance of 5HT3Ra expression levels (table 2). Then the measure of mRNA concentration of each sample was calculated and Mean, Standard deviation and P Value for 5HTR3A gene expression was determined.

Table 3 showed 2-tail t-test has illustrated significant expression changes in patient group.

As a result, there is a Confidence interval (CI) of relative concentration of HT3Ra in healthy and patient individuals. (Mean -0.14907, Mean Difference -0.14907) (Figure1). P value of this expression changes was 0.002 ($P<0.05$). Using ABI 3700 capillary system PCR products of 5-HT3A receptor sequenced and confirmed by DNA sequencer.

After sequencing, any sequence changes in control and patient individuals were observed. Figure 2 shows agarose gel electrophoresis of 5HTRA gene and related exons sequence which adapted with its sequence in NCBI. Sequencing analysis of PCR products had also showed some nucleotide changes that one of them was missense change (1091 ATA → AGA   Ile → Arg).
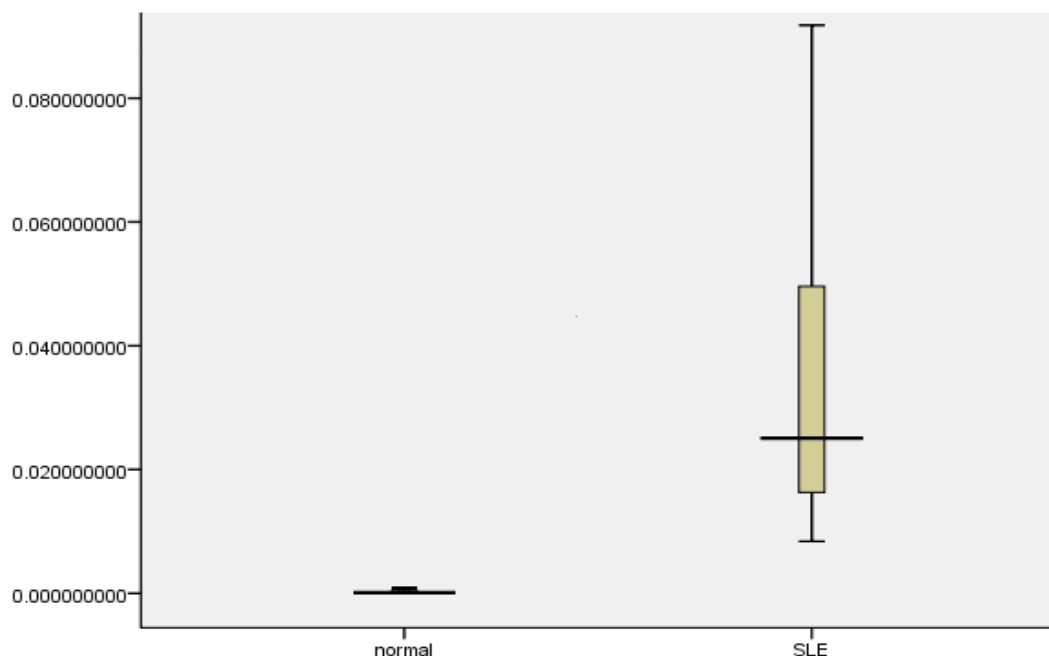
Figure 1: Confidence interval (CI) graph of relative concentration of HT3Ra in healthy and patient individuals. (Mean -0.14907, Mean difference -0.14907). P value of this expression changes was 0.002 (P<0.05).

DISCUSSION

Serotonin receptors type 3 functions as a ligand gate ion channel except other serotonin receptors that act as a G- protein coupled proteins[15]. These receptors have a role in electrical balance of cells and have cooperated with other receptors that act in this manner[16]. 5HT3Ra receptors are one of the main reasons in human neuropsychological disorders that are because of its functional role in cells[17]. Because receptors are studied less than other serotonin receptors, this study focused on this receptors for found relation between this receptor and immune diseases that not studied until now. Considering published data, it seems that 5HT3Ra expression has been changed in SLE patients. In this study, we therefore performed real time reverse transcriptase quantitative PCR by SYBR Green to investigate 5HT3Ra gene expression in SLE

patient in comparison with normal group. Some studies investigated the association of 5HTR genes expression with some autoimmune diseases including SLE. Xu et al study results supported that over expression of HTR1A might contribute to SLE[18]. Some other studies had also showed the association of serotonin and SLE. In Meyerhoff et al study Platelet serotonin levels were measured in 41 patients with systemic lupus erythematosus (SLE) and 36 normal controls. SLE patients had significantly lower mean serotonin levels (243 ± 131 versus 414 ± 175 ng/ 109 platelets, $P < 0.001$); the lowest levels occurred in those patients with active disease[19]. There are not any further publications about association of 5HTR3a gene expression with SLE. Ahangari et al had performed some studies about the association of 5HTR3a genes expression with Rheumatoid arthritis and schizophrenia[20].

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CDS: Putative 1      I  G  V  Y  F  V  V  C  M  A  L  L  V  R  S  L  A  E  T  I
        Query        TTGGTGTCTACTTTGTGGTGTGCATGGCTCTGCTGGTGAGAAGTTTGGCAGAGACCATCT
        Sbjct        ..........................................T.........C..........
CDS:5-hydroxytryptam I  G  V  Y  F  V  V  C  M  A  L  L  V  I  S  L  A  E  T  I
```
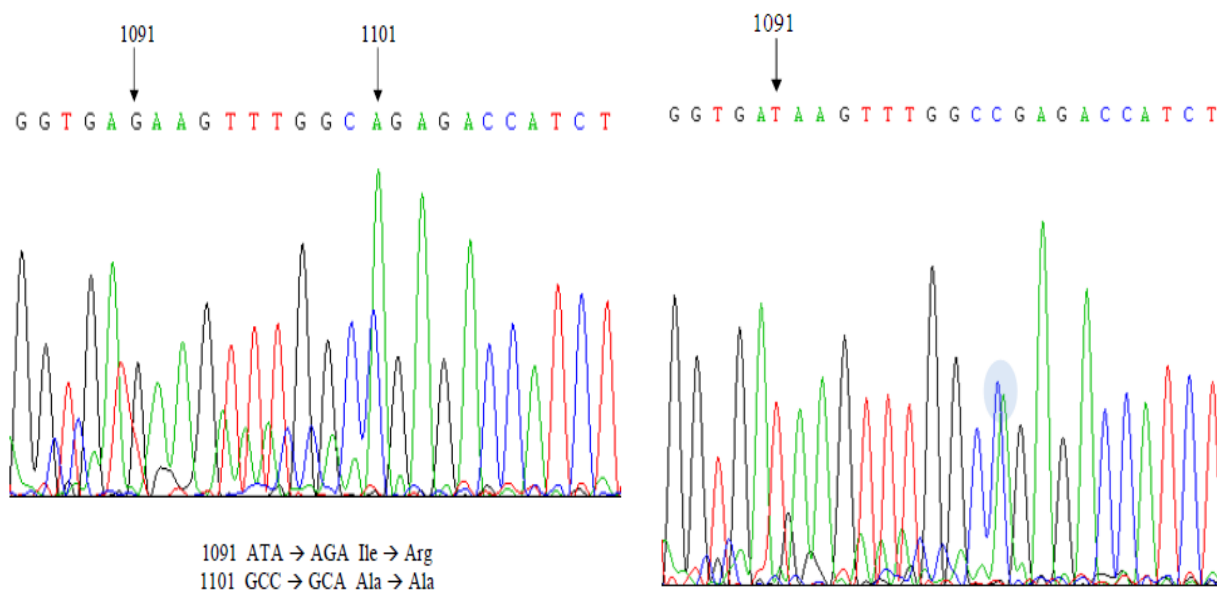
Figure 2: related exons sequence of 5HT3RA gene which adapted with its sequence in NCBI. Sequencing analysis of PCR products had also showed some nucleotide changes that one of them was missense change (1091 ATA → AGA   Ile → Arg).

Although Ahangari et al have shown decreased expression of this gene in Rheumatoid arthritis, and schizophrenia [20], Results of this study suggested over expression of 5HT3Ra in patients in contrast with normal group. Interestingly, some nucleotide changes have been found in 5HT3Ra gene in patients that one of them was missense change. In conclusion, this study supposed that over expression of 5HT3Ra gene in SLE patients lead to over activation of immune cells that result in over stimulation from serotonin blood serum that finally lead to autoimmune reaction that terminated in SLE with special mechanism.

Signaling pathway of 5HT3Ra shown that this receptor could be act as main element to make active or inactive pathways such as apoptosis, MAPK, long-term depression and cell proliferation. This receptor is a ligand gate ion channel (LGIC) that exchange Ca^+ (if 5HT3 receptor war homopentameric from 5HT3RA) and Na^+ inward and K^+ to outward [21]. Increasing of 5HT3Ra on cell surface of PBMCs result in increased rate of Ca^+ concentration in cells and finally lead to increased cell proliferation and apoptosis then may lead to auto reactivity and auto immunity

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