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Research Article

Evaluation of IFN-γ and T-bet Expression Levels as Possible Molecular Markers of Schizophrenia

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Abstract

Schizophrenia is an irritating mental disorder that affects around 1% of the world's population. The immune system contributes to the onset of the disease, particularly through production and secretion of some cytokines. In patients with schizophrenia, the balance of Th1 to Th2 ratio is often altered. In the present study, we investigated these changes by measuring the gene expression levels of IFN-γ and T-bet as Th1 indicators, as well as IL-4 and GATA-3 as representatives for Th2. Blood samples of schizophrenic patients (n=25) and healthy individuals (n=10) were obtained. Total RNA was extracted from leukocytes and cDNA synthesis was performed based on provided protocols. Real-time PCR technique was utilized for the assessment of gene expression levels. Results indicated a significant increase in the expression of IFN-γ and its transcription factor, T-bet, while IL-4 gene expression was reduced significantly. The expression level of GATA-3 gene revealed no meaningful changes. Altogether, results confirmed the relative shift of Th1 to Th2 status in the patient with schizophrenia and re-emphasize the importance of the inflammatory events in the incidence of the disease. Moreover, a new index was introduced based on the IFN-γ and T-bet genes expression, which can determine healthy condition with total accuracy of 79%.

***Keywords*:** Molecular marker, Schizophrenia, Th1, Th2, IFN-γ, T-bet

Introduction

Schizophrenia is a disturbance in the executive and sensory functions of the central nervous system (Ajami et al., 2014), which affects about one percent of the world's population (Rajasekaran et al., 2016). The onset of the disease usually occurs in late adolescence or early adulthood with a wide range of symptoms, including hallucinations, delusions, affective disorders and cognitive impairments (Srinivasan et al., 2016).

A substantial genetic contribution was demonstrated for familial cases of schizophrenia (Ayalew et al., 2012). Previous investigations were reported 41 to 65 percent risk of schizophrenia for monozygotic twins who have an affected brother or sister and 28 percent risk of disease for children with affected parents (Cardno and Gottesman, 2000). It has been illustrated that schizophrenia is a very complex genetic disorder and various genes are involved in its pathogenesis (Kumar et al., 2019). Some immune system disorders, such as infections and autoimmune inflammatory diseases could be considered as other risk factors (Benros and Mortensen, 2020; Benros et al., 2011; Eaton et al., 2006), which explain the impact of immune system imbalance on the pathology of the disease. Furthermore, it was evidenced that different types of cytokines play significant roles in the stimulation, production, and secretion of neurodegenerative modulators (Chang and Bistrian, 1998; Sonti et al., 1996). In various psychiatric disorders such as schizophrenia, changes in the balance of cytokine regulations can disrupt the balance of T-helper 1 (Th1) to T-helper 2 (Th2) cells (Macedo, 2019; Potvin et al., 2008).

Some cytokines play a role in the differentiation of T helper cells from $CD4^+$T cells. Interferon gamma (IFN-γ) is the major cytokine that mediates the differentiation of $CD4^+$T cells into Th1 ones (Lazarevic et al., 2013); and, simultaneously, prevents their differentiation into Th2 cells. In contrast, IL-4 promotes the differentiation of naive $CD4^+$T cells into Th2 cells (Dai et al., 2009; Myles et al., 2017). So, IFN-γ and IL-4 are Th1 and Th2

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specific cytokines, respectively (Annunziato et al., 1999; Katsikis et al., 1995). While, they are not suitable candidates for assessment of Th2 to Th1 ratio, since some other cells also produce and release them (Chakir et al., 2003).

T-bet is one of the main regulatory transcription factors which mediates the differentiation of $CD4^+$T cells to Th1 subset (Tullius et al., 2014). Also, it is a direct inhibitor of the GATA-3 gene expression (Kanhere et al., 2012). In turn, GATA-3 stimulates the differentiation of naïve $CD4^+$T cells into Th2 cells and prevents the differentiation of these cells to Th1 clones through the inhibition of STAT4 and T-bet. Considering the fact that T-bet and GATA-3 act as upstream factors for IFN-γ and IL-4 cytokine production, respectively (Wang et al., 2010), their expression levels were also investigated for accurate measurement of Th1 to Th2 ratio in schizophrenic patients.

Materials and Methods

Blood sample collection and RNA isolation

A total of 25 blood samples were obtained from 20 male and 5 female patients, diagnosed for schizophrenia according to the clinical interview, in the ages of 22 to 57. 10 healthy samples were also taken as controls. EDTA[1] was applied to prevent clotting of the samples. Leukocytes were obtained following the rupturing of red blood cells (RBCs). Therefore, 10 ml RBC lysis buffer (pH= 7.5) was added to 5 ml blood, after centrifuge at 5000 rpm for 20 min, pellet was isolated. For purifying leukocytes, the washing procedure was repeated twice. Total RNA extraction was carried out for leukocytes via Tripure reagent (Roche, Germany) according to standard protocols provided by the manufacturers. Then, quantitative and qualitative features of RNA samples were evaluated using nanodrop device, Picodrop. Also, the integrity of RNA samples was evaluated by agarose gel electrophoresis. In the following step, DNase I treatment (Sinaclon, Iran) performed based on the manufacturer's instructions, to ensure the elimination of genomic DNA.

Synthesis of cDNA and quantitative real-time PCR

A mixture of oligo (dT) and random hexamers was utilized for the synthesis of cDNA by the application of PrimScript™ RT reagent kit (TAKARA, Japan). Samples were diluted 25 folds before further applications. For assessment of IFN-γ, IL-4, T-bet and GATA-3 genes expression, specific primers were designed using GeneRunner and Primer-BLAST online software (Table 1). Gene expression experiments were performed via the Step One Plus Real-time PCR ™ device and Premix Ex Taq™ II SYBR reagent (TAKARA, Japan). The final volume of each reaction was 15 µl containing 7.2 µl of SYBR Premix Taq II (2X) (TAKARA, Japan), 0.3 µl ROX (50X), 3 pmol of each primer and 2 µl (diluted) of desired cDNA sample. DNA amplification performed using the following program: initial denaturation for 30 s, followed by 40 PCR cycles consisting of 95°C for 5 s, annealing and extension at 60°C for 30 s.

Statistical analyses

Relative gene expression levels were calculated through $2^{-\Delta Ct}$ (formula 1) (Schmittgen and Livak, 2008). Finally, data analysis was performed by Excel and R, using logistic regression.

Formula 1:

ΔCt Healthy= mean (Ct target-Ct control)

ΔCt Patient=mean (Ct target-Ct control)

Ratio= $2^{-\Delta Ct}$ Patient/ $2^{-\Delta Ct}$ Healthy

Considering the relationship of IFN-γ and T-bet gene expressions, Index 1 was introduced for different ratios of gene expression; and their differences were evaluated for patients versus healthy subjects.

$$\text{Index 1} = \frac{\text{IFN}\gamma + \text{Tbet}}{\text{IFN}\gamma - \text{Tbet}}$$

The strength and ability of the index were evaluated using logistic regression. The frequency of predicted classes versus observed ones was evaluated for model performance evaluations in the separation of dependent variable classes.

To predict the status of different individuals based on the model, predicted values were classified based on the default value of critical probability, which is equal to 0.5. The logistic regression model was evaluated based on different criteria including classification accuracy, classification specificity, classification sensitivity, and performance. Furthermore, the predictive power of the model was evaluated using the criterion of the area below ROC (Receiver Operating Characteristic) Curve (Hosmer et al., 2013; Metz, 1978).

[1] Ethylene diamine tetraacetic acid

Table 1. Sequences and characteristics of primer pairs which were applied for Real-time PCR experiments

Product length	Sequence	Accession number NCBI	Genes
136 bp	F:5'-GAATTGGAAAGAGGAGAGTGACAGA-3'	NG_015840.1	*IFN-γ*
	R:5'- GACATTCATGTCTTCCTTGATGGTC-3'		
130 bp	F:5'-GCTGCCTCCAAGAACACAACTG-3'	NG_023252.1	*IL-4*
	R:5'- TGTGCCTGTGGAACTGCTGTG -3'		
141 bp	F:5'- GACGGCGGATGTTCCCATT-3'	NG_012166.1	*T-bet*
	R:5'- TGTGCCTGTGGAACTGCTGTG -3'		
136 bp	F:5'- TCATTAAGCCCAAGCGAAGG -3'	NG_015859.1	*GATA3*
	R:5'-GTCCCCATTGGCATTCCTC-3'		
123 bp	F:5'- GTGAACCATGAGAAGTATGACAAC-3'	NG_007073.2	*GAPDH*
	R:5'- CATGAGTCCTTCCACGATACC-3'		

The criteria of the model performance were reliable while they were calculating based on new data which were not used in the fitted model. In the present study, the "leave-one-out" method was applied for validation of the regression model.

Results

Ratio of optical absorbance of RNA samples at 260/280 wavelengths was about 1.9 and agarose gel electrophoresis showed the integrity of RNAs with desirable quality. Comparative gene expression analysis between patient and healthy individuals represented increment in IFN-γ (*p*-value = 0.068) and T-bet (*p*-value = 0.007) expression levels. While, no significant changes observed in GATA-3 (*p*-value > 0.05) expression. The expression of IL-4 was not evidenced here.

Index 1, which shows combined effects of IFN-γ and T-bet genes in the frame of a numerical ratio, was significantly different between healthy and disease conditions, when it was evaluated based on the Mann-Whitney test (*p*-value = 0.015). Descriptive statistics of index 1 are presented in table 2.

Table 2. Descriptive statistics of index 1

	Mean	Min	Max
Healthy	1	-1.3	6.2
Schizophrenic cases	-2.8	-27	2

In the present study, IFN-γ, T-bet and index1 were introduced as independent variables which entered into the logistic regression model. Parameter estimates, odd ratios and statistical significance of regression coefficients are presented in table 3.

The regression equation is:

$$\mathrm{Log}\left(\frac{\mathrm{p}}{1-\mathrm{p}}\right) = 1.363 + 3.311(\mathrm{IFN}\gamma) - 0.68(\mathrm{Tbet}) - 0.832(\mathrm{index})$$

According to table 3, IFN-γ, T-bet and index 1 explain a significant amount of variations in the probability of healthy status. Although, IFN-γ gene and intercept were not significant according to Wald test, these variables were significant based on likelihood ratio test (*p*-value = 0.02) and the corresponding model with these variables showed a lower AIC (Akaike information criterion) than the reduced model.

Table 3. Parameter estimates, odd ratios and statistical significance of regression coefficients

Variables in model	Coefficient	Standard deviation	Odds ratio	*p*-value
Intercept	1.363	0.764	3.908	0.065
IFN-γ	3.311	2.311	27.417	0.068
T-bet	-0.68	0.383	0.506	0.007
Index1	-0.832	0.372	0.435	0.001

It was estimated that for every one-unit increase in the introduced index, the odds ratio is reduced to 0.43.

Table 4. Logistic regression model evaluation using leave-one-out validation method.

Accuracy classification	Classification feature	Sensitivity classification	Area under the ROC curve	R2Nagelkerke
0.79	0.25	1	0.76	0.19

According to the classification table which was obtained from the validation test (Table 4), the overall accuracy of the model was 79%. The area under the ROC curve was 76% and the pseudo-R2 value was 19%. These are indicatives of a fairly good prediction of the fitted model. Therefore, the introduced index in the present study, not only indicates a statistically significant difference at the confidence level of 5% (Mann-Whitney test) but also, it has a good classification capability for the diagnosis of patients with schizophrenia.

Discussion

Inflammatory events of the central and peripheral nervous systems are important determinants of various stages of schizophrenia (Khandaker et al., 2015). Perhaps, it can render acceptable molecular markers through deep monitoring of the immune system's performances in schizophrenic patients.

The immune system plays a significant function in the incidence, pathogenesis and treatment of mental illnesses (Rosenblat, 2019; Tomasik et al., 2016), in a cytokine-mediated manner (Chang and Bistrian, 1998; Sonti et al., 1996). The association of immune encountered genes' polymorphisms, especially for *IFN-γ*, *TNF-α*, *IL-4* and *IL-10* genes with the incidence of schizophrenia has been reported (Noto et al., 2015; Na and Kim, 2007). Also, modifications in the expression levels of several immune-related genes have been reported in schizophrenic patients (Chan et al., 2011; He et al., 2020; Kim et al., 2004; Momtazmanesh et al., 2019; Potvin et al., 2008). According to the "equilibrium hypothesis", there is a balance in the ratio of Th1 to Th2 cells in healthy individuals (Kidd, 2003). This equilibrium lost its balanced status during various psychiatric disorders (Solek et al., 2018; Cox et al., 2015; de Witte et al., 2014). It was demonstrated that in schizophrenic patients, serum expression levels of *IFN-γ* and *IL-4*, as an indicator of Th1 to Th2 ratio, increased in comparison to control cases (Kim et al., 2004). Moreover, the higher activity of Th1 cells is associated with the reduced activity and less production of IL-4 and IL-10, as the representatives of Th2 cells' activity (Mansur et al., 2012).

In the present study, the gene expression profile of schizophrenic patients regarding their cytokine balance was investigated. So, IFN-γ and IL-4 were selected as the main cytokines of Th1 and Th2 cells, respectively. The expression of *T-bet* and *GATA-3* transcription factors were also evaluated. Results indicated a significant increase in the activity of Th1 cells in comparison to Th2 population, which means that in patients with schizophrenia, inflammatory events are increased prominently (Kelsven et al., 2020). Several factors, such as severe infection, autoimmune disorders and high smoking rates can lead to genetic changes, especially in case of schizophrenia, which clarifies more than ever the function of inflammatory events in this disease (Shi et al., 2009; Stolz et al., 2019). Likewise, the therapeutic effects of anti-inflammatory drugs and genetic, biochemical and immunological findings indicate the important role of inflammation in schizophrenia (Muller et al., 2015; Upthegrove and Khandaker, 2020).

An important step in providing appropriate treatment strategies is to identify molecular biomarkers that can be applied for early detection or prediction of schizophrenia (Liu et al., 2017; Trovao et al., 2019; Vatankhah et al., 2019). Although the use of post-mortem brain biopsies provides an opportunity for direct work on patient's neurons, this is not an ideal method, for reasons such as reduced mRNA integrity, and consequently reduced credibility and usefulness of biomarkers (Modai and Shomron, 2016). On the other hand, neurological-based assessments are costly (Kahn and Sommer, 2015). Thus, in recent years, the search for blood sample-based biomarkers of schizophrenia has been considered as a valid alternative (Bahn and Chan, 2015; He et al., 2019; Tasic et al., 2019). These biomarkers were classified into different categories and were discovered through various approaches (Lai et al., 2016). Although a variety of blood markers were examined and introduced, more than 70% of these markers for schizophrenia are playing a role during inflammatory responses (Chan et al., 2011). It should be noted that only a small number of previous studies have been identified the sensitivity and specificity of their introduced markers (Al Awam et al., 2015; Sun et al., 2015; Li et al., 2012).

The significance of the present study is its ability for prediction of the disease condition using a logistic regression based on the measurement of candidate cytokines. Although our index is weak to identify normal individuals, which may be due to the small sample size and some other factors that affect the immune system. It could be promising to introduce a novel indicator for screening of schizophrenic patients with high accuracy. However, we are aware that there should be certainly more additional tests for better confirmation of the results. Nevertheless, it is the first study of its kind in Iran.

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Conflict of Interest
The authors report no conflicts of interest in this work.

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