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Over-expression of TGF-β1 gene in medication free Schizophrenia

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ABSTRACT

Background and purpose: Immunological pathways play a crucial role in developing and precipitating neuropsychiatric disorders. Although the exact pathogenesis of schizophrenia is unknown, the possible role of genetic and biomarker involvement of the immune system is gaining attention. Here we quantified the mRNA expression of cytokines as a key role player of the immune system from the peripheral blood mononuclear cells of patients with schizophrenia and healthy controls to identify the differentially expressed genes.

Methods: Sixteen medication-free schizophrenia patients and 16 healthy subjects were enrolled in the current study. To investigate the desired expression level of mRNAs including *TGF-β1, IL-1β, IL-23, TNF-α, NF-κB*, and *BDNF*, quantitative real-time PCR was performed using specific oligonucleotide primers and the *Applied Bio systems StepOne™ real time PCR system*. DNA methylation was also analyzed through methylation-specific polymerase chain reaction (MSP).

Results: TGF-β1 was significantly up-regulated in peripheral blood mononuclear cells of patients vs. healthy individuals (P value = 0.03). In addition, we found a significant correlation between the positive symptom scale and *TGF-β1* gene overexpression (r = 0.536, P = 0.039). However, we did not observe any statistically significant differences for the methylation status of CpG Islands 1 and 2 between the patients and normal group. No statistical significance was found either for gene expression of *IL-1β* (P = 0.32), *IL-23* (P = 0.12), *TNF-α* (P = 0.87), *NF-κB* (P = 0.07), and *BDNF* (P = 0.33).

Conclusions: Although the number of medication-free schizophrenia patients is extremely limited, our data highlighted the potential role of *TGF-β1* as a regulatory cytokine in complex inflammatory mechanism involved in medication-free schizophrenia. In addition, we observed that increased level of *TGF-β1* mRNA in this disease might not be under methylation as an epigenetic control element at the genomic level.

1. Introduction

Schizophrenia is a severe psychiatric disorder which is among the top ten leading causes of long-term disability worldwide (McGrath and Sasser, 2009). It affects approximately 1.1% of people over the age of 18 years and mostly persists during the patient's lifetime and places a significant burden on the global health (Altamura et al., 2014). Schizophrenia generally includes symptoms including psychosis, apathy, and withdrawal and is characterized by cognition disorder, as well as emotional and social interaction difficulties (Olgiati et al., 2009).

Although significant research attempts have been made, the pathophysiology of the disease is not well elucidated and a complex interplay of multiple genetic, epigenetic, and environmental factors are postulated for the cause of schizophrenia. Some molecular processes including synaptic machinery, mitochondrial-related transcripts, and immune response have been known to be altered in the cortex of patients with schizophrenia (Mirnics et al., 2000; Iwamoto et al., 2005; Saetre et al., 2007; Xu et al., 2012). One of the possible mechanisms of schizophrenia is deregulation of immune processes in the central nervous system (CNS) (Khandaker et al., 2015; Bowcut and Weiser, 2018). Indeed, the hereditary background of the disease suggests genetic etiologies as a primary etiology of these events (Arzaghi et al., 2011). Several studies have proposed the role of immune system deregulation in the pathophysiology of schizophrenia (Moga et al., 2017).

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Gene expression alterations of diverse cytokines as key elements of the immune system in both central nervous system (CNS) and peripheral blood are key players of immune-pathogenesis of schizophrenia (Allen et al., 2008; Muller et al., 2000; Pandey et al., 2018). Some studies have indicated abnormal gene and protein expression of inflammatory cytokines in the postmortem brain of schizophrenia patients (Pandey et al., 2018).

Meanwhile, the properties of antipsychotics on blood levels of cytokines in schizophrenia have been shown by several meta-analysis studies (Borovcanin et al., 2013; De Witte et al., 2014; Van Kesteren et al., 2017). *Transforming growth factor beta 1* or *TGF-β1* is a polypeptide member of the transforming growth factor beta superfamily of cytokines, which inhibits the production of *Th1* cytokines including *interferon-g (IFN-γ)*, *tumor necrosis factor-α (TNF-α)*, *IL2*, and *IL2R* (Grayson et al., 2006; Poulter et al., 2008; Gallego et al., 2018; Brown Jr, 2016). *TGF-*β1 is expressed in early embryonic structures such as notochord, floor plate, and development of midbrain dopaminergic neurons; so its expression is crucial for nerve-related diseases including schizophrenia (Farkas et al., 2003). *TNF-α* is one of the most important cytokines in systemic inflammation and some studies suggest *TNFα* and *TNFα*-related signaling pathways in the pathophysiology of schizophrenia (Hoseth et al., 2017). *Brain-derived neurotrophic factor (BDNF)* is also a neurotrophic factor which appears to play a role in inflammatory pathways in the central nervous system, and is one of the candidate genes in the pathogenesis of schizophrenia (Wynn et al., 2018; Janicijevic et al., 2018). The *transcription factor kappa B (NF-κB)* regulates multiple aspects of innate and adaptive immune functions and serves as a pivotal mediator of inflammatory responses in schizophrenia (Roussos et al., 2013).

One of the possible epigenetic mechanisms of gene expression regulation in the eukaryotic cells is DNA methylation (Siegfried and Simon, 2010). Methylation of cytokine coding genes in T cells can be critical regulatory factor to modulate cytokine gene expression (Ikegame et al., 2013). Analysis of DNA methylation signatures in the brain have highlighted that global DNA methylation in different brain regions is associated with higher cognitive functions (Alelú-Paz et al., 2016b). One possible mechanism of drug efficacy can be altered methylation pattern of schizophrenia patients. Thus, determining the methylation of cytokines before treatment can be considered in predicting gene expression profile for predicting treatment resistance and schizophrenia management (Gillespie et al., 2017).

Accordingly, we designed this study in medication-free patients with schizophrenia to find its cytokines overexpressed gene profile and immunological mechanism. Then, based on the targeted gene with expression alterations, the hypo/hypermthylation of its promoter region was checked to find the impact of methylation on the targeted gene overexpression.

2. Materials and methods

2.1. Study population

Fifteen patients in medication-free schizophrenia (DNS) and 15 healthy subjects frequency-matched by age and sex without familial relationship with patients and no previous history of psychiatric disorders in them and their families were enrolled in the study. All patients were diagnosed by Structured Clinical Interview for DSM-IV (SCID) in a psychiatric hospital. All patients were medication free and their psychopathological status was assessed by trained psychiatrist using Positive and Negative Syndrome Scale (PANSS) (Mittleman et al., 1997), to define positive, negative, and general psychopathology symptoms of schizophrenia. Positive symptoms refer to disordered thoughts, delusions, and visual and/or olfactory hallucinations. On the other hand, negative symptoms include anhedonia, apathy, emotional withdrawal, and stereotyped thinking. General psychopathology symptoms represent the severity of negative and positive symptoms. Subjects with chronic infections, allergies, history of cardiovascular disease, type 1 and 2 diabetes, epilepsy, rheumatoid arthritis, head trauma, multiple sclerosis, depression, bipolar disorder, and any illicit drug abuse and dependence except smoking were excluded. Once completely explained about the study, all participants signed written informed consent form. The study protocol was approved by Ethics Committee of Tehran University of Medical Sciences (TUMS).

2.2. RNA extraction and synthesis of cDNA

For this purpose, 5 ml of peripheral blood was collected from each participant in heparin-containing tubes and were processed for isolation of lymphocytes by Lymphocyte-H (Cedarlane Laboratories, Hornby, Ontario, Canada). RNA was extracted from peripheral blood mononuclear cells by TRIzol (Life Technogene) according to the manufacturer's instructions. The RNA pellets were stored at −80 °C once dissolved into DEPC treated water. RNA solution was qualified by measuring the ratio of OD260/280 on a Nano Drop spectrophotometer (NanoDrop Thermo Scientific 2000), with the solution with OD260/280 ratio < 1.6 discarded. RNA was reverse transcribed by First Strand cDNA Synthesis Kit (Thermo Science) according to the manufacturer's guidelines.

2.3. Quantitative real-time PCR

To investigate the desired mRNAs expression level including *TGF-β1*, *IL-1β*, *IL-23*, *TNF-α*, *NF-κB*, and *BDNF*, quantitative real-time PCR was performed using specific oligonucleotide primers (Table 1), SYBR Premix Ex Taq II kit (Takara, Japan), and an Applied Biosystem StepOne™ real-time PCR system (Applied Biosystems, CA, USA). Hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) housekeeping gene was used as internal control for normalization of gene expression data. Data Analysis was performed using the $2^{-\Delta\Delta CT}$ method. This gene has been suggested as the housekeeping gene and for schizophrenia it is usually recommended as the real -time PCR internal control (Silver et al., 2008).

2.4. DNA extraction and methylation-specific polymerase chain reaction (MSP)

Genomic DNA was extracted from 1.5 cc of whole blood collected in EDTA tubes using Phenol-chloroform extraction technique (Di Pietro et al., 2011). Further, 2 μg of genomic DNA was treated with the Epitect Bisulfite kit (Qiagen) according to the manufacturer's instructions. For analysis of DNA methylation of *TGFB1* gene, methylation-specific polymerase chain reaction (MSP) was performed for 2 CpG islands of the promoter region. The methylated and unmethylated TGFB primer sets are given in Table 2.

Table 1
Sequence of primers for real-time PCR quantification.

Gene	Primer pair sequences	Amplicon size
HPRT F	5′-CCTGGCGTCGTGATTAGTGAT-3′	131 bp
HPRT R	5′-AGACGTTCAGTCCTGTCCATAA-3′	
TGF-β1 F	5′- CGACTACTACGCCAAGGA -3′	150 bp
TGF-β1 R	5′- GAGAGCAACACGGGTTCA -3′	
NF-κB F	5′- AGTGCTGGAGTTCAGGATAAC -3′	193 bp
NF-κB R	5′- GAGAATGAAGGTGGATGATTGC-3′	
TNF-α F	5′-CCCAGGCAGTCAGATCATCTTC-3′	85 bp
TNF-α R	5′-AGCTGCCCCTCAGCTTGA-3′	
BDNF F	5′- ACCTGAACACTTATTGCTTTG -3′	114 bp
BDNF R	5′-CATTCGGCCTGAGTTTGG-3′	
IL-1β F	5′-ATGGCTTATTACAGTGGCAATGAG-3′	138 bp
IL-1β R	5′-GTAGTGGTGG TCGGAGATTCG -3′	
IL-23 F	5′-GGACAACAGTCAGTTCTGCTT-3′	115 bp
IL-23 R	5′-CACAGGGCTATCAGGGAGC-3′	

Table 2
Sequence of designed primer sets for MSP.

Primer set	Sequences	Amplicon size (Brustolim et al.)
Methylated TGFB1 CpG island 1- Forward	5′-GGGGCGGTTTTAAAATTTTTTGTCGATTTAGTC-3′	163
Methylated TGFB1 CpG island 1- Reverse	5′-AAAACCGAAAAAATACCCCGAACGAAACG-3′	
Unmethylated TGFB1 CpG island 1- Forward	5′-GGGGGTGGTTTTAAAATTTTTTGTTGATTTAGTTG-3′	169
Unmethylated TGFB1 CpG island 1- Reverse	5′-AACTCAAAACCAAAAAAATACCCCAAACAAAACA-3′	
Methylated TGFB1 CpG island 2- Forward	5′-GTCGTGTTCGTTTTGTATAATAGTATTCGC-3′	105
Methylated TGFB1 CpG island 2- Reverse	5′-GCGAATAACCTCCTTAACGTAATAATCG-3′	
Unmethylated TGFB1 CpG island 2- Forward	5′-AGGTTGTGTTTGTTTTGTATAATAGTATTTGTG-3′	111
Unmethylated TGFB1 CpG island 2- Reverse	5′-ACACACAAATAACCTCCTTAACATAATAATCA-3′	

2.5. Statistical analysis

All continuous variables were presented as means and standard deviation (SD). The Mann-Whitney U test was used for between-group comparisons of gene expressions. The association between TGF-β1 gene expression levels and severity of schizophrenia was analyzed using Spearman correlation. Statistical analysis was performed by SPSS version 15 (SPSS Inc. Chicago, IL, USA) with $P \leq 0.05$ considered as statistically significant.

3. Results

3.1. Demographic data of the study subjects

Among all patients with newly diagnosed schizophrenia, 15 patients tose who were medication free were enrolled in the study. There consisted of 7 women and 8 men. The healthy controls were matched in terms of gender and age with the patients. The mean ± standard deviation (SD) of the age was 34.25 ± 7.86 and 33.93 ± 4.78 years in the case and control groups, respectively. The demographic data and clinical features of the study subjects are summarized in Table 3.

3.2. Gene expression in subjects

Quantitative real-time PCR analysis is presented in Fig. 1. As can be seen, *TGF-β1* was upregulated in peripheral blood mononuclear cells of patients vs. healthy individuals. The growth of *TGF-β1* gene expression in DNS compared to the control group was significant (*P*-value = 0.03). In addition, we found a significant correlation between the positive symptom scale and *TGF-β1* gene expression ($r = 0.536$, $P = 0.039$). However, there were no significant correlations with the total PANSS score, neither with the negative symptoms nor with the general psychopathology scale.

No significant statistical differences were observed for gene expression of *IL-1β* (*P*-value = 0.32), *IL-23* (*P*-value = 0.12), *TNF-α* (*P*-value = 0.87), *NF-κB* (*P*-value = 0.07), and *BDNF* (*P*-value = 0.33) between the medication-free schizophrenia and healthy subjects.

Table 3
Demographic and clinical characteristics of subjects.

Qualitative variables	Medication free Schizophrenia patients	Normal controls
Sex (male/female)	56.2/43.8%	53.3/46.7%
Age (years, mean ± SD)	34.25 ± 7.86	33.93 ± 4.78
BMI (kg/m^2, mean ± SD)	21/48 ± 6.81	23.18 ± 4.78
Positive and negative syndrome scale (PANSS) (mean ± SD)		
Positive symptoms	26.37 ± 8.33	NA
Negative symptoms	22.87 ± 5.63	NA
General psychopathology	43.43 ± 5.52	NA
Total score	92.60 ± 15.01	NA

3.3. The status of promoter's CpG islands methylation

Methylation statue of *TGFB1* promoter CpG Island in the target cells is presented in Table 4, where DNS patients were monoallelically methylated in CpG Island 1 *TGFB1*. There were no statistically significant differences for the methylation status of CpG Islands 1 and 2 between the patients and normal group.

4. Discussion

Transforming growth factor betas (*TGF-βs*) are famous as multifunctional growth factors taking part in the completion of developmental process, disease, and tissue repair. *TGF-beta* family consists of three members (*TGF-beta1, 2, 3*) which show high similarity and homology and are produced by both glial and neuronal cells (Lawrence, 1996). In CNS, *TGF-β1* has neuro-protective functions and is important for injury-related cytokine, specially associated with astrocyte scar formation (Dobolyi et al., 2012). Neurogenesis during adulthood has been postulated to occur in the hippocampus of patients with schizophrenia through *TGF-β* signaling (Lie et al., 2005; Ageta et al., 2008). It is a fact that the number of schizophrenia patients who take no medications is extremely limited. In spite of this limitation in the targeted DNS sample, we presented the first report on the expression of *TGFB1* gene elevation in peripheral blood mononuclear cells of DNS patients. The importance of selecting patients who had not received any medication is the effect of antipsychotic drugs on changing the gene expression profile to the normal conditions (Crespo-Facorro et al., 2014; Lee et al., 2017). Benes et al reported a significant upregulation of *TGF-β* signaling pathway in hippocampus of patients with schizophrenia (Benes et al., 2007). Synaptic-neurodevelopmental model of schizophrenia proposes that excess levels of normal synaptic pruning occurring in certain brain regions may be involved in psychotic symptoms (Mirnics et al., 2001; Frydecka et al., 2015). Impaired excitatory and inhibitory balance in the hippocampal network leads to behavioral abnormalities similar to schizophrenia (Sun et al., 2010). *TGF-β* pathway is also involved in various aspects of neurodevelopment (Liu and Niswander, 2005), adult neurogenesis (Arolt et al., 2000; Ageta et al., 2008) and neuro-protection (Chiang et al., 2004).

This overexpression of *TGF-β1* can be the consequence of epigenetic regulatory factors such as promoter methylation. DNA methylation is an epigenetic mechanism which occurs with the addition of a methyl group to the cytosine and modifying the function of the genes, thereby affecting gene expression without changing the DNA sequence (Vanyushin, 2006; Riddihough and Zahn, 2010). Jaffe, A. E indicated that DNA methylation is the central change of brain development and is really important in schizophrenia. He identified 2,104 CpGs which differed between schizophrenia patients and controls, involved in the development and neuro-differentiation through mapping DNA methylation (Jaffe et al., 2016). Further, the epigenome analysis of several brain regions from schizophrenic patients with severe cognitive impairment using high-resolution DNA methylation array suggested 139 differentially methylated CpG sites (Alelú-Paz et al., 2016a). Accordingly, in the current study, we checked the methylation of two targeted

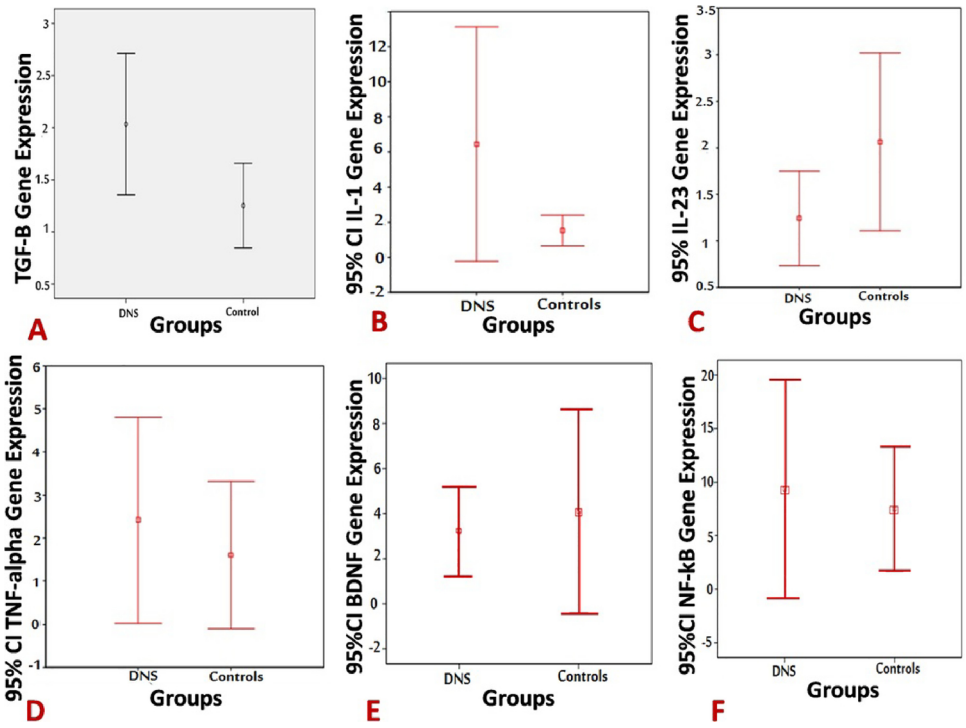

Fig. 1. Cytokines gene expression of peripheral blood mononuclear cells in patients with DNS vs. health subjects. The mean ± standard deviation (SD) of the age was varied from 26 to 42 years in medication free schizophrenia group (DNS) versus 28–37 years in control groups. Error-Bar shows mean ± SD [delta] mRNA expression normalized against HPRT internal control in patients with DNS and control group. (A) There was a significant increase in TGF-β1 gene expression DNS + vs. controls ($P < 0.035$). (B) IL-β1 mRNA expression error bar show higher mean ± SD in DNS vs. control group, but is not a significant different between them. (C) IL23 mRNA expression mean ± SD in DNS group was decreased, but not significant, compared to control. (D) mRNA expression augmentation of TNF-α1 is seen in the DNS group, but not significantly. (E) BDNF gene expression in peripheral blood mononuclear cells is not much difference in the two groups. (F) There is no significant difference for NF-κB mRNA expression. The error bars represent the standard deviation of mRNA expression and for TGF-β1, IL-β1, TNF-α1, and NF-κB the wider range of error bar of DNS in comparison with control can be seen but for IL23 and BDNF the narrower ones are seen. It can be the indicator of significant differences in expression between the control and DNS samples for each experiment (P-value < 0.05).

Table 4
Methylation statue of TGFB1 promoter in peripheral blood mononuclear cells of subjects.

Targeted region	Methylation		Unmethylation	
	DNS ($n = 15$)	Normal ($n = 15$)	DNS ($n = 15$)	Normal ($n = 15$)
CpG island 1	3[a]	0	15	15
CpG island 2	0	0	15	15

[a] 3 DNS patients were monoallelically methylated in CpGIsland 1 TGFB1.

promoter regions of *TGF-β1* gene with no significant hypo/hypermethylation observed. Our findings can suggest that other epigenetic or genetic alterations are responsible for changing the *TGF-β1 expression.* It was in 2006 that R. Shah and his coworkers indicated that *Activator protein 1 (AP1)* and *hypoxia* are key regulators of *TGF-β1* expression levels. They revealed that common *TGF-β1* promoter polymorphism (509C→T, *rs1800469*) is linked to an almost twofold increase of *TGF-β1* levels in plasma of individuals and to the risk, progression, and outcome of numerous diseases (Shah et al., 2006). There are some recommendations over the post-transcriptional regulation of *TGF-β1* expression by *miR-744* (Martin et al., 2011).

Over the past two decades, several studies have proposed the role of inflammation in the pathophysiology of schizophrenia and a number of hypotheses have been formulated based on cytokine-mediated mechanisms (Miller et al., 2011; Miller et al., 2013; Kirkpatrick and Miller, 2013). Overproduction of immune markers has been observed to correlate with having more severe psychotic symptoms (Drexhage et al., 2010; Hope et al., 2013). Aberrant cytokine levels particularly the imbalance between *T-helper type 1 (Th1)* and *type 2 (Th2)* cytokines, towards the predominance of *Th2*, has been implicated as one of the most important immune deregulation mechanisms in schizophrenia (Chiang et al., 2013). More than *TGF-β1* the mRNA expression of other cytokines has been checked in this study where no overexpression was observed in *IL-1β*, *IL-23*, *TNF-α*, *NF-κB*, and *BDNF*. According to our results, peripheral blood mononuclear cells mRNA levels of *IL-1β, IL-23, NF-κB* and *TNF-α* concentration even in age and gender adjusted model were not different in DNS and controls. The limitation of our research was that patients were not yet treated with antipsychotic medications when our targeted gene expression profile was evaluated. Accordingly, most patients were not likely to have experienced the first episode, so the average age was elevated. In contrast, some studies reported no difference (Erbagci et al., 2001) and some others highlighted a significant change in the serum level of these cytokines in schizophrenia patients vs. healthy controls (Di Nicola et al., 2013; Kim et al., 2000; Borovcanin et al., 2015). Very recently it has been suggested that *TNF-α* and *IL-6* are linked with the deficit syndrome and negative symptoms of chronic schizophrenia (Goldsmith et al., 2018). *TNF-α* was dysregulated in both post-mortem brain and peripheral blood, although negative findings have also been reported (Pandey et al., 2018; Pandey et al., 2015; Dean et al., 2013; Mohite et al., 2018). Interestingly, it was observed that it also affects the individuals with treatment-resistant schizophrenia (Mostaid et al., 2018). Lately, Zhang, Yi, et al. has provided preliminary evidence that the interaction of *BDNF* and *TNF-α* may develop susceptibility to schizophrenia and cognitive dysfunction (Zhang et al., 2018). The *brain-derived neurotrophic factor* is a protein product of *BDNF* coding gene and is a member of the neurotrophin family of growth factors supporting differentiation, maturation, and survival of neurons in the nervous system. It also shows a neuro-protective effect under adverse conditions such as glutamatergic stimulation, cerebral ischemia, hypoglycemia, and neurotoxicity (Bathina and Das, 2015). The *BDNF* expression in schizophrenic patients did not change compared to control subjects in our study, which was in agreement with some previous studies (Shimizu et al., 2003; Huang and Lee, 2006), in contrast to some other studies with significant changes in *BDNF* levels in some cerebral and serum regions (Ikeda et al., 2008). These contradictory results may be due to the different demographic characteristics including the age of onset of the disease as well as the use or non-use of drugs. In a study published in 2005, it was observed that various cofounders including age and gender have a particular effect on stored and circulating *BDNF* in the blood cells (Lommatzsch

et al., 2005). A positive relationship was reported between serum BDNF levels and cortex in rats (Karege et al., 2002). BDNF crossing the blood-brain barrier has also been confirmed in mice (Pan et al., 1998), though there is no evidence of such a two-way BDNF transmission in humans. In the study of Takahashi et al., the level of BDNF protein in the hippocampus and anterior cingulate cortex increased in patients with schizophrenia, while it did not change in the cortex of the prefrontal or occipital lobe (Takahashi et al., 2000).

The abnormalities of the *NF-κB* signaling pathway in schizophrenia were observed and provided evidence for an additional possible mechanism affecting the translocation of *NF-κB* signaling to the nucleus more than its overexpression (Roussos et al., 2013). Consequently, the *NF-κB* signaling pathway can be potentially an important therapeutic target in psychiatric disorders (Altinoz et al., 2016; Park and Hong, 2016; Ibi et al., 2017).

Taken everything into the consideration, our data corroborated the potential role of *TGF-β1* as a regulatory cytokine in complex inflammatory mechanisms involved in schizophrenia. In addition, we provided evidence that the increased level of *TGF-β1* mRNA in schizophrenia may not be under epigenetic control mechanism of methylation. Despite the limitations of this study including the small size of study population and using peripheral blood mononuclear cells instead of nerve tissue, our data suggest the *TGF-β1* expression profile as a marker which enhanced the risk for psychosis. Therefore, *TGF-β1* could be a valuable biomarker for schizophrenia.

Authors' contributions

SMA participated in the acquisition, analysis and interpretation of data and drafting manuscript. AAN participated in the design, patient acquisition and analysis and interpretation of data. MMA participated in acquisition of data, laboratory and genetic tests and analysis and interpretation. SE participated in acquisition of data, laboratory and genetic tests and analysis and interpretation. FKH has done the revised of the written manuscript. All authors read and approved the final manuscript.

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