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ORIGINAL ARTICLE

INVESTIGATION OF FASCICULATION AND ELONGATION PROTEIN ζ-1 (*FEZ1*) IN PERIPHERAL BLOOD REVEALS DIFFERENCES IN GENE EXPRESSION IN PATIENTS WITH SCHIZOPHRENIA

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ABSTRACT

Schizophrenia (SZ) is a chronic neuropsychiatric disorder characterized by affective, neuromorphological and cognitive impairment, deteriorated social functioning and psychosis with underlying molecular abnormalities, including gene expression changes. Observations have suggested that fasciculation and elongation protein ζ-1 (*FEZ1*) may be implicated in the pathogenesis of schizophrenia. Nevertheless, our current knowledge of the expression of *FEZ1* in peripheral blood of schizophrenia patients remains unclear. The purpose of this study was to identify the characteristic gene expression patterns of *FEZ1* in peripheral blood samples from schizophrenia patients. We performed quantitative reverse-transcriptase (qRT-PCR) analysis using peripheral blood from drug-free schizophrenia patients ($n = 29$) and age and gender-matched general population controls ($n = 24$). For the identification of *FEZ1* gene expression patterns, we applied a comparative threshold cycle (CT) method. A statistically significant difference of *FEZ1* mRNA level was revealed in schizophrenia subjects compared to healthy controls ($p = 0.0034$). To the best of our knowledge, this study is the first describing a down-regulation of *FEZ1* gene expression in peripheral blood of patients with schizophrenia. Our results suggested a possible functional role of *FEZ1* in the pathogenesis of schizophrenia and confirmed the utility of peripheral blood samples for molecular profiling of psychiatric disorders including schizophrenia. The current study describes *FEZ1* gene expression changes in peripheral blood of patients with schizophrenia with significantly down-regulation of *FEZ1* mRNA. Thus, our results provide support for a model of SZ pathogenesis that includes the effects of *FEZ1* expression.

Keywords: Fasciculation and elongation protein ζ-1 (*FEZ1*); gene expression; quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR); schizophrenia (SZ)

INTRODUCTION

Schizophrenia (SZ) is a severe mental disorder affecting approximately 1.0% of the human population worldwide [1]. Twin, family and adoption studies strongly support that genetic factors play an important role in the etiology of SZ. Currently, numerous genetic studies, including linkage scans and their meta-analyses, candidate gene association analyses, gene expression and genome-wide association studies (GWAS) have identified particular genes and chromosomal loci with the disorder [2-4]. Circulating blood is easily accessible material and has been suggested as an alternative to tissue samples for molecular profiling of human disease and disease risk [3,5]. This is based on the capacity of peripheral blood to reflect pathological

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changes in the body. As the brain tissue is not easily accessible for investigation, blood-based expression profiling is increasingly being undertaken to search potential biomarkers for SZ [6,7]. Fasciculation and elongation protein ζ-1 (*FEZ1*) is one of the first identified binding partners of Disrupted-in Schizophrenia 1 (*DISC1*), a susceptibility gene for major mental disorders including SZ in yeast two-hybrid screen of an adult human brain library [8]. Moreover, proteomic techniques revealed that *FEZ1* protein interacts with various intracellular partners, such as motor signaling, and structural proteins, one of which is *DISC1* [9]. Currently, the role of *FEZ1* in mammalian neuronal development *in vivo* is not well understood. The *FEZ1* null mice exhibit hyperactivity and enhanced responsiveness to psychostimulants [10], supporting a po-tential contribution of *FEZ1* dysfunction to SZ. Recently, additional evidence based on sequencing of *DISC1*-interacting partner genes including *FEZ1* revealed an increased burden of rare missense variants in SZ susceptibility in an isolated northern Swedish population [11]. In contrast, the *FEZ1* gene shows no association to SZ in Caucasian or African American populations [12]. However, no association was found in either population between specific haplotypes and any of the psychiatric disorders [13]. Interestingly, there is a significant reduction of *FEZ1* mRNA in both hippocampus and dorsolateral prefrontal cortex of SZ patients and also an association of the *DISC1* genotype and *FEZ1* mRNA levels [9]. All these findings raise the possibility that *FEZ1* and *DISC1* may assist in regulating both neuronal development and risk for SZ. We hypothesized that previously shown *FEZ1* mRNA levels in prefrontal cortex of SZ patients may be relevant to those in peripheral blood tissue. The aim of this study was to identify gene expression patterns of *FEZ1* in peripheral blood samples from SZ patients.

MATERIALS AND METHODS

Ethics Statement. This study and the informed consent forms were approved by the Medical University of Plovdiv Ethics Committee.

Participants. Written informed consent was obtained from 29 patients recruited at the State Psychiatry Hospital Pazardjik, Pazardzk, Pazardzk, Bulgaria and 24 healthy volunteers. Routine psychiatric examination, wide medical hystory and the Mini-International Neuropsychiatric Interviews were done by a certified psychiatrist to evaluate the diagnosis of paranoid SZ only, on Diagnostic and Statistical Manual of Mental Disorders (IVth edition) criteria and to exclude any mental disorder in the controls. Important inclusion criteria were that the participants had not received any medication (even *psychotropic*) 1 month before blood sampling and they had a standard breakfast, so they were assessed in a state of exacerbation. Persons with other chronic medical and current acute somatic/neurologic illness, alcohol or drug abuse/dependancy were also excluded. The sample population included 15 males/14 females diagnosed with SZ and 24 age- and gender-matched general population controls (12 males/12 females) with no evidence for any psychiatric or neurological disorder in first-grade relatives (Table 1).

Table 1. Age descriptive statistics.

Groups	*n*	Mean	SD	SE Mean
SZ	29	45.62	12.565	2.333
Controls	24	46.38	12.673	2.587

SD: standard deviation; SE: standard error; SZ: schizophrenia.

Blood Collection and RNA Isolation. Blood samples from the patients and control groups were collected in PAXgene Blood RNA collection tubes (PreAnalyticX GmbH, Hombrechtikon, Switzerland) that contain a reagent that lyses blood cells and immediately stabilizes intracellular RNA to preserve the gene expression profile. To reduce any potential bias in gene expression due to diurnal variation, blood was drawn in the morning, from all of the subjects. We used the PAXgene Blood miRNA Kit (PreAnalyticX) to extract total RNA from the blood samples [14]. Total RNA was then quantified by absorbance at A260 nm using Epoch Micro-Volume Spectrophotometer System (BioTek, Winooski, VT, USA) and the purity was estimated by the ratio A260/A280 nm. The absorbance ratio of 260 nm and 280 nm (A260/A280) was between 1.93 and 2.1 for all samples included for further analysis. The RNA integrity was confirmed by non denaturing agarose gel electrophoresis, which was then stored at –80 °C until further analysis. The resulting RNA was treated with RNase-free DNase I (Promega BioSciences, San Luis Obispo, CA, USA) according to the manufacturer's protocol and checked for DNA contaminations prior to copy DNA synthesis step.PAGE

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) Analysis of *FEZ1* mRNA Level. The qRT-PCR analyses were performed in at least three physically separate rooms in to order to reduce the chance for contamination. Copy DNA for the protein coding gene was synthesized from total RNA with oligo $(dT)_{18}$ primer using RevertAid First Strand cDNA Synthesis Kit according to the assay protocol (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription reactions contained 1 μg of total RNA samples, 1 μL oligo $(dT)_{18}$ primer and nuclease free water to a final volume of 12 μL, after incubation at 65 °C for 5 min., we added 4 μL 5X RT buffer, 1 μL RiboLock RNase Inhibitor (20 U/μL) (Thermo Fisher Scientific), 2 μL 10 mM dNTP Mix and 1 μL RevertAid MMuLV Reverse Transcriptase (200 U/μL) (Thermo Fisher Scientific), to final volume of 20 μL. A relative gene expression method was employed to determine gene expression levels. The reactions were set up in duplicate in a 96-well format using the 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and Maxima SYBER Green/Rox qRT-PCR Kit (Thermo Fisher Scientific). Amplification of the single amplicon coresponding to the *FEZ1* sequence was confirmed by monitoring the dissociation curve (melting curve analysis) and by agarose gel electrophoresis. The qRT-PCR forward and reverse primers for *FEZ1* used in this study were 5'-GGG ACT GCA TGA GAC CAT GT-3' and 5'-TTG AGG GCT GTA GCC AGA CT-3', respectively [15]. B-actin (*ACTB*) was used as an internal control for normalization. *ACTB*-specific qRT-PCR primers were as follows: forward 5'-AGT GTG ACG TGG ACA TCC GCA-3' and reverse 5'-GCC AGG GCA GTG ATC TCC TTC T-3'.

After performing the qRT-PCR analysis, the Ct values were measured, different methods could be used to determine the expression level of the target gene in the test sample relative to the calibrator sample. Here, we used the Livak and Schmittgen method [16], also known as the comparative 2^{-Ct} method. All the analyzed SZ samples showed mean Ct values range from 26.7 to 34.9 and mean control Ct values ranged from 28.0 to 31.3. The mean Ct values of the reference *ACTB* gene for SZ samples ranged from 16.6 to 21.7 and the range for control samples from 16.3 to 18.0, respectively.

First, for the normalization of the Ct values of the target genes to that of the reference gene, for both the test sample and the calibrator samples was made using the equation:

$$Ct_{(test)} = Ct_{(target,\ test)} - Ct_{(reference,\ test)}$$

$$Ct_{(calibrtator)} = Ct_{(target,\ calibrator)} - Ct_{(reference,\ calibrator)}$$

Second, normalization of the Ct of the test sample to the Ct of the calibrator was made using the equation:

$$Ct = Ct_{(test)} - Ct_{(calibrator)}$$

Finally, calculation of the expression ratio with the following equation:

2^{-Ct} = normalized expression ratio.

The result obtained is the fold increase (or decrease) of the target gene in the test samples relative to the calibrator sample.

Statistical Analyses. All statistical calculations were performed using the Statistical Package for the Social Sciences (SPSS) software, version 20.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) *t*-test of Ct was used to examine differences in expression levels of *FEZ1* mRNA across healthy controls and SZ subjects. A Spearman correlation analysis was also done for searching relation between expression levels and demographic characteristics of the tested individuals.

To investigate the characteristics of *FEZ1* gene expression as potential diagnostic biomarkers in SZ patients, a ROC (receiver operating characteristic) curve was done and AUC (area under the ROC curve) was calculated. Statistical tests were two-sided with a p value of 0.05.

The STRING Pathway Analysis of FEZ1 Protein Interactions. The FEZ1 interaction data for each validated protein was obtained using the STRING database (version 9.0; http://string-db.org/) (Figure 1).

RESULTS

In order to identify whether a key *DISC1* (interacting partner gene) *FEZ1* expression is altered in SZ patients we performed a detailed analysis of peripheral blood samples. To test this, we evaluated *FEZ1* mRNA levels in SZ patients.

As the data were normally distributed, we performed two-tailed *t*-test. A statistically significant difference of *FEZ1* mRNA level was revealed (p = 0.0068) (Figure 2). Relative quantification (RQ) calculations were done using the 2^{-Ct} method [16]. The results clearly demonstrate down-regulation of *FEZ1* mRNA levels in SZ patients. The data are presented

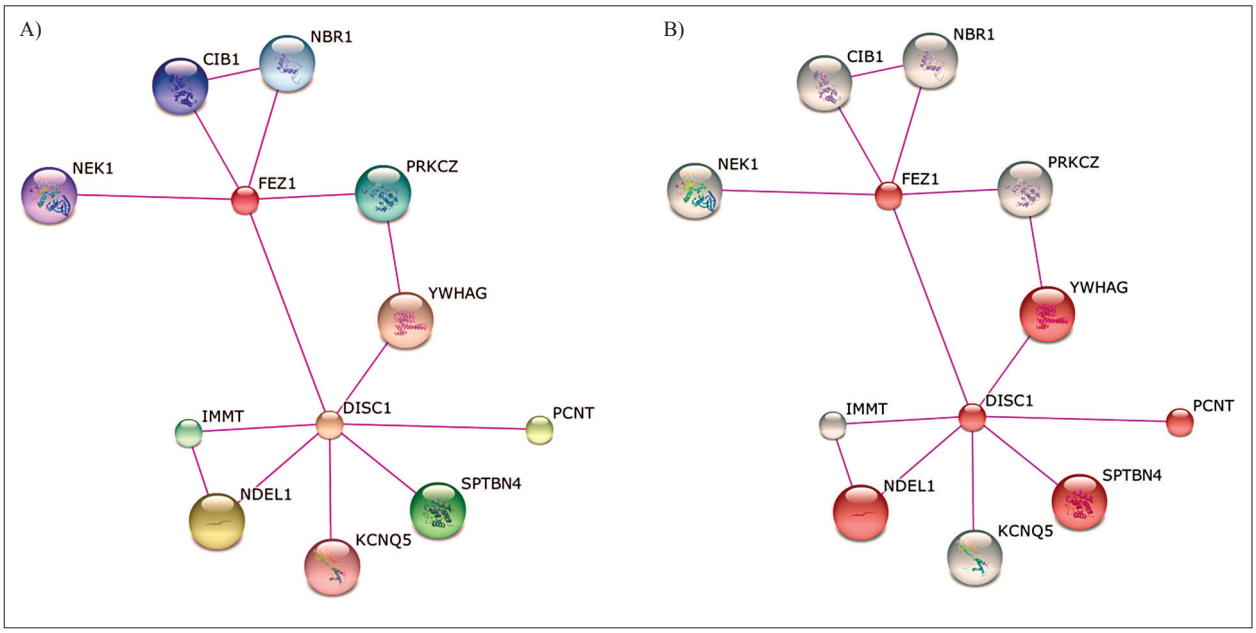

Figure 1. Network visualization of a key protein partners of FEZ1. A) The STRING screenshot shows an experimentally validated protein partners of FEZ1. B) Protein partners of FEZ1 (shown in red) in GO Biological Processes, term: neurogenesis.

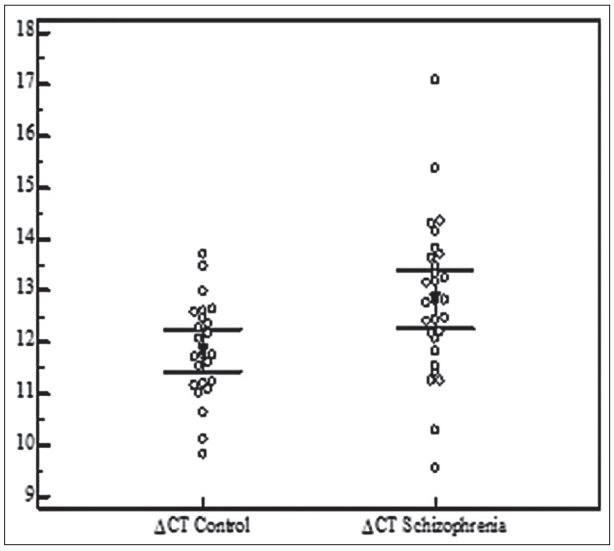

Figure 2. The figure shows the ΔCt values of *FEZ1* expression across the analyzed SZ patients and healthy controls compared with the cycle in which they were obtained. The qRT-PCR procedure was used to determine relative quantification levels of *FEZ1* mRNA (RQ *FEZ1*) in peripheral blood from 29 patient samples.

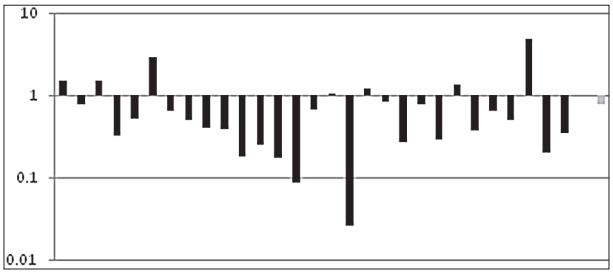

Figure 3. Presentation of the results as fold changes in gene expression normalized to an endogenous reference gene relative to the healthy control patients. Log transformed *FEZ1* RQ of individual SZ subjects (in black), where 1 on the y-axis is average expression in controls. The average change of RQ level is shown in gray.

as the fold change in gene expression normalized to an endogenous reference gene and relative to the healthy control patients (Figure 3).

The observed differences in individual expression levels clearly demonstrate the heterogeneity in the expression profile of the analyzed gene that is in agreement with the results observed in inter-patient variation in the expression studies by other authors. The PCR amplification of the specific products corresponding to *ACTB* and *FEZ1* amplicons was confirmed by monitoring the dissociation curve (Figure 4).

The Spearman test did not manage to reveal any significant correlation with age and sex in the tested groups ($p = 0.65/p = 0.54$). The ROC also displayed good characterization of state prediction with AUC = 0.728, ($p = 0.0011$) (Figure 5). All this was consistent with a key role of FEZ1 protein as interacting partner with DISC1 as SZ susceptibility genes (Figure 1A), and FEZ1 protein partners involved in neurogenesis

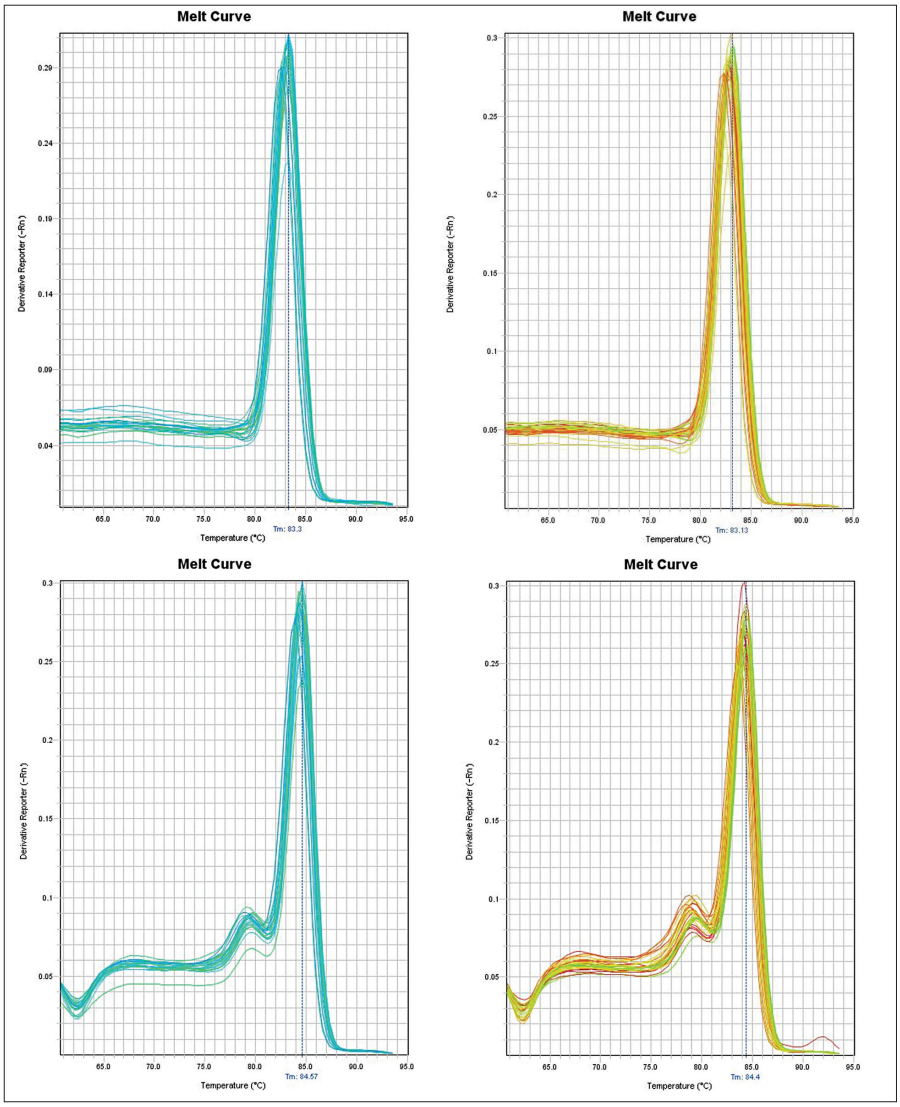

Figure 4. The qRT-PCR data showing a DNA melt profile result for amplification of the specific single product in qRT-PCR analysis. Specific single products corresponding to *ACTB* in SZ and healthy control patients top panel and *FEZ1* amplicons bottom panel, respectively, were confirmed by monitoring the dissociation curve (melting curve analysis). The melting dissociation, performed on *FEZ1*and *ACTB*, allowed confirmation of the specificity of the amplifications. The melting temperatures of *FEZ1* amplicons were 84 °C ± 1 °C, whereas *ACTB* had a melting temperature of 83 °C ± 1 °C.

as shown in Gene Ontology (GO) Biological Processes ($p = 0.063$) (Figure 1B).

DISCUSSION

Despite the identification of numerous SZ susceptibility genes, the pathology of SZ still remains unknown. At the molecular level, a large number of potential DISC1 binding partners have been identified from a yeast two-hybrid screen [12], many of which are also involved in neurodevelopmental processes implicated in the patho-physiology of psychiatric disorders. Experiments with primates and rodents demonstrate that *FEZ1* and *DISC1* have overlapping temporal and spatial expression patterns [17,18]. Both proteins are expressed in the pyramidal neurons of the developing hippocampus, the cerebral neocortex and the olfactory bulb. Moreover, disruption of the DISC1/FEZ1 interaction inhibits DISC1-stimulated neurite outgrowth in PC12 cells

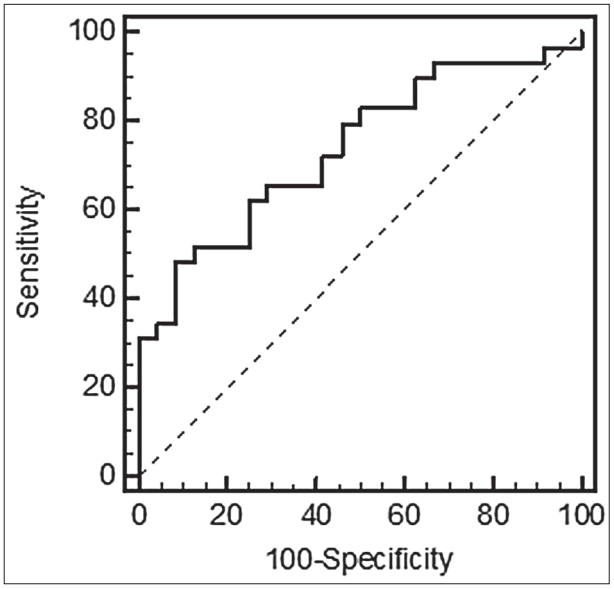

Figure 5. Performance of the *FEZ1* prediction model. The ROC for *FEZ1* expression was performed to evaluate the prediction accuracy. The dotted diagonal line represents random classification accuracy (AUC 0.5). The accuracy of *FEZ1* as a single predictor was AUC 0.728 [95% confidence interval (CI) minimum 0.589/ maximum 0.841; SE (standard error) 0.0698] with 55.2% sensitivity and 87.5% specificity between schizophrenic and healthy controls subjects.

[8], thus, decreased *FEZ1* levels could influence DISC1-stimulated functions.

On the one hand, this study is the first describing a *FEZ1* gene expression change in peripheral blood of patients with SZ; on the other hand, this change corresponds to the down-regulation in prefrontal cortex and hippocampus of schizophrenic patients [9]. Due to the fact that *FEZ1* interacts with *DISC1*, a susceptibility gene for major mental disorders to synergistically regulate dendritic growth of newborn neurons in the adult mouse hippocampus. We assume that any interaction related to another *FEZ1* partner would be compromised or at least influenced due to changes in the expression of the *FEZ1* transcript that would change the level of translated protein and its involvement in complexes associated with susceptibility for SZ functions (Figure 1).

Thus, our results provide support for a model of SZ pathogenesis that includes the regulatory effects on *FEZ1* gene expression in peripheral blood specific for patients with exacerbation of SZ. One obvious limitation of previous expression studies is the use of human postmortem brain tissue for quantitative analyses of gene expression profile, primarily because postmortem brain tissue from SZ patients is extremely rare and highly prized. Additionally, identification of gene expression profile can be complicated by a variety of confounding factors such as pH, drugs, cause of death, *etc.* In contrast, in attempts to overcome these limitations, we used RNA storage and extraction systems that block and preserve RNA for downstream expression study, so that the expression level that we identified really reflects the current physiological state of the analyzed patients. All these changes in expression levels probably are not due to gene polymorphisms, as such was not detected in a large SZ cohort [19], but can be attributed to various epigenetic mechanisms that alter distinct molecular pathways. As hypomethylation was observed in the exonic region of *HTR2A* and *MB-COMT* promoters in the DNA derived from saliva in SZ [20], it is possible that epigenetic factors leading to down-regulation of FEZ1 in schizophrenic brains may also reflect in peripheral blood and result in the reduction of the expression in these tissues. However, additional studies applying RNA sequencing analysis for identifying peripheral blood-based biomarkers that could represent brain expression and epigenetic aberrations remain a key step in implication of these findings in pathogenesis, diagnosis and future therapy.

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