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Evaluation of serum MicroRNA expression profiles in patients with panic disorder

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ABSTRACT

BACKGROUND: Studies on the role of microRNAs (miRNA) in anxiety disorders are limited. We aimed to determine the availability of miRNAs as biomarkers in serum and to demonstrate the changes of miRNAs expression in patients with panic disorder (PD).

METHODS: Thirty-five patients with PD and 35 healthy controls (HC) were evaluated with Structured Clinical Interview for DSM Disorders-I (SCID-I) and Panic Disorder Severity Scale (PDSS). In each group miRNA expression analysis was performed in venous blood by the Real-time Polymerase Chain Reaction (RT–PCR) method for genetic evaluation.

RESULTS: Compared with the HC group, eight miRNA expression levels were found different in the PD group. Five of them were upregulated and three of them were downregulated. There was no correlation between the levels of miRNA expression with PDSS total score and PDSS sub-items. However, miR-1297 and miR-4465 expression levels were significantly different between the two groups.

LIMITATIONS: There are some limitations in this research. Firstly the number of samples is small. Another limitation of our study is that the presence of medical illness and continuous drug use were not excluded when PD and HC groups were selected.

CONCLUSIONS: Our research is the first miRNA expression study in patients with PD which excluded psychotropic use and additional psychiatric disorders. In the PD group, miR-1297 and miR-4465 expression was upregulated than compared to the HC group. miR-1297 and miR-4465 regulate the GABAA gene regions that affect $GABA_A$ receptor subtypes that thought to play a role in the aetiology of PD.

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Introduction

Panic disorder (PD) is characterized by the presence of recurrent unexpected panic attacks and persistent concern about having additional panic attacks or their consequences and a significant maladaptive change in behaviour related to the attacks [1]. The lifetime prevalence of PD in the general population is 2–5% [2]. In first-degree relatives of patients with PD, the risk of lifelong PD was reported to be 3–17 times higher than that of relatives of people without PD, and the incidence of PD was reported as 5–17.3% [3,4]. Twin studies in many countries showed that genetic factors accounted for approximately 30–46% of the variance in PD [5,6]. In a study conducted on more than five thousand twins, inheritability of PD was determined as 0.28 [7]. Although the familial transition of PD has been clearly shown in some families, it should not be overlooked that there are limitations in differentiating the contributions between genetic and environmental factors in studies [8].

Although the mechanism of mutations or polymorphisms in DNA generally initiates the causal chain in the pathogenesis of psychiatric diseases, it is suggested that the mechanisms that control gene expression are also highly influential in the disease development process. Any step can be altered to the gene expression mechanism, from transcription of RNA to post-translational modifications of proteins [9]. One of the important mechanisms in regulating gene expression is epigenetic changes. Epigenetic changes are mitotically heritable changes in the genome that are not encoded within the DNA nucleotide sequence [10]. The most well-known epigenetic regimens are histone modification and DNA methylation. However, miRNAs, which provide epigenetic regulation at a post-transcriptional level, have emerged in recent years as potential players in the pathophysiology of mental illnesses [11].

miRNAs, which are typically 20–25 nucleotides long, have a key role in the post-transcriptional regulation of approximately 50% of all protein-coding genes [12]. miRNAs regulate protein synthesis post-transcriptionally, before or during the ribosomal translation process, by base pairing to target messenger

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RNAs (mRNAs) and either repress translation or induce mRNA degradation [13]. miRNAs functions include cellular proliferation and differentiation, apoptosis, movement, synaptic plasticity, and neurogenesis [13]. miRNAs have been investigated in schizophrenia, bipolar disorder, and major depression in psychiatry, and have also been studied twice in PD. These studies have shown that miR-488, miR-22, miR-491, miR-138, and miR-148 may play a role in panic disorder [14,15].

Genetic studies of anxiety disorders have been neglected compared with other psychiatric disorders. We also aimed to demonstrate the effect of miRNAs that provide epigenetic regulation at the post-transcriptional level in panic disorder [3], which has been shown to play the greatest role in genetic transmission from the anxiety disorder group.

In the present study, we aimed to study 16 miRNAs that regulate GABA and serotonin gene regions known to play a role in the aetiology of PD. In addition, this will be the first study on miRNA expression to consider variables such as age, sex, body mass index (BMI), additional psychiatric disease, and psychotropic use, which we think influence miRNA levels in PD.

Methods

At the beginning of the study, approval was obtained from the Scientific Research Ethics Committee of Manisa Celal Bayar University Faculty of Medicine. The study was conducted in the Manisa Celal Bayar University Psychiatry and Medical Genetics clinic. Thirty-five patients with PD who were diagnosed in accordance with the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) Structured Clinical Interview (SCID) [16] in a diagnostic evaluation interview, and 35 healthy controls (HC) were included in the study. The exclusion criteria included the presence of additional psychiatric illness in the patient group, current psychotropic drug use or less than 6 months ago, and presence of neurologic disease and mental retardation. Of the 211 patients with PD screened in the psychiatric outpatient clinic, 53 were excluded because of other axis I psychiatric disorders after SCID-I was applied, 42 were excluded because of current use of psychotropic drugs, 78 were excluded because of other axis I psychiatric disorders and psychotropic drug use, and 3 refused blood tests. Informed consent was received from the remaining 35 patients.

Informed consent was obtained from 35 healthy controls who were matched with the PD group in demographic variables (age, sex, height, weight, and BMI). All interviews and scales were performed with the HC and PD groups by the same psychiatrist. The Panic Disorder Severity Scale (PDSS) [17,18] was also administered to the patients with PD.

Genetic analyses

miRNA selection process

Seventeen miRNAs (hsa-miR-203a-3p, hsa-miR-216a-5p, hsa-miR-4465, hsa-miR-26b-5p, hsa-miR-26a-5p, hsa-miR-1297, hsa-miR-22-3p, hsa-miR-488-3p, hsa-miR-98-5p, hsa-miR-4458, hsa-miR-4500, hsa-miR-let-7a-5p, hsa-miR-let-7b-5p, hsa-miR-let-7d-5p, hsa-miR-let-7g-5p, hsa-miR-93-5p, and hsa-miR-16-5p) were selected based on their target genes according to the prediction algorithm tools: Targetscan (http://www.targetscan.org), Pictar (http://www.pictar.bio.nyu.edu), miRbase (http://microrna.sanger.ac.uk).

Collection of biologic material and genetic evaluation

Peripheral venous blood samples (3 cc) of the study patients were placed into 2 different serum separator tube (SST), which is containing special gel matrix that acts as a barrier between the blood cells and the serum, to create a serum for miRNA analysis. The samples were immediately transported to the genetics laboratory at room temperature. Serum samples were separated using centrifugation at 1200 *g* for 20 minutes and were transferred to cryotubes and stored at −80°C until collection of all samples was completed. Peripheral venous blood samples of the study patients were taken between 09:30 am and 11:30 am.

Small RNA isolation and cDNA preparation

Small RNA fractions were isolated from the patients' serum samples using miRCURY™ RNA Isolation Kits – Biofluids (Exiqon Life Sciences, Vedbaek, Denmark) by adding 2 µg RNA-free carrier glycogen per sample to the lysis solution. A complementary DNA (cDNA) reaction was performed with a total 10 ng using a Universal cDNA Synthesis Kit II (Exiqon Life Sciences, Vedbaek, Denmark).

Quantitative RT–PCR

Quantitative Real-time Polymerase Chain Reaction (RT–PCR) was performed using a Rotor-Gene Q (Qiagen, Hilden, Germany) and HotStarTaq Master Mix (Qiagen, Hilden, Germany) in combination with EvaGreen Dye (Biotium Inc., Hayward, CA, U.S.A.). For the determination of the expression level of individual miRNAs, miRNA sequence-specific primers were designed using primer design software [19]. hsa-miR-26a-5p, which was stably expressed according to Best Keeper [20], was used as the reference gene. All RT–PCR reactions had two replicates.

Data and statistical analysis

The SPSS for Windows 21.0 statistical package programme was used for statistical analysis. Descriptive statistics were used primarily in the analysis of the

data. The Chi-square test was used for categorical variables to examine the relationship between the groups and *t*-test (the Mann–Whitney U test if no normal distribution conditions were provided) and receiver operating curve (ROC) analysis was used for independent variables in the numeric variables. Pearson's correlation analysis (Spearman's correlation test if parametric conditions were not provided) was applied to examine the relationship between independent continuous variables.

miRNA expression fold change data gained from Rotor-Gene Q device software were analysed using the ΔΔCt method [21]. Data are presented as mean ± SE. A value of $p \leq .05$ was considered statistically significant. The nonparametric Mann–Whitney U test was used for the non-normally distributed group and the independent samples *t*-test was used for the normally distributed group to compare the miRNA expression fold change of the control group to the patient group.

Results

Two groups were formed in the study with a sampling of the PD and HC groups. There were 17 women and 18 men in both groups. No significant differences were observed in mean age, height, body weight, and BMI between the groups. Data are presented in Table 1.

No difference was found in the presence of past psychiatric disease ($p = .22$) and past psychiatric treatment ($p = .61$) between the PD and HC groups in their medical history. However, significant differences were present between the groups in terms of the presence of psychiatric illness in the family history ($p = .02$), current medical illness ($p = .002$), and drugs (i.e. antihypertensives, oral antidiabetics etc.) that they had to use constantly for their medical conditions ($p = .02$).

The average PDSS total score of the PD group ($n = 35$) was found as 16.88 and the standard deviation was 5.05. No significant correlation was determined between the subscale scores and total score of PDSS and miRNA expressions. Correlations between miR-98-5p and the first item of PDSS ($p = .06$), miR-488-3p, and the third item of PDSS ($p = .06$) were marked, but they did not reach significance.

Differences in expression levels of miR-1297 and miR-4465 between PD and HC groups were found to be statistically significant. Data are presented in Table 2.

Table 1. Comparison of panic disorder group (PD) and healthy control (HC) group in terms of age, height, body weight, and BMI.

	PD ($n = 35$)		HC ($n = 35$)		
Variable	Med	SD	Med	SD	p[a]
Age (years)	30.48	11.25	30.48	9.29	1.00
Height (cm)	169.09	8.88	169.09	9.25	1.00
Body weight (kg)	70.23	14.88	70.91	14.55	0.85
BMI (kg/m^2)	24.65	5.14	24.74	4.77	0.94

[a]Evaluated by independent samples *t*-test.

Table 2. Comparison of intergroup miRNA expression profiles.

Variable[a]	PD (*n*:35) Median (Interquartile 25–75)	HC (*n*:35) Median (Interquartile 25–75)	*p*(ES)[c]
miR-203a-3p	1.62 (1.33–2.23)	1.39 (1.20–2.07)	.32
miR-1297	6.49 (4.21–24.76)	17.87 (7.21–118.60)	**.002 (0.48)**
miR-98-5p	0.00 (0.00–0.11)	0.00 (0.00–0.04)	.26
miR-4458	0.00 (0.00–0.04)	0.01 (0.00–0.09)	.14
miR-488-3p	0.00 (0.00–0.02)	0.02 (0.00–0.05)	.45
let-7[a]	0.30 (0.21–0.61)	0.42 (0.21–0.82)	.46
Variable[b]	PD (*n*:35) Med ± ss	HC (*n*:35) Med ± ss	*p*(ES)[c]
miR-4465	0.10 ± 0.00	0.13 ± 0.01	**.01 (0.05)**
miR-26b-3p	0.21 ± 0.03	0.25 ± 0.04	.48

[a]Mann–Whitney U test was used because the variables did not show normal distribution.
[b]Independent samples *t*-test was used because the variables showed normal distribution.
[c]Cohen's effect size.

In the ROC analyses performed for miR-1297 and miR-4465, the area under the curve (AUC) for the miR-1297 was 71% (95% confidence interval (CI): [0.59–0.83], $p = .002$), and the AUC for miR-4465 was 72% (95% CI: [0.59–0.84], $p = .002$).

Discussion

In this study, expressions of 16 miRNAs that have been shown to regulate GABA and 5-HT gene regions were studied from the sera of patients with PD. After the initial pooling analysis, 8 miRNA expression levels were found to be different between the HC and PD groups. Three of 8 miRNAs (miR-26b-3p, miR-4458, let-7a) were found to be downregulated in patients with PD compared with the HCs, and 5 were up regulated. Between the two groups, miR-1297 and miR-4465 expressions were found to be significantly different.

There are two previous studies in the literature that investigated the association of miRNA with PD [14,15]. These two studies also addressed miRNA polymorphisms; studies on miRNA expression in panic disorder are not yet available in the literature. Thus, our study is the first in this area. In addition, patients using psychotropic drugs were not excluded in these two studies. Drugs that were used in the treatment of PD such as selective serotonin reuptake inhibitors and serotonin-norepinephrine reuptake inhibitors affect miRNA levels [22–27] This study was the first PD study to exclude the use of psychotropic medication

Previously, neither miR-1297 nor miR-4465 had been investigated in psychiatric disorders. Studies showed that miR-1297 played an important role in hepatocellular, colorectal, testicular, esophageal, and lung cancer, especially in oncologic diseases [28–32].

Results were obtained from two miRNA studies of PD that miR-488, miR-22, miR-491, miR-138, and miR-148 might play a role in PD [14,15]. MiR-1297 regulates GABRA4, GABRB2, GABRB3 and GABRG1 while miR-4465 regulates GABRA4 and GABRB2. All these gene regions encode the various subtypes of $GABA_A$ receptors [33,34].

GABA, the major inhibitory neurotransmitter in the central nervous system, is thought to play an important role in the development of anxiety and panic attacks. A study indicated that stress-associated anxiety and depression suicide risk was elevated in carriers of the *GABRA6* rs3219151 T allele [35]. Neurochemical mechanisms that interrupt GABAergic activity can cause anxiety [36,37] and various GABAergic drugs are effective in panic [38,39]. As a result of our study, we determined that miR-1297 and miR-4465 were upregulated in PD group compared with the HCs. These two miRNAs can be expected to regulate gene regions linked to benzodiazepines, which are potent anti-panic drugs. In addition, the gene regions that they affect regulate both benzodiazepine-sensitive $GABA_A$ receptors and benzodiazepine-insensitive $GABA_A$ receptors. Alcohol, binds to benzodiazepine-insensitive $GABA_A$ receptors [40] known to exhibit anxiolytic properties, and the association of alcohol use disorders in patients with PD is common [41,42]. In an alcohol withdrawal study, downregulation of GABRA4 expression is mediated in part by the induction of specific microRNAs [43]. Another study investigating candidate gene regions of PD, 8 of the GABAA receptor subtypes investigated were not associated with PD [44]. Another study found that GABRA5 and GABRB3 were associated with PD [45]. There are more studies investigating the relationship of PD with $GABAA_A$ receptor subtypes [46,47]. However, the results are inconsistent and new studies targeting the GABAergic system, $GABA_A$ receptor subtypes, and the miRNAs affecting them may be relevant in PD.

One of the hypotheses of our study was that altering the severity of PD correlated with miRNA expression levels in patients with PD. No significant correlation was seen between total scores and subscale scores of PDSS and miRNA expressions. The reason may be the small number of samples. However, in a study on PD and miRNA polymorphism, PDSS was used and the researchers found no correlation between PDSS total score and miRNA [15].

Another hypothesis was that miRNAs could be used as biomarkers in PD. However, because of the cost of miRNA analyses, and after the ROC analyses were performed (the AUC was not ideal as a biomarker), neither miR-1297 nor miR-4465 had ideal biomarker features in patients with PD. In this regard, studies with larger samples are needed.

There are some limitations to this research. First, the number of samples is small. Another limitation of our study is that the presence of medical illness and continuous drug use were not excluded when the PD and HC groups were selected. miRNAs have been shown to be affected by many medical diseases and drug use (for example diabetes mellitus, hypothyroid, arterial hypertension and antidiabetic drugs)[48–52]. For this reason, these two factors may have affected the results. Another limitation is that peripheral blood samples from the patients and healthy controls were not taken at the same time of day. Even though it is not clearly known, miRNA expressions may vary throughout the day. In addition, the hunger or resilience of subjects may affect the results because it was shown to be affected by blood glucose and insulin levels [53]. The final limitation is related to not using a severity scale to detect sub-threshold depressive and obsessive symptoms. The level and severity of accompanying depression, and other anxiety and obsessive symptoms may affect miRNA levels. General structural variations in microRNA sequences, and methodologic inconsistencies in technology used in studies to measure microRNA expression levels make it difficult to ascertain any robust data [54].

To our knowledge, this study was the first on miRNA expression in PD. Variables such as age, sex, BMI, and psychotropic drug use, which are thought to influence miRNA levels, were excluded. In order to understand $GABA_A$ receptor subtypes, the GABAergic system and miRNA association in PD, there is a need for further studies with larger samples that focus on the selection of miRNAs affecting the GABAergic system. It may also be useful to perform follow-up studies with large samples to predict the effect of drugs used in the treatment of PD and clinical improvement on miRNAs.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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