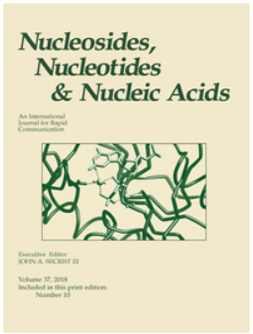

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A gene expression analysis of long non-coding RNAs NKILA and PACER as well as their target genes, NF-κB and cox-2 in bipolar disorder patients

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ABSTRACT

Bipolar disorder (BD) is a severe condition characterized by periods of mania and depression. Despite advances in the neurobiology of bipolar disorder, the exact etiology of the disease remains unclear. There is evidence that Inflammation is associated with bipolar disorder. COX-2 and NF-κB are two critical mediators in the inflammatory pathways. Long non-coding RNAs (lncRNAs) are a new class of non-coding RNAs that play a wide range of roles, especially in developing and maintaining normal brain functions. Two lncRNAs called PACER and NKILA control the expression of COX-2 and NF-κB genes, respectively. In this study, Expression levels of PACER and NKILA lncRNAs, as well as, COX-2 and NF-κB genes were measured in fifty patients with bipolar disorder and 50 healthy individuals by real-time PCR. Expression levels of NKILA and COX2 were considerably reduced in BD patients compared with healthy controls. Such significant downregulation in the expression of NKILA and PACER was only observed in male patients with BD compared with male healthy subjects. Also, according to the results of the ROC curve, the area under curve values for NKILA and COX2 were 0.68 and 0.52 respectively. Consequently, the NKILA gene could be considered a biomarker. By examining the degree of pairwise correlation between genes, all genes had a significant positive correlation with each other. Taken together, these results revealed a function for NKILA and PACER lncRNAs in the pathogenesis of BD.

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KEYWORDS
LncRNA; NKILA; PACER; NF-kB; COX-2; bipolar disorder; inflammation

Introduction

Bipolar disorder (BD) is a complex and severe psychological disease that burdens society. BD patients typically have manic and depressive moods associated with morbidity and suicide behaviors.[1,2] This disease has a

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global prevalence of 1.5–2.4% and is associated with mortality and morbidity.[3] The neurobiological studies revealed some pathobiological aspects of BD including genetic and biochemical factors. To date, despite the evidence that suggests the role of many genes in the pathophysiology of BD, the exact molecular basis of this disorder is still unknown.[4] Impaired regulation of intracellular pathways has been observed in BD. Inflammation is one of these pathways that is part of the immune system. The role of the immune system in BD is one of the most controversial issues in psychiatry-genetics.[4] Some studies demonstrate how the brains of people with BD are different from healthy people or people with other mental disorders.[5] Also, inflammatory cytokines have been shown to cause depressive-related behaviors, including changes in sleep and decreased activity.[1] Compared to healthy controls, adults with major depressive disorder (MDD) showed increased circulating proinflammatory cytokines.[6] Increased inflammatory markers have been observed in patients with BD, especially in people who are in the depressed or manic phase.[7] One of the main components of the immune system is the NF-κB pathway, which plays an essential role in the development of innate and acquired immunity.[7] One of the critical factors in regulating the NF-κB signaling pathway is NKILA, a non-coding functional RNA. It prevents the transfer of NF-κB to the nucleus, thereby preventing the activation of the inflammatory pathway.[8] LncRNAs are involved in several biological processes, many of which play a broad role in developing and maintaining normal brain functions.[9,10] There are different studies that show the role of lncRNAs in BD etiology.[11–15] Another critical mediator in the inflammatory pathway is COX-2. The various inflammatory mediators include prostaglandins (PG), thromboxane, and leukotrienes involved in the pathological process of multiple diseases. The production of various prostaglandins is driven by enzymes called cyclooxygenase (COX).[16] PACER lncRNA is located near the upstream of the COX-2 gene, and further experiments showed that this newly discovered lncRNA activates the expression of the COX-2 gene.[9] This study investigated the expression levels of lncRNA-NKILA and lncRNA PACER and their target genes, NF-κB and Cox-2, in blood samples of bipolar disorder patients and healthy controls. Dysregulation of these lncRNAs and their target genes may involve in the pathogenesis of BD through inflammatory pathways or might be applied as a biomarker of BD.

Materials and methods

Patients and healthy groups

A psychiatrist based on the DMS-V (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition) selected fifty people with BD to

participate in the study. All subjects were assessed using a semi-structured interview by experienced psychiatrists. The subjects were chosen from Imam Hossein Hospital of Shahid Beheshti University of Medical Sciences.

Patients were in the euthymic phase at the time of sampling. Blood samples were taken at the same time of the day without fasting. Exclusion criteria for patients were the existence of other psychiatric and personality disorders or physical illnesses such as multiple sclerosis and Parkinson's that may be associated with depression. Moreover, at any stage of sampling, if the patients suffer from other mental disorders or physical illnesses related to depression other than their illness, he/she will be excluded from sampling or participation in the work. Fifty people are considered as a control group with no history of BD and related diseases and are the same age and sex as the case group. In addition, healthy controls were excluded who had a first-degree biological relative with a history of psychopathology. All subjects with current substance abuse or cigarette smoking were excluded from the study. In this research, the age of cases with BD was between 17 and 56 years and the healthy control subjects were between 14–52 years of age. The female/male ratio in BD patients was 16:34 and in controls was 19:31. All methods were performed in accordance with the relevant guidelines and regulations. Informed written consent forms were signed by parents and/or legal guardians for study participation. The Institutional Ethics Committee of Jiroft University of Medical Sciences approved this study (ethic code: IR.JMU.REC.1399.014).

Sample collection and extraction of total RNA

5 ml of Peripheral blood was assembled in EDTA tubes. The blood samples were centrifuged at 3000 rpm for 10 min. Total RNA drew out from the PBMC using an RNAX kit according to the manufacturer's instructions. For RNA qualitative and quantitative analysis, gel electrophoresis and the spectrophotometer were used.

cDNA synthesis and quantitative PCR

According to the manufacturer's instructions, cDNA was synthesized by 3 µg of purified total RNA using Applied Biosystems High-Capacity cDNA Reverse Transcription Kits (PN: 4375575). Appropriate primers measured the expression levels of each lncRNA in comparison with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (Table 1). Quantitative real-time PCR was performed by ABI 7500 sequence detection systems (Applied Biosystem/MDS SCIEX, Foster City,

Table 1. Primers used in RT-qPCR.

Gene	Forward primer (5′ 3′)	Product size (bp)
PACER	F: TGGGGCGAGTAAGGTTAAGAAAG R: GGTGAAGGTACGGAGAACAGTAT	220
NKILA	F: GCTTAATGCAGTGTTCAGACCT R: CATTCCTCGCAATCACACCA	171
NF-κB	F: TACTCTGGCGCAGAAATTAGGTC R: ACTGTCTCGGAGCTCGTCTATTTG	265
COX2	F: ACGCTTTATGCTGAAGCCCTA R: GGCAGGATACAGCTCCACA	115
GAPDH	F: CCATGAGAAGTATGACAAC R: GAGTCCTTCC ACGATACC	105

CA, USA) using ten µl of BIOFACT™ 2X Real-Time PCR Master Mix (SYBR Green I), ten ng cDNA, 200 nM of concentration of each primer. All reactions were conducted at least in duplicate.

Statistical analysis

Finally, data normalization with the internal control gene and data analysis is performed with the Graphpad. Shapiro-Wilk test is used to check the normality of data distribution. Independent Student's t-test is used to compare the differences between the two groups. The Pearson test and Bonferroni correction for multiple comparisonsis used to check the correlation. Receiver Operating Characteristic (ROC) is used to analyze the specificity and sensitivity of the expression level of the desired genes to test genes as a biomarker. A significant value of $P < 0.05$ is considered. The expression level of genes is examined using the formula ($2^{-\Delta\Delta Ct}$) by the Livak method.[17]

Results

Gene expression of lncRNA PACER and COX2 as its target gene

The study of the expression of these genes showed that in patients compared to healthy individuals, the expression of the COX2 gene with a P-value <0.0001 decreased by an average of 2 times (Figure 1A), and therefore the difference in expression is statistically significant. PACER gene with P-value = 0.0825 was not statistically significant between the two groups. Moreover, the expression level of PACER was significantly decreased in the PBMCs obtained from the male BD patients compared to male controls (5.95 times, $P = 0.010$). However, no significant difference was observed in the expression level of COX2 between male BD patients and male controls ($P = 0.080$). Also, there was no significant difference in the expression levels of PACER ($P = 0.22$) and COX2 ($P = 0.45$) between the female BD patients compared to female controls (Table 2).

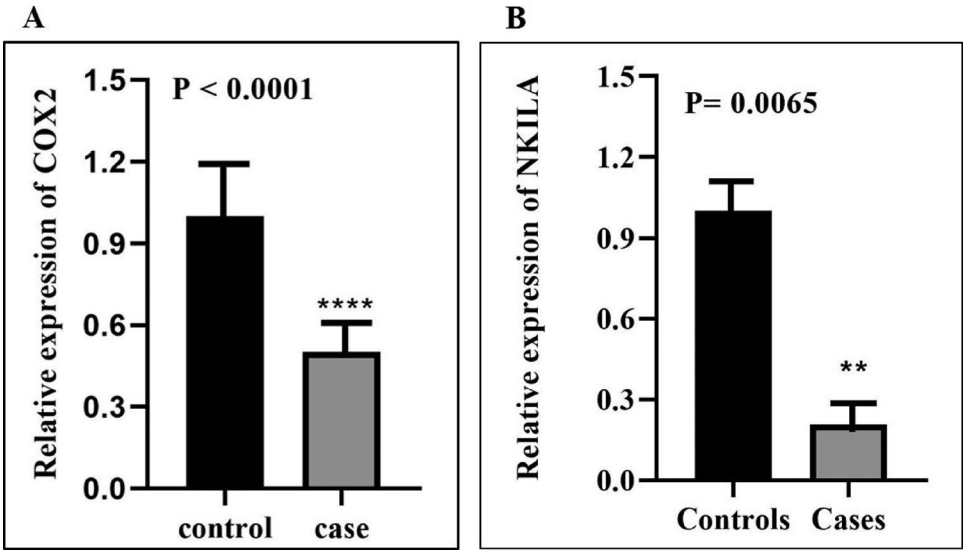

Figure 1. The expression analysis of genes in the PBMCs. The relative expression (Fold change) of COX2 (A) and NKILA (B). The expression of these genes was downregulated significantly in BD patients compared to controls. The relative expression level of transcripts was calculated using the formula $2^{-\Delta\Delta Ct}$.

Gene expression of lncRNA NKILA and NF-kB as its target gene

Examination of the expression of these two genes showed that the expression of NKILA lncRNA with a P-value equal to 0.0065 had a statistically significant difference between the patients and normal groups and decreased by an average of 5 times in patients (Figure 1B). However, the NF-kB gene with a P-value greater than 0.05 was not statistically significant. Results showed that the NKILA expression level but not NF-kB was significantly reduced in male BD patients compared to male controls (4.37 times, $P = 0.041$). The expression of these genes did not have any significant difference in the female with BD compared to healthy females (Table 2).

Pairwise correlation analysis

In the current study, the degree of correlation between genes was investigated in pairs using Graph Pad software and the Pearson correlation coefficient. The results of gene expression correlation in pairs show a significant positive correlation between genes in pairs ($P < 0.05$). Table 3 shows the pairwise correlation of genes. Also, the relationship between patient age, age of onset, and disease duration with the studied genes' expression were investigated. The results showed no significant relationship between these three factors and gene expression in BD individuals ($P > 0.05$) (Table 4).

Table 2. Relative expression of lncRNAs and their target genes in BD patients and healthy controls.

	PACER		NKILA		COX2		NF-kB	
	ΔCt means	P value	ΔCt means	P value	ΔCt means	P value	ΔCt means	P value
Total cases (n = 50) total controls (n = 50)	5.30/4.10	0.082	6.63/4.37	0.0065*	2.11/1.23	0.0001*	3.77/3.36	0.69
Male cases (n = 34) male controls (n = 31)	5.58/3.005	0.010*	6.52/4.39	0.041*	1.91/0.19	0.08	3.51/2.41	0.25
Female cases (n = 16) female controls (n = 19)	4.67/6.65	0.22	6.89/4.33	0.27	2.60/3.66	0.45	4.38/5.58	0.35

*Significant p-value < 0.05.

Table 3. Pairwise correlation between expression levels of lncRNAs in cases group.

Correlation	r	p-value
PACER - COX2	0.2136	$P < 0.0001$****
PACER - NKILA	0.2779	$P = 0.897$
PACER - NF-kB	0.4197	$P = 0.03$*
NKILA - NF-kB	0.4786	$P = 0.001$**
NKILA - COX2	0.4721	$P = 0.001$**
COX2- NF-kB	0.3257	$P = 0.21$

R: correlation coefficient.
*Significant p-value < 0.05.
**Significant p-value < 0.01.
****Significant p-value < 0.0001. P values are presented after Bonferroni correction.

The analysis of ROC curve

The sensitivity and specificity of expression of the studied genes as biomarkers were evaluated using the ROC curve in Graph Pad software. The results showed that the NKILA gene with a sub-curved area (AUC) of 0.68 and P = 0.0017 was statistically significant (Figure 2A), and the sensitivity and specificity of the NKILA gene were 76.74% and 56%, respectively. The current results show weak sensitivity and specificity. The sensitivity and specificity for the COX2 gene (AUC = 0.52 and P = 0.7119) are 76.74 and 40.91, respectively. This gene is not statistically significant (Figure 2B).

Discussion

Bipolar Disorder (BD) is a complex mental disease that consists of two phases of mania and depression that recur alternately. Given the relatively high prevalence of BD in communities and its association with other neurological diseases, understanding the genetic causes and cell

Table 4. Correlation analysis between expression levels of lncRNAs and demographic data in BD patients.

	PACER		NKILA		COX2		NF-kB	
Clinical data	R	P value	R	P value	R	P value	R	P value
Age	−0.079	0.61	0.119	0.40	−0.014	0.92	−0.028	0.85
Age at onset	−0.051	0.54	0.144	0.31	0.029	0.84	0.063	0.68
Disease duration	0.053	0.73	−0.017	0.90	0.042	0.78	0.094	0.54

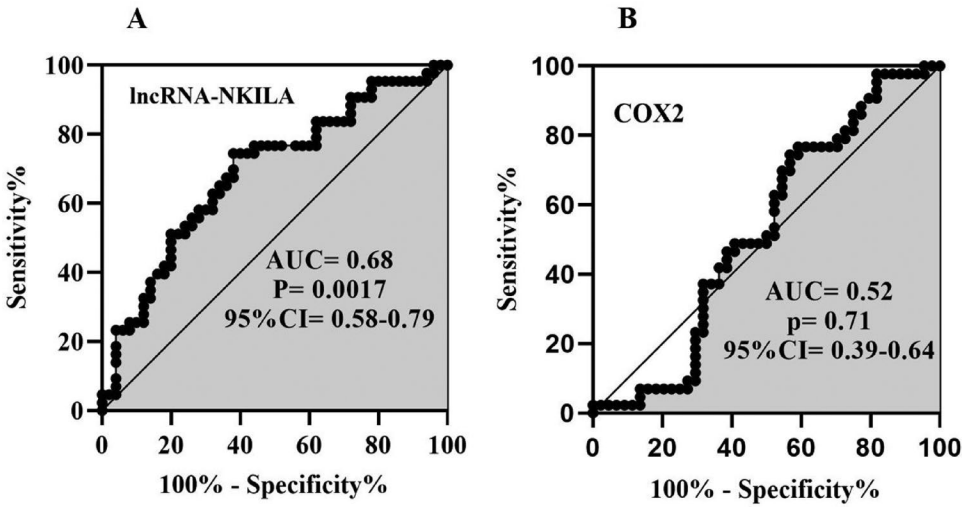

Figure 2. ROC curve analysis of lncRNA-NKILA (A) COX-2 (B). AUC: area under curve.

signaling pathways paves the way for a better understanding of BD and proper treatment and diagnostic strategies.[1] In the last few years, several studies have shown that the level of inflammatory factors, including circulating cytokines, increases in different phases of BD.[18,19] Also, high levels of IL-6 and TNF-alpha have been reported during mania. In the mania phase, the proinflammatory cytokines, IL-2, IL-6, IL-8, and INF-gamma, increase, while only IL-6 increases in the depressive phase. In addition, bipolar depression is characterized by an altered balance between IL-6 and the anti-inflammatory factor IL-10.[18] Furthermore, recent studies show that activation of the immune system may play a role in causing this disorder.[20] Although inflammation has been reported in bipolar patients, it is unclear how the immunological process affects the disorder.

As a result, in our study, we evaluated the expression level of NKILA and PACER and their target genes, NF-κB and Cox-2, in normal individuals and BD patients. Our analysis showed that only lncRNA NKILA and COX2 expression levels were significantly different in patients with bipolar disorder compared with healthy individuals. However, the expression level

of PACER and NKILA was significantly downregulated in the male BD patients compared to male controls. Also, by examining the degree of pairwise correlation between genes, all genes had a significant positive correlation with each other.

NF-κB gene has 24 exons and is located on the long arm of chromosome 4. The NF-κB signaling pathway, as part of the immune system, leads to the activation of proinflammatory cytokines and ultimately to inflammation.[21] Also, it is activated by acute stress and sleep disorders and may be involved in cellular responses to stressful life events.[22] So, it may be related to BD.[7, 21, 23] Although few studies have been conducted.

In addition, lncRNAs can control the expression of various genes and therefore play an essential role in biological processes, particularly in the immune system, brain growth, and function.[24,25] lncRNA-NKILA is a regulatory RNA that controls the expression of the NF-κB gene. This lncRNA reduces the inflammatory pathway by increasing the stability of the IKB/NF-κB complex in the cytoplasm and prevents NF-κB enter the nucleus.[21, 26] In this study, by reducing the expression of NKILA, the IKB/NF-κB complex may be less stable than normal and will cause more inflammation when NF-κB enters the nucleus. Unlike David J Miklowitz's study in which the expression of the NF-κB gene was increased in BD compared to healthy individuals,[7] our results indicated that the difference in expression of this gene between patients and healthy individuals was not significant, which could further highlight the role of the sample size and the involvement of factors other than the inflammatory pathway.

COX-2 is another actor in inflammatory pathways. This gene controls the production of various prostaglandins that play an essential role in inflammation by making an enzyme from the Cyclooxygenase (COX) family.[16] lncRNA-PACER, close to the chromosomal locus of the COX-2 gene, can act as an activator of this gene.[16, 27] In our results, the expression of COX-2 and Sex-specific expression of PACER genes decreased in patients. As mentioned, lncRNA-PACER can increase the expression of COX-2, and the decrease in its expression in our study can explain the reduced expression of COX-2 in bipolar patients. According to previous studies, increasing the expression of COX-2 due to the overproduction of prostaglandins can cause inflammation, reduce apoptosis and increase cell proliferation in cells.[28] In the present study, with a significant decrease in expression of COX-2 in BD patients, increased apoptosis of nerve cells due to decreased expression of this gene can be considered a possible cause. On the other hand, it can be considered that reducing the expression of this gene prevents the proliferation of nerve cells and causes changes in the structure of the brain, which can be a reason for

BD. However, to prove this hypothesis, future studies will be required by examining the expression of genes involved in the apoptotic pathway, neurons' growth, and their regulatory lncRNAs. That could shed light on some unknowns related to BD. Additionally, according to our results from the ROC curve and the significance of specificity and accuracy indices, only lncRNA-NKILA can be used as a diagnostic biomarker. However, further studies are needed to demonstrate the biomarker capability of this regulatory RNA. Finding a biomarker to diagnose the disease correctly, with high accuracy, and early on will help in the proper treatment of this disorder and will reduce the disability of BD patients in society.

In summary, the current study revealed that the expression levels of lncRNA NKILA and COX2, as well as Sex-specific expression of lncRNA PACER, were reduced in peripheral blood of BD patients. The current results propose a role for NKILA and PACER lncRNAs in the pathogenesis of the bipolar disorder. However, larger sample size and a large number of experimental and clinical tests are needed that demonstrate the curacy function of these lncRNAs in the neuropathology of BD.

Ethics approval and consent to participant

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent forms were obtained from all study participants. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences. All methods were performed in accordance with the relevant guidelines and regulations.

Disclosure statement

No potential conflict of interest was reported by the authors.

Authors' contributions

ZSF wrote the manuscript and revised it. MT designed and supervised the study. ZSF analyzed the data. RMA, SF and ZM performed the experiment. All authors read and approved the final version of manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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