Journal: Texila International Journal of Public Health
Publisher: Texila International Journal
ISSN: 2520-3134
Volume 13 Issue 4, 2025
DOI: 10.21522/TIJPH.2013.13.04.Art017

Evaluation The Correlation between Bmal1 and Clock Genes Expression with miRNA In kids With Bipolar Disorder

Zahra J. Ramadan[1], Owayes M. Hamed[1*], Elham kh. Abdullah[2]
[1]*Department of Biology, College of Science, University of Mosul, Mosul, Iraq*
[2]*Collage of Medicine, University of Ninevah, Mosul, Iraq*

Abstract

The study aimed to explore the influence of methylation levels of two clock genes, Bmal1 and Clock, and the association between gene expression and miRNA molecules (miRNA155 and miRNA211) in bipolar disorder. The study sample contains 40 kids whose ages ranged from 2 to 12 years with bipolar disorders, and 20 healthy kids as a control group. The study axes were divided into three parts. The first part involved determining the levels of gene expression for the Bmal1 and Clock genes using qRT-PCR. The second part is the determination of the levels of miRNA and snoRNA molecules that regulate Bmal1 and Clock genes using qRT-PCR. Third part: measuring methylation levels in the promoters of Bmal1 and clock genes using PCR. The results showed a significant decrease in the Bmal1 gene expression; it was the value of folding expression Bmal1 gene = 0.73, and a significant increase in the Clock gene; it was the value of folding expression Clock gene = 1.23. and the results showed a significant increase in miRNA gene expression; the value of folding expression for miRNA155/Bmal1 = 1.76, and a significant decrease in miRNA gene expression; the value of folding expression for miRNA211/Clock = 0.26. while the gene expression level for snoRNA molecule = 1.76 compared with control = 1. Conclusion: The results of this study demonstrate increases in the gene expression folding of the Clock gene and a significant decrease in miRNA211 gene expression which regulates the Clock gene.

Keywords: *Bmal1 gene, Clock gene, miRNA, mRNA, snoRNA and Methylation.*

Introduction

Circadian rhythms are generated through oscillatory physiological processes and couple biological function to the 24 h light-dark cycle. The original sensitivity to light mediates this synchronization [1]. Light-dark cues modify circadian rhythms by triggering an internal signaling cascade that includes several endogenously produced molecules, including proteins and neurotransmitters. These molecules can then create a signaling flood of their own, which may increase the initial light-dark cue [2]. Howbeit, because the participation of a wide diversity of signaling flood and their particular components, According to recent research, the transcriptional/translational feedback loops of many clock genes, inclusive of Period (Per) 1-3, form the circadian clock system, Cryptochrome (Cry) 1–2, Clock (circadian locomotor output cycles kaput), BMAL1 (brain and muscle aryl hydrocarbon receptor nuclear translocator–like protein 1), and Casein Kinase I epsilon (CKIε) [3]. Circadian rhythmicity is an essential biological characteristic that has recently accumulated greater interest in neuroscience and psychiatry [4]. On the other hand, it might be possible to predict future diseases if the causative

Received: 07.06.2025 ***Accepted: 13.08.2025*** ***Published on: 24.12.2025***
****Corresponding Author: owsbio31@uomosul.edu.iq***

miRNAs and clock genes general to patients with bipolar disorder and patients with severe diseases could be identified [5]. Studies on circadian rhythm bipolar disorders, in the line of epigenetic research, have exposed the pathogenic mechanisms of a rising number of diseases, which have expedited the evolution of new diagnostic and therapeutic applications in recent years [6]. It has been given that endogenous operators, such as malfunctions of the circadian clock and its entrainment acquired by disorders of the brainstem and/or hypothalamus, and exogenous operators, such as psychological tension, extreme light at night, and shortage of light through the day, are the cause of these bipolar disorders [7]. Also, a variety of operators may participate to circadian rhythm bipolar disorders [8-9]. Even though they work to adapt to social situations, patients with circadian rhythm bipolar disorders have defects with social problems like tardiness, absence, truancy, and withdrawal [10-11]. First epigenetic process occurs through many non-coding RNAs, which are very efficient in biological systems and compensate for their disability to be translated into proteins through alternative mechanisms [12-13]. Another epigenetic process is methylation, which is one of the most commonly studied and dominant epigenetic modifications. In contrast to other mechanisms (such as histone modification), it is highly stable and can be transmitted from parents to offspring [14]. DNA methylation profiling and epigenetic marking technologies can be used to detect and demonstrate differences in DNA methylation between patients and healthy controls, and thus to discuss the molecular biological mechanisms of disease evolution [15]. Therefore, some studies have been conducted to explore the possible roles of DNA methylation in bipolar disorder by using different designs or methods [16]. Inclusive of global DNA methylation, genome-wide DNA methylation, and site-specific/regional DNA methylation profiling, DNA methylation-related multi-omics data integration analysis and DNA methylation-related experiments [17].

Many recent research fields show that miRNAs also regulate clock components. The Period genes, Per1, Per2 and Per3, which are controlled in mammals by popular miRNAs, such as miR-24 [15]. Further, Per2 is likely to be controlled by miR-449a [18]. In addition, the studies display that Per3 is regulated by miR-103, moreover the rhythmic expression of miR-27b-3p and miR-155 performed a role in maintaining the rhythmic expression of Bmal1 mRNA and protein levels. Furthermore, miR-211 directly controlles Bmal1 and Clock by special mechanisms [19].

However, some snoRNAs act as RNA protect in pre-rRNA maturation, not as evidence for RNA modulation [20]. snoRNAs were first found in the 1960s during studies on ribosome biogenesis [21]. Further research detected that snoRNAs are contribute in modulate other types of RNA and widly role in RNA processing and the controlling of gene expression [22].

Materials and Methods

Case study: The current study included (40) male children suffering from bipolar disorder and (20) healthy male children who do not suffer from any problems and in the same age group, ranging from (2-12) years, who visited the “Rufaidah Medical Clinic” in a period of (3 months)

Blood sample collection and storage:(5.0) ml of venous blood was drawn from children with bipolar disorder and was divided into three parts:

1. The first part was placed in 1.5 ml Eppendorf tubes pre-filled with 750 µl Trizol for mRNA and non-coding RNA extraction.
2. The second part was placed in tubes containing an anticoagulant EDTA for DNA extraction for DNA Methylation test.

The gene expression levels of circadian rhythm genes, miRNAs, and snoRNAs were analyzed using qPCR.

RNA and non-coding RNA Extraction: Mixing 250 ml of the blood sample with 750 ml of Trizol solution, then using an RNA extraction kit supplied by the Transgenbiotech company, and then using a Nanodrop device to measure the purity of the extracted RNA.

Detecting the Gene Expression Levels for mRNA Molecules of Circadian Rhythm Genes by using the qPCR Technique

Specific primers of circadian rhythm genes and housekeeping gene used to detect the gene expression levels of mRNA molecules for circadian rhythm genes by Quantitative PCR as shown in Table 1:

Table 1. Primers of circadian rhythm genes and housekeeping gene for mRNA molecules:

Primer	Sequence
BMAL1-RT-F	GCCTACTATCAGGCCAGGCTCA
BMAL1-RT-R	AGCCATTGCTGCCTCATCATTAC
Clock-RT-F	TTGGCAAAATGTCATGAGCAC
Clock-RT-R	TTGCCCCTTAGTCAGGAACCT
PER1-RT-F	GGACACTCCTGCGACCAGGTACTG
PER1-RT-R	GGCAGAGAGGCCACCACGGAT
PER2-RT-F	CGCAGGGTGCGCTCGTTTGA
PER2-RT-R	GGGCTCTGGAACGAAGCTTTCG
PER3-RT-F	GGTCGGGCATAAGCCAATG
PER3-RT-R	GTGTTTAAATTCTTCCGAGGTCAAA
H.K-F	TGACCCAGATCATGTTTGAG
H.K-R	CGTACAGGGATAGCACAG

Detection the Gene Expression Levels for micRNA Molecules and snoRNA of Circadian Rhythm Genes by using the qPCR Technique

Specific primers of micRNA and snoRNA molecules (which regulated the circadian rhythm genes) and housekeeping gene used to detect the gene expression levels of micRNA and snoRNA molecules for circadian rhythm genes by Quantitative PCR as shown in the table (2):

Table 2. Primers of micRNA and snoRNA molecules and the housekeeping gene

micRNA	specific gene	Primers Sequence
micRNA 155 -F	Bmal1 gene	AAGCGACCTTAATGCTAATCGTGAT
micRNA 211 -F	Clock Gene	AACACGTGTTCCCTTTGTCATCC
micRNA 24 -F	Per1 Gene	AACACGTGTGCCTACTGAGCT
micRNA 449a-F	Per2 Gene	AACACGCTGGCAGTGTATTGTTAG
micRNA 103 -F	Per3 Gene	AACAAGAGCAGCATTGTACAGGG
snoRNA21 -F	All genes	AGCAGTCAGTAGTTGGTCCTTTG
snoRNA21 -R	All genes	CCATCAGTCCCGTCTTGAAAC
U –R	All gene	CAGTGCAGGGTCCGAGGT
U6 –F	H.K	GTGCTCGCTTCGGCAGCA
U6 –R	H.K	CAAAATATGGAACGCTTC

Calculating Gene Expression Folding

The following equation was used to determine the gene expression folding of the genes under study depending on the target gene's CT value [23] :

Gene Expression folding = 2 ΔΔCT

DNA Extraction for methylation: DNA was isolated from the blood of all (60) samples contained in the study using the DNA extraction kit provided by Transgenbiotech company, and then a Nanodrop device was used to measure the purity of extracted DNA .

Methylation-specific PCR in Promoters of the Bmal1 and Clock Genes

Bmal1 and clock Genomic DNA methylation-specific PCR analysis was altered using sodium bisulfite, We created a pair of primers for methylation alleles in the Bmal1 and Clock genes. Primer pairs for detection of the methylated sequences in the promoter of Bmal1 and Clock are shown in Table (3) [6].

Table 3. Primers of Bmal and Clock genes for methylation reaction

Gene	Primer	Sequence	Annealing	Band size
Bmal1 gene	F	ACTACTTTCCTACCACCAATCATTTAAC	55	376bp
	R	TGAGATTTTGGTAAATTAGGGATTTT		
Clock gene	F	CCTAAAAACTCTTTAACTTTCCCCC	55	271bp
	R	TTTTTTTAGGAGATGGGAGAAGATG		

The Modified DNA was amplified in a total volume of 20 ml . After the Methyl ion-specific PCR program was done, the PCR products were then loaded and electrophoresed on 2% agarose gels, stained with red safe dye, and visualized under UV illumination.

Results

Measuring the gene expression level of circadian rhythm genes

The result of the study shows there are changes in the level of gene expression for Bmal1, Clock , PER1, PER2, and PER3 genes in the patients compared to the control group, as shown in the following tables.

Table 4. The values of CT and the level of gene expression for the Bmal1 gene and the housekeeping gene for patients with bipolar disorder compare with the control group

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. Control	ΔΔCT	Folding
Control	28.43	22.78	5.65	5.65	0	1
Patients	31.19	24.88	6.3	5.65	0.65	0.73

The results of study showed increase CT value of patients compared with CT value of control, also increase ΔCT value of patients compared with control, the gene expression folding of patients value was 0.73 compared with control value 1, this decrease caused decrease in *Bmal1* activity and this cause the bipolar disorder.

Table 5. The values of CT and the level of gene expression for the Clock gene and the housekeeping gene for patients with bipolar disorder compare with the control group

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. Control	ΔΔCT	Folding
Control	29.53	22.78	6.75	6.75	0	1
Patients	31.52	24.88	6.64	6.75	- 0.10	1.23

The results of study showed increase CT value of patients compared with CT value of control, also increase ΔCT value of control compared with patients, the gene expression

folding of patients value was 1.23 compared with control value 1, this increase caused increase in *clock* gene activity and participate in bipolar disorder developments.

Table 6. The value of CT and the gene expression level for the Per1 gene and the Housekeeping gene in children with bipolar disorder compared to the control group.

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. Control	ΔΔCT	Folding
Control	27.14	22.71	4.43	4.43	0	1
Patients	30.72	24.39	6.32	4.43	1.89	0.33

The results of study showed increase CT value of patients compared with CT value of control, also increase ΔCT value of patients compared with control, the gene expression folding of patients value was 0.33 compared with control value 1, this decrease caused decrease in *PER1* activity and represent the main reason for bipolar disorder development.

Table 7. The value of CT and the gene expression level for the Per2 gene and the Housekeeping gene in children with bipolar disorder compared to the control group.

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. Control	ΔΔCT	Folding
Control	30.31	22.71	7.6	7.6	0	1
Patients	30.70	24.39	6.31	7.6	- 1.28	1.76

The results of study showed increase CT value of patients compared with CT value of control, also decrease ΔCT value of patients compared with control, the gene expression folding of patients value was 1.76 compared with control value 1, this increase caused increase in *PER2* activity and represent the main cause for bipolar disorder development.

Table 8. The value of CT and the gene expression level for the Per3 gene and the Housekeeping gene in children with bipolar disorder compared to the control group.

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. Control	ΔΔCT	Folding
Control	31.71	22.71	9	9	0	1
Patients	32.35	24.39	7.96	9	- 1.04	1.58

The results of study showed increase CT value of patients compared with CT value of control, also decrease ΔCT value of patients compared with control, the gene expression folding of patients value was 1.58 compared with control value 1, this increase caused increase in *PER3* activity and represent the main reason for bipolar disorder development.

Measuring the Gene Expression Level of Non-coding RNA Regulate the Circadian Rhythm Genes

The result of the study shown there are change in the level of expression for mi-RNA and snoRNA that regulate the circadian rhythm genes in the patients compared within control group as shown in the following tables.

Table 9. The value of CT and the gene expression level for micRNA molecules and the Housekeeping gene in children with bipolar disorder compared to the control group.

micRNA molecules	Gene target	No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. Control	ΔΔCT	Folding micRNA	Folding Target gene
micRNA 155	BMAL1 gene	Control	26.73	18.29	8.44	8.44	0	1	1
		Patients	25.22	18.06	7.15	8.44	- 1.28	1.76	0.73
micRNA 211	Clock gene	Control	26.71	18.76	7.95	7.95	0	1	1
		Patients	28.83	19.11	9.72	7.95	1.77	0.26	1.23

micRNA 24	PER1	Control	22.61	18.29	4.32	4.32	0	1	1
		Patients	24.65	18.06	6.62	4.32	2.30	0.41	0.33
micRNA 449a	PER2	Control	25.59	18.76	6.83	6.83	0	1	1
		Patients	28.24	19.11	9.13	6.83	2.30	0.20	1.76
micRNA 103	PER3	Control	15.13	18.29	- 3.16	- 3.16	0	1	1
		Patients	15.46	18.06	- 2.59	-3.16	0.59	0.56	1.58

The results of the study, as shown in figure (1), approved increase in the expression folding for miRNA155 in patients group, value was 1.76 as red column, this molecule will inhibit the expression of BMAL1 gene which value was 0.73, also show decrease in the expression folding for micRNA-211 in patients group, value was 0.26 as green column, this molecule will stimulate the expression of CLOCK gene which value was 1.23, and show decrease in the expression folding for micRNA-24 in patients group, value was 0.41 as purple column, this molecule will not show significant effect in the expression of PER1 gene which value was 0.33, and show decrease in the expression folding for micRNA-449a in patients group, value was 0.20 as blue column, this molecule will stimulate the expression of PER2 gene which value was 1.76, finally show decrease in the expression folding for micRNA-103 in patients group, value was 0.56 as blue column, this molecule will stimulate the expression of PER3 gene which value was 1.58.

Table 10. The values of CT and the level of gene expression for snoRNA and the housekeeping gene for patients with bipolar disorder compare with the control group

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. Control	ΔΔCT	Folding
Control	17.04	14.19	2.85	2.85	0	1
Patients	17.25	15.22	2.03	2.85	-0.82	1.76

The results of study showed increase CT value of patients compared with CT value of control, also decrease ΔCT value of patients compared with control, the gene expression folding of patients value was 1.76 compared with control value 1, this increase caused increase in genes activity for all genes and this represent the main reason for bipolar disorders development.

Results of Methylation-specific PCR for the Bmal1 and Clock Genes

The results of the test for the DNA methylation process in the promoter of the *Bmal1* and *Clock* gene, (100%) hyper-methylation in Bmal1 gene and (0%) no methylation in Clock gene, shown in the following Figures (1, 2).

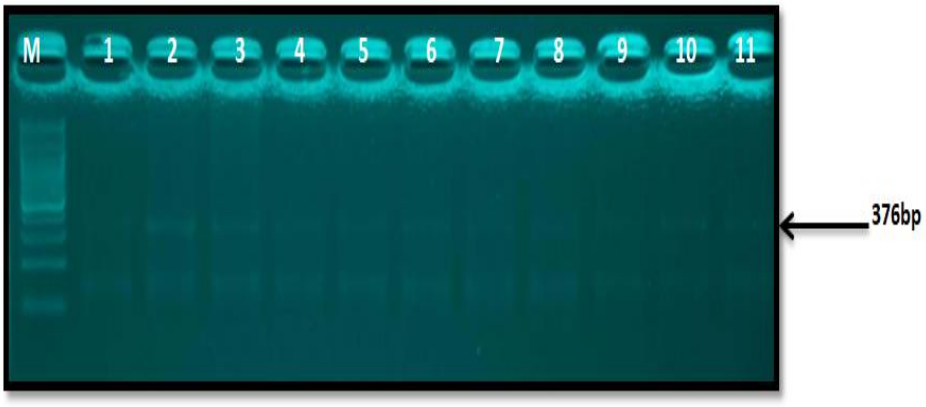

Figure 1. Results of Methylation-specific PCR reaction of Bmal1 gene

Figure 2. Results of Methylation-specific PCR reaction of Clock gene

Our results may provide a molecular basis for designing clinical protocols for Early diagnosis for bipolar disorders based on differences in the circadian clock between patient kids and healthy. On the hand, It has been shown that CpG methylation of promoter sequences, an epigenetic alteration, can inactivate promoter functions leading to dys-regulation and inhibition of gene expression, We further explored whether CpG methylation had occurred in the Bmal1 and Clock gene. The DNA methylation in the promoter of the Bmal1 gene was present in 100% of the samples and in the promoter of the Clock gene in 0% of the samples.

Discussion

The results of gene expression for circadian rhythm genes show significant changes in the level of folding expression, this variations could explain some phenotypic consequence, such as duration of sleep, waketime, addiction, insomnia, day preferences, etc. In our work, we noticed that the gene expression of BMAL1 gene were associated with sleep duration and waketime Heritability of sleep duration has been estimated as 46% of the variability and 44% for sleep quality. However, these values vary substantially with age, being less influential at younger ages. While the increase in expression of CLOCK gene will cause disruption in regulation of circadian rhythm genes and caused sleep disorder, on other hand, This cyclic process repeats, generating the characteristic oscillations of CLOCK and BMAL1 gene expression that underlie the circadian rhythm and the disruption in expression of CLOCK and BMAL1 genes will disruption this cyclic process and caused many disorders in sleep. By contrast, the variant in gene expression levels for period family genes approved comparable 24 h rhythmicity for PER1 gene under sleep/wake and stable routine sleep deprivation conditions, which agrees our information on PER1 gene expression. Also the daily expression of PER2 gene in whole blood cells in healthy men subjects. The peak phase of PER2 appeared in the early morning, while that of other circadian rhythm genes in the midnight. This study suggested that the keep under observation of PER2 gene in whole blood cells, which may be functionally necessary for the molecular control of the circadian pacemaker as well as in SCN, and be benefit to estimate internal synchronization.and the variant in PER3 gene expression correlated mental health and sleep disorder. In 2021, a study proved that PER3 expression strongly increase in ADHD group and the expression profile of circadian rhythm genes will variant in study samples. On other hand, many circadian-related miRNAs are change in both sleep disorders and neurodegenerative diseases, so these facts implicate that disruption in the expression of circadian miRNAs in individual with sleep disorder considered as biomarker for another mental disorder, in addition, control of the expression of miRNAs in the early stage of disease will be considered as treatment of mental disorders. Also the snoRNAs facilitate

modifications such as 2′-O-methylation, N-4-acetylation, and pseudouridylation, which impact not only ribosomal RNA (rRNA)andtheir synthesis but also different RNAs. The results of methylation process in promoter of BMAL1 and CLOCK gene approved that hyper methylation of BMAL1 gene will suprresed the expression of this gene and the hypomethylation of CLOCK gene will cause increase in xxpression level of this gene.

Conclusion

This studt suggested the mental disorders will related to disruption in gene expression for BMAL1, CLOCK, PER1, PER2 and PER3 genes and noncoding RNA molecules wich regulate the gene expression by stimulate or inhabite expression, also the epigenetic regulation by methylation at promoters of BMAL1 and CLOCK genes, all this factores coordinate the circadian rhythm genes and so cause mental disorders.

Acknowledgments

The authors thank Alsalam Specialized Hospital for supporting patient recruitment and sample collection. We also thank Mosul University for providing the facilities and resources to conduct this study. Special thanks to all the patients and their families who participated in this research, and to the dedicated laboratory staff for their assistance in sample processing and analysis. This study was conducted as part of ongoing efforts to better understand the molecular mechanisms underlying prostate cancer and improve therapeutic strategies.

Conflict of Interest

I hereby declare that I have no financial or commercial stake in any firm that the study described in the attached paper may have an impact on.

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