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The Role of miRNA Molecules and DNA-Methylation on Expression of Circadian Rhythm Genes in Kids with Mental Disorders

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Abstract

The variation of circadian rhythm genes is involved in mental disorders in kids, and the disturbance of circadian rhythm considered as a risk factor to the development of most mental disorders. Our aim was to study the estimated the role of miRNA molecules(miRNA24, miRNA449a, and miRNA102 on regulated for Per1, Per2, and Per3) and DNA methylation on gene expression of circadian clock genes. The study sample comprised of 40 patient of kids their ages range between (2 – 12 years) having mental disorders , and 20 sample of healthy kids as a control . The venous blood sample is distribute into two tubes: first tube (EDTA tube) to extracted DNA, second tube (Trizol tube) to extracted RNA. The study topics were divided into 3 sections: first, determining the level of gene expression for Per1, Per2, and Per3 genes using the qRT-PCR technique. Second section: determination of the level of Non-coding RNA molecules that regulate Per family genes using the qRT-PCR technique. The third section measures the percentage of methylation in the promoter of Per family genes using the PCR technique.

The results of the first section showed a significant decrease in the *Per1* gene expression; it was the value of folding expression cDNA *Per1* gene = 0.33, and a significant increase in the *Per2* and *Per3* genes; it was the value of folding expression cDNA *Per2* gene = 1.76 and folding expression cDNA *Per3* gene = 1.58. The results of the second section showed a significant decrease in miRNA gene expression; the value of folding expression cDNA miRNA24 *Per1* gene = 0.41, foldin expression cDNA miRNA449a *Per2* gene = 0.20, and folding expression cDNA miRNA102 *Per3* gene = 0.56. The results of the third section of the DNA methylation showed that DNA methylation in promoter of the *Per1* gene was present in 50% of the samples, in the promoter of the *Per2* gene, in 43% of the samples, and in the promoter of the *Per3* gene, in 0% of the samples.

Key words: circadian rhythm gene, miRNA , gene expression, methylation, q-PCR, Neurodevelopmental disorders.

دور جزيئات الـ miRNA وعملية مثيلة الـ DNA في التعبير الجيني لجينات الساعة البيولوجية لدى الاطفال الذين يعانون من الاضطرابات الذهنية

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الخلاصة

تتداخل تغييرات جينات الساعة البيولوجية مع الاضطرابات الذهنية لدى الأطفال. وقد يكون اضطراب إيقاع الساعة البيولوجية عامل خطورة في تطور العديد من الاضطرابات الذهنية. ان الهدف من هذه الدراسة هو ايجاد دور جزيئات الـ miRNA (miRNA24, miRNA449a, miRNA102) والتي تعمل على تنظيم جينات (Per1, Per2, Per3) وايضا ايجاد دور عملية مثيلة الـ DNA في التعبير الجيني لجينات الساعة البيولوجية. شملت الدراسة الحالية (40) طفل من الاطفال الذين يعانون من الاضطرابات الذهنية و (20) طفل من الاطفال الاصحاء الذين لا يعانون من اي مشاكل وبنفس الفئة العمرية التي تتراوح بين (2-12) سنة. تم تقسيم عينة الدم الوريدي الى جزأين، الجزء الاول تم وضعه في انابيب تحتوي مادة مانعة للتخثر EDTA لاستخلاص الحامض النووي الريبي منقوص الاوكسجين واجراء اختبار المثيلة. الجزء الثاني تم وضعه في انابيب تحتوي مادة الترابزول 750 مايكروليتر لإستخلاص الحامض النووي الريبي. تم تقسيم محاور الدراسة الى ثلاثة محاور، المحور الاول: تحديد مستوى التعبير الجيني لجينات الساعة البيولوجية (Per1, per2, Per3) باستخدام تقنية qRT-PCR، المحور الثاني: تحديد مستوى التعبير الجيني لجزيئات الحمض النووي الريبي غير المشفرة والتي تنظم جينات الساعة البيولوجية باستخدام تقنية qRT-PCR، اما المحور الثالث: تحديد نسبة المثيلة في بادئ جينات الساعة البيولوجية المدروسة باستخدام تقنية الـ PCR.

اظهرت نتائج المحور الاول من الدراسة انخفاض واضح في التعبير الجيني لجين Per1 حيث كانت قيمة التعبير الجيني تساوي (0.33) واطهرت زيادة واضحة في التعبير الجيني لجينات Per2, Per3 حيث كانت قيمة التعبير الجيني تساوي (1.76 و 1.58) على التوالي. بينما اظهرت نتائج المحور الثاني للدراسة انخفاض واضح في قيمة التعبير الجيني لجزيئات الـ miRNA (miRNA24 *Per1* gene = 0.41, miRNA449a *miRNA* *Per2* gene = 0.20, miRNA102 *Per3* gene = 0.56) وقد اظهرت نتائج المحور الثالث للدراسة بان عملية المثيلة عند بادئ الجين Per1

كانت تمثل 50% من عينات الدراسة، بينما عملية المثيلة عند بادئ الجين Per2 كانت تمثل 43%، وعملية المثيلة عند بادئ الجين Per3 كانت تمثل 0% من عينات الدراسة.

Introduction

Almost all species have internal timekeeping systems called circadian rhythms, which are essential for preserving homeostasis and fostering survival in the face of environmental change. The circadian clock, an internal oscillator that synchronizes a range of behavioral, physiological functions, and biochemical processes with daily cycles, controls these rhythms. Numerous ailments, including mental disorders and insomnia, are linked to disruptions in the circadian cycle [1]. Evolutionarily conserved across species, the circadian clock is produced by cell-autonomous transcriptional/translational feedback loops (TTFLs). Mammals have a major TTFL that has two directions: positive and negative. In the positive direction, BMAL1 and CLOCK form a heterodimer and bind to E-box areas on clock-controlled genes (CCGs), such as Periods (Per genes) [2]. As PER levels increase in the cytoplasm, they go to the nucleus and interact with the CLOCK:BMAL1 dimer to decrease CCG transcription through either displacement or blocking processes, depending on PER levels. There are perhaps nine circadian rhythm genes; Period family genes which contain *PER1*, *PER2* and *PER3* gene, Cryptochrome family genes which contain *Cry1* and *Cry2* gene. Also the core of circadian rhythm genes *BMAL1* and *CLOCK* genes, Also there are other genes like *TIMELESS* and *CASEIN KINASE 1e* genes, all this genes are control the human circadian rhythm [3]. The three period (PER) genes produce unique proteins with the PER-ARNT-SIM (PAS) domain that are mostly involved in nucleocytoplasmic function rather than direct DNA binding. helix-loop-helix transcription factors (PAS) are encoded by the CLOCK and BMAL1 genes [4]. A molecular clockwork made up of interconnected feedback loops for gene expression is created from the products of these genes. The PER genes play a crucial role in the auto-regulatory transcription and translation feedback loops of the circadian genes, which form the basis of the current concept of these oscillators. The TTFL's negative arm is made up of these interactions [5]. Additionally, the accurate creation and control of circadian rhythms in mammals depends on auxiliary feedback loops. Major health risks and mental issues can result from disruptions in this internal cycle. According to reports, sleep-related disorders affect at least 50% of global population [6]. An internal time-keeping system called the circadian clock regulates many physiological functions through the production of circadian rhythms in gene expression, which are then converted into cycles in metabolism and behavior. The SCN in the brain has the central clock, and other tissues and organ systems contain peripheral clocks, which together comprise the system [7]. The RNA World theory states that most of the information processing and metabolic changes required for biology to diverge from chemistry in the early history of life were performed by RNA or RNA-like substances [8]. RNA serves as a "photocopier" of DNA, a building block of proteins, a structural element of ribosomes and ribozymes, and a regulator of several biological processes, among other functions [9]. Thus, RNA may have been the precursor of life and evolved alongside it. Given the notion of RNA World and the assumed origin of the circadian clock in the first living species on Earth, it is logical to surmise that the

RNA regulation system and the circadian system coevolved and maybe interacted with one another. It has long been believed that non-coding RNAs that are unable to produce proteins are all "junk," but more recent research has challenged this notion [10].

Non-coding RNAs are, in fact, a popular subject in science right now. Numerous non-coding RNAs play an important role in biological systems and use different strategies to make up for their inability to be translated into proteins [11]. A class of non-coding RNAs known as microRNAs (miRNAs) serves as a post-transcriptional regulator. MiRNAs are involved in the regulation of multiple elements that are critical to the body's basic systems [12]. Recent data indicates that miRNAs control the circadian rhythm of gene expression and vice versa. Post-transcriptional and post-translational regulation, which is predicated on a well-functioning transcription and translation feedback system, accurately controls the molecular mechanism of the circadian clock [13]. Nonetheless, it has been proposed that a number of illnesses in the body are caused by the disruption of the circadian clock system. If there is a malfunction in the central nervous system, it may lead to neurological disorders such as sleep disorders, neurodegenerative diseases and Neurodevelopmental disorders (NDDs) which include Attention deficiency and hyperactivity disorder (ADHD), autism spectrum disorder (ASD) and Tourette syndrome (TS) [14 - 15]. Furthermore, patients with neurodevelopmental disorder, neurodegenerative diseases, and sleep disorders frequently have miRNAs that are improperly expressed in several tissues, the blood, and/or bodily fluids in common. These data suggest that miRNAs may serve as useful therapies as well as biomarkers of disease etiology due to their capacity to alter the expression of genes linked to or causative of diseases [15 - 22].

Materials and Methods

Case study

The current study included (40) male children suffering from mental disorders 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 time for (3 months) , based on approval form of a research protocol/ ministry of health and environment (form number 02/2024).

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

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

The gene expression level of *Per1*, *Per2* and *Per3* genes and Non-coding RNA was analyzed depend on q-PCR technique and the process include several steps:

RNA and non-coding RNA Extraction: After mixing 250 ml of blood sample with 750 ml of Trizol solution, an RNA extraction kit supplied by Transgenbiotech company is used, then Nano drop device use to measurement the purity of extract RNA.

The process of Non-coding RNA sedimentation

In this process the poly a polymerase (E. Coli) dose not reckon on the presence of the template and the ATP will catalyzed to incorporation into 3' end of RNA to form AMP, this step addition the E. Coli poly (a) tail to the 3' end of RNA. The high tailing efficiency of the enzyme Poly a polymerase working on add 20 ~ 200 A bases at the 3' end of RNA.

Application

1. For RNA 3' end labeling.
2. Poly (a) tails were added to RNA for cloning or affinity purification. For example, Non-coding RNA was added to Poly (a) to provide oligo-dT primer binding sites for cDNA synthesis.
3. Improve the translation efficiency of RNA in eukaryotic cells.

How to operate: the reaction components will preparation as following with total volume (20µl) , the component of PCR reaction with final reaction volume 20µl, the Master mix poly(A) 2 µl, ATP, GMP 1 µl, RNA TEMPLATE 10 µg, Poly(A) polymerase 1 µl and RNase free water 6 µl.

The process of converting the extracted mRNA and Non-coding RNA molecule into a cDNA: After the extraction of mRNA and Non-coding RNA is completed, it is converted into cDNA dependence on the reverse transcriptase enzyme activity, by used the Transgenbiotech company kit.

RT PCR reaction: specific housekeeping genes with the primer of gene use to:

detection the levels of gene expression for mRNA for PER genes. The sequence of primers use in RT-PCR reaction for mRNA molecules was *Per1*- RT Forward GGACACTCCTGCGACCAGGTACTG, *Per1*- RT Reversed GGCAGAGAGGCCACACGGAT, *Per2*- RT Forward CGCAGGGTGCGCTCGTTTGA, *Per2*- RT Reversed GGGCTCTGGAACGAAGCTTTCG, *Per3*- RT Forward GGTCGGGCATAAGCCAATG, *Per3*- RT Reversed GTGTTTAAATTCTTCCGAGGTCAAA, *Housekeeping*- RT Forward TGACCCAGATCATGTTTGAG, *Housekeeping*-RT Reversed CGTACAGGGATAGCACAG [16]. The component of reaction with final reaction volume 20µl was Ultra sybr q-PCR MIX with 10 µl volume, forward primer RT with 0.5 µl volume, Reversed primer RT with 0.5 µl volume, template of cDNA with 4 µl volume and distilled water with 5 µl volume. The program of RT-PCR for mRNA molecules was four stages; Pre denaturation at temperature 95°C for time period 10 min, and the Denaturation at temperatur

95°C for 15 sec, Annealing /Extension at 60°C for 1 min and Melting curve analysis at 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec.

detection the gene expression levels of miRNA molecules [16]. The sequence of primers use in RT-PCR reaction for miRNA molecules was miRNA 24 –F regulate Per1 Gene AACACGTGTGCTACTGAGCT, miRNA 449a-F regulate Per2 Gene AACACGCTGGCAGTGTATTGTTAG, miRNA 103–F regulate Per3 Gene AACAAAGAGCAGCATTGTACAGGG, universal-R for all genes CAGTGCAGGGTCCGAGGT, U6 –F for house keeping GTGCTCGCTTCGGCAGCA and U6 –R for house keeping CAAAATATGGAACGCTTC [16]. The component of reaction with final reaction volume 20µl, the Ultra syber q-PCR master mix was first component with 10 µl volume, forward primer RT with 0.5 µl volume, Reversed primer RT with 0.5µl volume, the template of cDNA with 4 µl volume and distilled water with 5 µl volume. The RT-PCR program for miRNA molecules was four stages; Pre denaturation at temperature 95°C for time period 10 min, Denaturation at temperature 95°C for time period 15 sec, Annealing /Extension at temperature 60°C for time period 1 min and Melting curve analysis at temperature 95°C for time period 15 sec, then temperature 60°C for time period 1 min, then temperature 95°C for time period 15 sec and temperature 60°C for time period 15 sec.

calculating gene expression folding

we calculated the folding of gene expression for all genes in this research by using double Δ CT method ($\Delta\Delta$ CT) based on the following equation: [17]

$$\text{Gene Expression folding} = 2^{-\Delta\Delta\text{CT}}$$

DNA Extraction for methylation: DNA was isolated from the blood of all (60) samples included in the study using the DNA extraction kit supplied by Transgenbiotech company, then Nano drop device use to measurement the purity of extract DNA.

Methylation-specific PCR in promoters of the *Per* family gene

Period family (*PER1*, *PER2* and *PER3*) Genomic DNA methylation-specific PCR analysis was altered using sodium bisulfite, and methylation-specific PCRs were carried out largely in accordance with the instructions [18]. We examined the methylation status of the promoter regions of the Period family genes to detected whether irregular CpG methylation for the area of promoter was the cause for distinctive Period family gene expression in children with mental disorders. We created a pair of primers for methylation alleles in each of the PER genes. To detection the methylation sequence we used this Primer pairs :Per1-F ATTTAGGTTTACGTGCGTTC, Per1-R CGACTCAAAAACGAAAATCG, its annealing temperature was 60 and band size was 298 bp. Per2-F GCGGTTTCGTTGCGGTTTAC, Per2-R GCCGACGCCGTTTCAAACCG, its annealing temperature was 60 and band size was 140 bp. Per3-F CGGTTTTTCGTTTCGAGTTCGC, Per3-R ACGATTAATCGTCGAAACCG, its annealing temperature was 60 and band size was 145 bp. In summary, 10 ml of 1 N NaOH was added to 40 ml of H₂O to denature about 4 mg of genomic DNA for 10 minutes. This

was followed by modification with 30 ml of 10 mM hydroquinone and 520 ml of 1.5 M sodium bisulfite (pH 5.0) at 50°C for 16 hours. Using a wizard DNA Transgenbiotech kit, 100 ml of prewarmed water (65–70°C) was used to elute the DNA samples. The eluent was mixed with fifty microliters of 1 N NaOH, and the combination was allowed to sit at room temperature for five minutes. The pellet was suspended in 45 milliliters of water after being precipitated with 150 milliliters of 100% isopropanol and cleaned with 70% ethanol [19]. The Modified DNA was amplified in a total volume reaction 20 µl: first component was DNA with 4 µl volume, while the Forward primer with 1 µl volume and the Reverse primer with 1 µl volume, also the PCR master mix with 10 µl volume and distilled water with 4 µl volume. The PCR was performed in a thermal cycler for 38 cycles and six stages; Initial denaturation at 98°C for 2 min one cycle, Denaturation at 94°C for 15 sec 35 cycle, Annealing at 55°C 15 sec 35 cycle, Extension at 72°C for 15 sec 35 cycle, Final extension at 72°C for 2 min one cycle and Stop reaction at 4°C for 4 min one cycle. After the Methylation-specific PCR program is done, PCR products were then loaded and electrophoresed on 2% agarose gels, stained with red safe dye and visualized under UV illumination [20].

Results and Discussion

Determined the gene expression folding for *PERs* family genes

The studies result shown clear contrast in the level of gene expression for *PERs* family genes in the patients group when compared with control group as shown in the following tables (1,2 and 3)

Table(1): gene expression for the *PER1* gene and the housekeeping gene

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

Table(2): gene expression for the *PER2* gene and the housekeeping gene

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

Table(3): gene expression for the *PER3* gene and the housekeeping gene

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

Light and other environmental elements have a significant impact on the central pacemaker of SCN. Conversely, hormones and neural variables impact the circadian clock activity in peripheral tissues, which are then regulated by the central pacemaker through a variety of communication pathways [2]. The period family genes (*PER1*, *PER2*, and *PER3*) will

regulation the central clock and peripheral tissue clock[18]. Many studies give an idea of that period family mutations was cause the disruption circadian clock, resulting in a shortened circadian period with decreased precision and stability[19].in our study , we found difference gene expression levels in the PER genes in kids have mental disorder, the gene expression folding of *PER1* (0.33) was decrease ,while the gene expression folding of *PER2*, *PER3* (1.76 , 1.58) was increase as compared with gene expression folding in healthy kids (1). Since expression of the period family genes act a major role in the circadian rhythm, this results proposed that the gene expression in circadian clock in the kids have mental disorder behaves differently from healthy kids.

Measuring the gene expression level of Non-coding RNA regulate the *PERs* family genes

The studies result shown clear contrast in the level of gene expression for mi-RNA that regulate the *PERs* family genes in the patients compared within control group as shown in the following tables (4, 5 and 6)

Table(4): gene expression for mi-RNA24 that regulate *PER1* gene and the housekeeping gene

No.	CT. target	CT. H.K	Δ CT. Target	Δ CT. control	$\Delta\Delta$ CT	Folding
Control	22.61	18.29	4.32	4.32	0	1
Patients	24.65	18.06	6.62	4.32	2.30	0.41

Table(5): gene expression for mi-RNA449a that regulate *PER2* gene and the housekeeping gene

No.	CT. target	CT. H.K	Δ CT. Target	Δ CT. control	$\Delta\Delta$ CT	Folding
Control	25.59	18.76	6.83	6.83	0	1
Patients	28.24	19.11	9.13	6.83	2.30	0.20

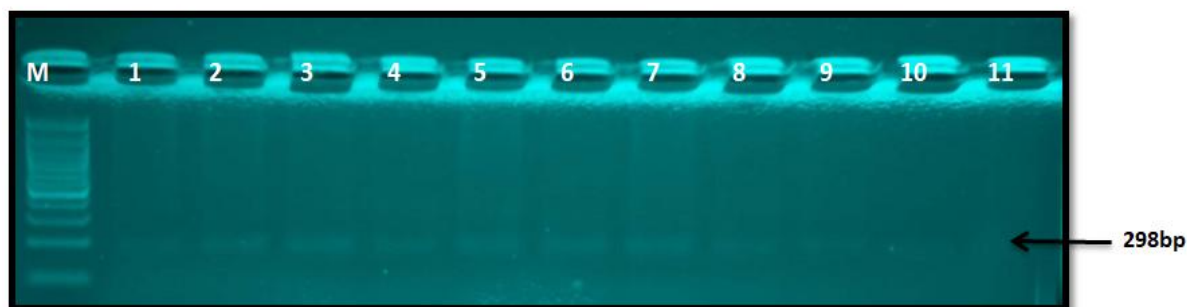
Table(6): gene expression for mi-RNA103 that regulate *PER3* gene and the housekeeping gene

No.	CT. target	CT. H.K	Δ CT. Target	Δ CT. control	$\Delta\Delta$ CT	Folding
Control	15.13	18.29	- 3.16	- 3.16	0	1
Patients	15.46	18.06	- 2.59	-3.16	0.59	0.56

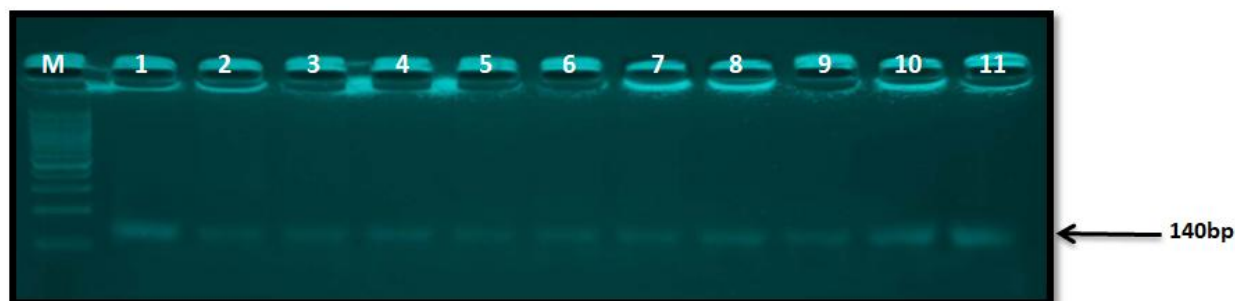
we also found different levels of miRNA molecules that regulate period genes family in kids have mental disorder and healthy kids [21], the gene expression level of miRNA24, miRNA449a and miRNA103 that regulate *PER1*, *PER2* and *PER3* gene was decrease (0.41, 0.20, 0.56) as compared with gene expression folding in healthy kids (1). This is indicative of heterogeneity in regulation of period family genes in kids with mental disorder. This results may suggested a molecular basis for scheming clinical protocols for Early diagnosis for mental disorders depended on differential in the circadian clock between patient kids group and healthy group.

Results of Methylation-specific PCR for the *Per* family genes

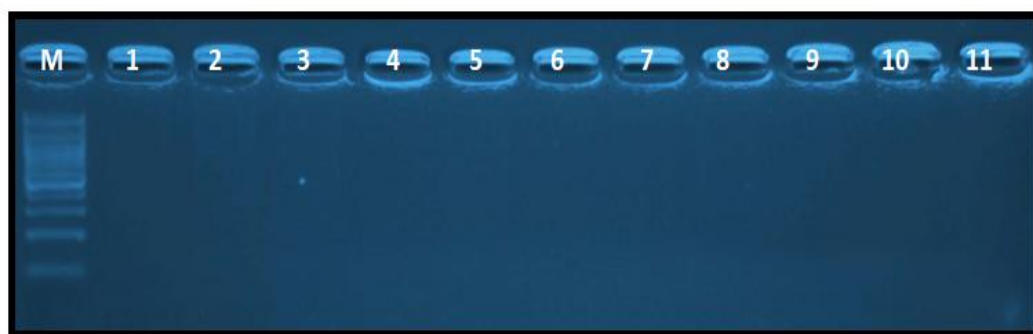
DNA methylation operation in the promoter of the *PERs* family gene, (50%) hyper-methylation in *PER1* gene, (43%) hyper-methylation *PER2* gene and (0%) no methylation in *PER3* gene, shown in the following figures (1, 2 and 3)



Figure(1): Results of Methylation-specific PCR reaction of *Per1* gene



Figure(2): Results of Methylation-specific PCR reaction of *Per2* gene



Figure(3): Results of Methylation-specific PCR reaction of *Per3* gene

On the hand, this results be revealed the methylation of promoter sequences, considered as an epigenetic alteration, this will disable promoter functions cause dys-regulation and suppression the gene expression, We also find whether CpG methylation had taken place in the period family genes. In order to the *PER1* promoter was additional repeatedly methylated (50%) and *PER2* (43%), *PER1* and *PER2* was the focus of next studies. No conection between *PER3* methylation (0%) and mental disorders.

In our study We propose that the different expression of the PER proteins between patient kids group and healthy group is slightly due to promoter methylation of the *PER1* and *PER2* gene, resulting in dis-regulation of the PER proteins, or disordering of the signal transduction pathway or cell cycle impress the PER protein expression. In the methylation study of promoter *PER1*, we construct that 20 cases showed methylation in the *PER1* promoter and 17 cases showed methylation in promoter but no methylation was detected in the promoter in the *PER3* gene. This difference in methylation resulting in disturbance of PERs gene expression and trouble in the circadian clock. On the other hand we proposed that period family genes inhibition act major importance role in the development of mental disorders.

Conclusion

The results of this study demonstrate increases in the the gene expression folding of *PER2*, *PER3* and decrease in the gene expression folding of miRNA449a and miRNA103 that regulate *PER2* and *PER3* gene, while there is no clear difference between the gene expression folding of *PER1* and the gene expression folding of miRNA24, also show that the methylation status has a significant correlation with the genes expression folding of the period family genes and miRNA molecules that regulate it.

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