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Research Article

Detection the genetic variation of circadian Rhythm genes and its correlation with sleepiness disorders in kids

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Abstract-Human health depends on sleep, which is still one of science's biggest mysteries. the circadian system is regulated the timing, duration and depth of sleep, and the hemostat of sleep. The main cue for the entrainment of circadian rhythms to the external environment is light, and the functions of SCN is a pacemaker responsible for this coordination process. The aim of study was find the correlation between sleepiness disorders and mutation in circadian rhythm genes. in this study we detection of the genetic variation of the circadian rhythm genes (*BMAL1*, *CLOCK*, *PER1*, *PER2* and *PER3* gene) by T-ARMS-PCR, the Case of study was 60 kids between the age of (2-12) years, divide into two categories the first contain 40 kids with sleepiness disorders while the second contain 20 sample include healthy kids and it have been considered as control. when we Detection of the genetic variation of the *BMAL1* rs3789327 gene by T-ARMS-PCR we found 60% of study samples are have mutant genotype and 35% are hetero-genotype, and when Detection of the genetic variation of the *Clock* rs6811520 gene by T-ARMS-PCR we found 92% of study samples are have mutant genotype and 5% are hetero-genotype, and when Detection of the genetic variation of the *PER2* rs2304672 gene by T-ARMS-PCR we found 75% of study samples are have mutant genotype and 20% are hetero-genotype, this indicate that three SNPs cause disturbance in circadian rhythm and this showed strong correlation with sleepiness disorders in kids, while *PER1* and *PER3* genes was show less correlation with disturbance in circadian rhythm because the percentage of mutant genotype for *PER1* gene was 10% and 5% for hetero-genotype, and the mutant genotype for *PER3* gene was 15% and 5% for hetero-genotype in the sample study. and the Hardy Weinberg equilibrium indicate that the P value for all genes studied was less than 0.5, this indicate the sample study is unstable and do not submit for the Hardy Weinberg equilibrium and the impact was environmental.

Keywords: Circadian Rhythum Genes, Mental Disorders, Bmal1gene, Clock Gene, Pers Family Genes.

I. INTRODUCTION

Human health depends on sleep, which is still one of science's biggest mysteries. the circadian system is regulated the timing, duration and depth of sleep, and the hemostat of sleep , which is have two-process

model [1]. S Process which reflects how the accumulates of sleep pressure during wakefulness and how discharged during the sleep. when the subject is awake it operates the internal timer which measures the tendency to fall asleep and when the subject is asleep the tendency to wake up. C Process (circadian rhythm) the ecologically appropriate the functions to restrict sleep during the day [2]. In mammals, the suprachiasmatic nucleus (SCN) at the hypothalamus is the pacemaker of circadian rhythm. if SCN lesion, in the sleep-wake cycle the circadian rhythm is completely eliminated, although 2–4 h periodicity persist of ultradian rhythms [3].The direct retinal input also receives via the retinohypothalamic tract (RHT) of the SCN, which enable entrain to external light/dark cues of the central clock [4]. According to the two-process model, it is the interaction of C and S process which determines when the person wake and sleep [5].the circadian rhythms is tightly regulated sleep. In optimal conditions, circadian rhythm is aligned with work, light/dark cycle, social obligations , and family [6]. However, environmental light/dark cycle changes (such as shift work, jet lag, nighttime light exposure etc.) and/or genetic abnormalities of the circadian system is impair proper entrainment, that result the chronic circadian rhythm sleep disorder (CRSD) [7,8]. The studies suggest that CRSD may be detrimental to mental function and physical health, with increase in the incidence of cardiovascular diseases, metabolic syndrome ,cancer, obesity, and metal disorders [9].

Circadian rhythms are endogenous biological processes, by which, all organisms can adapt to the environmental changes depend on the day-night cycle and adjust their behaviors accordingly and physiological functions [10,11]. The main cue for the entrainment of circadian rhythms to the external environment is light, and the functions of SCN is a pacemaker responsible for this coordination process. Moreover, hormones , body temperature or feeding/fasting entrained the circadian clock [12].fundamentally, it turns out that all cells and tissues in our body have circadian clocks [13].

Circadian Rhythm Sleep Disorders

Circadian rhythm sleep disorders (CRSDs) are conditions that the internal circadian rhythms are not properly aligned with the external environment. CRSDs are divided into four main parts, including advanced sleep phase disorder (ASPD), delayed sleep phase disorder (DSPD), irregular sleep-wake rhythm and non24-h sleep-wake disorder. Although the social , environmental and/or occupational schedules may lead to sleep disturbance, some kids may also be genetical predisposed to the development of CRSD [14,15,16]. It is estimated that ~33% of sleep quality variance and ~40% of sleep pattern variance are contributable to genetic differences [17,18]. Kids with advanced sleep phase disorder usually feel very sleepy and have to go to bed early in the evening (generally between 6–9 pm) and wake up very early in the morning (generally between 2–5 am) [19]. This disorders is a rare disorder with a strong genetic trait [20,21]. Delayed sleep phase disorder (DSPD) is characterized by a persistent and intractable delay of sleep onset and offset time comparing to normal person, generally more than 2 h. kids with DSPD are unable to fall asleep and wake up at socially acceptable times, resulting in excessive daytime sleepiness [22].

II. MATERIALS AND METHODS

Case of study

60 kids between the age of (2-12) years who were referred to “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). The sample were divide into two categories the first contain 40 kids with sleepiness disorders while the second contain 20 sample include healthy kids and it have been considered as control.

Blood sample collection and storage

1 ml of venous blood withdrawn from the kids and put in EDTA tube for DNA extraction.

DNA extraction

Blood DNA Extraction done using the protocol of transgenbiotech kit, this protocol is suitable for use with fresh or frozen whole blood, can be use blood treated with citrate or EDTA. R.B.Cs lysis is not necessary.

detection the concentration and purity of extracted nucleic acids

Biodrop spectrophotometer used to detect concentration and purity of nucleic acids.

Detection the genetic variation of circadian rhythm genes by using Tetra-ARMS-PCR

Four sets of primers for each genes of circadian rhythm genes used to detection the genetic variation depending on Tetra-ARMS-PCR technique as shown in the table (1):

Table (1) : The primers of circadian rhythm genes used in Tetra-ARMS-PCR

Gene	Rs	Primer	Sequence	Band size	Annealing
Bmal1 gene	Rs3789327	FO	GCTTTTCCTCAGCAATGCAAAATGGACA	348 bp	70 72 71
		RO	AGGCTCTGGACATTCCAACTCCATAGCA		
		FI	GGCCTCTGTGTCTTTTATTAACCAGCAACG	187bp	
		RI	CCAGTGGCAGGGTCACTCTTGGGAATAT	219bp	
Clock gene	Rs6811520	FO	GTTAATGATGAACCAACAGACTGGGAAT	333 bp	65
		RO	TGGAAAGCTCAAAATTGATAGCTGTTTT		
		FI	TTTGTTAGCTGAATACAGCTATGTCTGCC	185bp	
		RI	GGAACAGGCCTTTCCAATATAGAGGA	203bp	
Per1 gene	Rs3027177	FO	GAAGGTATCCTGGCAGGAGGGGGAGAGC	339 bp	75
		RO	GGCCTGTGTCAAGCAGGTGCAGGGTAAG		
		FI	TCCAGCTCCTCCAGGGTATAGGTGGCCA	206bp	
		RI	GAGGGCGAGCCTTGCTCCATGGAAAC	187bp	
Per2 gene	Rs2304672	FO	TCTCATGTCCACTGGAGCCACTGCTCAT	335 bp	73
		RO	GCAACAGAGCCAGACCCTGTCTCCAAAG		
		FI	GCGTATCCATTCATGCTGGGCTCAGC	223bp	
		RI	GCATCCCTCTGTTTGCCAGCTTCGATC	165bp	
Per3 gene	Rs228697	FO	GGAAGAGAATACGCAGCCCCCGGAACT	307 bp	74
		RO	CCTCGCTTTGTGCCTCCCACTTTTCCTC		
		FI	TATGACCGTTTTCCTGCCTGACCGCC	198bp	
		RI	ACGATGGCGACAACAGAGGACAGACTGC	163	

III. RESULTS

Detection of the genetic variation of the *BMAL1* rs3789327 gene by T-ARMS-PCR

The results of the T-ARMS-PCR reaction reveal that there is a correlation between children with sleepiness disorders and the genetic variation of the *BMAL1* gene at the location rs3789327 as shown in the figure (1).

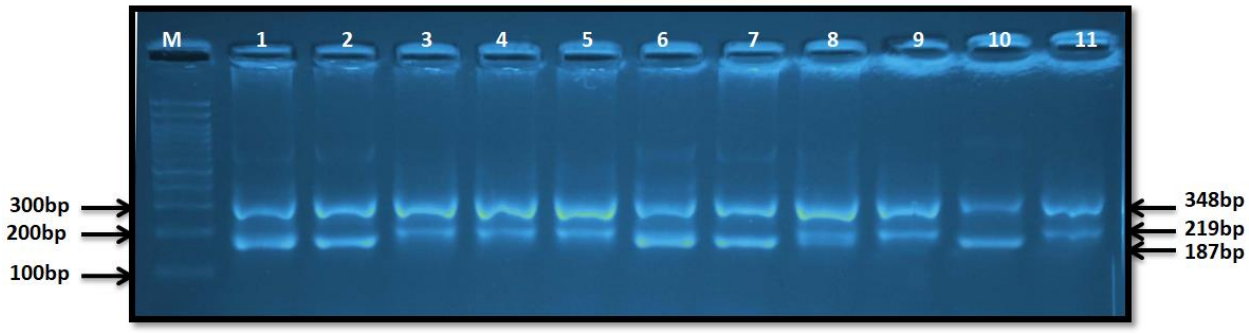

Figure (1): The PCR product for *BMAL1* gene separated by 2% agarose gel electrophoresis

Symbol M represents the DNA ladder and the samples (3, 4, 5, 9, 11) carrying the normal genotype, which contain two bands, 348 bp for main gene and 219 bp for normal allele, while the samples (1, 2, 6, 7, 10) carrying the mutant genotype, which contain two bands, 348 bp for main gene and 187 bp for mutant allele, but the sample (8) carrying hetero-genotype with three bands, 348 bp for main gene, 219 bp for normal allele and 187 bp for mutant allele.

Table (2): The percentage distribution observed of the different genotypes of the *BMAL1* gene at the location rs3789327 between control and children with sleepiness disorders, knowing that the G allele is the wild allele and the A allele is the mutant allele.

Genotypes	Patients		Control		P Value	OR	(95%Cl)
	NO.	%	NO.	%			
GG	14	35	17	85	P=0.0009	0.095	0.0237 to 0.381
GA	2	5	2	10	P=0.4725	0.4737	0.0617 to 3.638
AA	24	60	1	5	P=0.0018	28.5	3.462 to 234.6215

The G allele is normal, and the allele A is mutant.

According to the results, the allelic percentage was observed as well as the frequency of the different genotypes of the ***BMAL1*** gene at the location rs3789327.

The results for sleepiness disorder patients showed that the frequency value of the mutant genotype AA was 60%, which is the highest percentage compared to the wild genotype GG, which is 35%. The heterozygote GA rate was 5% compared with the control group, 85% for the wild genotype, 10% for the heterozygote type, and 5% for the mutant genotype.

The value of the OR for the mutant genotype was 28.5; It is higher than 1.0 within the probability level p = 0.0018, which is considered a risk factor for the development of the disease.

Table (3):The allele frequency of the *BMAL1* gene at the location rs3789327 between control and children with sleepiness disorders

Genotypes	**Patients**		**Control**		**P Value**	**OR**	**(95%Cl)**
Alleles	**NO.**	**%**	**NO.**	**%**			
G	**30**	**37.5**	**36**	**90**	**P = 0.0001**	**15.0**	**4.8560 to 46.3341**
A	**50**	**62.5**	**4**	**10**			

The results for sleepiness disorder patients showed that the frequency value of the mutant allele A was 62.5%, which is the highest percentage compared to the wild allele G, which is 37.5%. compared with control group which frequency value of mutant allele 10% and wild allele 90%.

The OR value of mutant allele A was 15.0; It is higher than 1.0 within the probability level p = 0.0001, which is considered a risk factor for the development of the disease.

Detection of the genetic variation of the *Clock* rs6811520 gene by T-ARMS-PCR

The results of the T-ARMS-PCR reaction reveal that there is a correlation between children with sleepiness disorders and the genetic variation of the *Clock* gene at the location rs6811520 as shown in the figure (2).

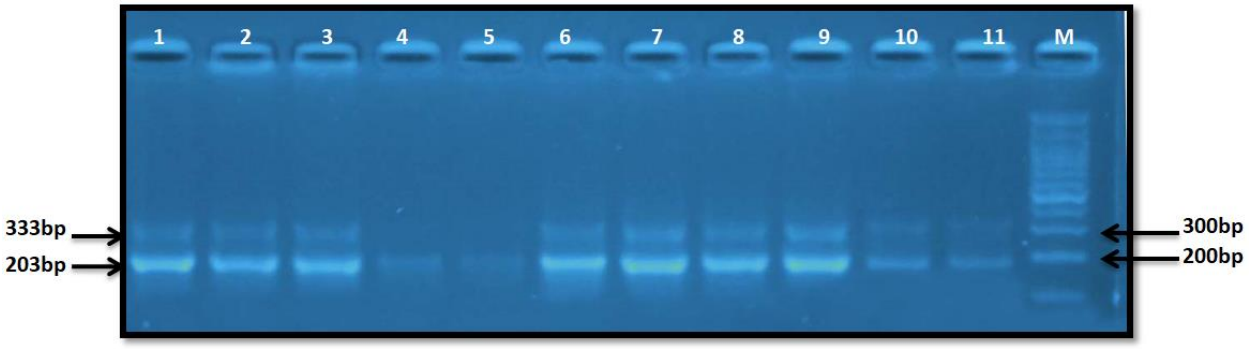

Figure (2): The PCR product for *Clock* gene separated by 2% agarose gel electrophoresis

Symbol M represents the DNA ladder and all the samples carrying the normal genotype, which contain two bands, 333 bp for main gene and 203 bp for normal allele.

Table (4): The percentage distribution observed of the different genotypes of the *Clock* gene at the location rs6811520 between control and children with sleepiness disorders, knowing that the C allele is the wild allele and the T allele is the mutant allele.

Genotypes	Patients		Control		P Value	OR	(95%Cl)
	NO.	%	NO.	%			
CC	1	2.5	18	90	P=0.0001	0.0028	0.0002 to 0.0335
CT	2	5	1	5	P=1.0	1.0	0.0852 to 11.7383
TT	37	92.5	1	5	P=0.0001	234.3333	22.8033 to 2408.0766

The C allele is normal, and the allele T is mutant.

According to the results, the allelic percentage was observed as well as the frequency of the different genotypes of the *Clock* gene at the location rs6811520.

The results for sleepiness disorder patients showed that the frequency value of the mutant genotype TT was 92.5%, which is the highest percentage compared to the wild genotype CC, which is 2.5%. The heterozygote CT rate was 5% compared with the control group, 90% for the wild genotype, 5% for the heterozygote type, and 5% for the mutant genotype.

The value of the OR for the mutant genotype was 234.333; It is higher than 1.0 within the probability level p = 0.0001, which is considered a risk factor for the development of the disease.

Table(5):The allele frequency of the *Clock* gene at the location rs6811520 between control and children with sleepiness disorders

Genotypes	Patients		Control		P Value	OR	(95%Cl)
Alleles	NO.	%	NO.	%			
C	4	5	37	92.5	P=0.0001	234.3333	49.8524 to 1101.4941
T	76	95	3	7.5			

The results for sleepiness disorder patients showed that the frequency value of the mutant allele T was 95%, which is the highest percentage compared to the wild allele C, which is 5%. compared with control group which frequency value of mutant allele 7.5% and wild allele 92.5%.

The OR value of mutant allele T was 234.333; It is higher than 1.0 within the probability level p = 0.0001, which is considered a risk factor for the development of the disease.

Detection of the genetic variation of the *PER1* rs3027177 gene by T-ARMS-PCR

The results of the T-ARMS-PCR reaction reveal that there is a correlation between children with sleepiness disorders and the genetic variation of the *PER1* gene at the location rs3027177 as shown in the figure (3)

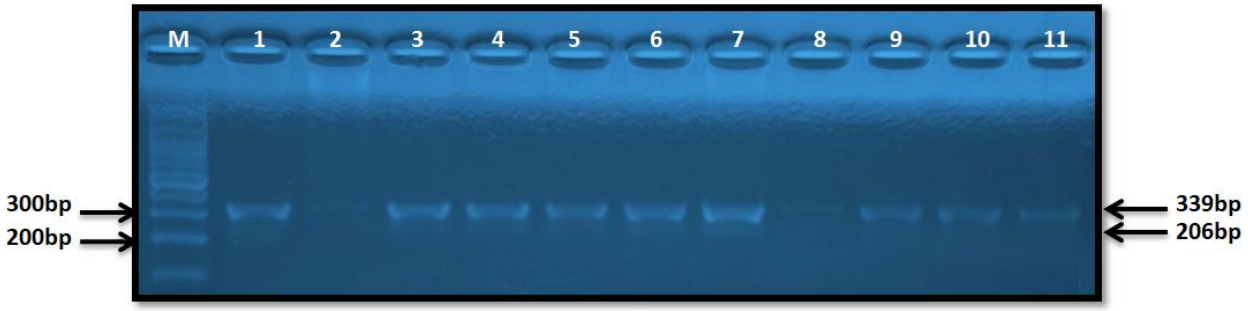

Figure (3): The PCR product for *PER1* gene separated by 2% agarose gel electrophoresis

Symbol M represents the DNA ladder and all the samples carrying the normal genotype, which contain two bands, 339 bp for main gene and 206 bp for normal allele.

Table (6): The percentage distribution observed of the different genotypes of the *PER1* gene at the location rs3027177 between control and children with sleepiness disorders, knowing that the A allele is the wild allele and the G allele is the mutant allele.

Genotypes	Patients		Control		P Value	OR	(95%Cl)
	NO.	%	NO.	%			
AA	34	85	17	85	P=1.0	1.0	0.2224 to 4.4963
AG	2	5	2	10	P=0.4725	0.4737	0.0617 to 3.638
GG	4	10	1	5	P=0.5171	2.1111	0.2201 to 20.2457

The A allele is normal, and the allele G is mutant.

According to the results, the allelic percentage was observed as well as the frequency of the different genotypes of the *PER1* gene at the location rs3027177.

The results for sleepiness disorder patients showed that the frequency value of the mutant genotype GG was 10%, which is the lowest percentage compared to the wild genotype AA, which is 85%. The heterozygote AG rate was 5% compared with the control group, 85% for the wild genotype, 5% for the heterozygote type, and 10% for the mutant genotype.

The value of the OR for the mutant genotype was 2.1111; It is higher than 1.0 within the probability level p = 0.5171, which is considered a risk factor for the development of the disease.

Table(7):The allele frequency of the *PER1* gene at the location rs3027177 between control and children with sleepiness disorders

Genotypes	Patients		Control		P Value	OR	(95%Cl)
Alleles	NO.	%	NO.	%			
A	70	87.5	36	90	P=0.6882	1.2857	0.3768 to 4.3867
G	10	12.5	4	10			

The results for sleepiness disorder patients showed that the frequency value of the mutant allele G was 12.5%, which is the lowest percentage compared to the wild allele A, which is 87.5%. compared with control group which frequency value of mutant allele 10% and wild allele 90%.

The OR value of mutant allele G was 1.2857; It is higher than 1.0 within the probability level p = 0.6882, which is considered a risk factor for the development of the disease.

Detection of the genetic variation of the *PER2* rs2304672 gene by T-ARMS-PCR

The results of the T-ARMS-PCR reaction reveal that there is a correlation between children with sleepiness disorders and the genetic variation of the *PER2* gene at the location rs2304672 as shown in the figure (4).

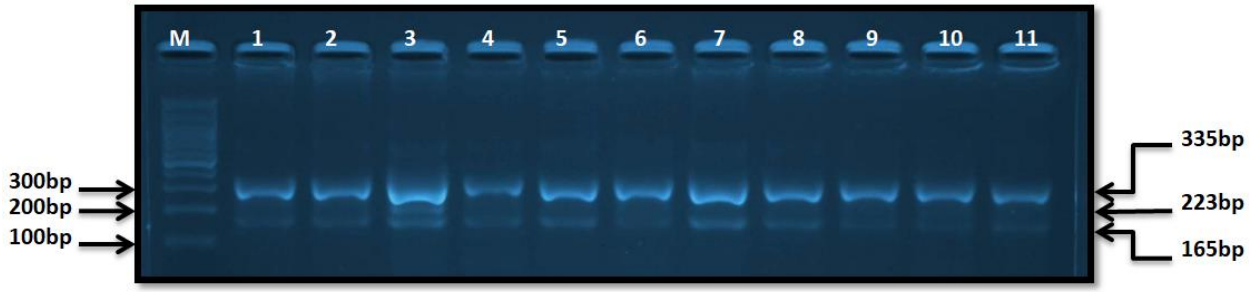

Figure (4): The PCR product for *PER2* gene separated by 2% agarose gel electrophoresis

Symbol M represents the DNA ladder and the samples (1, 2, 4, 5, 7, 8, 9, 11) carrying the mutant genotype, which contain two bands, 335 bp for main gene and 165 bp for normal allele, while the samples (3, 6, 10) carrying the hetero-genotype, with three bands, 335 bp for main gene, 223 bp for normal allele and 165 bp for mutant allele.

Table (8): The percentage distribution observed of the different genotypes of the *PER2* gene at the location rs2304672 between control and children with sleepiness disorders, knowing that the C allele is the wild allele and the G allele is the mutant allele.

Genotypes	Patients		Control		P Value	OR	(95%Cl)
	NO.	%	NO.	%			
CC	2	5	16	80	P=0.0001	0.0132	0.0022 to 0.0792
CG	8	20	2	10	P=0.3365	2.25	0.4305 to 11.7582
GG	30	75	2	10	P=0.0001	27.0	5.3071 to 137.3625

The C allele is normal, and the allele G is mutant.

According to the results, the allelic percentage was observed as well as the frequency of the different genotypes of the *PER2* gene at the location rs2304672.

The results for sleepiness disorder patients showed that the frequency value of the mutant genotype GG was 75%, which is the highest percentage compared to the wild genotype CC, which is 5%. The heterozygote CG rate was 20% compared with the control group, 80% for the wild genotype, 10% for the heterozygote type, and 10% for the mutant genotype.

The value of the OR for the mutant genotype was 27.0 ; It is higher than 1.0 within the probability level p = 0.0001, which is considered a risk factor for the development of the disease.

Table(9):The allele frequency of the *PER2* gene at the location rs2304672 between control and children with sleepiness disorders

Genotypes	Patients		Control		P Value	OR	(95%Cl)
Alleles	NO.	%	NO.	%			
C	12	15	34	85	P=0.0001	32.1111	11.0922 to 92.9593
G	68	85	6	15			

The results for sleepiness disorder patients showed that the frequency value of the mutant allele G was 85%, which is the highest percentage compared to the wild allele C, which is 15%. compared with control group which frequency value of mutant allele 15% and wild allele 85%.

The OR value of mutant allele G was 32.1111; It is higher than 1.0 within the probability level p = 0.0001, which is considered a risk factor for the development of the disease.

Detection of the genetic variation of the *PER3* rs228697 gene by T-ARMS-PCR

The results of the T-ARMS-PCR reaction reveal that there is a correlation between children with sleepiness disorders and the genetic variation of the *PER3* gene at the location rs228697 as shown in the figure (5)

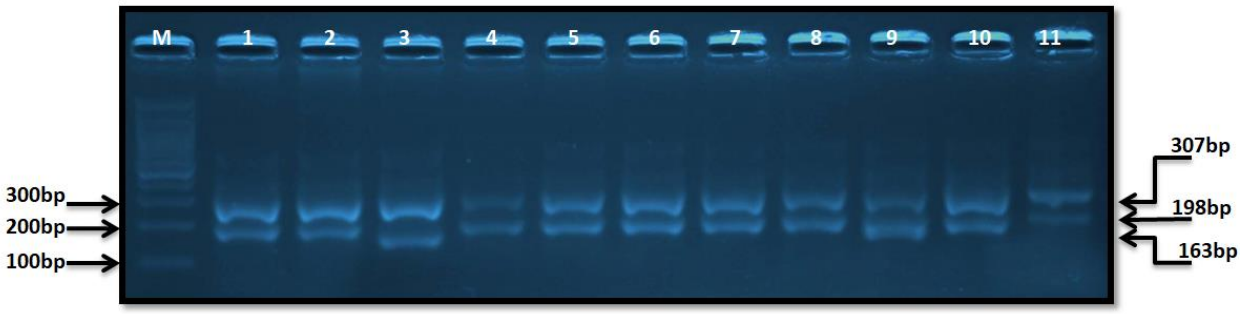

Figure (5): The PCR product for *PER3* gene separated by 2% agarose gel electrophoresis

Symbol M represents the DNA ladder and the samples (1, 2, 4, 5, 6, 7, 8, 10, 11) carrying the normal genotype, which contain two bands, 307 bp for main gene and 198 bp for normal allele, while the samples (3, 9) carrying the mutant genotype, which contain two bands, 307 bp for main gene and 163 bp for mutant allele.

Table (10): The percentage distribution observed of the different genotypes of the *PER3* gene at the location rs228697 between control and children with sleepiness disorders, knowing that the C allele is the wild allele and the G allele is the mutant allele.

Genotypes	Patients		Control		P Value	OR	(95%Cl)
	NO.	%	NO.	%			
CC	32	80	15	75	P=0.6582	1.3333	0.3727 to 4.7698
CG	2	5	3	15	P=0.2068	0.2982	0.0456 to 1.9514
GG	6	15	2	10	P=0.5936	1.5882	0.2904 to 8.6875

The C allele is normal, and the allele G is mutant.

According to the results, the allelic percentage was observed as well as the frequency of the different genotypes of the *PER3* gene at the location rs228697.

The results for sleepiness disorder patients showed that the frequency value of the mutant genotype GG was 15%, which is the lowest percentage compared to the wild genotype CC, which is 80%. The heterozygote CG rate was 5% compared with the control group, 75% for the wild genotype, 15% for the heterozygote type, and 10% for the mutant genotype.
The value of the OR for the mutant genotype was 1.5882 ; It is higher than 1.0 within the probability level p = 0.5936, which is considered a risk factor for the development of the disease.

Table(11):The allele frequency of the *PER3* gene at the location rs228697 between control and children with sleepiness disorders

Genotypes	**Patients**		**Control**		**P Value**	**OR**	**(95%Cl)**
Alleles	**NO.**	**%**	**NO.**	**%**			
C	**66**	**82.5**	**33**	**82.5**	**P=1.0**	**1.0**	**0.3683 to 2.7153**
G	**14**	**17.5**	**7**	**17.5**			

The results for sleepiness disorder patients showed that the frequency value of the mutant allele G was 17.5%, which is the lowest percentage compared to the wild allele C, which is 82.5%. compared with control group which frequency value of mutant allele 17.5% and wild allele 82.5%.

The OR value of mutant allele G was 1.0; It is equal the 1.0 within the probability level p = 1.0 , which does not considered a risk factor for the development of the disease.

Table(12): The Hardy Weinberg equilibrium results of circadian rhythm genes

Bmal1	**Genotype**	**GG**	**GA**	**AA**
	Observed genotype	**14**	**2**	**24**
	Expected genotype	**5.6**	**18.8**	**15.6**
	P-value= 0.001		**Chi squared valueX^2= 31.92177778**	
Clock	**Genotype**	**CC**	**CT**	**TT**
	Observed genotype	**1**	**2**	**37**
	Expected genotype	**0.1**	**3.7**	**23.1**
	P-value= 0.002737		**Chi squared valueX^2= 8.975069252**	
Per1	**Genotype**	**AA**	**AG**	**GG**
	Observed genotype	**34**	**2**	**4**
	Expected genotype	**30.6**	**8.8**	**0.6**
	P-value= 0.001		**Chi squared valueX^2= 23.80408163**	
Per2	**Genotype**	**CC**	**CG**	**GG**
	Observed genotype	**2**	**8**	**30**
	Expected genotype	**0.9**	**10.2**	**28.9**
	P-value= 0.172530		**Chi squared valueX^2= 1.86082276**	
Per3	**Genotype**	**CC**	**CG**	**GG**
	Observed genotype	**32**	**2**	**6**
	Expected genotype	**27.2**	**11.6**	**1.2**
	P-value= 0		**Chi squared valueX^2= 27.34656397**	

When execution the Hardy Weinberg equilibrium it indicate that the P value for *BMAL1* gene equal 0.001, *CLOCK* gene equal 0.002, *PER1* gene equal 0.001, *PER2* gene equal 0.172530 and *PER3* gene equal 0, so the P value for all genes are < 0.5, this indicate the sample study is unstable and do not submit for the Hardy Weinberg equilibrium and the impact was environmental.

IV. DISCUSSION

In this study we found there are many mutations in circadian rhythm genes , when we Detection of the genetic variation of the *BMAL1* rs3789327 gene by T-ARMS-PCR we found 60% of study samples are have mutant genotype and 35% are hetero-genotype, and when Detection of the genetic variation of the *Clock* rs6811520 gene by T-ARMS-PCR we found 92% of study samples are have mutant genotype and 5% are hetero-genotype, and when Detection of the genetic variation of the *PER2* rs2304672 gene by T-ARMS-PCR we found 75% of study samples are have mutant genotype and 20% are hetero-genotype, this indicate that three SNPs cause disturbance in circadian rhythm and this showed strong correlation with sleepiness disorders in kids, while *PER1* and *PER3* genes was show less correlation with disturbance in circadian rhythm because the percentage of mutant genotype for *PER1* gene was 10% and 5% for hetero-genotype, and the mutant genotype for *PER3* gene was 15% and 5% for hetero-genotype in the sample study. the Hardy Weinberg equilibrium indicate that the P value for all genes studied was less than 0.5, this indicate the sample study is unstable and do not submit for the Hardy Weinberg equilibrium and the impact was environmental.

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