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Per3 length polymorphism in patients with type 2 diabetes mellitus

Abstract

Background: A number of observations support the involvement of circadian clock genes in the regulation of metabolic processes. One of these circadian genes, *Per3*, exhibits a variable number tandem repeat length polymorphism, consisting of two alleles, namely four and five repeat alleles, in its exon 18. The objective of this study was to examine the existence of *Per3* variants in patients with type 2 diabetes mellitus (T2DM) as compared to a non T2DM control group.
Methods: Intravenous blood samples were collected to obtain white blood cells from 302 T2DM patients and 330 non-diabetic, age- and sex-matched, individuals. *Per3* genotyping was performed on DNA by polymerase chain reaction.
Results: Frequency of five repeat allele was higher, and that of four repeat allele lower, in T2DM patients as compared to non-diabetic controls (χ^2=6.977, p=0.0082)
Conclusions: The results indicate an association of *Per3* five repeat allele with T2DM occurrence and suggest that individuals with five repeat allele may be at a greater risk for T2DM as compared to those carrying the four repeat allele.

Keywords: circadian rhythms; clock gene; metabolism; *Per3* polymorphism; type 2 diabetes mellitus.

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Introduction

The mammalian circadian system is composed of a central pacemaker, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, and peripheral clocks present in almost every cell in the organism [1]. The SCN master clock is synchronized by Zeitgebers (photic and non-photic). Non-photic Zeitgebers also affect peripheral oscillators found in organs such as the pancreas, liver, skeletal muscle, heart, adipose tissue, intestine, and retina [2–4].

Circadian clock genes are known to regulate circadian periodicity through a transcription-translation feedback loop, the circadian clock gene *Per3* playing an essential role in such a feedback loop. *Per3* consists of 21 exons of which the exon 18 contains a polymorphic repeat domain, with four (*Per 3*[4]) or five (*Per3*[5]) copies of a tandem repeat region with a length of 54 bp sequence encoding 18 amino acids [5]. Several studies have reported associations of *hPer3* alleles with a number of physiopathological conditions, including preference of timing in sleep/wake cycles [6, 7], delayed sleep phase syndrome (DSPS) [5–8], and sleep homeostasis [9]. In *Per3* mutant mice, circadian period and phase underwent changes in liver and other peripheral tissues [10]. *Per3* has also been reported to regulate adipocyte fate [11].

In humans, the onset of T2DM is recognized to be multifactorial and related to, among other processes, the timing and extent of food intake and physical exercise [12–14], sleep duration, and circadian desynchronization [15–18]. Yet, whether there is any association of the *Per3* alleles with T2DM and obesity in humans remains to be explored. The objective of the study was to investigate the existence of *Per3* length polymorphism in type 2 diabetes mellitus (T2DM) patients as compared to a group of sex- and age-matched non-diabetic controls.

Materials and methods

Sampling subjects

Blood samples were collected from 302 T2DM individuals (age=50.7±10.3) and 330 healthy non-T2DM individuals (age=49.9±9.1) who served as controls. Among 302 T2DM individuals, 184 were males (age=52.1±9.8) and 118 were females (age=48.8±10.8). In controls, 184 were males (age=50.4±8.9) and 146 females (age=49.4±3.0). All patients were living in Madurai, Tamil Nadu, India. Type 1 diabetes patients were excluded from the study.

Control subjects were initially screened randomly based on plasma glucose fasting level below 99 mg/dL and were excluded from participation if they had diabetes or any family history of diabetes [19]. The individuals were categorized as T2DM based on the guidelines prescribed by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bethesda, MD, USA, http://diabetes.niddk.nih.gov/DM/pubs/diagnosis/index.aspx). All the patients with diabetes were under usual medication. All the information with regard to the protocol was explained and written consents were obtained from participants. The guidelines for the ethical treatment of patients in chronobiological studies [20] were followed and the experimental protocol was reviewed and approved by the Institutional Ethical Committee of the Madurai Kamaraj University, Madurai, India.

Per3 genotyping

Genomic DNA was isolated from the blood samples using the phenol-chloroform method. The polymerase chain reaction (PCR) was performed using the specific primers as described in [21]. The PCR reaction was carried out in 10 μL tubes containing 1 μL of 1×PCR reaction buffer, 0.5 μL (0.5 mM) of dNTPs mix, 1.5 μL (0.4 mM) Forward/Reverse Primer mix, 0.05 μL (0.025 U) *Taq* polymerase enzyme (Genet Bio, Daejeon, Korea), 2 μL (100 ng) of genomic DNA and 4.95 μL of Milli-Q water. The PCR amplification conditions included an initial step of 94°C for 5 min, 40 cycles of amplification (94°C for 30 s, 60.6°C for 30 s, 72°C for 30 s) and a final extension at 72°C for 5 min. The resultant PCR products showed three genotypes based on their DNA size, characterized as: (i) *Per3*[5/5] homozygous allele (401 bp); (ii) *Per3*[4/4] homozygous allele (347 bp); and (iii) *Per3*[4/5] heterozygous allele (347 bp and 401 bp). Subsequently, 5% of long repeat and short repeat alleles were sequenced for confirmation.

Statistical analysis

Distribution of four and five repeat allele frequencies in the population was determined using the Hardy-Weinberg exact test [22]. Deviation from Hardy-Weinberg equilibrium and the association of allele frequencies of four and five repeat alleles with T2DM and control were analyzed by Chi-square Testing using the Statistical Program for Social Science (SPSS) software (Version 20.0; IBM Corp, Armonk, NY, USA); p-values lower than 0.05 were taken as evidence of statistical significance.

Results

Table 1 summarizes genotype and allelic distribution of *Per3* variants in both groups of individuals. Genotype distribution in the three groups was within the limits of Hardy-Weinberg equilibrium. No significant deviation was found between the observed and expected allele frequencies for T2DM and control groups (p>0.05). The observed genotype frequencies in control and T2DM groups were 41.2% and 33.8% for *Per3* homozygous four repeat allele (4/4), 47% and 47.3% for *Per3* heterozygous allele (5/4) and 11.8% and 18.9% for *Per3* homozygous five repeat allele (5/5), respectively. An increased prevalence of five repeat homozygotes was seen in T2DM patients as compared to non-diabetic controls (odds ratio=1.95, confidence interval 95%=1.21–3.15, p=0.006).

The allelic distribution of *Per3* variants in both groups of individuals is summarized in Table 2. Frequency of five repeat allele was higher, and that of four repeat allele lower, in T2DM patients as compared to non T2DM individuals (χ^2=6.977, p<0.0082).

Discussion

The foregoing results indicate that the *Per3* five repeat allele could be related to T2DM occurrence and suggest

Table 1 Genotypic distribution of the 4-/5- length polymorphism of *Per3* in type 2 diabetes mellitus (T2DM) patients and controls.

Group	n			Genotype[a]
		4-/4-	4-/5-	5-/5-
T2DM	302	102 (34%)	143 (47%)	57 (19%)
Control	330	136 (41%)	155 (47%)	39 (12%)
Odds ratio (95% confidence interval)		1	1.23 (0.87–1.73) p=0.24	1.95 (1.21–3.15) p=0.006

[a]4-, 4-repeat allele; 5-, 5-repeat allele.

Table 2 Four and Five repeat allele distribution of *Per3* in type 2 diabetes mellitus (T2DM) patients and controls.

Group	n	Allele (%)		X²	p-Value
		4-	5-		
T2DM	302	347 (57%)	257 (43%)		
Control	330	427 (65%)	233 (35%)	6.977	0.0082
Odds ratio (95% confidence interval)		0.74 (0.59–0.92)	1.36 (1.08–1.71)	IC 95%(1.081726–1.703075)	
		p<0.01	p<0.01		

4-, 4-repeat allele; 5-, 5-repeat allele.

that individuals with five repeat allele could be at a greater risk for T2DM as compared to individuals carrying the four repeat allele. Previously, a single nucleotide polymorphism (SNP) identified in *CRY2,* another circadian clock gene, but not in *PER3,* correlated with T2DM [23]. The distribution of *Per3* alleles and genotypes were found to be in Hardy-Weinberg equilibrium, suggesting that genotypic and allele frequencies are constant in the population.

Although knowledge of *Per3's role* as a component of time-keeping system in humans is limited, a number of reports indicate that *hPer3* can be involved in the functioning of sleep [8, 24, 25] and of certain metabolic processes [11, 26]. *Per3* alleles seem to influence sleep homeostasis and sleep/wake timing in individuals [8, 9] and a strong correlation has been found between the amount of sleep time and *Per3* protein secretion in individuals [27]. Compared to four repeat individuals, five repeat individuals tend to experience a greater sense of sleep pressure and, further, tend to demonstrate a poorer cognitive performance when they are sleep deprived. Interestingly, five repeat *Per3* allele has been linked to DSPS in a Brazilian population [7] as well as to a delayed acrophase of melatonin secretion [27]. Exposure of blue light decreased melatonin levels to a greater extent in individuals carrying the five repeat *Per3* allele than in four repeat individuals [28]. In contrast, no correlation has been found between *Per3* genotypes and preference of sleep/wake timing [29, 30].

A recent study attributed a major role of *Per3* in the time keeping of peripheral oscillators present in the liver [10]. *Per3* could also have a possible functional role in the regulation of adipogenesis as constitutive expression of *Per3* blocked the process of adipogenesis in mesenchymal stem cells, whereas elimination of *Per3* promoted it [11]. Moreover, *Per3* mutant mice exhibited a remarkable difference in their body mass and body composition [26] and had more adipose tissue content than wild type mice [11].

Per3 is down regulated when cells are maintained under gluco-lipotoxic conditions in vitro [31].

A link between prolonged sleep disturbance and the increasing worldwide prevalence of the metabolic syndrome has been proposed [15, 17, 32]. For instance, it has been reported that clock gene expression is impaired in individuals with metabolic syndrome, and, further, that impairments in transcript levels of PER3 in human leukocytes occur in diabetic subjects and gestational diabetic women [33, 34]. Interestingly, mRNA of PER3 has been found to be inversely proportional to the glycated hemoglobin levels in T2DM patients [31, 34]. Taken together, these findings suggest that impaired *Per3* may influence glucose metabolism.

Presence of three phenotypic variants in *Per3* could be due to differential phosphorylation of proteins. The polymorphic repeat consists of a region of putative phosphorylation sites and phosphorylation would give rise to phenotype difference in individuals. Third repeat insertion/deletion would influence the phosphorylation level, thus modifying the behavior of an individual resulting in three different behavioral phenotypes [6].

The present study had several limitations that need to be considered in any interpretations of its findings. Besides its preliminary nature and the necessity for it to be confirmed in larger samples of patients and in different ethnic populations, the present study did not investigate the association between the onset of T2DM, body mass index and alcohol dependence among patients and the genotypic variants of *Per3*. Such a study is needed to substantiate the existence of a direct link between the polymorphic variants and their possible association with diabetes. Moreover it remains to be explored whether *Per3* directly influences T2DM or indirectly through altered sleep/wake cycles or by any other unknown mechanism that remains unidentified. Indeed, besides circadian changes, other causative factors for the onset of T2DM must be considered, including insulin resistance in liver

and abnormal adipogenesis [35], less physical inactivity [14], sleep loss [17, 36], sedentary lifestyle [14, 37], and high amount of food intake and/or high calorie food consumption [16]. In any event, the findings reported herein are compatible with the hypothesis that *Per3* five repeat allele is associated with a greater risk of T2DM.

Conflict of interest statement

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