Sleep Science

ORIGINAL ARTICLE

Screening for polymorphisms in the HIOMT gene and associations with circadian phenotypes

Rastreamento de polimorfismos no gene HIOMT e associações com os fenótipos circadianos

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ABSTRACT

HIOMT is a gene that encodes hydroxyindole-O-methyltransferase, the final enzyme in the melatonin synthesis pathway. As the timing of melatonin synthesis is different for morning and evening people, it is possible that polymorphisms in genes coding for the enzymes which participate in melatonin synthesis can influence this hormone synthesis and release patterns that may result in different circadian outputs. The aim of this study was to search for polymorphisms in the HIOMT gene and to verify possible associations between genetic variations in this gene and circadian phenotypes in a Brazilian population sample. Among the 44 extreme morning and the 48 extreme evening people, ten polymorphisms were found, being two of them not described so far. Haploview analyses showed linkage disequilibrium between pairs of polymorphisms in the promoter B region. Also, the haplotype AG (rs4446909, rs5989681) is associated with evening preference. The analysis of these data indicates that polymorphisms in the HIOMT gene exhibit a possible trend to influence circadian phenotypes in this Brazilian population sample, possibly affecting the rate and/or level of melatonin synthesis.

Keywords: circadian rhythm, genetics behavorial, melatonin, polymorphism genetic.

RESUMO

O HIOMT é um gene que codifica para a hidroxindole-O-metiltransferase, a enzima final na via de síntese da melatonina. Uma vez que a temporização da síntese de melatonina é diferente para pessoas matutinas e vespertinas, é possível que polimorfismos nos genes codificantes para as enzimas que participam da síntese da melatonina possam influenciar os padrões de síntese e liberação deste hormônio, resultando em diferentes respostas circadianas. O objetivo deste estudo foi buscar por polimorfismos no gene HIOMT e verificar a existência de possíveis associações entre as variações genéticas neste gene e os fenótipos circadianos em uma amostra da população brasileira. Entre os 44 indivíduos matutinos extremos e os 48 vespertinos extremos, dez polimorfismos foram encontrados, sendo dois deles ainda não descritos até o momento. Análises do Haploview mostraram um desequilíbrio de ligação entre pares de polimorfismos na região do promotor B. Ainda, o haplótipo AG (rs4446909, rs5989681) está associado com a preferência vespertina. A análise destes dados indica que polimorfismos no gene HIOMT exibem uma tendência na influência sobre os fenótipos circadianos na amostra da população brasileira investigada, podendo afetar a taxa e/ou o nível da síntese de melatonina.

Descritores: genética comportamental, melatonina, polimorfismo genético, ritmos circadianos.

INTRODUCTION

Chronotypes are related to the preference for doing activities in specific times of the day. Although most of the population prefers intermediates hours to wake up and go to sleep, to perform physical and intellectual activity, some people prefer the earlier hours - the *morning* ones - and other ones willing do it later on the day - the *evening* subjects[1]. Several physiological events, such as central temperature variation and hormone secretion, like melatonin, exhibit a different expression pattern in morning or evening subjects[2]. Melatonin is an hormone secreted by the pineal gland predominantly during the dark phase, and is mainly known to be both a circadian marker which signals the beginning and end of the night, and a photoperiod-dependent molecule, which is important for seasonal adaptation in animals[3-6].

The synthesis of melatonin begins with the uptake of tryptophan by the pinealocytes (pineal gland cells), followed by a series of enzymatic reactions that ultimately produce melatonin. The enzyme arylalkylamine N-acetyltransferase (AA-NAT) is involved in the rate-limiting step of melatonin synthesis and is responsible for acetylating serotonin to form N-acetylserotonin. Hydroxyindole-O-methyltransferase (HIOMT) or acetylserotonin-O-methyltransferase (ASMT) is the final enzyme that is responsible for the o-methylation of N-acetylserotonin to form melatonin[7,8].

The *HIOMT* gene, also called *ASMT*, was isolated from human pineal glands in 1993[9] and it is located on the pseudoautosomal regions of the human X (Xp22.3) and Y (Yp11.3) chromosomes[10]. The structural analysis of the *HIOMT*

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gene was performed in 1994, and two distinct promoters, A and B, are associated with the transcription of this gene[11]. Promoter A does not seem to drive the expression of *HIOMT* in the brain and pineal tissues, or in most of the retinas tested. However, promoter B drives its expression in all analyzed tissues, obtained from pineal, brain and retina. Also, *HIOMT* has three different final transcripts resulting of alternative splicing of the primary message, and while the promoter A seems to signal the transcription of only two of them, the promoter B is able to drive all of the three.

Melatonin has been used as a strong circadian phase marker; for instance, the melatonin phase of secretion is delayed in evening people when compared to morning people[2]. Although studies have shown associations between polymorphisms in the *AA-NAT* gene, diurnal preference, and Delayed Sleep Phase Disorder[12-14], to date, no study has reported any associations between *HIOMT* gene polymorphisms and circadian phenotypes.

Because the timing of melatonin synthesis is different in morning and evening people, it is possible that polymorphisms in the genes that code for enzymes participating in the melatonin synthesis may influence individual patterns of synthesis and release. Therefore, the aim of this study is to analyze the promoter region and the exons of the *HIOMT* gene to search for polymorphisms in a sample of the Brazilian population and to search for associations between these polymorphisms and the diurnal preference.

MATERIAL AND METHODS

Subjects and Chronotyping

After answering to the Horne & Östberg questionnaire[15] for the determination of their diurnal preferences, the 5% lowest and the 5% highest HÖ scores were selected for genetic analysis. The groups of 44 extreme morning subjects (24.34 ± 4.26 years; 70% females; 81.8% Caucasians) and 48 extreme evening subjects (23.46 ± 4.70 years; 65% females; 77.1% Caucasians) were selected among a larger group of 1245 undergraduate students from São Paulo city, matching in age, gender and self-declared ethnicity ($p > 0.05$), for participating in this study.

Polymorphism Screening

Blood samples were collected from all participants, and the genomic DNA was extracted from their white blood cells through the salting out of the cellular proteins and precipitation with a saturated NaCl solution[16]. The DNA was purified using ethanol to eliminate impurities that might interfere in the next procedures.

Ten pairs of primers (Table 1) were designed in the intronic regions to flank all the nine exons and the promoter B region of the *HIOMT* gene, using the *Primer 3* software, available at: http://frodo.wi.mit.edu/primer3/. The reference sequence of the gene used to design the primers was ENSG00000196433, and the transcript sequence used was ENST00000381241, both of which available at www.ensembl.org/index.html.

All fragments were amplified by PCR (Polymerase Chain Reaction) and were visualized by electrophoresis in 1% agarose gels. The annealing temperatures used for the amplification of each exon and of the promoter region, and the expected product sizes are described in Table 1.

The PCR products were subjected to a denaturation-renaturation method for further analyses using DHPLC (Denaturing High-Performance Liquid Chromatography) to search for polymorphisms. DHPLC analyses were performed on the WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE, USA) equipped with a 3500HT DNASep Cartridge (Transgenomic). The PCR products were first analyzed at 50°C, a non-denaturing temperature, to ensure sufficient quantity and specificity. Based on the size and on the sequence of the PCR product, the navigator software (Transgenomic) calculated the temperatures at which 40-80% of the DNA molecules were double stranded - these temperatures were then used in the heteroduplex screening (Table 1). For some products, more than one temperature value was necessary to screen the entire sequence of the amplicon. DNA was eluted at a flow rate of 0.9 ml/min with buffer A (0.1 M triethylammonium acetate or TEAA and 0.25% acetonitrile) and buffer B (0.1 M TEAA and 25% acetonitrile). This linear acetonitrile profile permitted the fixation and release of the DNA from the cartridge and the detection of the homoduplex or heteroduplex profile by an ultraviolet detector.

Heteroduplex samples were sequenced directly using an ABI PRISM 377 (California, USA) to identify the polymorphisms in the *HIOMT* gene. Polymorphisms found at frequencies higher than 10%, were considered as high frequency polymorphisms (Table 2), and were validated using the TaqMan SNP (Single Nucleotide Polymorphism) Genotyping Assays (Applied Biosystems, CA, USA). Polymorphisms found at frequencies lower than the established threshold, considered as low frequency polymorphisms, were rescreened through DHPLC: one homoduplex sample was directly sequenced and defined as standard. When this standard was mixed in equal volumes with each of the homoduplex samples, if a heteroduplex pattern emerged, it indicated that the sample was homozygous but different from the standard, signing a polymorphic allele.

Statistical Analyses

The Hardy-Weinberg equilibrium test was used to verify whether the frequencies of the polymorphisms were in equilibrium. The Chi-square (χ^2) test was used to compare the allelic frequencies between the groups (Table 2), and the software Haploview[17] to perform haplotype and linkage disequilibrium (LD) analyses and permutation tests with 1,000 replicates[17,18]. The significance level was 0.05. The ESEfinder 3.0 tool[19] was used to identify possible functional exonic splicing enhancers that would be recognized by serine/arginine-rich proteins: SF2/ASF, SC35, and SRp40.

The study was approved by the Committee on Ethics at Universidade Federal de São Paulo (UNIFESP), Brazil, # 476/04.

Table 1. Primer sequences, PCR annealing temperatures used, sizes of the PCR products and DHPLC temperatures used in the polymorphism screening analyses.

HIOMT Gene	Primers	Temperature Annealing	PCR Product	DHPLC Temperature
Promoter region	F: 5'GTTGAACTTCCAGCCTCCAG 3` R: 5'GGATTGGAGACAAGATGGGA 3`	57°C	469 bp	63°C
Exon 1	F: 5'CCAGCAGGCTCTGTGCTC 3' R: 5'CAAGGGGGAATAGACTTCCG 3'	64°C and 60°C*	180 bp	63°C
Exon 2	F: 5'CAATGCTTTCCTCCCTAGCC 3' R: 5'CCATCCTGGCTCACACAGT 3'	61°C and 58°C*	377 bp	61.5°C
Exon 3	F: 5'GCAGGAACTCGGAATCTCAC 3' R: 5'CACCTGGCAGACGCCGTGAG 3'	67.3°C - 58.3°C* (variation = 0.5°C/cycle)	183 bp	59.5°C, 62.6°C and 64.5°C
Exon 4	F: 5'CCTGGGCTACAGAGCTGAAA 3' R: 5'TACTCAAATGGCACAACCCA 3'	61°C	278 bp	50°C
Exon 5	F: 5'TCTTGACAAGCGTGGTTTTG 3' R: 5'CTGCATTCTGATGCTTTGACA 3'	52.8°C	249 bp	59.4°C
Exon 6	F: 5'AAAAGGATGCGTTCATGTCC 3 R: 5'GGACACAGGGAGGGGAAC 3'	60°C	155 bp	Direct sequencing
Exon 7	F: 5'GTCAAACGGGCTGTGTCC 3' R: 5'GGACTGGATGTTTCTGGGAA 3'	58°C	239 bp	61.3°C
Exon 8	F: 5'GTCAAATGGGATTGGATTGC 3' R: 5'GACACGGGGCAGAATTGTAC 3'	63.8°C, 62.8°C and 61.8°C*	233 bp	62.2°C
Exon 9	F: 5'ATGGTTCACTGGGACTTTGG 3' R: 5'GCTTCTTGTTCTTGGTGTTCA 3'	65.8°C, 63.8°C and 60.8°C*	377 pb	57.7°C, 60.5°C and 62°C

F: Forward; R: Reverse; * *Touchdown* PCR.

Table 2. Allelic frequencies of the nine SNPs found in the analyzed Brazilian population, for the morning and evening groups.

Polymorphism	Allelic frequencies (44 morning subjects)	Allelic frequencies (48 evening subjects)	Chi square	*p* value
rs4446909 (G/A)	0.281 (A)	0.193 (A)	1.207	0.272
rs5989681 (G/C)	0.281 (C)	0.295 (C)	0.025	0.874
rs56690322 (G/A)	0.177 (G)	0.182 (G)	0.007	0.933
rs6644635 (C/T)	0.417 (T)	0.489 (T)	0.363	0.547
rs28515673 (G/A)*	0.033 (A)	0.017 (A)	0.330	0.568
IVS2 + 14 G > A*	0 (A)	0.017 (A)	0.990	0.319
rs28675287 (T/C)	0.177 (C)	0.125 (C)	0.712	0.399
rs17844917 (C/T)	0.125 (T)	0.114 (T)	0.044	0.833
rs11346829 (G/-)	0.094 (-)	0.148 (-)	0.998	0.318

* The allelic frequencies for this polymorphism were calculated using a sample of 30 individuals per group.

RESULTS

A total of ten polymorphisms were identified along all nine exons with their respective intronic flanking regions and also the promoter region (Table 3). The frequencies of all of them are in Hardy-Weinberg equilibrium. Polymorphism reference numbers were taken from www.ensembl.org/index.html. Among the polymorphisms sequenced and identified in this study, one represents a variable number of tandem repeats, or VNTR; one is a deletion (G/-); six of them are transitions (G/A or C/T changes); and the remaining two represent transversions (G/C and C/A).

Four polymorphisms along the promoter region, one in the exon 2, one in the exon 5, and two SNPs in the exon 8 (Table 3), previously described in the literature, were also found in this Brazilian sample, and besides these ones, two new, unpublished polymorphisms were found in the investigated sample (Table 3). The first one constitutes of a cytosine to adenine substitution in the second intron of the gene, represented through the notation "IVS2 + 14 C > A", following the guidelines from Dunnen & Antonarakis[20].

The second new polymorphic allele found is an intronic VNTR, constituted of 11-16 repetitions of the sequence TGAAA in the intron 3. As DHPLC is able to identify deletion or insertion at 50°C, the same temperature used to analyze the quality of the PCR products as described in the methods, we decided to sequence this region for all of the samples. Five samples were excluded from the analysis because of the poor quality of their sequences. About 40% of the samples from each group had the allele with the sequence TGAAA repeated 14 times. However, we did not find any differences between groups regarding this VNTR. The two new polymorphisms found, plus the SNP rs28515673 in the intron 2 (Table 3), were detected at low frequencies in the investigated groups, not constituting candidates for association with diurnal preference.

Table 3. SNPs found in our study and in database analysis.

	Databank analysis	Our results
Promoter region	rs4446909 rs5989681 rs56690322 rs6644635	rs4446909 (G/A) rs5989681(G/C) rs56690322(G/A) rs6644635(C/T)
Exon 1	rs17149149	No polymorphisms identified
Exon 2	rs28515673 rs6588802 Not identified	rs28515673 (G/A) Not identified A new intronic SNP (C/A)
Exon 3	rs5989834	No polymorphisms identified
Exon 4	No polymorphisms described	A new intronic VNTR
Exon 5	rs28675287	rs28675287 (T/C)
Exon 6	rs6588809 rs28613362 rs7471973	No polymorphisms identified
Exon 7	rs4521942	No polymorphisms identified
Exon 8	rs17844917 rs11346829	rs17844917 (C/T) rs11346829 (G/-)
Exon 9	No polymorphisms described	No polymorphisms described

The haplotype analysis output shows a linkage disequilibrium between pairs of polymorphisms in the promoter region (Figure 1), and we observed a moderate increased frequency of the AG haplotype (rs4446909, rs5989681) in the evening group when compared to the morning group, 5.5% and 0.2% respectively (χ^2 = 4.475, p = 0.034). However, the permutation test did not confirm the association.

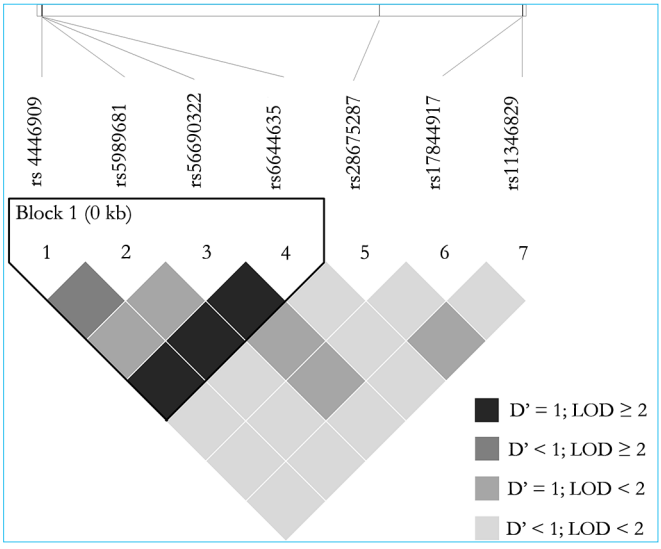

Figure 1. Haplotype block structure for the seven SNPs found in higher frequencies along the HIOMT gene. Linkage disequilibrium between pairs ofpolymorphisms can be verified in the promoter region (D' ≤ 1, LOD ≥ 2).

DISCUSSION

Melatonin is recognized for its property to promote both synchronization and circadian phase shifting, exhibiting "chronobiotic" effects[21]. Indeed, the melatonin secretion peak takes time earlier in morning when compared to evening people[2,22]. This scenario sets the stage for the assumption that genetic variability in the enzymes involved in melatonin production and secretion, as well as in the genes encoding for those enzymes, has an important role in the establishment of the circadian traits.

Previous studies have demonstrated that a non synonymous mutation along *AA-NAT*, a gene participating in the melatonin synthesis pathway, is associated with Delayed Sleep Phase Disorder in a Japanese population sample[13], and that a single-nucleotide polymorphism in the promoter region of this same gene may seems to associate with sleep patterns in students from Singapore[12]. About fifteen polymorphisms (Table 3, databank analysis) have been detected previously along the promoter region and the exons and introns of the last gene participating on the melatonin synthesis chain, the Hydroxyindole-*O*-methyltransferase - *HIOMT*.

In this study, two new polymorphisms were found in the analyzed sample, possibly due to the ethnic origin of the Brazilian population. The Brazilian population is formed mainly of an European/Portuguese and Brazilian/Indigenous background[23,24], mixed at first with a variety of African groups and later, in the beginning of the 20th century, with some European ethnicities, mainly Italian and Spanish people, and with Asian ethnicities, mainly the Japanese[23,25]. In the city of São Paulo, it has been estimated that about 7% of the population has a genetic contribution from native DNA[26]. Therefore, the new polymorphisms found could represent specific variations preserved in the course of evolution, and might be related to the adaptation to specific environmental conditions, such as temperature and photoperiod.

In silico analysis demonstrated that the allele C of the SNP rs28675287 abolishes an exonic splicing enhancer consensus motif for the splicing factor SC35[19,27]. As this SNP is located between exons 5 and 6, a conserved and perhaps evolutionary important locus, it may be involved in the splicing of exon 6 in three of the four possible transcripts for the *HIOMT* gene (www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000196433;r=X:1733894-1761974).

The haplotype blocks found in the genome are useful targets when looking for associations between SNPs and phenotypes, as they sign alleles in linkage disequilibrium, *i.e.*, chromosomal regions with little evidence of recombination, presenting alleles in different frequencies than those expected if they segregate with independent assortment[18]. We observe here an association between the haplotype AG (rs4446909, rs5989681) in the promoter B region and diurnal preference, with this haplotype present at a higher frequency in the evening group when compared to the morning subjects ($\chi^2 = 4.475$; $p = 0.034$). This same haplotype has been associated with a lower risk for recurrent depression in Polish patients, maybe linking mood and sleep disorders[28], and also is found in higher frequencies in autistic individuals than in control subjects, being associated with a decrease in *HIOMT* transcripts in the blood cells of this patients[29]. The polymorphism rs4446909 (G/A) is located inside of the putative transcription factor binding site (TFBS) GATA-1, and it is next to OCT-1, a ubiquitous TFBS. By the other hand, the polymorphism rs5989681 seems not to be near any important TFBS. However this association should be look with care because the permutation test did not confirm it.

The relatively low number of subjects participating in this study can be considered a limiting factor, and the ethnic composition of the groups may carry some stratification bias. Plus, among the Brazilian population, the *ethnicity* concept is not necessarily related to ancestry but, more often, to appearance. Besides the fact that each Brazilian individual is a unique mosaic of foreign and native genomes - making it impossible to predict genomic ancestry through skin color nor the opposite, - self-related determination of one's ethnicity has a strong socioeconomic component[30,31]. Although, the study design included an homogeneous group of patients, carefully diagnosed, and *morning* and *evening* groups composed by individuals in the extreme phenotypes, reducing the stratification effects. Here, we reported the first screening for polymorphisms in the *HIOMT* gene in a sample of Brazilian population, indicating a possible trend to influence circadian phenotypes, which may exert some effect on the determination of circadian profiles through changes in the melatonin synthesis.

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