Association between *CLOCK*, *PER3* and *CCRN4L* with non-small cell lung cancer in Brazilian patients

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Received July 9, 2013; Accepted March 24, 2014

DOI: 10.3892/mmr.2014.2224

Abstract. Circadian rhythms comprise of daily oscillations in a variety of biological processes and are regulated by an endogenous clock. Disruption of these rhythms has been associated with cancer progression, and understanding natural oscillations in cellular growth control, tumor suppression and cancer treatment, may reveal how clock and clock-controlled genes are regulated in normal physiological functioning. To investigate the association between clock genes and non-small cell lung cancer (NSCLC), we genotyped three tag SNPs (*rs938836*, *rs17050680*, *rs3805213*) in the Nocturnin gene (*CCRN4L*), five SNPs (*rs228727*, *rs228644*, *rs228729*, *rs707467*, *rs104620202*) in the period 3 (*PER3*) gene and one SNP (rs6855837) in the *CLOCK* gene, in 78 Brazilian patients with NSCLC. One tag SNP in *CCRN4L* (*rs3805213*) and another tag SNP from *PER3* (*rs228729*) demonstrated a significant correlation with genotype and allele frequency in lung cancer ($P=4.4x10^{-3}$ and $P=5.7x10^{-2}$; P=0.004 and P=0.02, respectively). The results of our study suggest these polymorphisms in the *CCRN4L* and *PER3* genes may represent a risk factor in the occurrence and development of NSCLC in Brazilian patients.

Introduction

Circadian rhythms are daily oscillations in physiological processes driven by endogenous clocks, that exist in the brain and peripheral tissues and which are synchronized to external environmental cycles such as day and night (1). They regulate numerous biological functions in the human body, including sleep and wakefulness, body temperature, blood pressure, hormone production, digestive secretion and immune activity (2).

The molecular mechanisms of 24 h timekeeping and circadian rhythm generation in the central clock of the body are based on interactive positive and negative transcriptional-translational feedback loops generated by circadian clock genes (3). Up to 10% of the genome of cells is expressed, at the RNA level, in a circadian-coordinated manner, with each tissue exhibiting unique tissue-specific expression profiles (4). The circadian clock controls cellular processes, including proliferation, apoptosis, DNA repair, metabolism, detoxification and the DNA damage response (5). Alterations in clock genes may disrupt this rhythmic control, resulting in abnormalities in cell proliferation, apoptosis, DNA damage response and metabolism, which may subsequently contribute to coronary heart attacks, depression and tumor promotion (4,6). Therefore, circadian clock regulation is a critical component of disease pathogenesis.

Cancer may be a circadian associated disorder, as the extensive evidence gathered in mice and human studies, has suggested. Earlier investigations have revealed that the cycle of cell division is under circadian control, that the rate of tumor growth demonstrates a daily circadian rhythm and shift work or jet lag may be contributing factors to the increased incidence of cancer and mortality rate in humans (7,8).

At the molecular level, the circadian clock is composed of the products of at least eight core genes (Clock, Ck1ε, Cry1, Cry2, Per1, Per2, Per3 and Bmal1), which are organized in a transcriptional-translational regulatory network (9,10). Molecular-genetic analysis studies in vertebrates have provided evidence that consistent alterations in gene expression critically controls the expression patterns of further output genes (clock-controlled genes), such as Nocturnin (*CCRN4L/NOC*), albumin D-box binding protein (DBP), RORα and REV-ERBα (11,12).

Lung cancer is the leading cause of cancer-related mortality worldwide due to its high incidence, malignant behavior and an lack of effective treatments that are able to improve disease prognosis and survival rate (13). It is, therefore, a necessity to unravel and better understand the

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Key words: *CCRN4L*, *CLOCK*, *PER3*, single-nucleotide polymorphism, NSCLC

Table 1. Position of all SNPs used in this study.

SNP	Chromosome	Chromosome position	Gene	Gene location	Ancestral allele
rs938836[a]	4	139939653	*CCRN4L*	Intron 1-2	G
rs17050680[a]	4	139946648	*CCRN4L*	Intron 1-2	A
rs3805213[a]	4	139965724	*CCRN4L*	Intron 2-3	G
rs6855837	4	56319244	*CLOCK*	Exon 14	G
rs228727	1	7847836	*PER3*	Intron 3-4	A
rs228644	1	7866083	*PER3*	Intron 9-10	G
rs228729[a]	1	7845695	*PER3*	Intron 2-3	G
rs707467	1	7861684	*PER3*	Intron 7-8	T
rs10462020	1	7880683	*PER3*	Exon 15	T

[a]tag SNP. SNP, single nucleotide polymorphism; *PER3*, period 3.

molecular mechanisms associated with cancer development and progression (14).

In the present study, we analyzed polymorphisms in *CLOCK*, period 3 (*PER3)* and *CCRN4L* genes and investigated, in a case-control study, their association with non-small-cell-lung cancer (NSCLC) in Brazilian patients.

Materials and methods

Subjects. The study's cohort consisted of 78 patients with primary diagnosis of NSCLC, all eligible for surgery and with no previous history of chemotherapy or radiotherapy. The age range was 25-82 years (mean, 60.1±12.6; median, 62 years). Patients were recruited from a reference center of thoracic surgery from the Hospital Julia Kubitscheck (Minas Gerais, Brazil). Local Ethics Committee approval was obtained and all participants signed a written informed consent form. The study protocol was also approved by the Ethics Committee of the Universidade Federal de Minas Gerais (Minas Gerais, Brazil; ETIC 473-05). As a control group for CCRN4L, CLOCK and PER3 genotyping, we studied 74 healthy individuals with no cancer history and all >55 years old.

Polymorphism genotyping. Genomic DNA was isolated from lung tissue samples according to a proteinase K-based protocol (15). Peripheral blood samples from the control group were collected in vacuum tubes and genomic DNA was isolated using the high salt method (16). HapMap database (www.hapmap.org) as well as previous published literature were used for selecting single-nucleotide polymorphisms (SNPs) covering *CCRN4L*, *CLOCK* and *PER3* genes; 20 ng of DNA of each patient was used for TaqMan SNP genotyping assays (*rs938836*, *rs17050680*, *rs3805213*, *rs6855837*, *rs228727*, *rs228644*, *rs228729*, *rs707467*, *rs10462020*) according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA; Table I). Genotyping was performed by Real-Time PCR (RT-PCR) using allelic discrimination in the 7500 RT-PCR System (Applied Biosystems). PCR parameters involved an initial denaturation at 95˚C for 10 min followed by 50 cycles at 95˚C for 15 sec and 60˚C for 1 min. Each reaction contained 5.0 μl of mix, 0.1 μl of probe, 3.9 μl of deionized water and 60 ng of DNA. We retyped at least 10% of the samples for quality control.

Table II. Patient characteristics.

Variable	no.	%
Age		
Median	60.1	
Range	25-82	
Sex		
Male	48	61.5
Female	30	38.5
Histological subtype		
ADC	39	50
SCC	25	32
Others	14	18
Smoking history		
Smokers	62	79.5
Never smokers	16	20.5

ADC, adenocarcinoma; SCC, squamous cell carcinoma.

Statistical analysis. Allele and genotype frequencies were compared between case and control groups with the χ^2 test using the UNPHASED software program (v.3.0.13, https://sites.google.com/site/fdudbridge/software/unphased-3-1). HAPLOVIEW 4.1 software (Broad Institute, Cambridge, MA, USA) was used to evaluate pairwise linkage disequilibrium (LD) matrices between each tag SNP to examine the LD block structure and Hardy-Weinberg equilibrium (HWE; significance cutoff, 0.05). FAMHAP software (famhap.19β, http://famhap.meb.uni-bonn.de/; Germany) was used for haplotype association analysis. The odds ratios (ORs) and 1,000 permutations were performed using UNPHASED. We performed 1,000 permutations in each test to estimate the global significance of the results and to validate the expectation-maximization values. P<0.05 was considered to indicate a statistically significant result.

Table III. Genotype and allele frequencies of SNPs in lung cancer cases and control subjects.

		Patients (n=78)		Controls (n=74)				
Gene	Polymorphism	no.	%	no.	%	P-value[a]	Odds-R	95% CI
CCRN4L	*rs938836/190+2328G>A*							
	GA	57	73	60	81		1	1
	AA	21	27	14	19	0.23	1.58	0.73-3.40
	G	57	36.5	60	40.5		1	1
	A	99	63.5	88	59.5	0.47	1.18	0.74-1.88
CCRN4L	*rs17050680/190+9323A>G*							
	GG	6	7.6	5	7		1	1
	GA	22	27.8	25	34.7	0.66	0.73	0.19-2.73
	AA	51	64.6	42	58.3		1.01	0.28-3.55
	G	34	21.5	35	24.3		1	1
	A	124	78.5	109	75.7	0.56	1.17	0.68-2.00
CCRN4L	*rs3805213/461-69C>T*							
	TT	8	10	6	7.7		1	1
	TC	38	47.5	19	24.3	0.004 (0.014)	1.5	0.45-4.94
	CC	34	42.5	53	68		0.48	0.15-1.50
	T	54	33.7	31	19.8		1	1
	C	106	66.3	125	80.2	0.005 (0.02)	0.48	0.29-0-81
CLOCK	*rs6855837*							
	TT	2	2.5	1	1.4		1	
	TG	6	7.7	3	4	0.53	1	0.06-5.99
	GG	70	89.8	70	94.6		0.5	0.04-5.64
	T	10	6.4	5	3.4		1	
	G	146	93.6	143	96.6	0.21	0.51	0.17-1.53
PER3	*rs228727*							
	TT	16	21	12	14.8		1	
	TC	33	43.4	36	44.4	0.56	0.68	0.28-1.66
	CC	27	35.6	33	40.8		0.61	0.24-1.51
	T	65	42.7	60	37		1	
	C	87	57.3	102	63	0.30	0.78	0.50-1.23
PER3	*rs228644*							
	GG	25	31.2	36	44		1	
	GA	37	46.2	33	40.2	0.22	1.61	0.80-3.2
	AA	18	22.6	13	15.8		1.99	0.82-4.79
	G	87	54.4	105	64		1	
	A	73	45.6	59	36	0.07	1.43	0.95-2.33
PER3	*rs228729*							
	TT	17	21	9	11		1	
	TC	39	48.1	31	37.8	0.02 (0.010)	0.66	0.26-1.69
	CC	25	30.9	42	51.2		0.31	0.12-0.81
	T	73	45	49	29.9		1	
	C	89	55	115	70.1	0.004 (0.008)	0.51	0.32-0.81
PER3	*rs707467*							
	CC	4	5.3	4	5.2		1	
	CA	20	26.7	20	25.6	0.98	1	0.21-4.56
	AA	51	68	54	69.2		0.94	0.22-3.97
	C	28	18.7	28	18		1	
	A	122	81.3	128	82	0.87	0.95	0.53-1.70
PER3	*rs10462020*							
	TT	62	79.5	56	71.8		1	
	TG	15	19.2	19	24.4	0.40	0.71	0.33-1.53
	GG	1	1.3	3	3.8		0.30	0.03-2.97
	T	139	89.1	131	84		1	
	G	17	10.9	25	16	0.18	0.64	0.33-1.24

[a]Adjusted P-value from a 1,000 permutation test. CI, confidence interval; *PER3*, period 3.

Table IV. *PER3* haplotype frequency in lung cancer patients and controls.

Haplotype ID	rs228729	rs228727	rs707467	rs228644	rs10462020	Case freq	Control freq	Freq	P-value[a]
A	C	C	A	A	T	0.075	0.092	0.083	-
B	C	C	A	G	G	0.073	0.156	0.114	-
C	C	C	A	G	T	0.022	0.128	0.074	-
D	C	T	A	G	T	0.168	0.142	0.151	-
E	C	T	C	G	T	0.189	0.173	0.181	-
F	T	C	A	A	T	0.380	0.268	0.328	-
G	T	T	A	G	T	0.062	0.030	0.046	0.015 (0.020)[a]

[a]Adjusted P value. Freq, frequency; PER3, period 3.

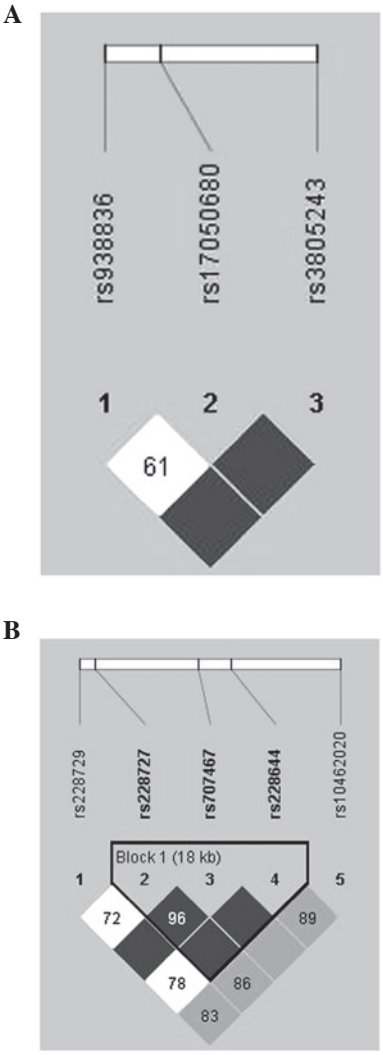


Figure 1. LD block structure in the *CCRN4L* and *PER3* genes. (A) The upper panel shows the location of polymorphisms (*rs938836*, *rs17050680* and *rs3805243*) in *CCRN4L*. The lower panel shows the output of HAPLOVIEW (V.4.1). Black squares indicate statistically significant LD between the pair of SNPs as measured by the D_ statistic. Darker colors indicate higher values of D_, up to a maximum of 1 of LD. (B) The upper panel shows the location of polymorphisms (*rs228727*, *rs228644*, *rs228729*, *rs707467*, *rs10462020*) in the *PER3* gene. The lower panel shows the LD plot, each square represents a pairwise LD relationship between the five SNPs and the block generated for the SNPs *rs228727*, *rs707467*, *rs228644* is marked. The block generated (block 1) under confidence interval algorithm of HAPLOVIEW is marked. LD, linkage disequilibrium; PER3, period 3.

Results

Patient characteristics (Table II). There were 30 female and 48 male participants with a median age of 60.1 years. Of the 78 tumors analyzed in this study, 25 (32%) were diagnosed as squamous cell carcinoma (SCC), 39 (50%) as adenocarcinoma (ADC) and 14 (18%) as other subtypes of NSCLC. Sixty-two patients were former or current-smokers and sixteen were never-smokers. The control group had 74 individuals, 40 female and 34 male with a median age of 75.4±7.96 years (range, 65-96 years).

Allele and genotype frequency of all SNPs (Table III). All samples were in HWE for eight SNPs, except for *rs938836* (*CCRN4L*) and *rs6855837* (*CLOCK*), 2.096×10^{-13} and 0.007, respectively. One *CCRN4L* marker (tag SNP *rs3805213*) showed a significant association with NSCLC to allele and genotype frequency (P=0.005 and P=0.004, respectively). For these tag SNPs, the T/C heterozygosity showed a significant association with increased risk for logarithm of the odds (LOD; 47.5%) correlated to comparison group (24.3%; P=0.004, OR =1.5 (0.45-4.94), χ^2=10.9, df=2; Table III). Allelic analysis of tag SNP *rs3805213* demonstrated a risk higher between allele T and LOD (33.7%) related to comparison group (19.8%; P=0.005, χ^2=7.81, df=1). Following the permutation tests, the values remained significant (Table III).

Another tag SNP tested from the *PER3* gene (*rs228729*) also showed a significant association with the disease to allele and genotype frequency (P=0.004 and P=0.02, respectively; Table III). In the control group, analyses of *rs228729* in T/T homozygous individuals showed a higher frequency (21%) compared with patients (11%; P=0.02, OR=1, χ^2=7.77, df=2; Table III). Allelic analysis for this PER SNP showed a higher risk between allele T and LOD (45%) related to the comparison group (29.9%; P=0.004, χ^2=8.06, df=1). Following the permutation tests, the values remained significant (Table III). No association was detected for the other markers of *PER3*, *CLOCK* and *CCRN4L* (*rs938836*, *rs17050680*, *rs6855837*, *rs228727*, *rs228644*, *rs707467* and *rs10462020*).

Haplotype analysis. The analysis revealed a strong association with rs228729 (PER3) allele T and the increased risk to LOD. The T-T-A-G-T haplotype (*rs228729*, *rs228727*, *rs707467*, *rs228644*, *rs10462020*, respectively) occurred more in patients

(62%) than in the comparison group (3%; P=0.015; Table IV). These results reinforce the presence of the allele T (*rs228729*) increased risk for LOD. To avoid genotyping errors (quality control) at least 10% of the samples were retyped.

Pairwise LD (Fig. 1). The analysis of *CCRN4L* demonstrated a significant LD between *rs17050680* and *rs3805213,* and also among *rs938836* and *rs3805213* (D'=1.0). Similarly, there was a moderate LD in *CCRN4L rs9302648* and *rs17050680* polymorphisms (D'=0.61). The Fig. 1B shows one LD block comprising only of three SNPs of the *PER3* gene tested (*rs228727*, *rs707467* and *rs228644*; D'=1.0). The *rs228729* and *rs10462020* were not included in the block.

Discussion

The present study was based on case-control association evaluating the clock-controlled gene *CCRN4L* and clock genes, including *CLOCK* and *PER3* with NSCLC. We analyzed nine SNPs (*rs938836*, *rs17050680*, *rs3805213*, *rs6855837*, *rs228727*, *rs228644*, *rs228729*, *rs707467*, *rs10462020*) in these genes in 78 Brazilian patients with NSCLC.

PER3 is a circadian regulation protein that affects the cell cycle, growth and differentiation. Previous studies have also demonstrated an association between the *PER3* gene and tumorigenesis. Wang *et al* determined the association between PER3 expression and colon cancer incidence/progression, as compared with normal tissue. As a 2.8-fold decrease in PER3 mRNA levels in colon cancerous tissue was observed (17). Climent *et al* (18) demonstrated that mice deficient in PER3 had increased susceptibility to breast cancer induced by carcinogen treatment or by overexpression of Erbb2. Zienolddiny *et al* (19) revealed that SNPs in the *PER3* gene were associated with decreased breast cancer risk and an association with prostate and colorectal cancer risk has also been identified (20,21). In our analysis, we identified a statistically significant association for SNPs *rs228729* [P=0.02, OR=0.51 (0.32-0.81), χ^2=7.8, df=2] in the *PER3* gene with NSCLC suggesting that PER3 may be correlated with the development of lung cancer. Furthermore, one LD block comprising of three SNPs of the *PER3* gene (*rs228727*, *rs707467* and *rs228644*) was observed and *rs228729* demonstrated a significant association with the disease, however was not located in this block.

Zieker *et al* (22) conducted a comparative microarray analysis to investigate the relative mRNA expression of clock-controlled genes (including Noc, hPER2, hCRY1, SMAD5) throughout a 24 h period in cell samples obtained from oral mucosa. The detected circadian expression profile of *CCRN4L* constituted a protective feature during daytime upregulation of the gene. The authors also suggested that this gene may be involved in specific tumor suppressor functions in human oral mucosa. In the present study, we identified an association between SNP *rs3805213* in CCRN4L and NSCLC [P=0.004, OR=1.5 (0.45-4.94), χ^2=10.9, df=2], suggesting a possible correlation between this protein and NSCLC. The *rs3805213* of CCRN4L gene did not form LD block with others SNPs despite demonstrating an association with disease.

Our results did not reveal a correlation between the rs6855837 in CLOCK gene and NSCLC. Huang *et al* (23) suggested that CLOCK gene expression seems to be altered in certain human cancer types compared with healthy corresponding tissues, possibly as a result of other events, such as promoter methylation. Therefore, further studies are required to evaluate the involvement of CLOCK genes in the tumorigenesis of NSCLC.

Our study provides preliminary evidence that polymorphisms in the *CCRN4L* and *PER3* genes may represent a risk factor in the occurrence of NSCLC in Brazilian patients. Further large scale genetic studies are needed to confirm these observations, since the sample size in the present study was relatively small. Furthermore, functional studies may clarify the role of other variants and haplotypes in patient susceptibility to NSCLC.

Acknowledgements

This study was supported by grants from CNPq, FAPEMIG and CAPES (Brazil).

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