

Testing the Circadian Gene Hypothesis in Prostate Cancer: A Population-Based Case-Control Study

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Abstract

Circadian genes are responsible for maintaining the ancient adaptation of a 24-hour circadian rhythm and influence a variety of cancer-related biological pathways, including the regulation of sex hormone levels. However, few studies have been undertaken to investigate the role of circadian genes in the development of prostate cancer, the most common cancer type among men (excluding nonmelanoma skin cancer). The current genetic association study tested the circadian gene hypothesis in relation to prostate cancer by genotyping a total of 41 tagging and amino acid-altering single nucleotide polymorphisms (SNP) in 10 circadian-related genes in a population-based case-control study of Caucasian men ($n = 1,308$ cases and 1,266 controls). Our results showed that at least one SNP in nine core circadian genes (rs885747 and rs2289591 in *PER1*; rs7602358 in *PER2*; rs1012477 in *PER3*; rs1534891 in *CSNK1E*; rs12315175 in *CRY1*; rs2292912 in *CRY2*; rs7950226 in *ARNTL*; rs11133373 in *CLOCK*; and rs1369481, rs895521, and rs17024926 in *NPAS2*) was significantly associated with susceptibility to prostate cancer (either overall risk or risk of aggressive disease), and the risk estimate for four SNPs in three genes (rs885747 and rs2289591 in *PER1*, rs1012477 in *PER3*, and rs11133373 in *CLOCK*) varied by disease aggressiveness. Further analyses of haplotypes were consistent with these genotyping results. Findings from this candidate gene association study support the hypothesis of a link between genetic variants in circadian genes and prostate cancer risk, warranting further confirmation and mechanistic investigation of circadian biomarkers in prostate tumorigenesis. [Cancer Res 2009;69(24):9315–22]

Introduction

The universal 24-hour oscillation, which arises from an ancient adaptation to the rotation of the earth, is one of the fundamental components of our biological world and has a profound impact on biochemical, physiologic, and behavioral processes in almost all living organisms. Disrupted circadian rhythm caused by “light-at-night” was hypothesized over a decade ago to explain, in part, the

high risk of human breast and prostate cancers in industrialized societies (1, 2). An expert panel assembled by the IARC recently concluded that “shift-work that involves circadian disruption is probably carcinogenic to humans” based on “limited evidence in humans” and “sufficient evidence in experimental animals for the carcinogenicity of light during the daily dark period” (3).

The role of circadian disruption in prostate tumorigenesis has been supported by observations of pilots, flight attendants, and shift workers who are more likely to have disrupted circadian cycles due to abnormal work hours. Studies from pilot cohorts have shown an excess risk of prostate cancer (4–6) that increases with increasing number of flight hours (6). Similarly, male rotating-shift workers have a higher risk of prostate cancer compared with day- or night-only workers (7, 8). Furthermore, studies of the blind who have a reduced or abolished sensitivity to environmental light exposures have observed a reduced risk of prostate cancer (9, 10), which provides additional evidence suggesting a role for circadian disruption in the etiology of prostate cancer.

Circadian rhythms are controlled and maintained by several core circadian genes via transcription-translation feedback loops that include positive activators, e.g., *CLOCK*, *neuronal PAS domain protein 2* (*NPAS2*), and *aryl hydrocarbon receptor nuclear translocator-like* (*ARNTL*), negative effectors, e.g., two *cryptochrome* (*CRY1* and *CRY2*) and three *period* (*PER1*, *PER2*, and *PER3*) genes, and modulators [*casein kinase 1*, ϵ (*CSNK1E*); see Kondratov and colleagues (11) for an extensive discussion on the molecular organization of the circadian system]. Emerging data have shown that circadian genes are also involved in regulating cancer-related biological pathways such as cell proliferation and apoptosis by controlling the expression of tumor suppressor genes, cell cycle genes, and genes encoding caspases and transcription factors (reviewed in ref. 12).

Given the potential role of circadian genes in tumorigenesis, it has been hypothesized that genetic variations in these genes could be associated with an individual's susceptibility to cancer, especially hormone-related cancers such as prostate cancer (13). In the current study, we tested the circadian gene hypothesis by systematically genotyping genetic variants in 10 circadian genes in subjects from two population-based case-control studies of prostate cancer.

Subjects, Materials, and Methods

Study population. The study population consists of participants from two population-based case-control studies in residents of King County, Washington (study I and study II), which have been described previously (14, 15). Briefly, subjects diagnosed with histologically confirmed prostate cancer were ascertained from the Seattle-Puget Sound Surveillance Epidemiology and End Results cancer registry. In study I, cases were diagnosed

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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between January 1, 1993, and December 31, 1996 and were 40 to 64 y of age at diagnosis. In study II, cases were diagnosed between January 1, 2002, and December 31, 2005 and were 35 to 74 y of age at diagnosis. Overall, 2,244 eligible prostate cancer patients were identified, 1,754 (78.2%) were interviewed, and blood samples yielding sufficient DNA for genotyping were drawn from 1,457 (83.1%) cases who completed a study interview. Demographic and clinical characteristics did not differ between cases that did and did not provide a DNA sample. A comparison group of controls without a self-reported physician's diagnosis of prostate cancer was identified using one-step random digit telephone dialing. Controls were frequency matched to cases by 5-y age groups and recruited evenly throughout each ascertainment period for cases. A total of 2,448 men were identified who met the eligibility criteria, 1,645 (67.2%) were interviewed, and blood samples were drawn and DNA were prepared from 1,352 men (82.2%) using standard protocols. Demographic characteristics did not differ between controls that did and did not provide a DNA sample. For the current analyses, only Caucasian participants with DNA were included (1,308 cases and 1,266 controls).

Data collection. Subjects in both studies completed in-person interviews conducted by trained male interviewers using similar standardized questionnaires. Questions pertained to the time before reference date, which was the date of prostate cancer diagnosis for cases and for controls, was a preassigned random date that approximated the distribution of cases' diagnosis dates. The questionnaire collected information on social and demographic factors, family structure and cancer history, and medical history including prostate cancer screening history. Clinical information on cases, including Gleason score, stage of disease, and serum prostate-specific antigen (PSA) level at diagnosis, was obtained from the cancer registry. All study procedures were reviewed and approved by the Fred Hutchinson Cancer Research Center institutional review board and written informed consent was obtained from all study subjects before participation.

Single nucleotide polymorphism selection and genotyping. Single nucleotide polymorphisms (SNP) were identified using the Tagger algorithm (16), which is implemented in the Haploview interface (17) of HapMap's genome browser, release 22. SNP sets were identified to capture all of the variation found within each circadian gene with $r^2 \geq 0.8$. In addition to the tagging SNPs, seven SNPs that cause amino acid changes were included in the genotyping panel. We also examined data from the National Cancer Institute (NCI) Cancer Genetic Markers of Susceptibility (CGEMS) project⁵ (18), and of the eight SNPs found to be significantly associated with prostate cancer risk in CGEMS ($P_{\text{global}} < 0.05$), six were included in the current study. Due to SNP compatibility limitations associated with the genotyping assay, two were unable to be included in the current study. Genotyping for all SNPs was performed at the National Human Genome Research Institute (Dr. Ostrander's laboratory) using the SNPlex Genotyping System (Applied Biosystems, Inc.) according to the manufacturer's protocol. The details of this assay have been described previously (19, 20). Briefly, the genotyping system uses multiplex PCR technology and capillary electrophoresis to determine the genotypes of up to 48 different SNPs per SNPlex array. The GeneMapper software package (Applied Biosystems) was used to assign genotypes for each SNP. Replicate samples ($n = 141$) were interspersed throughout all genotyping batches, and the concordance levels for blind duplicate samples were >99% for all SNPs assayed. All genotyping scores, including quality control data, were rechecked by different laboratory personnel, and the accuracy of each assay was confirmed.

Statistical analysis. All statistical analyses were performed using SAS version 9.1 (SAS Institute), unless otherwise noted. A χ^2 test was used to test for departures from Hardy-Weinberg equilibrium (HWE) for each SNP in the control population. SNPs not in HWE ($P < 0.01$) were excluded from subsequent analyses. A goodness-of-fit χ^2 test was used to determine whether the data were best described using a recessive, dominant, or codominant model. Because no SNPs were significant under a recessive model only, and using the homozygous common plus heterozygous genotypes

Table 1. Distribution of selected characteristics of prostate cancer cases and population-based controls

	Cases (<i>n</i> = 1,308)		Controls (<i>n</i> = 1,266)		<i>P</i> *
	<i>n</i>	%	<i>n</i>	%	
Age at reference date (y)					
35–49	102	7.8	107	8.4	
50–54	188	14.4	178	14.1	
55–59	325	24.8	343	27.1	
60–64	395	30.2	334	26.4	
65–69	153	11.7	160	12.6	
70–74	145	11.1	144	11.4	
Body mass index					
<25.0	429	32.8	389	30.7	
25.0–29.9	637	48.7	618	48.8	
≥30	242	18.5	259	20.5	0.34
Smoking history					
Never	522	39.9	541	42.7	
Former	631	48.2	561	44.3	
Current	155	11.9	164	13.0	0.13
Family history of prostate cancer [†]					
No	1025	78.4	1124	88.8	
Yes	283	21.6	142	11.2	<0.0001
PSA screening history					
Never	135	10.3	167	13.2	
DRE only	223	17.1	483	38.1	
PSA	950	72.6	616	48.7	<0.0001
PSA level* (ng/mL)					
0–3.9	178	13.6	1175	92.8	
4–9.9	722	55.2	74	5.8	
10–19.9	190	14.5	15	1.2	
≥20	118	9.0	2	0.2	
Missing	100	7.7	0	0.0	<0.0001
Gleason score					
2–4	67	5.1			
5–6	680	52.0			
7 = 3+4	355	27.1			
7 = 4+3	76	5.8			
8–10	126	9.6			
Missing	4	0.3			
Stage of disease					
Local	1022	78.1			
Regional	254	19.4			
Distant	32	2.5			
Aggressiveness [§]					
Less	873	66.7			
More	435	33.3			

Abbreviation: DRE, digital rectal examination.

* χ^2 test.

[†]Family history of cancer in a first-degree relative.

[‡]Serum PSA at diagnosis for cases and at interview for controls.

[§]Less aggressive: localized stage, Gleason score of ≤7 (3+4), and a diagnostic PSA level of <20 ng/mL; More aggressive: regional or distant stage, or Gleason score of ≥7 (4+3) or a diagnostic PSA level of ≥20 ng/mL.

as the referent category did not dramatically alter the risk estimates for any SNP, all genotyping results are presented under both a codominant and dominant model. Adjusted odds ratios (OR) with 95% confidence intervals (CI) and two-sided *P* values were calculated by unconditional logistic regression to estimate the relative risk associated with each genotype.

⁵ <http://cgems.cancer.gov/>

Adjustments were made to control for age and family history of prostate cancer in first-degree relatives. In addition, an indicator variable for study (I or II) was tested for confounding effects in the overall model, and no evidence of confounding was detected. Further, an interaction term for study and the combined variant genotypes for all significant SNPs in the overall population was tested, and no strong effect modification was identified ($P > 0.01$). As such, the study covariate was not included in the final models. In addition to the overall associations, separate risk estimates were obtained for less aggressive and more aggressive tumors using multinomial logistic regression. Tests for interaction were also conducted using a Wald χ^2 comparing the parameter estimates obtained for each genotype in less

aggressive versus more aggressive cases. A binomial test was conducted to determine the probability of obtaining as many or more significant SNPs by chance alone, as were actually observed in our analysis.

Haplotype estimates were calculated by the PHASE program (21) using the genotyping information from all markers in each gene. ORs and 95% CIs for each haplotype were determined by unconditional multivariate logistic regression, using all other haplotypes as the referent category. The same covariates that were included in the main effects model were also used in the haplotype model. Separate risk estimates were not obtained for haplotypes with a frequency <1%, but these individuals remained in the analysis as members of the reference group.

Table 2. List of genetic variations in circadian genes included in the analysis

dbSNP ID	Gene	SNP type*	Allele (major/minor)	MAF	P for HWE	Location
rs2278749	<i>ARNTL</i>	Tagging	G/A	0.184	0.320	11p15
rs2290035	<i>ARNTL</i>	Tagging	A/T	0.457	0.227	11p15
rs3816360	<i>ARNTL</i>	Tagging	G/A	0.353	0.706	11p15
rs4757144	<i>ARNTL</i>	Tagging	A/G	0.409	0.901	11p15
rs6486122	<i>ARNTL</i>	Tagging	T/C	0.335	0.810	11p15
rs7950226	<i>ARNTL</i>	Tagging	G/A	0.457	0.356	11p15
rs11133373	<i>CLOCK</i>	Tagging	C/G	0.341	0.162	4q12
rs11133376	<i>CLOCK</i>	Tagging	T/C	0.462	0.000	4q12
rs1399953	<i>CLOCK</i>	Tagging	G/A	0.266	0.850	4q12
rs17776421	<i>CLOCK</i>	Tagging	G/A	0.383	0.776	4q12
rs10778528	<i>CRY1</i>	Tagging	T/G	0.430	0.650	12q23-q24.1
rs10778534	<i>CRY1</i>	Tagging	T/C	0.362	0.063	12q23-q24.1
rs12315175	<i>CRY1</i>	Tagging	T/C	0.194	0.954	12q23-q24.1
rs7297614	<i>CRY1</i>	CGEMS	T/C	0.392	0.759	12q23-q24.1
rs11605924	<i>CRY2</i>	Tagging	A/C	0.472	0.035	11p11.2
rs12281674	<i>CRY2</i>	Tagging	A/G	0.030	0.893	11p11.2
rs2292910	<i>CRY2</i>	Tagging	C/A	0.316	0.245	11p11.2
rs2292912	<i>CRY2</i>	Tagging	C/G	0.217	0.619	11p11.2
rs135757	<i>CSNK1E</i>	CGEMS	G/A	0.245	0.160	22q13.1
rs1534891	<i>CSNK1E</i>	CGEMS	C/T	0.124	0.001	22q13.1
rs1997644	<i>CSNK1E</i>	Tagging	A/G	0.467	0.039	22q13.1
rs5757037	<i>CSNK1E</i>	Tagging	G/A	0.369	0.039	22q13.1
rs6001093	<i>CSNK1E</i>	CGEMS	T/C	0.184	0.172	22q13.1
rs1369481	<i>NPAS2</i>	Tagging	G/A	0.300	0.315	2q11.2
rs1562313	<i>NPAS2</i>	CGEMS	G/A	0.208	0.782	2q11.2
rs17024926	<i>NPAS2</i>	Tagging	T/C	0.329	0.504	2q11.2
rs2305160	<i>NPAS2</i>	AA change	C/T	0.340	0.418	2q11.2
rs4074920	<i>NPAS2</i>	Tagging	A/G	0.245	0.041	2q11.2
rs7565018	<i>NPAS2</i>	Tagging	A/G	0.236	0.725	2q11.2
rs895521	<i>NPAS2</i>	CGEMS	G/A	0.240	0.258	2q11.2
rs965519	<i>NPAS2</i>	Tagging	A/G	0.153	0.657	2q11.2
rs2253820	<i>PER1</i>	Tagging	G/A	0.154	0.881	17p13.1-p12
rs2289591	<i>PER1</i>	Tagging	G/T	0.243	0.495	17p13.1-p12
rs885747	<i>PER1</i>	Tagging	C/G	0.456	0.983	17p13.1-p12
rs11695472	<i>PER2</i>	Tagging	A/C	0.269	0.072	2q37.3
rs7602358	<i>PER2</i>	Tagging	T/G	0.238	0.221	2q37.3
rs1012477	<i>PER3</i>	Tagging	C/G	0.143	0.179	1p36.23
rs10462020	<i>PER3</i>	AA change	T/G	0.192	0.120	1p36.23
rs11121023	<i>PER3</i>	Tagging	G/A	0.219	0.584	1p36.23
rs228697	<i>PER3</i>	AA change	C/G	0.099	0.781	1p36.23
rs2640909	<i>PER3</i>	AA change	T/C	0.295	0.327	1p36.23
rs2291739	<i>TIMELESS</i>	AA change	T/C	0.411	0.002	12q12-q13
rs774027	<i>TIMELESS</i>	AA change	T/A	0.453	0.456	12q12-q13
rs774047	<i>TIMELESS</i>	AA change	C/T	0.451	0.511	12q12-q13

Abbreviation: MAF, minor allele frequency.

*AA, amino acid.

Table 3. Significant associations between genetic variants in circadian genes and prostate cancer risk

Genotype	All		Less aggressive		More aggressive		<i>P</i> *	
	Controls	Cases	Cases	Cases	Cases	Cases		
	<i>n</i>	<i>n</i>	OR [†] (95% CI)	OR [†] (95% CI)	OR [†] (95% CI)	OR [†] (95% CI)		
<i>PER1</i> , rs885747, tag								
C/C	367	375	Reference	239	Reference	136	Reference	
C/G	615	639	1.00 (0.83–1.20)	432	1.05 (0.86–1.30)	207	0.90 (0.70–1.16)	0.24
G/G	257	238	0.90 (0.72–1.14)	170	1.01 (0.78–1.31)	68	0.71 (0.51–0.99)	0.05
C/G+G/G	872	877	0.97 (0.82–1.15)	602	1.04 (0.86–1.27)	275	0.84 (0.66–1.07)	0.10
<i>PER1</i> , rs2289591, tag								
G/G	702	696	Reference	487	Reference	209	Reference	
G/T	461	459	1.00 (0.84–1.18)	296	0.91 (0.75–1.10)	163	1.19 (0.94–1.51)	0.04
T/T	68	79	1.24 (0.88–1.75)	47	1.05 (0.71–1.55)	32	1.70 (1.08–2.66)	0.05
G/T+T/T	529	538	1.03 (0.87–1.20)	343	0.93 (0.78–1.11)	195	1.25 (1.00–1.57)	0.02
<i>PER2</i> , rs7602358, tag								
T/T	706	669	Reference	436	Reference	233	Reference	
T/G	421	464	1.15 (0.97–1.36)	327	1.24 (1.03–1.50)	137	0.98 (0.77–1.25)	0.07
G/G	76	74	1.01 (0.72–1.42)	45	0.94 (0.64–1.39)	29	1.13 (0.72–1.79)	0.46
T/G+G/G	497	538	1.13 (0.96–1.33)	372	1.19 (1.00–1.43)	166	1.00 (0.80–1.26)	0.16
<i>PER3</i> , rs1012477, tag								
C/C	914	899	Reference	584	Reference	315	Reference	
C/G	290	331	1.15 (0.96–1.39)	238	1.28 (1.04–1.56)	93	0.92 (0.71–1.21)	0.02
G/G	31	27	0.85 (0.50–1.45)	20	0.97 (0.54–1.73)	7	0.63 (0.27–1.45)	0.33
C/G+G/G	321	358	1.12 (0.94–1.34)	258	1.25 (1.03–1.52)	100	0.89 (0.69–1.16)	0.02
<i>CSNK1E</i> , rs1534891, cgems								
C/C	941	966	Reference	641	Reference	325	Reference	
C/T	292	266	0.88 (0.73–1.06)	183	0.91 (0.73–1.12)	83	0.82 (0.62–1.08)	0.50
T/T	8	23	2.65 (1.16–5.95)	18	3.09 (1.32–7.21)	5	1.70 (0.55–5.29)	0.25
C/T+T/T	300	289	0.93 (0.77–1.12)	201	0.97 (0.79–1.19)	88	0.85 (0.64–1.11)	0.35
<i>CRY1</i> , rs12315175, tag								
T/T	805	778	Reference	515	Reference	263	Reference	
T/C	387	406	1.09 (0.92–1.30)	279	1.14 (0.94–1.38)	127	1.01 (0.79–1.29)	0.38
C/C	47	63	1.47 (0.99–2.18)	44	1.55 (1.01–2.39)	19	1.30 (0.75–2.26)	0.53
T/C+C/C	434	469	1.13 (0.96–1.34)	323	1.18 (0.98–1.42)	146	1.04 (0.82–1.32)	0.33
<i>CRY2</i> , rs2292912, tag								
C/C	763	812	Reference	555	Reference	257	Reference	
C/G	414	392	0.88 (0.74–1.05)	252	0.83 (0.68–1.00)	140	1.00 (0.79–1.27)	0.14
G/G	61	49	0.79 (0.54–1.17)	34	0.81 (0.52–1.25)	15	0.76 (0.43–1.37)	0.86
C/G+G/G	475	441	0.87 (0.74–1.03)	286	0.82 (0.69–0.99)	155	0.97 (0.77–1.22)	0.19
<i>ARNTL</i> , rs7950226, tag								
G/G	367	317	Reference	213	Reference	104	Reference	
G/A	588	631	1.22 (1.01–1.48)	430	1.24 (1.01–1.54)	201	1.19 (0.90–1.56)	0.76
A/A	262	276	1.22 (0.97–1.53)	177	1.16 (0.90–1.51)	99	1.33 (0.97–1.83)	0.44
G/A+A/A	850	907	1.22 (1.02–1.46)	607	1.22 (1.00–1.49)	300	1.23 (0.95–1.59)	0.94
<i>CLOCK</i> , rs11133373, tag								
C/C	520	575	Reference	377	Reference	198	Reference	
C/G	571	533	0.85 (0.72–1.01)	353	0.86 (0.71–1.04)	180	0.82 (0.65–1.04)	0.71
G/G	131	134	0.91 (0.69–1.19)	102	1.06 (0.79–1.42)	32	0.62 (0.41–0.95)	0.02
C/G+G/G	702	667	0.86 (0.73–1.01)	455	0.90 (0.75–1.08)	212	0.79 (0.63–0.99)	0.26
<i>NPAS2</i> , rs1369481, tag								
G/G	600	677	Reference	456	Reference	221	Reference	
G/A	535	484	0.81 (0.68–0.96)	326	0.81 (0.67–0.98)	158	0.80 (0.63–1.02)	0.95
A/A	104	93	0.80 (0.59–1.09)	60	0.77 (0.55–1.09)	33	0.86 (0.57–1.32)	0.62
G/A+A/A	639	577	0.81 (0.69–0.95)	386	0.80 (0.67–0.96)	191	0.81 (0.65–1.02)	0.92
<i>NPAS2</i> , rs895521, cgems								
G/G	709	778	Reference	519	Reference	259	Reference	
G/A	466	423	0.85 (0.71–1.0)	291	0.87 (0.73–1.05)	132	0.79 (0.62–1.00)	0.42
A/A	64	50	0.71 (0.48–1.05)	32	0.68 (0.44–1.06)	18	0.77 (0.44–1.32)	0.70
G/A+A/A	530	473	0.83 (0.70–0.97)	323	0.85 (0.71–1.02)	150	0.79 (0.62–0.99)	0.52

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Table 3. Significant associations between genetic variants in circadian genes and prostate cancer risk (Cont'd)

Genotype	All		Less aggressive		More aggressive		P*	
	Controls	Cases	Cases	Cases	Cases	OR [†]		
	n	n	OR [†] (95% CI)	n	OR [†] (95% CI)	n		OR [†] (95% CI)
<i>NPAS2</i> , rs17024926, tag								
T/T	563	499	Reference	330	Reference	169	Reference	
T/C	536	594	1.26 (1.06–1.49)	394	1.26 (1.04–1.53)	200	1.25 (0.99–1.59)	0.95
C/C	139	153	1.23 (0.94–1.60)	111	1.34 (1.00–1.78)	42	1.01 (0.68–1.49)	0.17
T/C+C/C	675	747	1.25 (1.07–1.47)	505	1.28 (1.07–1.53)	242	1.20 (0.96–1.51)	0.62

*Test for homogeneity of ORs for less versus more aggressive disease groups.
[†]Adjusted for age and family history of prostate cancer in a first-degree relative.

Results

Compared with controls, prostate cancer cases reported a higher proportion of first-degree relatives with a history of prostate cancer, a greater frequency of PSA screening and they had higher serum PSA levels ($P < 0.001$; Table 1). Genotyping was completed for 44 SNPs in nine core circadian genes and one circadian-related gene, *TIMELESS*. A list of these SNPs, including the minor allele frequency detected in this study population and P values for HWE, is presented in Table 2. The minor allele frequency of each SNP was very similar to that found in the CEPH HapMap population,⁶ which such as this study, is population-based. Three SNPs (rs11133376 in *CLOCK*, rs1534891 in *CSNK1E*, and rs2291739 in *TIMELESS*) did not fit HWE ($P < 0.01$) and were thus excluded from further analysis.

Genotypes of circadian genes and prostate cancer risk. Of the remaining 41 SNPs, 17 were significantly associated with risk of prostate cancer (either overall risk or risk of more aggressive disease), whereas approximately six ($41 \times 0.05 \times 3$) would be expected by chance alone ($P = 0.0001$; Table 3). Specifically, assuming a dominant model three SNPs located in *NPAS2* (rs895521: OR, 0.83; 95% CI, 0.70–0.97; rs1369481: OR, 0.81; 95% CI, 0.69–0.95; and rs17024926: OR, 1.25; 95% CI, 1.07–1.47) and rs7950226 in *ARNTL* (OR, 1.22; 95% CI, 1.02–1.46) were significantly associated with prostate cancer risk, as was the homozygous rare genotype of rs1534891 in *CSNK1E* (OR, 2.65; 95% CI, 1.16–5.95). We also analyzed these SNPs for interaction with family history of prostate cancer in a first-degree relative. Only one SNP, rs17024926 in *NPAS2*, showed significant interaction ($P_{\text{interaction}} = 0.013$ for the group with any variant allele), with a significant association observed only among men with no family history (OR, 1.36; 95% CI, 1.15–1.63). In addition, because heterodimerization between the protein products of *ARNTL* and *NPAS2* is required for proper functioning, and each of these genes harbored at least one significant SNP, we tested for interactions among each of the significant variants in these genes (rs7950226 in *ARNTL* combined with rs1369481, rs895521, or rs17024926 in *NPAS2*). In each case, elevated relative risks were observed for individuals carrying the risk allele at both loci, but the interactions were not statistically significant.

In addition to the main effects model, a multinomial regression analysis was conducted to determine whether any of the circadian gene variants were associated with a composite variable indicating aggressive disease. For these analyses, controls were compared simultaneously to prostate cancer cases stratified into two groups: (a) less aggressive [localized stage, Gleason score of ≤ 7 (3+4), and a diagnostic PSA level of < 20 ng/mL] and (b) more aggressive [regional or distant stage, or Gleason score of ≥ 7 (4+3) or a diagnostic PSA level of ≥ 20 ng/mL]. These results showed that eight SNPs were significantly associated with risk of less aggressive prostate cancer, including five assuming a dominant model: rs1012477 in *PER3* (OR, 1.25; 95% CI, 1.03–1.52), rs2292912 in *CRY2* (OR, 0.82; 95% CI, 0.69–0.99), rs7950226 in *ARNTL* (OR, 1.22; 95% CI, 1.00–1.49), and SNPs rs17024926 and rs1369481 in *NPAS2* (OR, 1.28; 95% CI, 1.07–1.53; OR, 0.80; 95% CI, 0.67–0.96, respectively); two assuming a recessive model: rs1534891 in *CSNK1E* (OR, 3.09; 95% CI, 1.32–7.21) and rs12315175 in *CRY1* (OR, 1.55; 95% CI, 1.01–2.39); and, finally, the heterozygous genotype of rs7602358 in *PER2* (OR, 1.24; 95% CI, 1.03–1.50). Four SNPs were significantly associated with risk of more aggressive prostate cancer, including two assuming a dominant model: rs11133373 in *CLOCK* (OR, 0.79; 95% CI, 0.63–0.99), and rs895521 in *NPAS2* (OR, 0.79; 95% CI, 0.62–0.99); and the homozygous rare genotypes of two SNPs in *PER1* [rs885747 (OR, 0.71; 95% CI, 0.51–0.99) and rs2289591 (OR, 1.70; 95% CI, 1.08–2.66)]. In total, the effect of one or more genotypes in four SNPs varied significantly according to disease aggressiveness ($P_{\text{interaction}} < 0.05$), including two SNPs in *PER1* (rs2289591 and rs885747), one SNP in *PER3* (rs1012477), and one SNP in *CLOCK* (rs11133373). Of note, the effect of all five SNPs that were significantly associated with prostate cancer risk in the full population did not vary significantly by aggressiveness. Genotyping results, including prostate cancer risk estimates for each of the nonsignificant SNPs, are provided in Supplementary Table S1.

Haplotypes of circadian genes and prostate cancer risk. In addition to the single-SNP analyses, haplotypes were estimated using the genotyping data from all SNPs in each gene. The haplotype analysis identified seven haplotypes in two different genes that were significantly associated with prostate cancer risk in the full population (Table 4). These included four different haplotypes in *ARNTL*, with a combined frequency of 13.94% in the population, as well as three haplotypes in *NPAS2* with a combined frequency of 16.11%. The effect of one common haplotype in *PER1* (24.82%) varied significantly according to aggressiveness as it was

⁶ <http://www.hapmap.org/>

Table 4. Significant associations between haplotypes of circadian genes and prostate cancer risk and aggressiveness status

Haplotype	Frequency %	Controls <i>n</i>	All		Less aggressive		More aggressive		<i>P</i> [*]
			Cases	OR [†]	Cases	OR [†]	Cases	OR [†]	
			<i>n</i>	95% CI	<i>n</i>	95% CI	<i>n</i>	95% CI	
<i>ARNTL</i>									
GAGGTA	6.00%	124	176	1.42 (1.12–1.80)	117	1.40 (1.08–1.82)	59	1.46 (1.06–1.82)	0.81
GTGGTA	5.36%	115	153	1.32 (1.02–1.69)	105	1.36 (1.03–1.79)	48	1.23 (0.87–1.75)	0.59
ATAATA	1.38%	26	43	1.65 (1.01–2.71)	29	1.65 (0.96–2.83)	14	1.65 (0.85–3.18)	0.99
GTAGCA	1.20%	38	22	0.56 (0.33–0.96)	17	0.65 (0.36–1.17)	5	0.39 (0.15–0.99)	0.30
<i>NPAS2</i>									
GAGGTAAC	11.63%	259	322	1.25 (1.05–1.49)	216	1.25 (1.03–1.52)	106	1.26 (0.99–1.61)	0.93
AAAGTAAT	2.34%	71	46	0.64 (0.44–0.94)	27	0.56 (0.36–0.88)	19	0.80 (0.48–1.33)	0.25
GAGGCGGT	2.14%	67	40	0.58 (0.39–0.87)	26	0.57 (0.36–0.90)	14	0.62 (0.35–1.11)	0.78
<i>PER1</i>									
CGT	24.82%	599	623	1.05 (0.92–1.19)	394	0.96 (0.83–1.11)	229	1.23 (1.03–1.48)	0.01

*Test for homogeneity of ORs for less versus more aggressive disease groups.

†Adjusted for age and family history of prostate cancer in a first-degree relative.

significantly associated with prostate cancer risk in the more aggressive disease group (OR, 1.23; 95% CI, 1.03–1.48; $P_{\text{interaction}} < 0.05$) but not in the less aggressive disease group (OR, 0.96; 95% CI, 0.83–1.11).

Discussion

In this hypothesis-driven association study of 10 candidate circadian genes, three, *ARNTL*, *CSNK1E*, and *NPAS2*, were significantly associated with susceptibility to prostate cancer in the full population, and SNPs in an additional six genes, *CLOCK*, *CRY1*, *CRY2*, *PER1*, *PER2*, and *PER3*, were significantly associated with a subgroup composed of less or more aggressive cancers. These results support the hypothesis of a link between genetic variations in circadian genes and prostate cancer, and are consistent with previous genetic association findings from breast cancer (22), prostate cancer (23), and non-Hodgkin's lymphoma (NHL; ref. 24).

It is not unexpected that alterations in these genes might have a broad impact on cancer susceptibility because as many as 10% of all genes in the mammalian genome are under some form of circadian control, including a number of transcripts with relevance to tumorigenesis (25). In addition, the molecular clockwork of the circadian system operates via interacting feedback loops among each of the core genes. As such, disturbances in one gene could result in phenotypic consequences across the entire system. As a result of this interconnectedness, if alterations to one or a few of the circadian genes were in fact causally associated with cancer risk, this phenotype could potentially be elicited by perturbing any one of the core circadian genes, with the consequence that a number of circadian gene variants could be significantly associated with disease risk without being directly causal.

Our results are also congruent with findings from a recently released NCI genome-wide association study on prostate cancer that showed some significant associations between variants in circadian genes and prostate cancer risk. The CGEMS project genotyped 550,000 SNPs in 1,182 prostate cancer cases and 1,174 controls from the Prostate, Lung, Cervical, and Ovarian Cancer Screening

Trial (18). A total of 104 SNPs in circadian genes were included in the CGEMS project. Eight of these, located in four genes, *NPAS2* (two SNPs), *CSNK1E* (three SNPs), *CRY1* (two SNPs), and *CRY2* (one SNP), were significantly associated with prostate cancer risk ($P < 0.05$ for all SNPs), including two (*NPAS2* rs895521 and *CRY2* rs12281674) with $P \leq 0.01$. Six of these eight variants were genotyped in the current study, and of these, two, *CSNK1E* rs1534891 and *NPAS2* rs895521, were also significantly associated with prostate cancer risk. There are several possibilities as to why the results of the two studies differed. First, our study is population-based and geographically homogenous, whereas CGEMS is neither. Because prostate cancer rates vary by geography and prostate cancer is a genetically heterogeneous disease, it is not surprising that different genetic variants are highlighted in these two studies. Nevertheless, both studies support a role of circadian genes in prostate cancer, which warrants further confirmation and validation in future studies.

The clock-cancer connection in prostate cancer is further supported by discoveries demonstrating that the circadian clock may function as a tumor suppressor at the systemic, cellular, and molecular levels through its involvement in cell proliferation, apoptosis, cell cycle control, and DNA damage response (26–28). For example, mice with mutant *mPer2* had impaired DNA damage responses to γ irradiation, which made them more cancer prone (26). *CSNK1E* also functions in promoting cell proliferation by stabilizing β -catenin (29). β -Catenin can interact with transcription factors of the T cell-specific transcription factor/lymphoid enhancer factor-1 family to regulate transcription (30) and promote tumorigenesis (31). *PER1* also plays an important role in regulating growth and DNA damage control, and it interacts with proteins in the cell-cycle pathway (32). A recent study also showed that the circadian gene *NPAS2* affects several gene pathways associated with the DNA damage response, including cell cycle, apoptosis, and DNA repair (33). As such, polymorphisms in circadian genes may change their protein expression and activity, thereby affecting downstream cancer-related biological pathways.

Apart from their tumor suppressor role, circadian genes might affect prostate cancer risk by mediating levels of serum androgen (13). This in turn may act as a carcinogen by increasing cellular proliferation, thereby increasing the chance of random DNA copy errors. The prostate is an androgen-dependent organ, because androgens are essential for its normal growth and maintenance. Animal studies have shown that large amounts of androgens given to rodents can induce prostate cancer (34). A human study has also observed that long-term exposure to high levels of androgens can promote the development of prostate cancer (35), although a large pooled analysis of 18 prospective studies did not support this finding (36). Further, men taking the drug finasteride, which reduces androgen exposure within the prostate, compared with men on placebo were shown to have about a 25% lower prevalence of prostate cancer over a 7-year period (37). Levels of testosterone, the principle circulating androgen, have also been shown to play a pivotal role in the differentiation and maintenance of prostate cancer cells (38). The impact of circadian rhythms on the expression of androgens has been previously documented in animals (39). In men, a circadian pattern in the levels of serum total testosterone has also been observed, and this circadian rhythm becomes blunted with normal aging (40). Circadian rhythms in plasma levels of cortisol, DHEA, δ 4-androstenedione, testosterone, and dihydrotestosterone have been detected in healthy young men (41). In addition, circadian gene variants have been correlated to alterations in serum sex hormone levels, including testosterone and sex hormone-binding globulin (42). These observations show the potential impact of the circadian rhythm on androgen expression, and suggest that circadian gene variants may alter the risk of hormonally mediated cancers by influencing the regulation of hormone pathways.

Among all 10 circadian genes examined, *NPAS2* gives the most robust association with prostate cancer risk. Importantly, *NPAS2* has been linked to the risk of two other cancer types: hormone-related breast cancer (43) and immune-related non-Hodgkin's lymphoma (44). The possible involvement of *NPAS2* in tumorigenesis has been supported by an animal study demonstrating that ARNTL/*NPAS2* heterodimers directly suppress transcription of the oncogene *c-myc* (26). A recent *in vitro* study further showed that the circadian gene *NPAS2* affects several pathways associated with DNA damage response, including cell cycle, apoptosis, and

DNA repair (33). Although the explicit mechanisms underlying these associations remain unclear, there is strong evidence indicating that *NPAS2* may serve as a biomarker for an individual's risk of cancer. As such, further study is needed to elucidate the functional significance of *NPAS2* variants, and to further understand their role in carcinogenesis.

In conclusion, the genotype and haplotype approaches, using both tagging and amino acid-altering SNPs in this population-based case-control study, provide comprehensive evidence supporting a role for the molecular clockwork pathway in prostate tumorigenesis. The strengths of this study include the relatively large sample size available for genotyping and the novel findings associated with the emerging area of circadian genetics in cancer. In addition, whereas previous epidemiologic studies have noted a link between circadian disruption and prostate cancer risk, no previous study has systematically examined the effects of variants in circadian genes on prostate cancer susceptibility. A major limitation is one that is shared with most genetic epidemiology studies, i.e., the lack of functional data relating the variants under study to a well-defined phenotypic effect. Nevertheless, the genetic polymorphisms identified in this study will build a molecular basis for future mechanistic studies and phenotypic assessments of circadian genes in relation to prostate cancer. Given the newly identified role of the circadian clock in tumorigenesis, unraveling the association between circadian genes and prostate cancer will add to our understanding of a fundamental aspect of cellular processes in tumorigenesis, and may facilitate the development of novel risk and prognostic biomarkers for prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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