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Common Genetic Variation in Circadian Rhythm Genes and Risk of Epithelial Ovarian Cancer (EOC)

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Abstract

Disruption in circadian gene expression, whether due to genetic variation or environmental factors (e.g., light at night, shiftwork), is associated with increased incidence of breast, prostate, gastrointestinal and hematologic cancers and gliomas. Circadian genes are highly expressed in the ovaries where they regulate ovulation; circadian disruption is associated with several ovarian cancer risk factors (e.g., endometriosis). However, no studies have examined variation in germline circadian genes as predictors of ovarian cancer risk and invasiveness. The goal of the current study was to examine single nucleotide polymorphisms (SNPs) in circadian genes *BMAL1*, *CRY2*, *CSNK1E*, *NPAS2*, *PER3*, *REV1* and *TIMELESS* and downstream transcription factors *KLF10* and *SENP3* as predictors of risk of epithelial ovarian cancer (EOC) and histopathologic subtypes. The study included a test set of 3,761 EOC cases and 2,722 controls and a validation set of 44,308 samples including 18,174 (10,316 serous) cases and 26,134 controls from 43 studies participating in the Ovarian Cancer Association Consortium (OCAC). Analysis of genotype data from 36 genotyped SNPs and 4600 imputed SNPs indicated that the most significant association was rs117104877 in *BMAL1* (OR = 0.79, 95% CI = 0.68-0.90, $p = 5.59 \times 10^{-4}$]. Functional analysis revealed a significant down regulation of *BMAL1* expression following *cMYC* overexpression and increasing transformation in ovarian surface epithelial (OSE) cells as well as alternative splicing of *BMAL1* exons in ovarian and granulosa cells. These results suggest that variation in circadian genes, and specifically *BMAL1,* may be associated with risk of ovarian cancer, likely through disruption of hormonal pathways.

Introduction

Almost every human cell contains an autonomous circadian clock that synchronizes gene transcription in a daily oscillation for many physiological processes allowing for adaptation to the 24 hour environmental day/night cycle. Circadian genes are known to regulate a variety of cellular processes including the cell cycle, apoptosis, and DNA damage repair [1]. Disruption in circadian gene expression, whether due to genetic variants or environmental factors (e.g., light at night, shiftwork), is associated with increased incidence and invasiveness of a variety of human cancers [2-5] such that in 2007 the International Agency for Research on Cancer classified shift work that involves circadian disruption as "a probable carcinogen" in humans [6]. Disruption of circadian rhythms is also associated with disturbances in menstrual function; female shift workers compared to non-shift workers are more likely to report menstrual irregularity and longer menstrual cycles [7]. Moreover, a recent study found that working nightshifts (i.e., 12:00-4:00 AM) was associated with an increased risk of serious and mucinous, invasive and borderline ovarian tumors in women who were 50 years of age and older [8]. Nevertheless, some studies have failed to find an association between shiftwork and cancer risk [9-11].

The molecular mechanism of the mammalian circadian rhythm is a transcriptional-translational-post-translational autoregulatory feedback loop [12]. The core of the loop consists of CLOCK and BMAL1 proteins, that form a dimer which binds to the E-box region in promoters of period (*PER1*, *PER2*, *PER3*) and cryptochrome (*CRY1*, *CRY2*) genes. Following transcription and translation, PER and CRY proteins form a complex with casein kinase 1 epsilon (CSNK1E) and translocate into the nucleus. Here they bind to BMAL1/CLOCK complex and inhibit their own transcription, which completes the basic auto regulatory loop. PER and CRY proteins are then tagged for proteasomal degradation *via* phosphorylation by CSNK1E and casein kinase 1 delta (CSNK1D) and subsequently by ubiquitination. This cycle lasts approximately 24 h. The BMAL1/CLOCK heterodimer also up regulates the transcription of Rev-erbα and Rora. Their protein products interact with ROR elements (RORE) in the promoter of *BMAL1* gene, upregulating (RORα) or downregulating (REV-ERBα) its transcription [12,13].

Circadian rhythm genes in the hypothalamic suprachiasmatic nucleus (SCN) and reproductive tissues control the timing and length of the ovulatory cycle and pregnancy by their influence on hormones [14]. Estradiol, synthesized in the ovary in response to the stimulation by gonadotropins from the hypothalamic-pituitary-gonadal (HPG) axis, influences the expression of circadian rhythm genes, and in a complex loop-back mechanism the circadian rhythm proteins interfere with estradiol signaling [15]. Overexpression of *CLOCK* transcription factors may play a role in the pathogenesis of endometriosis [16], which is a risk factor for some subtypes of ovarian cancer [17-19]. Infertility is observed in knockout *BMAL1*, *PER1*, and *PER2* mice [20-22]. These data are consistent with human studies indicating that genetic variation in *BMAL1* is associated with increased rates of miscarriage [23]. Nulliparity is a well-established risk factor for ovarian cancer, although it is currently unclear whether this association is due to infertility or other biological factors (e.g., increased ovulation) [24-27].

Variation in circadian genes has been associated with cancer susceptibility and outcomes. *CLOCK1*, *CRY1*, *CRY2*, *NPAS2*, *PER1*, *RORA* and *TIMELESS* variants are associated with breast cancer risk [5,28-33], while polymorphisms in *BMAL1, CLOCK1*, *CRY1*, *CRY2*, *CSNK1E*, *NPAS2, PER1*, *PER2*, and *PER3* are associated with prostate cancer risk [34-36]. *CRY2* and *NPAS2* variation is associated with risk of non-Hodgkin's lymphoma [37,38] while polymorphisms in *CLOCK1* are associated with colorectal cancer susceptibility [39]. *PER1* and *CLOCK1* variation is associated with glioma risk and outcome [40] and *PER3* polymorphisms have been associated with hepatocellular carcinoma survival [41]. Interestingly, variation in many of these genes is also associated with dysregulation of circadian behaviors, including sleep and activity patterns [42,43], although data are conflicting [44,45]. To date, however, there are no published studies on the association of variation in circadian genes with ovarian cancer risk and invasiveness.

The goal of the current study was to examine variants in seven key circadian rhythm genes (*BMAL1*, *CRY2*, *CSNK1E*, *NPAS2*, *PER3*, *REV1, TIMELESS*) and two transcription factors (*KLF10* and *SENP3*) activated by circadian rhythm gene expression as risk factors for epithelial ovarian cancer, histopathologic subtype, and invasiveness. SNPs were evaluated in a two-stage design: a discovery stage using two genome-wide association studies (GWAS) and a replication stage with approximately 44,000 cases and controls from 43 studies that comprise the Ovarian Cancer Association Consortium (OCAC).

Materials and Methods

Sample and procedure

The discovery set included 3,761 EOC cases and 2,722 controls in two ovarian cancer GWAS in North America and the United Kingdom (UK). Details of these studies have been previously published [46]. In brief, the North American study was comprised of four case-control studies genotyped using the Illumina 610-quad Beadchip Array™ (i.e., 1,814 cases and 1,867 controls) as well as a single case-control study genotyped on the Illumina 317K and 370K arrays (i.e., 133 cases and 142 controls). The UK study was comprised of four case-only studies genotyped on the Illumina 610-quad Beadchip Array™ and two common control sets genotyped on the Illumina 550K array (i.e., 1,814 cases and 713 controls). The North American and UK studies were analyzed separately and the results combined using fixed effects meta-analysis.

The replication sample consisted of 14,525 invasive EOC cases and 23,447 controls from 43 sites in the Ovarian Cancer Association Consortium (OCAC). An additional 1,747 participants with tumors of low malignant potential were also analyzed. The sample consisted of only participants with European ancestry due to small numbers belonging to other racial groups.

Gene and SNP selection

Seven essential circadian genes (*BMAL1*, *CRY2*, *CSNK1E*, *NPAS2*, *PER3*, *REV1, TIMELESS*) and two key transcription factor genes activated by circadian genes (*KLF10*, *SENP3*) were selected *a priori* for examination. On the Illumina 610quad, 241 tagSNPs in these genes were identified. The selection of SNPs for replication was informed by ranking of minimal p-values across four sets of results: 1) North American all histologies, 2) North American serous histology, 3) combined GWAS meta-analysis all histologies, and 4) combined GWAS meta-analysis serous histology. Of the 241 SNPs, 37 SNPs were significant in the GWAS discovery set.

Statistical analysis

Demographic and clinical characteristics of cases and controls were compared using t-tests for continuous variables and chi-square tests for categorical variables. Unconditional logistic regression, treating the number of minor alleles carried as an ordinal variable (i.e., log-additive model), was used to evaluate the association between each SNP and ovarian cancer risk. Per-allele log odds ratios (OR) and their 95% confidence intervals (CI) were estimated. Models were adjusted for study site and population substructure by including study-site indicators and the first five eigenvalues from principal components analysis. The number of principal components was based on the position of the inflexion of the principal components scree plot.

To maximize statistical power, the combined COGS dataset was used to perform SNP-specific analyses for all invasive EOC, the four main histological subtypes (serous, endometrioid, clear cell and mucinous), and tumors of low malignant potential (LMP). Odds ratios specific for each histological subtype were estimated by comparing cases of each subtype to all available controls as reference. Associations with a two-sided p value < 0.05 and a false discovery

rate (FDR) q-value [47] < 0.10 were considered to be statistically significant.

Imputation analyses

These analyses were based on imputed genotypes from the four ovarian cancer GWAS studies (US GWAS, UK GWAS, COGS and Mayo clinic) with a total of 15,398 invasive EOC case subjects and 30,816 control subjects of white-European ancestry. Imputation of each dataset into the 1000 Genomes Project was performed using IMPUTE2 software [48]. We used the 1000 Genomes Project v3 as the reference with pre-phasing of the data using SHAPEIT [49]. SNP log-additive model meta-analysis was carried out for combining results across studies. Only imputed SNPs with $r^2 > 0.25$ for each study were used in the analyses.

Functional analyses

An *in vitro* model of early-stage ovarian cancer has been previously described [45]. Briefly, Illumina HT12 gene expression microarrays were used to profile the transcriptome of 3D models of normal ovarian cells immortalized with *TERT* and overexpressing *cMYC* and a mutant *KRAS* or *BRAF* allele.

Results

Sample descriptives

All invasive cancers combined and the four main histological subtypes serous (n = 8,369), endometrioid (n = 2,067), clear cell (n = 1,024) and mucinous (n = 943) were analyzed. Sample characteristics are described in table 1. As expected, significant differences were observed between cases and controls on ovarian cancer risk factors including age, family history of ovarian cancer, age at menarche, body mass index (BMI), history of oral contraceptive use, endometriosis, and number of full term births (p values < 0.05). The proportion of serous histological subtype (57.6%) was higher than the other subtypes (14.2% endometrioid, 7.1% clear cell, 6.5% for mucinous, and 14.6% other).

Genotyped variants

A total of 36 SNPs demonstrated p values < 0.05 in the screening stage and passed quality control. Of these, two in *SENP3* (i.e., rs11656383, rs3499590) were rare variants (i.e., MAFs < 0.01) and were dropped from further analyses. Of the remaining 34 SNPs, 14 were associated with risk of overall EOC, histopathological subtype, and/or invasiveness (Table 2). Seven remained significant after applying the criterion of FDR < 0 .10. Specifically, one SNP was associated with risk of all invasive EOC, rs2513928 in *KLF10* (OR = 0.95, 95% CI = 0.92-0.98, $p = 1.75 \times 10^{-3}$). Four SNPs in *KLF10* were associated with risk of serous EOC (rs2513928: OR = 0.94, 95% CI = 0.91-0.98, $p = 2.42 \times 10^{-3}$; rs2511703: OR = 1.05, 95% CI = 1.02-1.09, $p = 6.54 \times 10^{-3}$; rs3191333: OR = 1.05, 95% CI = 1.02-1.10, $p = 6.72 \times 10^{-3}$; rs2513927: OR = 1.05, 95% CI = 1.01-1.09, $p = 1.18 \times 10^{-2}$). As shown in figure 1, linkage disequilibrium (LD) between the four significant SNPs in *KLF10* was low to moderate. Risk of endometrioid EOC was associated with *SENP3* rs6608 (OR = 1.13, 95% CI = 1.04-1.23, $p = 4.43 \times 10^{-3}$), *CSNK1E* rs135750 (OR = 1.13, 95% CI = 1.03-1.23, $p = 7.09 \times 10^{-3}$), *REV1* rs3792152 (OR = 0.92, 95% CI = 0.86-0.98, $p = 9.61 \times 10^{-3}$), and *BMAL1* rs10732458 (OR = 1.32, 95% CI = 1.07-1.63, $p = 9.64 \times 10^{-3}$). No SNPs were significantly associated with EOC invasiveness nor were any SNPs significantly associated with risk of mucinous or clear cell EOC after applying the criterion of FDR < 0.10.

Imputed variants

A total of 4600 imputed SNPs in the nine genes of interest

Table 1: Sample demographic and clinical characteristics (n= 37,972).

Characteristics	Controls (n = 23,447) N (%)	Invasive Cases (n = 14,525) N (%)	p-value[2]
Age (years) Mean ± SD	55.6 ± 11.9	58.1 ± 11.3	<. 0001
< 40	2027 (8.7)	748 (5.2)	<. 0001
40-49	4771 (20.6)	2544 (17.6)	
50-59	7403 (31.9)	4537 (31.3)	
60-69	6098 (26.3)	4324 (29.8)	
≥ 70	2892 (12.5)	2343 (16.2)	
Family history of ovarian cancer[1]			
No	15425 (92.0)	8634 (82.4)	<. 0001
Yes	1351 (8.0)	1849 (17.6)	
Age at menarche (years)			
Mean ± SD	12.9 ± 1.7	12.8 ± 1.6	0.0314
< 12	3128 (19.3)	1856 (19.2)	0.0772
12	3602 (22.2)	2257 (23.4)	
13	4357 (26.9)	2621 (27.1)	
≥ 14	5112 (31.6)	2923 (30.3)	
Body mass inde × (kg/m^2)			
< 25	3834 (48.2)	2528 (45.1)	0.0006
25-29	2332 (29.3)	1681 (30.0)	
≥ 30	1797 (22.6)	1396 (24.9)	
Oral contraceptive use			
No	6136 (37.5)	4203 (43.7)	<. 0001
Yes	10230 (62.5)	5419 (56.3)	
Histological subtypes	N/A		
Serous		8369 (57.6)	
Endometroid		2067 (14.2)	
Clear Cell		1024 (7.1)	
Mucinous		943 (6.5)	
Others[3]		2122 (14.6)	

[1]for the first degree relatives

[2]t-test for a continuous variable and chi-square test for a categorical variable

[3]Include mi × ed cell, other specified epithelial, undifferentiated, unknown (but known to be epithelial), nonepithelial, other or unknown if epithelial, or missing

Table 2: Associations between Genotyped SNPs in Circadian Genes and EOC Incidence Overall, in Histological Subtypes, and Invasiveness.

					All invasive		Serous		Clear cell	
Gene	**SNP**	**Chr**	**Min/Maj**	**MAF**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
BMAL1	rs1026071	11	G/A	0.30	0.98 (0.95-1.01)	2.26 × 10-01	1.00 (0.96-1.04)	9.38 × 10-01	**0.88 (0.8-0.98)**	**1.55 × 10-02**
BMAL1	rs10732458	11	A/G	0.02	1.11 (0.99-1.23)	6.91 × 10-02	1.10 (0.96-1.25)	1.64 × 10-01	1.19 (0.88-1.6)	2.52 × 10-01
BMAL1	rs10832027	11	G/A	0.33	0.98 (0.95-1.02)	3.48 × 10-01	1.00 (0.96-1.04)	9.79 × 10-01	0.92 (0.84-1.01)	9.15 × 10-02
BMAL1	rs1562438	11	A/G	0.29	0.98 (0.95-1.02)	3.07 × 10-01	1.00 (0.96-1.05)	8.46 × 10-01	**0.88 (0.80-0.97)**	**1.35 × 10-02**
BMAL1	rs16912751	11	G/A	0.05	0.98 (0.92-1.05)	6.23 × 10-01	0.96 (0.88-1.04)	3.42 × 10-01	1.13 (0.93-1.37)	2.18 × 10-01
BMAL1	rs2896635	11	T/A	0.33	0.98 (0.95-1.02)	3.14 × 10-01	1.00 (0.96-1.04)	9.57 × 10-01	0.93 (0.84-1.02)	1.17 × 10-01
BMAL1	rs3789327	11	G/A	0.48	1.01 (0.98-1.04)	5.34 × 10-01	1.01 (0.97-1.04)	7.88 × 10-01	1.04 (0.95-1.14)	4.17 × 10-01
BMAL1	rs3816360	11	A/G	0.34	1.00 (0.96-1.03)	7.75 × 10-01	1.02 (0.98-1.06)	4.36 × 10-01	**0.91 (0.82-1.00)**	**4.31 × 10-02**
BMAL1	rs4757151	11	A/G	0.47	1.00 (0.97-1.04)	7.76 × 10-01	1.01 (0.98-1.05)	5.46 × 10-01	0.97 (0.89-1.06)	5.20 × 10-01
BMAL1	rs6486122	11	G/A	0.32	0.98 (0.95-1.02)	2.83 × 10-01	1.00 (0.96-1.04)	9.53 × 10-01	0.92 (0.83-1.01)	8.10 × 10-02
BMAL1	rs7117836	11	A/G	0.02	1.10 (0.99-1.22)	8.49 × 10-02	1.09 (0.96-1.24)	1.65 × 10-01	1.19 (0.89-1.59)	2.46 × 10-01
BMAL1	rs7947951	11	A/G	0.32	0.99 (0.95-1.02)	3.60 × 10-01	1.00 (0.96-1.04)	9.13 × 10-01	0.92 (0.84-1.01)	9.30 × 10-02
CRY2	rs11038695	11	A/G	0.08	1.05 (0.99-1.11)	1.11 × 10-01	1.03 (0.97-1.11)	3.40 × 10-01	0.99 (0.84-1.17)	9.25 × 10-01
CSNK1E	rs135750	22	G/C	0.15	1.04 (1.00-1.09)	6.14 × 10-02	1.03 (0.98-1.08)	3.12 × 10-01	1.00 (0.89-1.13)	9.73 × 10-01
KLF10	rs12547834	8	G/A	0.07	0.96 (0.90-1.02)	1.43 × 10-01	0.94 (0.88-1.02)	1.20 × 10-01	1.02 (0.85-1.21)	8.49 × 10-01
KLF10	rs3191333	8	A/G	0.37	**1.04 (1.01-1.07)**	**2.42 × 10-02**	**1.05 (1.02-1.10)**	**6.72 × 10-03**	1.04 (0.95-1.14)	3.95 × 10-01
KLF10	rs980112	8	A/G	0.10	0.97 (0.92-1.02)	1.98 × 10-01	0.96 (0.90-1.03)	2.42 × 10-01	1.06 (0.92-1.23)	4.08 × 10-01
KLF10	rs2388232	8	G/A	0.27	1.01 (0.97-1.04)	7.92 × 10-01	1.00 (0.96-1.04)	9.22 × 10-01	**1.11 (1.01-1.23)**	**2.91 × 10-02**
KLF10	rs2511703	8	G/A	0.43	**1.04 (1.01-1.07)**	**1.83 × 10-02**	**1.05 (1.02-1.09)**	**6.54 × 10-03**	1.00 (0.91-1.09)	9.55 × 10-01
KLF10	rs2513927	8	A/G	0.49	**1.04 (1.01-1.07)**	**1.86 × 10-02**	**1.05 (1.01-1.09)**	**1.18 × 10-02**	1.00 (0.91-1.10)	9.79 × 10-01
KLF10	rs2513928	8	G/A	0.46	**0.95 (0.92-0.98)**	**1.75 × 10-03**	**0.94 (0.91-0.98)**	**2.42 × 10-03**	0.94 (0.85-1.02)	1.50 × 10-01
KLF10	rs2511660	8	A/G	0.22	0.97 (0.94-1.01)	1.57 × 10-01	0.96 (0.92-1.00)	6.95 × 10-02	0.99 (0.89-1.10)	8.56 × 10-01
KLF10	rs2511718	8	A/G	0.12	0.98 (0.94-1.03)	4.57 × 10-01	0.98 (0.92-1.04)	4.47 × 10-01	1.06 (0.93-1.22)	3.68 × 10-01
NPAS2	rs1053091	2	A/G	0.02	1.05 (0.93-1.19)	4.14 × 10-01	1.10 (0.96-1.27)	1.83 × 10-01	1.12 (0.79-1.59)	5.17 × 10-01
NPAS2	rs13012930	2	A/G	0.17	**0.96 (0.92-1.00)**	**4.80 × 10-02**	**0.95 (0.91-1.00)**	**4.11 × 10-02**	0.98 (0.87-1.10)	6.86 × 10-01
NPAS2	rs3768988	2	G/A	0.06	1.01 (0.95-1.07)	8.18 × 10-01	1.02 (0.94-1.10)	6.44 × 10-01	1.01 (0.84-1.22)	9.09 × 10-01
NPAS2	rs7573323	2	A/G	0.03	0.97 (0.88-1.07)	5.47 × 10-01	0.99 (0.88-1.11)	8.61 × 10-01	0.87 (0.65-1.18)	3.73 × 10-01
PER3	rs228644	1	A/G	0.40	1.00 (0.97-1.03)	9.23 × 10-01	1.00 (0.96-1.03)	8.38 × 10-01	0.97 (0.89-1.07)	5.45 × 10-01
PER3	rs228682	1	G/A	0.40	1.00 (0.97-1.03)	7.83 × 10-01	0.99 (0.96-1.03)	7.32 × 10-01	0.97 (0.88-1.06)	4.84 × 10-01
PER3	rs228698	1	A/G	0.04	1.00 (0.93-1.08)	9.73 × 10-01	0.99 (0.90-1.08)	7.67 × 10-01	0.90 (0.71-1.14)	3.79 × 10-01
PER3	rs697693	1	A/G	0.19	0.99 (0.95-1.03)	5.55 × 10-01	0.98 (0.94-1.03)	5.02 × 10-01	1.07 (0.96-1.19)	2.46 × 10-01
REV1	rs3792152	2	A/G	0.44	0.97 (0.94-1.00)	6.47 × 10-02	0.97 (0.94-1.01)	1.34 × 10-01	0.99 (0.90-1.08)	7.96 × 10-01
SENP3	rs6608	17	A/G	0.17	**1.05 (1.00-1.09)**	**3.35 × 10-02**	1.04 (0.99-1.09)	1.42 × 10-01	1.01 (0.90-1.14)	8.81 × 10-01
TIMELESS	rs7302060	12	G/A	0.41	0.99 (0.96-1.02)	3.53 × 10-01	0.98 (0.94-1.01)	2.09 × 10-01	0.97 (0.88-1.06)	4.77 × 10-01

		Endometriod		Mucinous		LMP vs. controls		Invasive vs. LMP	
Gene	**SNP**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
BMAL1	rs1026071	0.98 (0.91-1.05)	5.17 × 10-01	0.94 (0.85-1.05)	2.63 × 10-01	0.99 (0.92-1.07)	8.95 × 10-01	1.00 (0.92-1.08)	9.17 × 10-01
BMAL1	rs10732458	**1.32 (1.07-1.63)**	**9.64 × 10-03**	1.02 (0.72-1.44)	9.12 × 10-01	0.77 (0.58-1.02)	6.51 × 10-02	**1.44 (1.09-1.92)**	**1.17 × 10-02**
BMAL1	rs10832027	0.99 (0.93-1.06)	8.48 × 10-01	0.95 (0.86-1.05)	2.75 × 10-01	1.00 (0.93-1.07)	9.17 × 10-01	1.00 (0.92-1.07)	9.04 × 10-01
BMAL1	rs1562438	0.97 (0.90-1.04)	4.12 × 10-01	0.94 (0.85-1.05)	2.74 × 10-01	1.00 (0.93-1.08)	9.79 × 10-01	0.99 (0.92-1.07)	8.80 × 10-01
BMAL1	rs16912751	0.90 (0.78-1.05)	1.97 × 10-01	1.11 (0.91-1.36)	2.94 × 10-01	0.88 (0.75-1.04)	1.40 × 10-01	1.12 (0.95-1.33)	1.73 × 10-01
BMAL1	rs2896635	0.99 (0.92-1.06)	7.20 × 10-01	0.95 (0.86-1.05)	3.04 × 10-01	1.00 (0.93-1.07)	9.49 × 10-01	0.99 (0.92-1.07)	8.21 × 10-01
BMAL1	rs3789327	1.00 (0.94-1.07)	9.84 × 10-01	0.95 (0.86-1.04)	2.53 × 10-01	1.01 (0.94-1.08)	8.63 × 10-01	1.01 (0.94-1.08)	8.01 × 10-01
BMAL1	rs3816360	0.99 (0.92-1.06)	7.74 × 10-01	0.94 (0.85-1.04)	2.52 × 10-01	1.02 (0.94-1.09)	6.67 × 10-01	0.99 (0.92-1.06)	7.53 × 10-01
BMAL1	rs4757151	0.99 (0.92-1.05)	6.91 × 10-01	1.06 (0.97-1.17)	1.97 × 10-01	0.98 (0.91-1.05)	5.61 × 10-01	1.03 (0.96-1.11)	3.73 × 10-01
BMAL1	rs6486122	0.99 (0.92-1.06)	6.90 × 10-01	0.95 (0.86-1.05)	3.12 × 10-01	0.99 (0.92-1.07)	8.62 × 10-01	0.99 (0.92-1.07)	8.83 × 10-01
BMAL1	rs7117836	**1.24 (1.01-1.54)**	**4.40 × 10-02**	1.06 (0.76-1.48)	7.36 × 10-01	**0.76 (0.57-1.00)**	**4.82 × 10-02**	**1.45 (1.09-1.92)**	**9.81 × 10-03**
BMAL1	rs7947951	0.99 (0.93-1.06)	8.17 × 10-01	0.95 (0.86-1.05)	2.94 × 10-01	1.00 (0.93-1.07)	9.34 × 10-01	0.99 (0.92-1.07)	8.78 × 10-01
CRY2	rs11038695	1.09 (0.97-1.22)	1.48 × 10-01	0.97 (0.82-1.15)	7.19 × 10-01	1.07 (0.94-1.21)	2.88 × 10-01	0.98 (0.86-1.11)	7.02 × 10-01
CSNK1E	rs135750	**1.13 (1.03-1.23)**	**7.09 × 10-03**	1.06 (0.93-1.20)	3.90 × 10-01	1.02 (0.93-1.12)	6.98 × 10-01	1.03 (0.93-1.13)	6.10 × 10-01
KLF10	rs12547834	0.99 (0.87-1.13)	8.75 × 10-01	0.86 (0.71-1.04)	1.22 × 10-01	0.97 (0.84-1.11)	6.56 × 10-01	0.99 (0.86-1.14)	8.87 × 10-01
KLF10	rs3191333	1.03 (0.96-1.10)	3.84 × 10-01	0.95 (0.86-1.05)	3.01 × 10-01	0.99 (0.92-1.06)	7.70 × 10-01	1.05 (0.97-1.13)	2.21 × 10-01
KLF10	rs980112	0.95 (0.85-1.06)	3.49 × 10-01	0.90 (0.76-1.05)	1.85 × 10-01	0.99 (0.88-1.12)	8.95 × 10-01	0.98 (0.87-1.11)	7.73 × 10-01
KLF10	rs2388232	0.99 (0.92-1.06)	7.81 × 10-01	0.98 (0.88-1.08)	6.40 × 10-01	1.03 (0.96-1.12)	3.86 × 10-01	0.97 (0.90-1.05)	4.71 × 10-01
KLF10	rs2511703	1.05 (0.98-1.12)	1.34 × 10-01	0.95 (0.87-1.05)	3.17 × 10-01	0.98 (0.91-1.05)	5.75 × 10-01	1.06 (0.98-1.13)	1.32 × 10-01
KLF10	rs2513927	1.05 (0.99-1.13)	1.11 × 10-01	0.94 (0.85-1.03)	1.71 × 10-01	0.98 (0.92-1.05)	5.94 × 10-01	1.06 (0.99-1.13)	1.24 × 10-01

KLF10	rs2513928	0.95 (0.89-1.01)	1.20 × 10-01	1.02 (0.93-1.12)	6.88 × 10-01	0.96 (0.90-1.03)	2.56 × 10-01	0.99 (0.92-1.06)	6.95 × 10-01
KLF10	rs2511660	1.02 (0.94-1.10)	6.80 × 10-01	0.96 (0.85-1.07)	4.38 × 10-01	1.06 (0.98-1.15)	1.43 × 10-01	**0.92 (0.85-1.00)**	**4.47 × 10-02**
KLF10	rs2511718	0.96 (0.87-1.06)	4.28 × 10-01	0.95 (0.82-1.09)	4.52 × 10-01	1.01 (0.91-1.13)	8.02 × 10-01	0.97 (0.87-1.09)	6.41 × 10-01
NPAS2	rs1053091	0.84 (0.64-1.12)	2.45 × 10-01	1.02 (0.71-1.47)	9.00 × 10-01	1.10 (0.85-1.44)	4.69 × 10-01	0.93 (0.71-1.22)	5.88 × 10-01
NPAS2	rs13012930	1.02 (0.93-1.11)	7.23 × 10-01	0.91 (0.80-1.03)	1.31 × 10-01	1.04 (0.95-1.14)	3.73 × 10-01	0.92 (0.84-1.01)	9.47 × 10-02
NPAS2	rs3768988	0.93 (0.81-1.07)	3.02 × 10-01	1.01 (0.84-1.22)	8.90 × 10-01	1.09 (0.95-1.26)	2.06 × 10-01	0.93 (0.80-1.07)	2.83 × 10-01
NPAS2	rs7573323	0.92 (0.74-1.13)	4.13 × 10-01	0.83 (0.60-1.16)	2.78 × 10-01	0.83 (0.66-1.05)	1.12 × 10-01	1.18 (0.93-1.49)	1.76 × 10-01
PER3	rs228644	0.97 (0.91-1.04)	3.76 × 10-01	1.07 (0.97-1.17)	1.82 × 10-01	0.99 (0.92-1.06)	6.91 × 10-01	1.02 (0.95-1.09)	6.69 × 10-01
PER3	rs228682	0.97 (0.91-1.04)	3.51 × 10-01	1.07 (0.97-1.17)	1.90 × 10-01	0.98 (0.92-1.06)	6.37 × 10-01	1.02 (0.95-1.09)	6.65 × 10-01
PER3	rs228698	1.04 (0.89-1.23)	6.04 × 10-01	1.08 (0.86-1.36)	4.89 × 10-01	0.96 (0.81-1.15)	6.66 × 10-01	1.01 (0.85-1.21)	8.76 × 10-01
PER3	rs697693	0.99 (0.91-1.08)	8.61 × 10-01	0.92 (0.81-1.04)	1.67 × 10-01	1.04 (0.95-1.13)	3.81 × 10-01	0.96 (0.88-1.05)	3.45 × 10-01
REV1	rs3792152	**0.92 (0.86-0.98)**	**9.61 × 10-03**	0.99 (0.90-1.09)	8.32 × 10-01	0.98 (0.91-1.05)	4.87 × 10-01	1.01 (0.94-1.09)	7.65 × 10-01
SENP3	rs6608	**1.13 (1.04-1.23)**	**4.43 × 10-03**	1.00 (0.88-1.14)	9.90 × 10-01	1.01 (0.92-1.10)	9.00 × 10-01	1.04 (0.94-1.14)	4.79 × 10-01
TIMELESS	rs7302060	1.01 (0.95-1.08)	7.22 × 10-01	0.97 (0.88-1.07)	5.10 × 10-01	**0.93 (0.87-1.00)**	**4.86 × 10-02**	1.06 (0.99-1.14)	1.09 × 10-01

SNP: Single Nucleotide Polymorphism, Chr: Chromosome, Min/Maj: Minor and Major Allele, MAF: Minor Allele Frequency, LMP: Low Malignant Potential, OR: Odds Ratio

Note: odds ratio is calculated based on per-minor allele, bolded SNPs indicate an association of $p < 0.05$ with overall EOC or histologic subtype.

Table 3: Associations between the Top Imputed SNP in Each Gene with Good Imputation Quality ($r^2 > 0.8$) and EOC Incidence Overall.

Gene	SNP	Min/Maj	MAF	OR (95% CI)	p
BMAL1	rs117104877	G/A	0.017	0.79 (0.68-0.90)	5.59 × 10-4
CRY2	rs10838527	G/A	0.082	1.05 (0.99-1.11)	7.66 × 10-2
CSNK1E	rs111427515	G/T	0.008	1.25 (1.06-1.47)	6.60 × 10-3
KLF10	rs2511699	A/G	0.461	0.96 (0.93-0.99)	4.13 × 10-3
NPAS2	rs732375	T/A	0.134	1.07 (1.02-1.11)	3.76 × 10-3
PER3	rs228640	A/G	0.297	1.04 (1.01-1.07)	1.24 × 10-2
REV1	rs3792146	T/C	0.547	1.03 (1-1.06)	2.71 × 10-2
SENP3	rs143094271	A/G	0.023	0.86 (0.77-0.95)	4.01 × 10-3
TIMELESS	rs2638286	C/T	0.030	1.05 (0.96-1.15)	2.56 × 10-1

SNP: Single Nucleotide Polymorphism, Min/Maj: Minor and Major Allele, MAF: Minor Allele Frequency, OR: Odds Ratio

Note: odds ratio is calculated based on per-minor allele

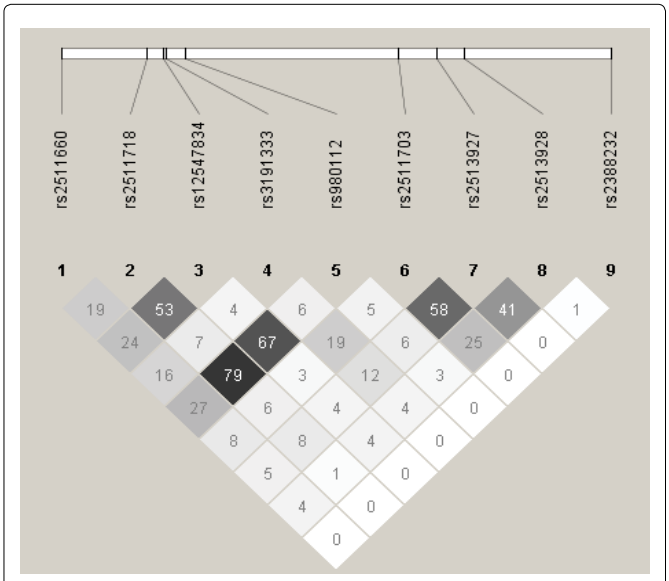

Figure 1: Linkage Disequilibrium (r^2) among Single Nucleotide Polymorphisms in *KLF10*.

(*BMAL1*, *CRY2*, *CSNK1E*, *NPAS2*, *PER3*, *REV1, TIMELESS, KLF10*, *SENP3*) were then examined for association with all invasive EOC. A total of 304 SNPs across all nine genes met criteria for statistical significance ($p < 0.05$). Top hits in each gene with good imputation quality [$r^2 > 0.8$] are shown in table 3. Across all genes, the most significant imputed SNP was rs117104877 in *BMAL1* (OR = 0.79, 95% CI = 0.68-0.90, $p = 5.59 \times 10^{-4}$).

Evaluating the functional role of BMAL1 in ovarian cancer

The role of *BMAL1* in ovarian cancer was examined using *in silico* analysis of existing biological datasets in ovarian normal and tumor tissues and an *in vitro* cell biology model of early stage ovarian cancer development. We evaluated gene expression in normal fallopian tubes (n = 8) compared to high-grade serous ovarian carcinomas (HGSOCs, n = 489) using data from The Cancer Genome Atlas (TCGA), but there was no evidence that *BMAL1* was differentially regulated in EOCs as compared to normal tissue (Figure 2).

BMAL1 expression was further investigated in an early stage transformation model of EOC based on overexpression of *CMYC* in the ovarian surface epithelium (OSE) [50]. *BMAL1* was significantly down regulated in this model, but down regulation was not enhanced by expression of a mutant *KRAS* allele (Figure 2b). Risk associated SNPs were located within intronic regions of *BMAL1* (Figure 2c) and clustered around a commonly described enhancer, suggesting that risk SNPs may influence enhancer activity. Rs2896635 in particular coincides with an enhancer used in many cell types, including an enhancer that is active in ovarian stromal cells that targets the *BMAL1* gene [51]. This suggests that non-cell autonomous signaling pathways may be involved in risk at this locus.

Discussion

Circadian genes appear to play an important role in regulating reproductive cycles, including ovulation, the length of the estrous cycle, and maintenance of pregnancy. The current study examined variation in nine key genes involved in circadian rhythm regulation or their transcription (*BMAL1*, *CRY2*, *CSNK1E*, *KLF10, NPAS2*, *PER3*, *REV1, SENP3, TIMELESS*) as predictors of epithelial ovarian cancer risk, histopathologic subtype, and invasiveness. We found that 14 of the 34 genotyped SNPs in the discovery set were associated with risk of overall EOC, histopathological subtype, and/or invasiveness at $p < 0.05$. Seven remained significant after applying the criterion of FDR < 0.10. Specifically, risk of overall and serous EOC was associated with variants in *KLF10* while risk of endometrioid EOC was associated with variants in SENP3, *CSNK1E, REV1,* and *BMAL1*. Of 4600 imputed variants in the nine genes of interest, 304 were found to be associated with overall EOC risk at p <. 05. Significant variants were found in all nine genes with the most significant located in *BMAL1*. Additional functional analyses of *BMAL1* indicated that it was down regulated as a consequence of overexpressing cMYC in the OSE, although differential regulation was not observed in HGSOCs compared to normal fallopian tube tissue. Taken together, these results suggest that circadian rhythm genes may play a role in the development of EOC, particularly the genes *KLF10* and *BMAL1*.

While previous research has implicated circadian genes in the development of several types of human cancer, the current study is the first to our knowledge to examine relationships with risk of ovarian cancer. Findings regarding the Krüppel-like factor 10

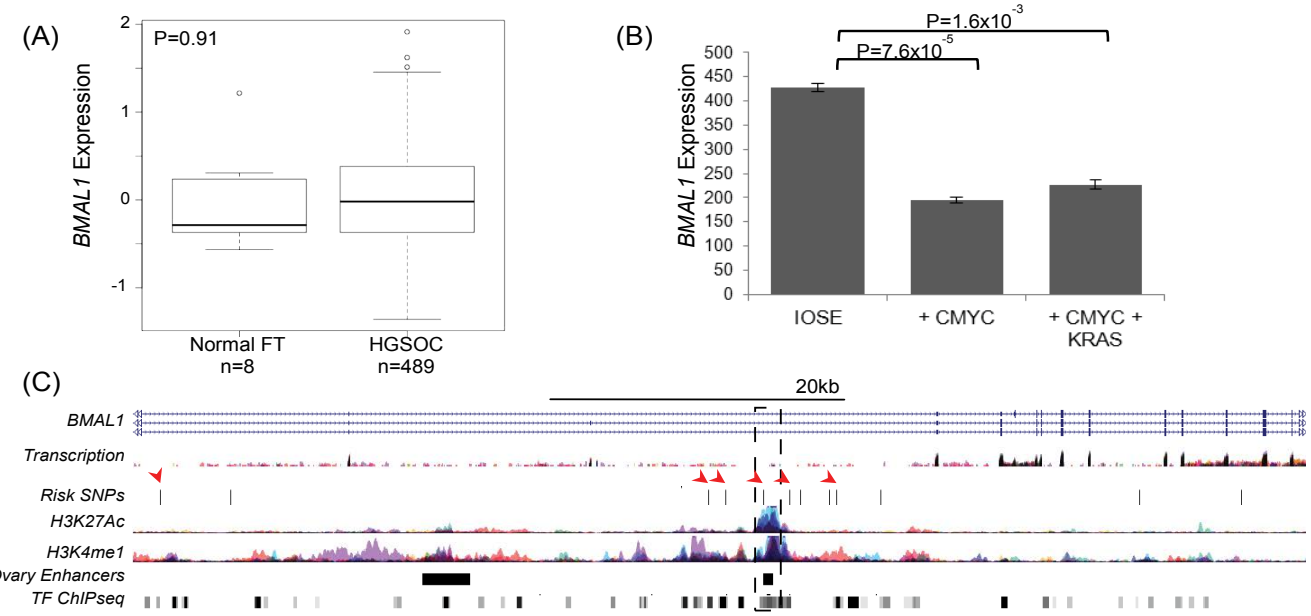

Figure 2: (A) *BMAL1* is not differentially expressed in TCGA expression data for 8 normal fallopian tubes and 489 high-grade serous EOCs; however, in an early stage model of ovarian cancer, (B) *BMAL1* is downregulated in partially transformed ovarian epithelial cells overexpressing *cMYC*. *BMAL1* downregulation is *cMYC* dependent, and not enhanced by the expression of a mutant KRAS allele. (C) 6 SNPs at the *BMAL1* locus coincide with marks of active regulatory elements (H3K27Ac and H3K4me1) or transcription factor binding sites (TF ChiPseq) (arrows). One SNP, rs2896635 coincides with a commonly used enhancer that is active in ovarian stromal tissue (dashed box), and which targets the *BMAL1* gene. ENCODE data and data from [44].

(*KLF10*) gene are consistent with a sizable body of experimental data indicating that *KLF10* acts to inhibit cellular proliferation and induce apoptosis in a variety of cell types via regulation of transforming growth factor beta (TGFβ) and in turn SMAD [52-58]. *KLF10* is a circadian transcriptional regulator that links the molecular clock to energy metabolism [59]. *KLF10* displays robust BMAL1-dependent circadian expression; the *KLF10* promoter recruits BMAL1 and is transactivated by the CLOCK/BMAL1 dimer through a conserved E-box response element. To our knowledge the role of *KLF10* in human ovarian cancer has not been investigated, although estrogen is known to increase *KLF10* gene transcription [60,61]. *KLF10* expression is reduced in breast tumors relative to normal tissue and is inversely correlated with stage of disease [62,63]. The *KLF10-TGFβ-SMAD* pathway has been implicated in the development of several other human cancers including those of the prostate, pancreas, kidney, lymphoma, and brain [53,64-67].

Our findings regarding *BMAL1* are interesting in light of data suggesting that this gene may regulate the p53 tumor suppressor pathway. Specifically, silencing of *BMAL1* gene expression prevents cell cycle arrest upon p53 activation in human fibroblast cells [68] and mouse colon and fibroblast cells [69]. These data are consistent with research suggesting that *BMAL1* is transcriptionally silenced via hypermethylation in hematologic malignancies; reintroduction of *BMAL1* causes growth inhibition, while *BMAL1* depletion by RNA interference increases tumor growth [70]. The BMAL1 protein also has been shown to bind to the promoter region of *VEGF* where it regulates transcription and promotes angiogenesis [71].

Evidence suggests that, controlling for stage, histological subtype, and grade, low *BMAL1* and *CRY1* expression together significantly predict lower overall survival in ovarian cancer patients [72]. Previous research also suggests significantly lower *BMAL1* and *CRY1* expression in EOC cells compared to normal ovarian tissue [72]. The current study demonstrated downregulation of *BMAL1* when cMYC was overexpressed in an early stage ovarian cancer transformation model, resulting in increasing ovarian epithelial cell transformation. Nevertheless, we did not observe differential regulation of *BMAL1* when comparing EOC cells to normal fallopian tube tissue. Our findings suggest that down regulation of *BMAL1* may be an early event in ovarian carcinogenesis and that *BMAL1* is a novel cMYC target. SNPs statistically significant in the current study lie within intronic sequences of the *BMAL1* gene and mechanisms by which they impact *BMAL1* expression have yet to be elucidated. Nevertheless, our data suggest that this risk locus may modulate ovarian cancer risk by altering the ovarian stromal microenvironment, for example by influencing the character of ovarian fibroblasts or granulosa cells, both of which express *BMAL1*. In conclusion, our results highlight the significance of circadian rhythm gene variation in EOC susceptibility and suggest an early role for the *BMAL1* gene in EOC pathogenesis.

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