Analysis of Genetic Variation in Circadian Rhythm Genes and Risk of Ovarian Cancer.

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Abstract

Background

Disruption in biological clock due to genetic variations is associated with increased occurrence of cancers such as breast, ovary, prostate, gastrointestinal and hematological malignancies. Circadian rhythm genes regulate the process of ovulation in the ovaries and are highly expressed in ovarian tumors; whereas disturbance in the circadian rhythm pathway is significantly associated with causative risk factors (i.e. endometriosis, PCOS, etc.) of ovarian cancer. Nevertheless, very few studies have been conducted till date where candidate SNPs of circadian rhythm genes proved as the main prognosticators of ovarian cancer risk and intrusiveness. The main purpose of this study was to investigate some common single nucleotide polymorphisms (SNPs) in circadian rhythm genes (rs475715 of *BMAL 1/ARNTL*, rs1026071, and rs228644 of *PER3*, rs3792152 of *REV1*, and rs7302060 of *TIMELESS*) as causative markers of ovarian cancer risk of in the population of Jammu and Kashmir in India.

Results

Our study included a total of 600 samples (200 cases and 400 age and sex-matched controls). Analysis of the genotype data from the selected SNPs indicated most significant association of rs3792152 of *REV1* (OR=1.6, with 95% CI=0.12-1.2, $p=0.0003$) and rs4757151 of *BMAL 1/ARNTL* (OR=1.847, with 95% CI=1.406-2.426, $p=9.15E-06$) with the ovarian cancer. The functional putative analysis revealed a significant regulatory effect of both these variants on other genes.

Conclusion

These results suggest that some SNPs in circadian rhythm genes, particularly *BMAL 1/ARNTL* and *REV1*, might be associated with the risk of ovarian cancer in the J&K population of North India.

Background

Ovarian cancer (OC) is one of the most common cancer globally, with more than 239,000 newly diagnosed cases and 152,000 deaths each year [1]. OC ranks 3rd in gynecological malignancies after cervix and uterine cancer [2]. OC has the worst prognosis and the peak death rate. Even though ovarian cancer has a lesser pervasiveness in assessment with breast cancer, it is three times more fatal, and it is foretold that, by the year 2040[3], the death rate of OC will increase significantly. The high death rate in OC patients is mainly due to, asymptomatic and undisclosed progression of the ovarian tumor, deferred beginning of signs and symptoms, and the absence of suitable screening that affects diagnosis of the progressive stages [3]. Despite countless developments in early diagnosis and treatment, OC survival has shown only borderline increase due to the intricacy and heterogeneity of molecular pathways involved, specifically in invasion, relapse of ovaries, and metastasis. Hence, it is crucial to investigate biomarkers to clarify the molecular processes for enlightening the diagnosis of OC. In women, the clock genes under the influence of hormones regulate the ovulation [4]. It was reported that estradiol hormone in ovary is under the influence of gonadotropins which may regulate the expression of clock genes associated with ovarian cancer [5]. From the previous findings, the accruing indication has recommended that circadian clock disturbance is an influential aspect of tumor instigation and proliferation. Epidemiological studies have proved that night workers have raised risk of various cancers (ovary, breast, prostate, and rectal cancer)[5–8] signifying a probable functional association between the biological clock and cancer formation.

It has been proved that abnormal expression of circadian rhythm gene is strongly associated with various types of cancers[9]. Various Preceding reports have also confirmed that the anomalous expression of biological clock genes is strongly correlated with the prognosis of cancer patients [10, 11]. It has been reported that circadian genes play an active role in tumor formation and cancer cell proliferation[10].

Single-nucleotide polymorphism (SNP) is considered as an important genetic biomarker for the early prediction of risk, their response to treatment, and the proliferation of cancer cells [12]. Reported studies have proved that there were several SNPs of circadian rhythm genes (*BMAL 1*, *PER3*, *PER2*, *CRY*, *TIMELESS*, *REV1*) which are significantly associated with the development and progression of various types of cancers (breast ovary, prostate, etc.) [13–17]. Moreover, developing evidence has revealed that SNPs of circadian genes are significantly involved in cancer predisposition [18, 19]. In the present study, we replicated and assessed the effects of candidate variants of circadian rhythm genes (*BMAL 1* rs475715, *PER3* rs228644, *REV1* rs379215,2 and *TIMELESS*rs7302060) as a case-control study from J&K region of India, which was previously found to be associated with ovarian cancer in North American population by Jim et al [20]

Methods

Sample collection

This case's control study from the J&K population was conducted in the school of Biotechnology, SMVDU, Katra. The cases included females with a histologically confirmed ovarian cancer. The cases have been obtained from various hospitals and clinics of J&K. Controls were age-matched to the cases. The cases with no familial history of cancer were included in the study. All subjects included in this study were unrelated women of J&K whereas their descendants have lived in the J&K for at least 5 generations. Overall, 600 samples including 200 incident cases of ovarian cancer and 400 population-based controls were enrolled in the study. Written informed consent was obtained from all subjects recruited in this study. During sample collection, pre-designed questionnaire was used to get the information which included age, BMI, hormonal status, age at menarche, menopausal status, histology of tumors, oral contraceptive use and breast nodules (Table 1) from both cases and controls. Patients who undertook radio/chemotherapy were excluded from the study.

Table 1
Clinical details of cases and controls

Characteristics	Cases (200)	Controls (400)	P value
Age (years) Mean ± S. D	59.2 ± 10.1	56.7 ± 14.4	0.02
BMI Mean ± S. D	22.6 ± 4.52	25.4 ± 4.89	9.74E-12
Menopausal Status			
Premenopausal	124	276	0.33
Post-menopausal	74	124	
Stage			
(I/II)	78	-	-
III/IV	110	-	
Age at menarche (years)			
> 12	107	215	0.02
< 12	93	185	
Histology of tumors			
Epithelial	138	-	-
		-	
Germ cell	9	-	
Sex cord stromal cell	33	-	
Metastasis	20	-	
Oral Contraceptive use			
Yes	80	165	0.1
No	120	165	
Breast Nodules			
Yes	22	-	
No	162		

Selection of SNP

Total four crucial selected circadian gene variants (*BMAL1* / *ARNTL* rs475715 and rs1026071, *PER3* rs228644, *REV1* rs3792152, and *TIMELESS* rs7302060) previously reported [20] to be associated with ovarian cancer were investigated in the current study. The details of SNPs were mentioned in **Additional File1: Table S1**. The SNPs were selected for genotyping only based on their M.A.F (Minor allele frequency) value (> 0.03 in Gujrati Indians). Linkage disequilibrium (LD) SNPs were excluded from this study. The primers (amplification and extension) were designed by Sequenom Mass ARRAY® Assay Design 3.0 Software (Sequenom, San Diego, USA). The primers were mentioned in additional file 1: Table S3.

Genotyping and quality control

From the 600 women (200 cases and 400 controls) who provided blood, sufficient DNA was extracted by using manufacturer protocol (Qiagen DNA isolation Kit cat no. 51206). All these subjects were genotyped by the (Sequenom Mass ARRAY platform) using the 384 well chip according to the standardized protocol which were replicated from the study [21]. Software Sequenom Typer was used for the analysis and management of data.

Statistical analysis

The odds ratios (OR) with their 95% confidence intervals (CI) as well as Hardy Weinberg equilibrium were estimated by Plink v1.07. Clinical characteristics of cases and controls were compared using the chi-square t-test for variables (Table 1). For the results, SNPs that were not following Hardy Weinberg equilibrium (H.W.E) were not included in further analysis.

Logistic regression & stratification analysis was used to estimate the risk or to estimate the association with Odds Ratio at 95% confidence interval and respective level of significance as p-value by using SPSS software. The power of the study was calculated statistically by PS software version 3.1.2 [22].

Putative visualization of variants in the human genome was also done by freely available Insilco tools (SNIPA and Haploreg) [23] [24]. Both tools were used to find the functional annotations i.e., to predict the expression quantitative trait locus (eQTL) of risk associated variants and to find the high linkage disequilibrium SNPs ($r^2 > 0.8$) with selected candidate variants.

Results

Four histopathological types of cases were analyzed. The sample characterized are discussed in Table 1. As estimated, significant differences were observed between cases and controls on ovarian cancer risk factors including age, BMI, age at menarche, breast nodules, oral contraceptive use, and menopausal status (P values < 0.05) (Table 1).

A total of 5 SNPs in association with circadian rhythm was included in the current 2 SNPs (*REV1* rs3792152 & *BMAL1* rs4757151) of circadian rhythm were found to be associated with the risk of ovarian cancer (OR = 1.6, with 95% CI = 0.12–1.2, $p = 0.0003$) and (OR = 1.847, with 95% CI = 1.406–2.426, $p = 9.15E-06$) (Table 2). After logistic regression with age and BMI, rs3792152 of *REV1* having genotype AA was found to be associated with increased risk of ovarian cancer (AA + AG vs. GG: adjusted OR = 1.97, 95% CI 1.25–3.1, $p = 0.003$) and rs4757151 of *BMAL1* having genotype AA was also associated with the increased risk of ovarian cancer (AA + AG vs. GG: adjusted OR = 2.4, 95% CI (1.48–3.87), $p = 0.0003$).

Table 2
Allele frequency of SNPs in ovarian cancer

S. No	GENE	SNPS	CASES	CONTROLS	ALLELE OR	P VALUE	DOMINANT OR	P VALUE	HWE
1	REVI	rs3792152	A = 0.5775 G = 0.4225	A = 0.4571 G = 0.5429	1.6(1.2–2.08)	0.0001	1.97(1.25–3.1)	0.003	0.9184
2	ARNTL /BMAL1	rs1026071	G = 0.3289 A = 0.6711	G = 0.3077 G = 0.6923	1.1(0.83–1.45)	0.48	1.27(0.86–1.86)	0.222	0.2334
3	ARNTL /BMAL1	rs4757151	A = 0.5458 G = 0.4542	A = 0.3941 G = 0.6059	1.84(1.4–2.42)	9.15E-04	2.4(1.48–3.87)	0.00034	0.7339
4	PER3	rs228644	A = 0.4218 G = 0.5782	A = 0.4716 G = 0.5284	0.81(0.59–1.11)	0.204	0.69(0.41–1.19)	0.186	0.00261
5	TIMELESS	rs7302060	C = 0.4085 T = 0.5915	C = 0.3469 T = 0.6531	1.3(0.97–1.73)	0.07	1.48(0.96–2.28)	0.072	0.8051

The SNPs rs1026071 of *BMAL1* (OR = 1.1, 95% CI (0.83–1.45), $p = 0.48$) & variant rs7302060 (OR = 1.3, 95% CI (0.97–1.73), $p = 0.07$) of *TIMELESS* gene was found not associated with the ovarian cancer (Table 2). The variant rs228644 of *PER3* was not following HWE (hardy Weinberg equilibrium) and thus was not analyzed further.

The putative visualization of significantly associated variants (rs3792152 of *REVI* & rs4757151 of *BMAL1*) was done by Haploreg v4.1 (<https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php>) and SNIPA (<https://snipa.helmholtz-muenchen.de/snipa3/>). These tools predict the possible mechanism underlying the identified associations and determine proxy variants of the identified variants. Haploreg identified that both variants rs3792152 of the *REVI* gene and rs4757151 of the *BMAL1* genes were located in enhancer histone marks. Both risks associated with intronic variants were predicted to change the regulatory binding motifs (**Additional File1: Supplementary Figures S1 and Supplementary Figure S2**). SNIPA identified that the intronic variant rs3792152 was predicted to have a direct regulatory effect on *C2orf15*, *LIPT1*, *LYG1*, *MITD1*, *REV1*, *TSGA10*, *TXNDC9* through eQTL (Expression quantitative trait loci) with *REV1* gene, whereas intronic variant rs4757151 was predicted to have a direct regulatory effect on transcripts (*RN7SKP151* & *BTBD10*) through eQTL *ARNTL* (**Additional File1: Supplementary Table S2 and Supplementary Figures S3 and Supplementary Figure S4**).

Discussion

In this case-control study, we reported the significant association of ovarian cancer with the variants of circadian rhythm gene pathway, predominantly with already reported ovarian cancer risk associated circadian gene variants (*BMAL1* / *ARNTL* rs475715 and rs1026071, *PER3* rs228644, *REV1* rs3792152, and *TIMELESS* rs7302060) [20]. We examined variation in the four most common genes of the circadian pathway (*REV1*, *ARNTL/BMAL1*, *TIMELESS*, and *PER3*) as prognosticators

of ovarian cancer risk and invasiveness. We found that two out of five variants were associated with the risk of ovarian cancer. Specifically, the risk of ovarian cancer was associated with variant rs475715 of *BMAL1/ARNTL* & rs3792152 of *REV1*, whereas other variant rs228644 of *PER3*, rs7302060 of *TIMELESS*, and rs1026071 of *ARNTL* were found to be not associated with ovarian cancer in our studied region.

Biological clock in humans called circadian clock / circadian rhythm, autonomously oscillate with a period near 24 hours. The mechanism of the circadian clock is based on the positive/negative response circlets which are produced by core circadian clock genes. The monitoring feedback loop of circadian rhythm consists of *PER*, *CRY*, *CLOCK*, and *BMAL1* proteins having a function of regulations in the transcription/translation process. The heterodimer (*BMAL1/CLOCK*) complex inhibiting or repressing the *PER/CRY* genes activity in the nucleus region where the monitoring feedback loop formation is completed after the complex (*BMAL1/CLOCK*) formation which regulates the transcription of *Rev-erba* and *Rora* (nuclear receptors)[25].

Findings regarding the circadian rhythm seem to be initiated by both transcriptional and post-transcriptional mechanisms which induce gene expression [26, 27]. The transcription of *PER* and *CRY* genes is initiated by two transcription factors *CLOCK* and *BMAL1 / ARNTL*. After reaching the grave concentration the *PER / CRY* reduces the effect of *CLOCK/ BMAL1* facilitated initiation of their particular genes in a negative feedback loop. It was found that both complex *PER / CRY* and *CLOCK/ BMAL1* intricate with each other and bound to chromatin. The regular daily oscillations in clock gene is contributed by protein degradation, phosphorylation, and nuclear entry [28]. The regulation of *CRY* and *PER* gene expression generates genetic and biochemical evidence [29] but the regulation of the *CLOCK/ BMAL1* gene is very much less known. Various studies reported that *PER* and *CRY* gene establishes a positive feedback loop in the process of *BMAL1* transcription [28–30]

REV1 (REV1-DNA directed polymerase) is a nuclear receptor that acts as a transcriptional repressor in the circadian pathway, where activates and inhibits the transcription of the *BMAL1* gene [31]. The *BMAL1* transcription is regulated by *REV-ERB* alpha, thus it acts as a connector link through which components of negative and positive limb constitute to form a molecular link. It determines the length of the period and phase-shifting properties of the biological clock [30]. It was proved that *BMAL1* deficient cells due to DNA damage lead to arrest in cell cycle and reveal a possible modulatory effect on tumor suppressor genes i.e. *P53*. It has been reported that the knockdown of the *BMAL1* gene induces cell growth, reduced programmed cell death which appears to play a role in carcinogenesis [32].

Our study is the first replicative case-control association study of clock genes. Jin et al [20] reported that the gene expression of the *BMAL1* gene has been controlled by *cMYC* where the overexpression of *cMYC* leads to the downregulation of *BMAL1*. So, it has been suggested that *BMAL1* gene variants were significantly associated with the risk of ovarian cancer. The circadian gene variants are associated with prostate cancer which was reported in the GWAS study [33]. In some populations of the world, it has been reported that night workers have disturbed biological clocks where the findings of various studies proved the night working women's and men's are more prone to cancers (Breast, ovary, and prostate cancer) [19, 33–35]. Our results indicated that *BMAL1* and *REV1*: rs475715 & rs3792152 both intronic variants, were associated with ovarian cancer risk. Functional prediction implicated that rs47515 has direct eQTL effect and regulates the expression of *C2orf15*, *LIPT1*, *LYG1*, *MITD1*, *REV1*, *TSGA10*, and *TXNDC9* genes, whereas rs3792152 regulates the *RN7SKP151* & *BTBD10* with *BMAL1* gene. These findings suggest that it is located within a region that directly affects expression potentially through the modulation of the histone markers in the enhancer region. Yeh et al, 2014 [36] proved that in the ovarian cancer cell (in CP70 and MCP2) the H3K27 (histone mark) is supplemented in the promoter region of *ARNTL / BMAL1* gene, Whereas the presence of inhibitor (GSK126) of EZH2 region reestablished the expression of *ARNTL/ BMAL1* gene in ovarian cancer cells (in CP70 and MCP2). They also confirmed that there is a sensitivity of chemotherapy drug (cisplatin) in ovarian cancer cells after increasing the expression of the *ARNTL / BMAL1* gene. With these findings, it was confirmed that *BMAL1 / ARNTL* may act as a tumor suppressor by regulating the p53 tumor suppressor pathway in

ovarian cancer [20, 36]. So, our results climax the implication of circadian rhythm gene variation in ovarian cancer susceptibility and suggest an early role for the *BMAL1* and *REV1* gene in ovarian cancer pathogenesis.

Our results also suggest that the circadian gene variant may play a significant role in the etiology of ovarian cancer. From the literature survey, the identification of a significant association between circadian genes and ovarian cancer is still unpredictable. To the best of our knowledge, the case-control association studies between circadian gene variants and ovarian cancer were investigated in very few studies (20,25–29). Few of them have found an association with ovarian cancer and other cancers at the variant level [20, 37–39]. Even though various previous research studies have implicated circadian genes in the progression of cancers in women. Nevertheless, our study possibly requires more variants of the circadian rhythm pathway to highlight significant associations between certain circadian genes and the risk of aggressive ovarian cancer.

Conclusion

This study fortifies the current indication supporting the premise of a link between circadian rhythm genes and ovarian cancer risk. Additional studies with larger sample size as well as functional validation of risk associated gene variants is warranted to confirm those findings. Besides these it has been reported that women working during nightshifts are associated with several cancer but its probable role in finding the association between circadian genes and ovarian cancer risk should not be justified till date so this study proposes the need for further studies to investigate the association of carcinogenic effects of circadian disruption in relation with environmental factors, such as night-workers, regular exposure to stressed conditions, irregular diet patterns, and electromagnetic (EM) waves, which disturb circadian rhythm or biological clock by fluctuating the melatonin levels.

Abbreviations

eQTL: expression quantitative trait locus.

M.A.F: Minor allele frequency

LD: Linkage disequilibrium

Declarations

Availability of data and materials

Correspondence and requests related to manuscript should be addressed to R.S or R.K. There is no any copyright material in this manuscript. The data has been incorporated is a result of analysis.

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Author contribution

R.S, R.K and S.V planned the study. S.V, A.B, G.R.B, B.S and D.B collected the samples. S.V, R.S, A.B and GRB performed experiment in lab. S.V analyzed the results and drafted manuscript. R.S, R.K, A.B, S.S, R.A.Q, H.R and G.C provided critical comments regarding manuscripts. A.W, J.S and H.R provided samples for the study. All authors read and approved the final manuscript.

Ethical Approval

This study was approved by the Institutional Review Board committee of SMVDU with wide reference no. SMVDU/IERB/14/28. Experimental protocols conducted in this study strictly followed the guidelines set by the Institutional Ethical Review Board (IERB) SMVDU.

Competing interests

On behalf of all authors declare that they have no competing interests.

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