Period3 Structural Variation: A Circadian Biomarker Associated with Breast Cancer in Young Women

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Abstract

Circadian disruption has been indicated as a risk factor for breast cancer in recent epidemiologic studies. A novel finding in circadian biology is that genes responsible for circadian rhythm also regulate many other biological pathways, including cell proliferation, cell cycle regulation, and apoptosis. Therefore, mutations in circadian genes could conceivably result in deregulation of these processes and contribute to tumor development, and be markers for susceptibility to human cancer. In this study, we investigated the association between an exonic length variation in a circadian gene, *Period3* (*Per3*), and breast cancer risk using blood samples collected from a recently completed breast cancer case-control study in Connecticut. There were 389 Caucasian cases and 432 Caucasian controls included in our analysis. We found that the variant *Per3* genotype (heterozygous + homozygous 5-repeat alleles) was associated with an increased risk of breast cancer among premenopausal women (odds ratio, 1.7; 95% confidence interval, 1.0-3.0). Our finding suggests that the circadian genes might be a novel panel of potential biomarkers for breast cancer and worth further investigation. (Cancer Epidemiol Biomarkers Prev 2005;14(1):268–70)

Introduction

Genetic determinants have been found to be responsible for a fundamental biological phenomenon: a universal 24-hour oscillation in biochemical, physiologic, or behavioral processes. Transcription-translation feedback loops among products of circadian genes synchronize with the environment to generate endogenous rhythms and regulate molecular clockwork in the suprachiasmatic nuclei (the mammalian central pacemaker) and peripheral tissues. These circadian rhythms affect expression of about 2% to 10% of mammalian genes (1). More importantly, emerging data have shown that circadian genes are also involved in regulating cell proliferation and apoptosis by controlling expression of tumor suppressor genes and cell cycle genes, as well as genes that encode the caspases and transcription factors (2). Given a potential role of circadian genes as tumor suppressors, genetic polymorphisms in the circadian genes have been speculated to be associated with increased susceptibility to human cancers (2), which was first stated explicitly for breast cancer by Stevens and Rea (3).

One of the circadian genes, *Period* (*Per3*), belongs to the *Period* gene family that is a central component in the clockwork mechanism. The *Per3* gene has a structural variation that is a polymorphic repeat region with four or five copies of a 54-bp repetitive sequence in the exon 18 (GenBank accession no. AB047686). This length variation results in insertion/deletion of 18 amino acids and has been found to be associated with delayed sleep phase syndrome and diurnal preference (4, 5). A missense polymorphism in the *Per3* gene (647 Val/Gly) was also found to be associated with self-reported morningeveningness scores (6). These findings suggest an impact of functional genetic variations in the *Per3* gene on circadian rhythm. Recently, functional disruption of these circadian genes has been associated with cancer through the regulation of cell proliferation and apoptosis. For example, loss of the *Per2* gene can activate c-Myc signaling pathways leading to genomic instability and cell proliferation. *Per2* dysfunction can also impair *p53*-mediated apoptosis and consequently result in genomic instability and the accumulation of damaged cells (2). Thus, circadian genes may be connected to fundamental cellular processes that affect cancer risk. In this project, we explored the association between this structural polymorphism in the *Per3* gene and breast cancer risk using a breast cancer case-control study.

Materials and Methods

Study Population. We used the blood samples collected from a recently completed breast cancer case-control study in Connecticut. Detailed information about study population was described elsewhere (7). Briefly, the cases were histologically confirmed breast cancer patients from New Haven County and Tolland County, Connecticut. The cases, aged 30 to 80 years old, had no previous diagnosis of cancer except for nonmelanoma skin cancer. For New Haven County, eligible cases were identified from the major hospital of the county, the Yale-New Haven Hospital, through the computer database system at the Department of Surgical Pathology. Controls were also randomly selected from the computer database system from women who were histologically confirmed without breast cancer. The participation rates were 77% for cases and 71% for controls in New Haven County. For Tolland County, because there was no major county hospital in this county, newly diagnosed breast cancer cases were identified from area hospital records by the Rapid Case Ascertainment system at the Yale Comprehensive Cancer Center. Controls from Tolland Country were recruited through random digit dialing methods for those under age 65 and randomly selected from Health Care Financing Administration files for those aged 65 and over. The participation rates were 74% for cases and 64% for controls in Tolland County.

The study pathologist reviewed all the pathologic diagnoses for breast cancer patients and benign breast disease controls. Breast carcinomas were classified as carcinoma *in situ*, invasive ductal, or lobular carcinoma, and were staged according to the American Joint Committee on Cancer (AJCC) staging system (8).

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Interviewing. Informed consent was obtained from all study participants before the collection of epidemiologic data by personal interview. The 45-minute interview was administered by trained interviewers following institutional guidelines for human subjects. Data on smoking habits, alcohol consumption, and hormone replacement therapy of case and control subjects were obtained. Other information was also collected, including menstrual and reproductive factors (age at menarche, age at first pregnancy, age at menopause, parity, lifetime lactation history), family history of breast cancer, lifetime occupational history, body mass index, hair dye use, residence history, as well as dietary intakes of fat, fiber, and soy products.

Menopausal status was assessed at the time of diagnosis. Women with hysterectomy or bilateral oophorectomy were considered as postmenopausal women, whereas very few women with dubious menopausal status were considered as missing data. At the completion of the interview, blood was drawn into sodium-heparinized tubes for DNA isolation and subsequent molecular analysis.

***Per3* Genotyping.** Genomic DNA was extracted using standard method and PCR assay was used to determine the length polymorphism in *Per3*. The PCR primers used were forward, 5′-TGGCAGTGAGAGCAGTCCT-3′, and reverse, 5′-AGTGGCAGTAGGATGGGATG-3′. PCR was performed in a 25-μL volume made up of 1 μL (20 ng) diluted genomic DNA, 5 μL of primer mix (1.25 μmol/L), 1 μL 10 mmol/L deoxynucleotide triphosphate mix, 2.5 μL 10× buffer [500 mmol/L KCl, 100 mmol/L Tris-HCl (pH 9.0), 1% Triton X-100], 12 μL distilled water, 2.5 μL of 25 mmol/L $MgCl_2$, and 1 μL Taq polymerase (5 units/μL). A positive DNA control and a negative water control were included with each PCR plate of samples. The PCR cycling conditions were 3 minutes at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C, with a final step at 72°C for 3 minutes to allow for the complete extension of all PCR fragments. The products were resolved and separated for 30 minutes at 220 V on a 2% agarose gel stained with ethidium bromide. After electrophoresis, homozygous alleles with 5 repeats were represented by a DNA band with size at 247 bp. Whereas homozygous alleles with 4 repeats were represented by a DNA band with size at 193 bp, heterozygotes displayed a combination of both alleles (247 and 193 bp). Approximately 5% of the samples were duplicated to assure quality control in genotyping; two reviewers separately did genotype scoring to confirm results.

Statistical Analysis. All statistical analyses were done using STATA statistical software. Only Caucasians were included in the analyses, because there were so few study subjects for other ethnic groups. The study subjects with homozygous 4-repeat allele were used as reference group in the analyses. Odds ratios (OR) with 95% confidence intervals (CI) were calculated to estimate the relative risk associated with the variant genotypes. The adjusted odds ratios were calculated by logistic regression with control for age (as a continuous variable), family history of breast cancer, family income (<$11,000, $11,000-$20,000, >$20,000, unknown), body mass (as a continuous variable), age at menarche, age at first birth, and county.

Results

This study included 389 breast cancer cases and 432 controls. All of our study subjects were Caucasians. The *Per3* genotype distributions for cases and controls are shown in Table 1. Overall, the cases exhibited a slightly higher frequency of the homozygous 5-repeat genotype (9.25%) than the controls (6.48%). Genotyping results from all of the duplicated samples had 100% agreement. The distribution of genotypes in the controls deviated from the expected Hardy-Weinberg values because of a deficiency of the homozygous 5-repeat allele ($P = 0.04$).

Overall, we found a slightly increased breast cancer risk associated with the homozygous 5-repeat allele (OR, 1.5; 95% CI, 0.9-2.5); however, this association was not statistically significant after adjustment for age, family history of breast cancer, family income, body mass, age at menarche, age at first birth, and county (Table 1).

Among premenopausal women, an elevated breast cancer risk was associated with both homozygous 5-repeat allele (OR, 1.8; 95% CI, 0.6-5.3) and heterozygous alleles (OR, 1.7; 95% CI, 1.0-3.0). When we combined the variant 5-repeat alleles (heterozygous + homozygous), we observed a significant 1.7-fold (95% CI, 1.0-3.0) elevated breast cancer risk in women with variant alleles compared with those having homozygous 4-repeat allele.

Among postmenopausal women, a similar association was not evident. We only detected a slightly increased risk (OR, 1.4; 95% CI, 0.7-2.6) associated with homozygous 5-repeat allele after adjustment.

Discussion

This study shows that the structural variation in the *Per3* gene may affect breast cancer risk especially among young women, suggesting a potential role of circadian genes in breast tumorigenesis. Our results are consistent with findings from previous animal model studies, which showed that mice with mutations in the circadian gene *Per2* were more prone to tumor development and deficiencies in DNA damage responses (9). Functional genetic variations may dysregulate protein expression and activity of these circadian genes, and consequently disrupt circadian rhythm that might be associated with tumor development. In support of this speculation, animal model studies have indicated that circadian disruption from constant light exposure can accelerate breast epithelial stem-cell proliferation, alter mammary-gland development, and increase the formation of chemically induced mammary tumors (10). However, a later experiment found a reduced mammary tumor burden in rats exposed to constant light (11); the difference between the two experiments is that constant light began *in utero* in one (10), and at age 26 days in the other (11). In addition, disruption in the circadian rhythm was associated with accelerated growth of malignant tumors in mice, supporting a role of the circadian clock in tumor progression (12).

Table 1. *Per3* genotypes and breast cancer risk stratified by menopausal status

Genotypes*	Cases, *N* (%)	Controls, *N* (%)	OR† (95% CI)
Overall			
−/−	180 (46.2)	206 (47.7)	1.0
+/−	173 (44.5)	198 (45.8)	1.1 (0.8-1.4)
+/+	36 (9.3)	28 (6.5)	1.5 (0.9-2.5)
+/− and +/+	209 (53.8)	226 (52.3)	1.1 (0.8-1.5)
Premenopausal Women			
−/−	33 (37.5)	76 (51.0)	1.0
+/−	47 (53.4)	64 (43.0)	1.7 (1.0-3.0)
+/+	8 (9.1)	9 (6.0)	1.8 (0.6-5.3)
+/− and +/+	55 (62.5)	73 (49.0)	1.7 (1.0-3.0)
Postmenopausal Women			
−/−	147 (48.8)	130 (45.9)	1.0
+/−	126 (41.9)	134 (47.4)	0.9 (0.6-1.2)
+/+	28 (9.3)	19 (6.7)	1.4 (0.7-2.6)
+/− and +/+	154 (51.2)	153 (54.1)	0.9 (0.7-1.3)

*−/− Homozygous 4-repeat allele; +/− heterozygous alleles; +/+ homozygous 5-repeat allele.
† Adjusted for age, family history of breast cancer, family income, body mass, age at menarche, age at first birth, and county.

Associations between circadian disruptions and breast cancer have also been observed in epidemiologic studies of human shift workers, which were based on the idea that light exposure at night would disrupt circadian rhythms (3, 13–15). Those studies found that breast cancer risk increases with the number of years that individuals spent working at night. On the other hand, breast cancer risk has been reported to be lower in blind women compared with sighted women (16). Reduction of breast cancer risk was also reported to be associated with the degree of visual impairment in a follow-up study in Finland (17). These previous findings together with evidence from our molecular epidemiologic study suggest that both environmental and genetic factors involved in circadian rhythms may play a role in breast tumorigenesis.

A possible molecular mechanism of functional impact of the *Per3* length variation might be explained by the location of this variation, which is involved in the phosphorylation process. Stability and nuclear translocation of the Per3 protein are regulated by phosphorylation, which is conducted by another circadian protein casein kinase I . Phosphorylation by casein kinase I is enhanced by prephosphorylation of a lead serine or threonine residue in the recognition motif. The decreased number of amino acid residues as phosphorylation substrates in the shorter variant of the Per3 protein possibly has a functional impact on phosphorylation-dependent activity. In fact, studies from delayed sleep phase syndrome and diurnal preference have suggested a functionality of this *Per3* length variation in circadian rhythm (4, 5). However, the distribution of genotypes for the *Per3* polymorphism in our study deviates from the expected Hardy-Weinberg values ($P = 0.04$). This departure might be due to the relatively small sample size of our study because the frequency of the homozygous 5-repeat allele is as low as 6.48% in the controls (28 of 432). With such a low frequency rate, number changes in this group may have a profound impact on the test of Hardy-Weinberg equilibrium.

The association between this *Per3* length variation and breast cancer risk is more evident among young women compared with old women in our study. One reason accounting for this finding might be that many more causes in old women would attenuate any estimate of relative risk. Compared to young women, old women have longer and higher cumulative exposures to environmental factors in addition to inherited genetic risk factors. It has long been suggested that breast cancer occurrence among young women is more likely related to inherited risk, whereas the disease among old women may be more likely related to environmental exposures. Therefore, genetic risk factors should be more evident in young women than in old women. Given the fact that there is substantial inherited risk of breast cancer especially in young women that cannot be explained by mutations in *BRCA1* and *BRCA2* (18), other genes, such as circadian clock genes that have a role in many fundamental biological processes, might be promising candidates accounting for part of the inherited breast cancer risk.

Different findings between pre- and postmenopausal women in our study also suggest that circadian gene products may interact with female hormones in breast tumorigenesis, although the underlying mechanisms are not clear now. High expression of *mPer3* has been found in hypothalamic regions, which provides hormonal regulation, suggesting a role of *mPer3* in the daily rhythmic secretions of hormones. Also, rhythmic expression levels of *mPer3* were found outside the suprachiasmatic nucleus in the organum vasculosum lamina terminalis, which is involved in the daily surge rhythms of luteinizing hormone and follicle-stimulating hormone (19). Moreover, hormone changes associated with menopausal status may play an important role in breast cancer. For example, differences in breast cancer outcome following screening for pre- and postmenopausal women have been observed (20, 21). In addition, a few studies have found a relationship between menstrual cycle timing and outcome of breast cancer resection (22–24). Therefore, it is plausible that menopausal status may be an important factor in the relationship between the *Per3* polymorphism and breast cancer risk.

In summary, our study shows that genetic variations in circadian genes may confer inherited susceptibility to breast cancer, which is also indirectly supported by results from observational epidemiologic studies. The sample sizes limit the analyses to explore other potential risk factors in this study, so large molecular epidemiologic studies are warranted to further examine this functional *Per3* polymorphism associated with breast cancer in multiethnic groups. Nevertheless, genetic polymorphisms in circadian genes may be a promising biomarker of an individual's susceptibility to breast cancer, given the central role of the circadian clock mechanisms in cell proliferation, cell cycle regulation, and apoptosis throughout organisms.

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