

The Core Circadian Gene *Cryptochrome 2* Influences Breast Cancer Risk, Possibly by Mediating Hormone Signaling

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Abstract

As transcriptional regulators, circadian genes have the potential to influence a variety of biological pathways, including many cancer-related processes. *Cryptochrome 2* (*CRY2*) is essential for proper circadian timing and is a key component of the circadian regulatory feedback loop. Here, we report findings from genetic, epigenetic, loss-of-function, and transcriptional profiling analyses of *CRY2* in breast cancer. Six single-nucleotide polymorphisms in *CRY2* were identified for genotyping in a case-control population ($n = 441$ cases and $n = 479$ controls), and three single-nucleotide polymorphisms (rs11038689, rs7123390, and rs1401417) were significantly associated with postmenopausal breast cancer risk, with significant effect modification by menopausal status [dominant model for rs11038689: odds ratio (OR), 0.71; 95% confidence interval (95% CI), 0.51-0.99; P for trend = 0.028; homozygous variants for rs7123390: OR, 0.44; 95% CI, 0.22-0.86; P for trend = 0.028; and rs1401417: OR, 0.44; 95% CI, 0.21-0.92; P for trend = 0.017]. Interestingly, this association was only evident in women with estrogen and progesterone receptor (ER/PR)-negative breast tumors but not with ER/PR-positive tumors. Breast cancer patients also had significantly higher levels of *CRY2* promoter methylation relative to controls, which is consistent with tissue array data showing lower levels of *CRY2* expression in tumor tissue relative to adjacent normal tissue. Furthermore, *in vitro* analyses identified several breast cancer-relevant genes that displayed altered expression following *CRY2* knockdown. These findings suggest a role for *CRY2* in breast tumorigenesis and provide further evidence that the circadian system may be an important modulator of hormone-related cancer susceptibility. *Cancer Prev Res*; 3(4): 539–48. ©2010 AACR.

Introduction

The human circadian rhythm is a fundamental aspect of human physiology, and a wide range of biological processes are influenced by the circadian clock, including body temperature, energy metabolism, hormone secretion, and sleep-wake cycles (1). Circadian disruption is associated with a variety of adverse effects, including metabolic disruption, the promotion of oxidative stress, and alterations in immune function (2). Increasing evidence also suggests that the circadian system may play a critical role in various cancer-related processes (3, 4), and the IARC recently concluded that shift work that involves circadian disruption is “probably carcinogenic to humans” (5).

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At the molecular level, the maintenance of circadian rhythm involves a complicated interplay between environmental and endogenous cues, and many circadian processes are regulated by a relatively small set of core circadian genes (6). These genes form a negative and positive feedback system, which allows for fairly tight control of circadian gene expression, thereby ensuring that circadian gene products will be active during the appropriate phase of the circadian cycle (7–9). Recent evidence indicates that several of the core circadian genes are involved in transcriptional regulation, and as many as 2% to 10% of all mammalian genes may be regulated to some degree by the circadian oscillatory mechanism, suggesting the potential for broad consequences of disruptions to this system (10–12).

One such core circadian gene, *cryptochrome 2* (*CRY2*), is essential to the maintenance of circadian rhythm through its role in the negative arm of the feedback loop and may function more broadly as a transcriptional repressor (13–17). *CRY2* has also been shown to be involved in cellular proliferation, including roles in DNA damage checkpoint control (18) and regulation of genes important for cell cycle progression (19, 20). The role of cryptochromes in DNA damage response and susceptibility to carcinogenesis remains controversial, however, as *Cry1*^{-/-}, *Cry2*^{-/-} transgenic mice did

not display a cancer-prone phenotype in response to ionizing radiation exposure (19). Here, we report findings from genetic and epigenetic epidemiologic analyses of *CRY2* in breast cancer, as well as a loss-of-function investigation into the effects of *CRY2* knockdown on breast cancer-relevant gene expression.

Materials and Methods

Study subjects

The study population consists of subjects enrolled in a previous breast cancer case-control study conducted in Connecticut. Details about subject recruitment and participant characteristics have been described in previous publications (21, 22). Briefly, cases were incident, histologically confirmed breast cancer patients (International Classification of Diseases for Oncology, 174.0-174.9) between the ages of 30 and 80 with no previous diagnosis of cancer other than nonmelanoma skin cancer, who were alive at the time of the interview. Cases were identified either from computerized patient information at Yale-New Haven Hospital (YNHH) in New Haven County, Connecticut, or from nearby Tolland County, Connecticut, via hospital records by the Rapid Case Ascertainment Shared Resource at the Yale Cancer Center. YNHH controls were patients at YNHH who underwent breast-related surgery for histologically confirmed benign breast diseases. Tolland County controls were identified either through random digit dialing (for subjects younger than 65 y) or through the Health Care Finance Administration files (for subjects age 65 y and older). After obtaining approval from each participant's hospital and physician, potential subjects were contacted by letter and then by telephone, and those who agreed to participate were interviewed by a trained interviewer. Among YNHH subjects, participation rates were 71% for controls and 77% for cases, and among Tolland County subjects, participation rates were 61% for controls and 74% for cases. A standardized, structured questionnaire was used to obtain several participant characteristics, including family history of cancer, reproductive history, diet, and demographic factors. At the conclusion of the interview, blood was drawn into sodium-heparinized tubes and DNA was isolated from peripheral blood lymphocytes (PBL) for use in genotyping and methylation analyses. Estrogen receptor (ER) and progesterone receptor (PR) status was determined immunohistochemically at YNHH, as previously described (23), with an H score of >75 considered receptor positive. A total of 441 cases (93%) and 479 controls (95%) had DNA samples available for genetic analysis in the current study. The mean age of cases was 57 y, whereas controls had a mean age of 55 y. Both cases and controls were predominantly Caucasian (91% and 92%, respectively), with very few African Americans (6% and 6%) or other races (3% and 2%). With the exception of menopausal status (cases were 76.6% postmenopausal compared with 65.6% in controls; $P < 0.001$), no measured factor differed significantly by case-control status.

Single-nucleotide polymorphism selection and genotyping

Single-nucleotide polymorphisms (SNP) were identified in the HaploView interface of the HapMap genome browser, Release 22,⁴ using the Tagger algorithm (24). Applying the pairwise tagging approach, which provides a list of the minimum number of SNPs that can represent all of the markers in a given region at a specified level of correlation, five SNPs (rs11038689, rs7123390, rs2292912, rs10838524, and rs11605924) with minor allele frequencies of >0.1 were identified as representative of all variation found within the exonic and intronic regions of the *CRY2* gene, with $r^2 \geq 0.8$. One additional intronic SNP (rs1401417), which had been previously identified as significantly associated with prostate cancer risk (25), was also included in the genotyping pool.

Genotyping for all SNPs was done at Yale University's W.M. Keck Foundation Biotechnology Research Laboratory using the Sequenom MassARRAY multiplex genotyping platform (Sequenom, Inc.) according to the manufacturer's protocol. Duplicate samples from 100 study subjects and 40 replicate samples from each of two blood donors were interspersed throughout each batch for all genotyping assays. The concordance rates for quality control samples were >95% for all assays. All genotyping calls, including quality control data, were rechecked by different laboratory personnel, and genotyping scores were reproduced with 100% accuracy.

CpG island identification and methylation analysis

Using the CpG Island Searcher web tool,⁵ one CpG island was identified that spans the promoter region, the first exon, and part of the first intron of the *CRY2* gene (-450 to +750). The MethPrimer program⁶ was then used to design methylation-specific PCR primers within the identified CpG island region. To distinguish methylated and unmethylated DNA sequences, genomic DNA samples isolated from PBLs were bisulfite treated using the EZ DNA Methylation kit (Zymo Research) according to the manufacturer's protocol. On bisulfite treatment, unmethylated cytosines are converted into uracil, whereas methylated cytosines remain unchanged. After the conversion, the presence of methylation was determined by quantitative PCR using primers specific to the methylated or unmethylated sequence, and the Power SYBR Green kit (Applied Biosystems) according to the manufacturer's protocol. The primer sequences used to detect unmethylated DNA were GTTTGTGGA-TAGTTTTAGTTTGT (forward) and CACCTAACAAAT-TAACCCAAAAACA (reverse), and the primers for methylated DNA were GTTTGC GGATAGTTTTAGTTTGC (forward) and CCTAACGATTAACCCAAAAACG (reverse).

⁴ http://www.hapmap.org/cgi-perl/gbrowse/hapmap22_B36

⁵ <http://www.cpgislands.com/>

⁶ www.urogene.org//methprimer

A methylation index (MI) was determined for each subject using the following formula: $MI = [1/(1 + 2^{-(CT_u - CT_{me})})] \times 100\%$, as previously described (26), where CT_u is the average cycle threshold obtained from duplicate quantitative PCRs using the unmethylated primers and CT_{me} is the average cycle threshold obtained using the methylated primers. Because radiotherapy and chemotherapy can affect DNA methylation, only patients from the breast cancer population who had not undergone these treatments were included in this portion of the analysis ($n = 80$), along with an equal number of age-matched controls. Each PCR was done in duplicate using both the unmethylated and the methylated primers for a total of four reactions per subject. One or more of these reactions failed (no amplification or SD of >1 across replicates) in samples from four of the cases and were thus excluded, leaving a final sample of 76 cases and 80 controls.

Cell culture and treatments

Human breast adenocarcinoma cells (MCF-7) were obtained from the American Type Culture Collection. Cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 0.01 mg/mL bovine insulin, and 1% penicillin/streptomycin (Sigma-Aldrich). Small interfering RNA (siRNA) oligos targeting *CRY2* (sense, 5'-UGCUUCAUUCGUUCAUUGUUAAGCCG-3'; antisense, 5'-GGCUUACAUAUGAACGAAUGAAGCA-3') and a scrambled sequence negative control siRNA (sense, 5'-CUUCCUCUCUUCUCUCCCUUGUGA-3'; antisense, 5'-UCACAAGGGAGAGAAA-GAGGAAGGA-3') were designed and manufactured by Integrated DNA Technologies. Each oligo was diluted in Opti-MEM serum-free medium (Invitrogen), complexed with Lipofectamine RNAiMax transfection reagent (Invitrogen), and reverse transfected with $\sim 100,000$ cells in 12-well plates at a final concentration of 10 nmol/L. Forty-eight hours after transfection, cells were harvested and RNA was isolated for determination of *CRY2* knockdown and subsequent whole-genome expression microarray.

RNA isolation, knockdown assessment, and whole-genome expression microarray

RNA was extracted from cells treated with negative control and *CRY2*-targeting siRNA oligos using the RNA Mini kit (Qiagen), with on-column DNA digestion, according to the manufacturer's instructions for mammalian cells. The purified RNA was then used as a template for first-strand cDNA synthesis using the AffinityScript cDNA kit (Stratagene) with oligo(dT) primers. Quantitative real-time PCR was done using the Power SYBR Green PCR master mix (Applied Biosystems) with gene-specific primers and a standard thermal cycling procedure on an ABI 7500 instrument (Applied Biosystems). The primers used for *CRY2* amplification were ACCGGGACTCTGTCTACTG (forward) and GCCTGCACTGCTCATGCT (reverse). RNA quantity was normalized using *HPRT1* content, and *CRY2* silencing was quantified according to the $2^{-\Delta\Delta Ct}$ method. Biological replicates of RNA extracted from

CRY2 knockdown and normal cells were interrogated by whole-genome microarray (Agilent, Inc. 44K chip, done by MoGene, LC). All microarray data have been uploaded to the Gene Expression Omnibus database, and these data can be accessed by referencing accession number GSE14617. As the focus of the current study was on breast cancer and hormone signaling, we did a search for breast cancer-related genes using two online resources—the Breast Cancer and Estrogen Receptor Signaling pathway gene list from SABiosciences and the hormone-related genes identified by the Breast and Prostate Cancer Cohort Consortium⁷—to compile a targeted list of transcripts for analysis.

Expression analysis of *CRY2* in breast tumor tissues

We searched the ArrayExpress⁸ database (27) using the Atlas of Gene Expression function to identify an expression array comparison involving breast tumor tissue and adjacent normal tissue. Using the keywords Gene, "*CRY2*" and Conditions, "Breast Cancer", and setting the species filter to *Homo sapiens*, one array was identified (accession number E-TABM-276). The investigators of this study collected samples from breast tumors and the surrounding zones (1, 2, 3, and 4 cm away from the boundary of the tumor; ref. 28). For the current analysis, we focused on patients for which expression data were available for both tumor samples and distant normal tissue (3-4 cm away), and conducted a paired analysis of *CRY2* expression levels. Further details about tissue collection and the experimental protocol of this array are available at the ArrayExpress database or from the primary publication (28).

Statistical analysis

All statistical analyses were done using the SAS statistical software, version 9.1 (SAS Institute). *CRY2* knockdown was assessed using the $2^{-\Delta\Delta Ct}$ method with RNA content normalized to the housekeeping gene *HPRT1*. For the case-control analyses, allelic distributions for all SNPs were tested by goodness-of-fit χ^2 for compliance with Hardy-Weinberg equilibrium. Odds ratios (OR) and 95% confidence intervals (95% CI) were determined by unconditional multivariate logistic regression. Covariates included in the final model were age (continuous), race (Caucasian, African-American, other), family history of breast cancer in a first-degree relative (no or yes; in mother, sister, or daughter), body mass index (<21 , 21-24.9, 25+ kg/m²), smoking (ever or never), family income per person 10 y before diagnosis or interview ($<\$20,000$, $\$20,000$ - $24,999$, $\$25,000$ +, or unknown), age at menarche (<13 , 13-14, 15+ y), lifetime months of lactation (0, 1-10, 11+), number of live births (0, 1-3, 4+), and study site (New Haven County or Tolland County). Information on hormone use was not available

⁷ <http://epi.grants.cancer.gov/BPC3/gene.html>

⁸ www.ebi.ac.uk/arrayexpress

for this population and thus could not be controlled for. Other factors, including alcohol consumption and age at first birth, did not influence the effect estimates and were thus excluded from the final models. An interaction term was added to the model, and the *P* value associated with the Wald test comparing the parameter estimates for each association in premenopausal and postmenopausal women was used to assess whether the effect of each SNP was modified by menopausal status. Haplotypes were determined using the PHASE program (29), and haplotypes with a frequency of <3% were not individually analyzed but were maintained in the analysis population as controls. Of the 12 haplotypes identified, 98% of the sample chromosomes came from the 4 most frequent haplotypes, which were then analyzed by unconditional multivariate logistic regression, using all other haplotypes as the comparison group, and including the same group of covariates as the main-effects models. The sign rank test was used for comparison of *CRY2* expression in paired samples of tumor tissue and adjacent normal tissue, and expression level by ER/PR status was compared using the Wilcoxon two-sample test. Due to the multiple comparisons involved in the microarray analysis, adjustments were made to control for false-positive findings. A correction was applied to each observation using the Benjamini-Hochberg false discovery rate adjustment, as previously described (30), to obtain a new adjusted *P* value (*Q*).

Results

SNPs in *CRY2* are associated with postmenopausal breast cancer risk

Six SNPs were identified for genotyping, including five internal tagging SNPs identified through HaploView and one SNP that had previously been identified as significantly associated with prostate cancer risk (25). Genotyping failed for one SNP (rs10838524), which was thus excluded from further analysis. For each of the five remaining SNPs, compliance with Hardy-Weinberg equilibrium was examined among the controls, and no significant departures were observed ($P < 0.10$). ORs and 95% CIs were determined for each of the five remaining SNPs. A test of homogeneity indicated significant ($P < 0.05$) effect modification by menopausal status for three of these SNPs when comparing homozygous genotypes. As such, overall associations are stratified by menopausal status. For the three SNPs for which menopausal status was an effect modifier, significant associations were observed with postmenopausal breast cancer risk (rs11038689: dominant model OR, 0.71; 95% CI, 0.51-0.99; rs7123390: homozygous variant OR, 0.44; 95% CI, 0.22-0.86; rs1401417: homozygous variant OR, 0.44; 95% CI, 0.21-0.92; Table 1). A haplotype analysis of the five variants showed that only one haplotype, which contained the three significant variants from the main-effects model, was significantly associated with breast cancer risk in postmenopausal women (frequency, 22.5%; OR, 0.72; 95% CI, 0.56-0.94), whereas no haplotypes were significantly associated with premenopausal breast cancer (data not shown).

Note that despite the fact that each SNP was chosen using the haplotype-based Tagger algorithm (24), we found significant correlation among the three significant SNPs (Supplementary Table S1). As such, each of these SNPs may be serving as markers for the same causal SNP, and therefore, the three significant associations may be interpreted as approximating one single association.

In an attempt to further explore these associations in a separate population, we also analyzed a genome-wide association study of postmenopausal breast cancer initiated by National Cancer Institute's Division of Cancer Epidemiology and Genetics in collaboration with the Nurses' Health Study (31). This data set,⁹ termed the Cancer Genetic Markers of Susceptibility study, contains four markers within the genomic region of *CRY2*. None of these markers is significantly associated with breast cancer risk in this population. However, only one of the four markers, rs11605924, was also included in our study. The Cancer Genetic Markers of Susceptibility-estimated OR for homozygotes (1.02) was similar to our estimate among postmenopausal women (1.22), and both found no significant association with breast cancer risk. In addition, the minor allele frequency among the controls for this SNP matches very closely with the minor allele frequency among controls in our population (46.5% and 46.4%, respectively).

Association of *CRY2* and postmenopausal breast cancer risk by hormone receptor status

Because ER/PR status is an important clinical factor in predicting breast cancer prognosis and dictating treatment options, we did a separate analysis of each SNP with the population stratified according to joint ER/PR status, where known. Interestingly, the homozygous or combined heterozygous/homozygous variant genotypes for four of the five SNPs (rs11038689, rs11605924, rs7123390, and rs1401417) were significantly associated with breast cancer risk in women with ER/PR-negative tumors, but none was associated with ER/PR-positive disease (H score > 75; Table 2). Similarly, the *P* value for trend associated with increasing number of variant alleles was statistically significant ($P < 0.05$) for each of these four SNPs in the ER/PR-negative stratum, whereas no significant associations were observed among ER/PR-positive tumors despite the approximately equal number of subjects in each category. These data suggest that the effect of *CRY2* on breast cancer risk may be significantly influenced by the presence of endogenous hormones, and *CRY2* may be particularly relevant for ER- and PR-negative tumorigenesis. Note that due to sample size limitations, this analysis was done for postmenopausal women only.

⁹ <http://cgems.cancer.gov/data/>

Table 1. Association of *CRY2* SNPs with breast cancer risk by menopausal status

Genotype	Premenopausal			Postmenopausal			Interaction <i>P</i> *
	Cases (n)	Controls (n)	OR [†] (95% CI)	Cases (n)	Controls (n)	OR [†] (95% CI)	
rs11038689							
A/A	55	97	Reference	197	167	Reference	
A/G	37	53	1.31 (0.74-2.29)	101	116	0.74 (0.52-1.04)	0.100
G/G	7	5	2.95 (0.82-10.66)	13	20	0.54 (0.26-1.13)	0.044
A/G or G/G	44	58	1.43 (0.83-2.46)	114	136	0.71 (0.51-0.99)	0.038
Trend			<i>P</i> = 0.101			<i>P</i> = 0.028	0.018
rs11605924							
A/A	31	35	Reference	90	90	Reference	
A/C	43	76	0.64 (0.33-1.25)	138	145	0.91 (0.62-1.34)	0.316
C/C	24	44	0.69 (0.33-1.44)	86	68	1.22 (0.77-1.93)	0.097
A/C or C/C	67	120	0.66 (0.36-1.22)	224	213	1.00 (0.69-1.45)	0.158
Trend			<i>P</i> = 0.334			<i>P</i> = 0.410	0.104
rs2292912							
C/C	60	90	Reference	183	188	Reference	
C/G	28	57	0.71 (0.40-1.29)	104	95	1.17 (0.82-1.67)	0.254
G/G	11	8	1.75 (0.56-5.44)	28	22	1.67 (0.85-3.28)	0.495
C/G or G/G	39	65	0.80 (0.45-1.41)	132	117	1.23 (0.88-1.74)	0.437
Trend			<i>P</i> = 0.950			<i>P</i> = 0.129	0.437
rs7123390							
G/G	53	90	Reference	168	144	Reference	
G/A	35	58	1.03 (0.59-1.81)	125	126	0.84 (0.60-1.18)	0.503
A/A	8	7	2.05 (0.65-6.44)	15	28	0.44 (0.22-0.86)	0.029
G/A or A/A	43	65	1.13 (0.66-1.94)	140	154	0.77 (0.56-1.07)	0.215
Trend			<i>P</i> = 0.396			<i>P</i> = 0.028	0.065
rs1401417							
G/G	55	100	Reference	198	169	Reference	
G/C	35	55	1.27 (0.72-2.23)	104	116	0.77 (0.55-1.09)	0.180
C/C	7	5	2.87 (0.79-10.44)	12	23	0.44 (0.21-0.92)	0.017
G/C or C/C	42	60	1.39 (0.81-2.40)	116	139	0.72 (0.52-1.00)	0.056
Trend			<i>P</i> = 0.122			<i>P</i> = 0.017	0.016

*Wald test of equality of the parameter estimates for each association in premenopausal and postmenopausal women.

†Adjusted for age, race, family history of breast cancer, body mass index, parity, years of breast-feeding, age at menarche, and study site.

CRY2 promoter methylation is associated with breast cancer

To determine whether *CRY2* promoter methylation was associated with breast cancer risk, we did methylation-specific PCR for all patients who had not undergone radiotherapy or chemotherapy, as well as an equal number of controls frequency matched by age. Although only untreated cases could be used for methylation analysis, no differences were detected among several characteristics between untreated and treated cases (Supplementary Table S2). In both premenopausal and postmenopausal women, cases had higher MIs, although the difference was only statistically significant among postmenopausal women, where the mean MI among controls was 22.32% and the mean MI among cases was 30.59% (*P* = 0.049; Table 3). Although it is unclear how *CRY2* promoter methylation

influences gene expression, the fact that cases had a higher degree of methylation in this region implies that *CRY2* may operate as a tumor suppressor.

Decreased expression of *CRY2* in breast tumor tissue, especially in ER/PR-positive cancers

To determine whether *CRY2* expression is altered in breast tumor samples, we identified a transcriptional profiling array from the Atlas database, which examined gene expression in breast tumor tissue and adjacent normal breast tissue. As expected based on our methylation findings, *CRY2* levels were significantly lower in tumor tissue (mean normalized expression = 13.4) compared with adjacent normal tissue (mean normalized expression = 77.2; *P* = 0.023; Fig. 1). The tissue samples were also categorized according to their histology ("invasive carcinoma," "cystic

Table 2. Association of *CRY2* SNPs with postmenopausal breast cancer by ER/PR status

Genotype	Controls (n)	ER/PR-positive		ER/PR-negative	
		Cases (n)	OR* (95% CI)	Cases (n)	OR* (95% CI)
rs11038689					
A/A	167	38	Reference	38	Reference
A/G	116	21	0.82 (0.44-1.53)	13	0.48 (0.23-0.99)
G/G	20	2	0.36 (0.08-1.70)	1	0.21 (0.03-1.73)
A/G or G/G	136	23	0.74 (0.40-1.36)	14	0.44 (0.22-0.89)
Trend			0.200		0.018
rs11605924					
A/A	90	20	Reference	12	Reference
A/C	145	24	0.73 (0.36-1.51)	20	1.15 (0.50-2.63)
C/C	68	16	1.12 (0.49-2.55)	21	2.49 (1.03-5.99)
A/C or C/C	213	40	0.84 (0.43-1.65)	41	1.55 (0.72-3.30)
Trend			0.834		0.035
rs2292912					
C/C	188	37	Reference	31	Reference
C/G	95	18	0.88 (0.45-1.73)	19	1.15 (0.57-2.31)
G/G	22	7	2.03 (0.64-6.48)	3	1.05 (0.22-4.94)
C/G or G/G	117	25	1.00 (0.53-1.90)	22	1.14 (0.58-2.26)
Trend			0.558		0.765
rs7123390					
G/G	144	29	Reference	35	Reference
G/A	126	29	1.30 (0.70-2.42)	16	0.53 (0.27-1.06)
A/A	28	2	0.30 (0.06-1.40)	2	0.27 (0.06-1.27)
G/A or A/A	154	31	1.08 (0.59-1.97)	18	0.48 (0.25-0.93)
Trend			0.530		0.023
rs1401417					
G/G	169	39	Reference	40	Reference
G/C	116	21	0.79 (0.42-1.48)	14	0.50 (0.25-1.01)
C/C	23	2	0.31 (0.07-1.42)	2	0.39 (0.08-1.87)
G/C or C/C	139	23	0.70 (0.38-1.29)	16	0.49 (0.25-0.95)
Trend			0.129		0.040

*Adjusted for age, race, family history of breast cancer, body mass index, parity, years of breast-feeding, age at menarche, and study site.

change," and "normal" tissue) and hormone receptor status (ER and PR, positive and negative). For each of the histologic subgroups, we also compared *CRY2* expression in ER/PR-negative tumors with that in tumors with

either ER or PR expressed. In each case, ER/PR-negative tumors had higher *CRY2* levels, although this difference was only statistically significant in the cystic change subgroup (mean *CRY2* expression in ER- or PR-positive samples = 52.8; mean *CRY2* expression in ER/PR-negative samples = 113.1; $P = 0.027$).

Table 3. Methylation of the *CRY2* promoter by case/control and menopausal status

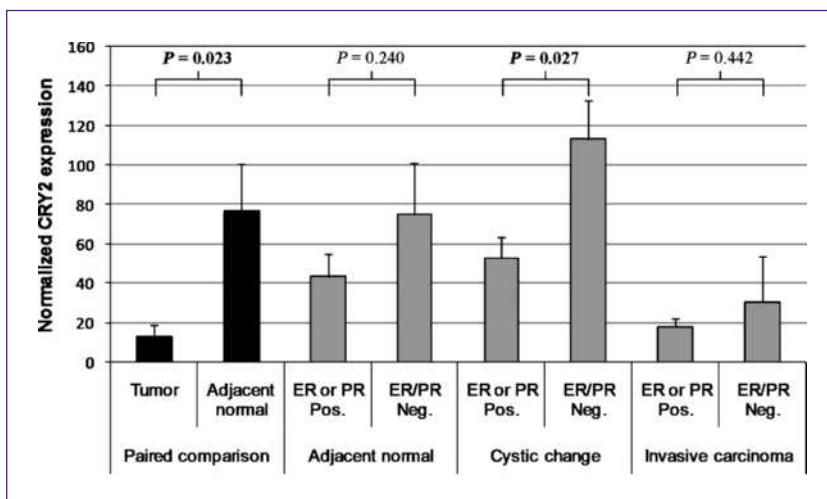
	Premenopausal		<i>P</i> *	Postmenopausal		<i>P</i> *
	MI (%)			MI (%)		
	Mean	SEM		Mean	SEM	
Controls	29.34	5.27		22.32	2.24	
Cases	34.93	8.16	0.552	30.59	3.50	0.049

**t* test comparing mean MI in cases and controls.

Breast cancer-relevant transcripts are influenced by *CRY2* knockdown *in vitro*

A whole-genome expression microarray revealed several breast cancer-related genes that displayed significantly altered expression in MCF-7 cells treated with *CRY2*-targeting siRNA oligos relative to cells treated with negative control siRNAs. To focus our analysis on the most highly relevant transcripts, we identified 131 unique breast cancer-related and hormone-related transcripts using online resources. Of these, 43 appeared at low intensity in one or both biological replicates of the microarray, were

Fig. 1. *CRY2* expression in breast tissue taken at mastectomy from a publicly available breast tumor tissue array. *CRY2* expression was compared in paired samples of tumor tissue and adjacent normal tissue (3–4 cm from the tumor boundary) extracted from the same patient. In addition, *CRY2* expression was compared in tissue expressing either ER or PR versus ER/PR-negative samples from each of three histologic subtypes. *CRY2* expression was lower in tumor tissue than in adjacent normal tissue, and ER/PR-negative samples had consistently higher *CRY2* levels than those expressing either ER, PR, or both, although this difference was only statistically significant in the cystic change histologic subtype.



thus deemed unreliable estimates of gene expression, and were excluded from further analysis. Twenty-two of the remaining 88 transcripts (25%) had significantly altered expression following *CRY2* knockdown, representing a variety of biological pathways including regulation of cell differentiation, proliferation, motility, angiogenesis, and apoptosis, in addition to sex hormone regulation and estrogen signaling (Table 4). Although it remains unclear why variants in *CRY2* might be especially relevant for ER/PR-negative tumors in postmenopausal women, these data represent an intriguing set of observations that warrant further investigation.

Discussion

Although recent evidence suggests an important role for circadian genes in transcriptional regulation and cancer-related processes, the extent to which individual genes may serve as risk or prognostic biomarkers for cancer has yet to be fully elucidated. Together with *CRY1* and the Period genes, *CRY2* forms the negative arm of the circadian feedback loop and is essential for proper maintenance of circadian timing. As such, *CRY2* has the potential to influence, directly or indirectly, the expression and availability of gene products in a variety of biological pathways (13, 32, 33). The results presented here suggest that variants in *CRY2* may significantly influence breast cancer susceptibility, which is consistent with recent observations from genetic association studies that showed that SNPs in *CRY2* are associated with risk of non-Hodgkin's lymphoma (34), and functional genetic variations in two other circadian genes, *PER3* and *NPAS2*, were significantly associated with breast cancer risk (35, 36).

An interesting aspect of the epidemiologic portion of this study was the effect of menopausal and hormone receptor status on the association between *CRY2* SNPs and breast cancer risk. Because ER/PR-negative tumors are more common among premenopausal women, the implication from these data is that menopausal status is likely a

primary modifier of the gene-disease association, independent from the effects of ER/PR status. However, the further observation that *CRY2* variants were most strongly associated with ER/PR-negative tumors may be of particular interest for future investigations, given that ER/PR-negative tumors tend to be more aggressive, are not generally treatable with selective ER modulators such as tamoxifen, and are thus associated with decreased survival (37). Although several previous studies have suggested that hormones, including estrogen, may influence the expression of genes in the circadian system (38–40), the mechanisms underlying the observed differences in the effect of *CRY2* SNPs on breast cancer risk remain unclear and warrant further investigation. Of note, we did not have information on hormone replacement therapy use in our population and were thus unable to control for it in the analysis. Although hormone replacement therapy use is associated with elevated risk of breast cancer, in order for this exposure to operate as a confounder in our study, it would have to be associated with *CRY2* genotypes. Although this is possible by chance, we feel that confounding by hormone replacement therapy use is unlikely to explain our results.

Compared with genetic studies of circadian genes in tumorigenesis, epigenetic changes, such as promoter methylation, remain a relatively unexplored area in the field of cancer research. The findings from our current study of *CRY2* are among the first evidence suggesting that promoter methylation status in circadian genes could be a potential biomarker for cancer susceptibility. Our results show that *CRY2* promoter methylation, which may lead to decreased gene expression, was elevated in breast cancer cases relative to controls. Several previous studies have also noted significant downregulation of circadian genes associated with various cancers. For example, the expression level of several circadian genes, including *CRY2*, was significantly diminished among those suffering from chronic myeloid leukemia compared with healthy individuals (41). Decreased expression of *CRY2* was also observed in hepatocellular

Table 4. Breast cancer–relevant transcripts that displayed significantly altered expression following *CRY2* knockdown

Gene ID	RefSeq	Description	Fold change	Q
<i>AKR1C3</i>	NM_003739	Sex hormone metabolism, proliferative signaling	1.95	0.0028
<i>BCL2</i>	NM_000633	Regulation of apoptosis, prognosis, and therapeutic response	-1.47	0.0487
<i>C3</i>	NM_000064	Estrogen signaling	2.09	0.0007
<i>CCND1</i>	NM_053056	Cell cycle regulation, tumor progression	1.51	0.0087
<i>CD44</i>	NM_000610	Cellular adhesion, tumor metastasis	1.58	0.0028
<i>CD47</i>	NM_198793	Induction of apoptosis	1.59	0.0255
<i>CDKN1A</i>	NM_000389	Cell cycle regulation	1.71	0.0001
<i>FAS</i>	NM_000043	Regulation of apoptosis, tumor progression, and prognosis	1.81	0.0003
<i>GNAS</i>	NM_080425	Regulation of apoptosis, proliferation, and tumor progression	1.90	0.0261
<i>HSPB1</i>	NM_001540	Regulation of estrogen signaling	1.79	0.0000
<i>IL6</i>	NM_000600	Promotion of tumor growth and invasion	8.17	0.0000
<i>IL6R</i>	NM_000565	Promotion of tumor growth and invasion	6.06	0.0385
<i>INHA</i>	NM_002191	Regulation of cell differentiation and proliferation	3.61	0.0000
<i>INHBA</i>	NM_002192	Regulation of cell differentiation and proliferation	2.00	0.0004
<i>KIT</i>	NM_000222	Proto-oncogene, cell growth and differentiation	-2.03	0.0000
<i>LHB</i>	NM_000894	Sexual reproduction, promotes mammary tumorigenesis	-1.85	0.0050
<i>RAC2</i>	NM_002872	Cell growth regulation, immune response, chemotaxis	-1.87	0.0016
<i>SERPINA3</i>	NM_001085	Estrogen signaling, serine protease inhibitor	1.63	0.0126
<i>TFF1</i>	NM_003225	Estrogen-inducible, promotes cell migration	3.48	0.0000
<i>TFF3</i>	NM_003226	Estrogen-inducible, promotes cell migration	-1.58	0.0023
<i>THBS1</i>	NM_003246	Cell-cell interactions, control of tumor metastasis	2.21	0.0000
<i>TNFAIP2</i>	NM_006291	Induced by tumor necrosis factor, angiogenesis	1.70	0.0086

NOTE: Fold change and false discovery rate–adjusted *P* values (Q) represent the mean of biological replicate microarray assays.

carcinomas, and this reduction was not caused by genetic mutations but by several factors, including promoter methylation (42). Together, these findings suggest that methylation changes, in addition to genetic variants in circadian genes, could serve as novel cancer biomarkers. However, despite these promising initial findings, further study is needed to confirm this association, as very few cases in our sample were untreated, leaving a fairly small sample size available for this portion of the study.

Because the methylation analysis was done using DNA samples isolated from PBLs, it remains unclear whether epigenetic changes measured in this surrogate tissue are an accurate reflection of the methylation status in breast tissue. A previous study has shown strong correlation between methylation in PBLs and colon tissue (κ statistic = 86.5%; $P < 0.0001$; ref. 43), and a large proof-of-principle study found significant associations between the level of PBL methylation in several ER- α target genes and breast cancer risk, highlighting the potential for epigenotyping to be useful in estimating risk, even when measured in peripheral blood cell DNA (44). If hypermethylation in the *CRY2* promoter region does in fact lead to diminished gene expression, our data indicate that *CRY2* may operate as a tumor suppressor, as increased methylation was associated with increased breast cancer risk. This suggestion is corroborated by the transcriptional profiling array, show-

ing that *CRY2* expression was lower in tumor tissue than in adjacent normal tissue. This array also showed that *CRY2* was elevated in ER/PR-negative breast tissue samples, which is in keeping with the general suggestion that variants in *CRY2* had the biggest effect on breast cancer risk in women with ER/PR-negative tumors.

Among the microarray findings, the strong induction of trefoil factor 1 (*TFF1*) in cells with reduced *CRY2* (fold change = 3.5; $Q < 0.0001$) is of particular interest. *TFF1* is strongly estrogen regulated, and aberrant expression of this transcript can influence several cancer-related pathways, including proliferation, apoptosis, anoikis, angiogenesis, and migration and invasion (45). In addition, although it has been suggested that *TFF1* encourages cell cycle delay at the G₁-S transition, it has also been shown to increase the levels of cyclin D1 (*CCND1*), a key stimulator of cell cycle progression (46). Interestingly, *CCND1* was also significantly induced following *CRY2* knockdown (fold change = 1.51; $Q = 0.009$). Although the nature of these relationships remains unclear, these data are consistent with the overall suggestion that *CRY2* may have tumor suppressor properties, and provide the intriguing suggestion that *CRY2* may be involved in hormone signaling, with the potential to regulate, directly or indirectly, transcripts with well-established relevance for breast tumorigenesis.

In summary, our study shows a genetic association of the circadian gene *CRY2* with breast cancer risk, which is potentially modified by menopausal and ER/PR status. Moreover, an epigenetic association between *CRY2* promoter hypermethylation and increased breast cancer risk was also detected, suggesting that *CRY2* may operate as a tumor suppressor. This role was further supported by an observed decreased expression of *CRY2* in breast tumor tissues and an *in vitro* loss-of-function analysis, which showed that several breast cancer-relevant transcripts displayed altered expression following *CRY2* knockdown. These data suggest a novel role for *CRY2* in hormone signaling and breast tumorigenesis and provide evidence supportive of the hypothesis that the circadian system may be an important modulator of breast cancer risk. However,

additional large studies will be required to further elucidate the role of *CRY2* in breast cancer susceptibility.

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No potential conflicts of interest were disclosed.

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