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Research Article

Clock-Cancer Connection in Non–Hodgkin's Lymphoma: A Genetic Association Study and Pathway Analysis of the Circadian Gene Cryptochrome 2

Aaron E. Hoffman,[1] Tongzhang Zheng,[1] Richard G. Stevens,[2] Yue Ba,[1] Yawei Zhang,[1] Derek Leaderer,[1] Chunhui Yi,[1] Theodore R. Holford,[1] and Yong Zhu[1]

[1]Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut and [2]Department of Community Medicine and Health Care, University of Connecticut Health Center, Farmington, Connecticut

Abstract

Circadian genes have the potential to influence a variety of cancer-related biological pathways, including immunoregulation, which may influence susceptibility to non–Hodgkin's lymphoma (NHL). However, few studies have examined the role of circadian genes in lymphomagenesis. The current study examined Cryptochrome 2 (*CRY2*), a core circadian gene and transcriptional repressor, as a potential circadian biomarker for NHL. We first performed genetic association analyses of tagging single nucleotide polymorphisms (SNP) in *CRY2* and NHL risk using DNA samples from a population-based case-control study (n = 455 cases and 527 controls). Three SNPs were found to be significantly associated with risk of NHL when combining all subtypes [dbSNP IDs, odds ratios (ORs), and 95% confidence intervals: rs11038689, OR, 2.34 (1.28–4.27), P = 0.006; rs7123390, OR, 2.40 (1.39–4.13), P = 0.002; and rs1401417, OR, 2.97 (1.57–5.63, P = 0.001)]. Each of these associations remained significant when restricting the analysis to B-cell cases and when further restricting to follicular lymphomas. An analysis of *CRY2* diplotypes confirmed these significant findings. To further determine the functional effect of *CRY2*, we silenced the gene *in vitro* and performed a whole genome expression microarray. A pathway-based analysis showed that genes significantly altered by *CRY2* knockdown formed networks associated with immune response and hematologic system development. In addition, these genes were predicted to have significant effects on several disease processes, including cancer (B-H $P = 3.75E^{-9}$) and hematologic disease (B-H $P = 8.01E^{-8}$). In conclusion, both genetic association and functional analyses suggest that the circadian gene *CRY2* may play an important role in NHL development. [Cancer Res 2009;69(8):3605–13]

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). Several observational studies indicate that individuals who do not maintain a normal sleep/wake cycle may be at increased risk for several cancer types, and after considering evidence from epidemiologic and experimental studies, the IARC recently concluded that shift work that involves circadian disruption is "probably carcinogenic to humans" (2). Although much of the current epidemiologic evidence has focused on breast cancer, it has recently been hypothesized that circadian disruption may also affect risk for non–Hodgkin's lymphoma (NHL), possibly through its influence on immunoregulation (3).

Limited indirect evidence suggests that genetic components of the circadian system may have a role in processes relevant for NHL tumorigenesis. For example, reduced expression of the circadian gene *PER2* has been detected in lymphoma cell lines and in samples drawn from patients with acute myeloid leukemia (4), and a recent genetic association study showed that a nonsynonymous polymorphism in the core circadian gene *NPAS2* is associated with decreased risk of NHL, especially B-cell lymphoma (5). In addition, several studies have established an important role for circadian rhythm in the maintenance of proper immune function. First, it has been shown that several key components of the immune system are under circadian regulation, with circadian rhythmicity present in nearly all aspects of immune response (6–11). Specifically, circadian rhythms have been observed in natural killer (NK) cells, which are an essential component of the innate immune system against infections and cancerous growth (12). Second, disruption of circadian rhythms can cause aberrant immune cell trafficking and abnormal cell proliferation cycles (13). Moreover, disruption of the circadian rhythms in NK cells and phagocytic activity has been observed in malignant melanoma cells, leading to a discoordination between the two immune system components that is not observed in healthy humans (14).

Overall, these preliminary studies suggest that circadian disruption has the potential to significantly affect a number of mechanisms that may influence NHL susceptibility, most notably through its role in influencing immune response. However, although immune dysfunction remains the only well-established risk factor for NHL (15, 16), immunodeficiency is seen only in a subset of NHL patients. As such, if an association between circadian disruption and lymphomagenesis can be firmly established, there remains the additional question of whether the relationship is maintained outside of pathways related to immune system function. Further study into these associations is therefore warranted and are apropos to investigations into the potential for circadian gene variants to serve as a novel panel of NHL risk biomarkers.

The current study investigates the role of the core circadian gene *CRY2* in NHL tumorigenesis. *CRY2* is essential to the maintenance of circadian rhythm through its role in the negative arm of the circadian feedback loop, and may have a broader regulatory role as a transcriptional repressor (17, 18). *CRY2* has also

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

Requests for reprints: Yong Zhu, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT 06520. Phone: 203-785-4844; Fax: 203-737-6023; E-mail: yong.zhu@yale.edu.

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been shown to be involved in cell cycle regulation, including roles in DNA damage checkpoint control (19) and regulation of genes important for cell cycle progression (20). Here, we report findings from an epidemiologic analysis of the association between genetic variants in *CRY2* and risk of NHL. In addition, we performed a whole genome expression microarray to determine the effect of *CRY2* silencing on the expression of cancer-related genes, and to determine whether *CRY2* influences biological pathways that may be relevant for lymphomagenesis.

Patients, Materials, and Methods

Case-control study of NHL. The study population has previously been described (21). Briefly, all participants were female residents of Connecticut, and cases were incident, histologically confirmed NHL (ICD-O, M-9590-9642, 9690-9701, 9740-9750) identified through Yale Cancer Center's Rapid Case Ascertainment between 1996 and 2000. Population-based controls younger than age 65 y were recruited by random digit dialing, and controls older than age 65 y were identified through Health Care Financing Administration files. Five-year age strata were constructed, and controls were frequency matched to cases by intermittently adjusting the number of controls selected from each stratum. Participation rates were as follows: 72% for cases, 69% for random digit dialing controls, and 47% for controls identified by health care financing records.

Data collection. The study was approved by Institutional Review Boards at Yale University, the Connecticut Department of Public Health, and the National Cancer Institute. Participation was voluntary, and written informed consent was obtained. Those who agreed were interviewed by trained study nurses either at the subject's home or at a convenient location, and after the administration of a questionnaire, subjects provided a 10-mL peripheral blood sample. Genomic DNA was isolated from peripheral blood lymphocytes for each study subject.

Single nucleotide polymorphism selection and genotyping. Single nucleotide polymorphisms (SNP) were identified using the Tagger algorithm (22), which is implemented in the Haploview interface (23) of HapMap's genome browser, Release 22.[3] Five SNPs (rs10838524, rs11038689, rs11605924, rs2292912, and rs7123390) were identified as representative of all variations found within the exonic and intronic regions of the *CRY2* gene using the CEU population returning SNPs with minor allele frequency of $\geq$0.2 and r^2 of $\geq$0.8. In addition, one intronic SNP (rs1401417) that had been identified as significantly associated with prostate cancer risk in a previous study (24) was also included in the genotyping pool. Genotyping for all SNPs was performed 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 2 blood donors were interspersed throughout each batch for all genotyping assays. Genotyping failed for one SNP (rs10838524). The concordance rates for QC samples were over 95% for the remaining assays. Genotyping call rates were as follows: 97.0% for rs11038689, 97.5% for rs11605924, 97.7% for rs2292912, 96.6% for rs7123390, and 97.8% for rs1401417. All genotyping scores, including quality control data, were rechecked by different laboratory personnel and the accuracy of each assay was confirmed.

Cell culture and treatments. Human breast adenocarcinoma cells (MCF-7) were used to determine the effect of *CRY2* knockdown on pathways related to lymphomagenesis. MCF-7 cells were chosen rather than cells derived from lymphoma tissue, as lymphoma cells would likely begin with aberrant immune signaling, thus causing difficulty in interpreting the effects of *CRY2* knockdown on immunoregulation. Cells were obtained from American Type Culture Collection and were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 0.01 mg/mL bovine insulin, and 1% penicillin/streptomycin (Sigma-Aldrich). siRNA oligos targeting *CRY2* (Sense, 5′-UGCUUCAUUCGUUCAAUGUUAAGCCGG-3′; Antisense, 5′-GGCUUAACAUUGAACGAAUGAAGCA-3′) and a scrambled sequence negative control siRNA (Sense, 5′-CUUCCUCUCUUUCUCUCCCUUGUGA-3′; Antisense, 5′-UCACAAGGGAGAGAAAGAGGGAAGGA-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 ~100,000 cells in 12-well plates at a final concentration of 10 nmol/L in growth medium without penicillin/streptomycin. Cells were harvested 48 h after transfection for subsequent analysis.

RNA isolation and quantitation. *CRY2* silencing efficiency was determined by qPCR of RNA samples isolated using the RNA Mini kit (Qiagen), with on-column DNA digestion, according to the manufacturer's instructions for mammalian cells. First-strand cDNA was synthesized from purified RNA using the AffinityScript cDNA kit (Stratagene) with oligo-dT primers. Quantitative real-time PCR conditions were prepared using a 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 as follows: L, ACCGGGGACTCTGTCTACTG; R, GCCTGCACTGCTCATGCT. *CRY2* knockdown was assessed using the $2^{-\Delta\Delta Ct}$ method with RNA content normalized to the housekeeping gene *HPRT1*.

Gene expression microarray and pathway analysis. Gene expression differences in *CRY2* knockdown and normal cells were interrogated by whole genome microarray (Agilent, Inc., 44k chip, performed by MoGene). RNA was isolated in duplicate (biological replicates) from cells treated with *CRY2*-targeting or scrambled negative siRNA. Transcripts were identified as "differentially expressed" if they fit the criteria of Benjamini-Hochberg–adjusted *P* value of <0.01 in both biological replicates and mean fold change of >|2|. Transcripts with differential expression were investigated for network and functional interrelatedness using the Ingenuity Pathway Analysis software tool (Ingenuity Systems).[4] This software scans the set of input genes to identify networks using information in the Ingenuity Pathways Knowledge Base, an extensive, manually curated database of functional interactions extracted from peer-reviewed publications (25). A Fisher's exact test, based on the hypergeometric distribution, is then performed to determine the likelihood of obtaining at least the same number of molecules by chance (i.e., from a random input set), as actually overlap between the input gene set and the genes present in each identified

[3] http://www.hapmap.org/cgi-perl/gbrowse/hapmap22_B36/ accessed on January 15, 2008.

[4] http://www.ingenuity.com

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Table 1. Distribution of selected characteristics by case-control status

Variable	Cases (n = 455)	Controls (n = 527)	P
	n (%)	n (%)	
Mean age (y)	61.88	62.34	0.607
Race			
Caucasian	438 (96.26)	496 (94.12)	
African-American	13 (2.86)	14 (2.66)	
Other	4 (0.88)	17 (3.22)	0.108
Family history of cancer in first degree relatives			
No	96 (21.10)	130 (24.67)	
NHL	9 (1.98)	2 (0.38)	
Other cancer	350 (76.92)	395 (74.95)	0.030
Case pathology			
All B cell	365 (80.22)		
Diffuse large B cell	135 (36.99)		
Follicular	105 (28.77)		
SLL/CLL	54 (14.79)		
Marginal Zone	30 (8.22)		
Other	41 (11.23)		
All T cell	33 (7.25)		
NOS	58 (12.75)		

Abbreviation: NOS, not otherwise specified.

network. All microarray data have been uploaded to the Gene Expression Omnibus database. Raw and processed data related to this experiment can be accessed via their Web site[5] by referencing accession #GSE14617.

Statistical analysis. All statistical analyses were performed using the SAS statistical software (SAS Institute), unless otherwise noted. For the case-control analyses, allelic distributions for all SNPs were tested by goodness-of-fit χ^2 for compliance with Hardy-Weinberg equilibrium. A χ^2 test was also used to determine whether any of the variants were associated with case-control status, using either the full table (homozygous wild-type, heterozygous, and homozygous variant), a dominant model, or a recessive model. Odds ratios (OR) and 95% confidence intervals (CI) were determined for each SNP disease association by unconditional multivariate logistic regression, including the following covariates: age, race, and family history of cancer in a first-degree relative. Haplotype estimates were calculated by the PHASE program, which reconstructs haplotypes from population genotyping data (26). Diplotypes were constructed based on the pair of haplotypes estimated for each individual, and ORs and 95% CIs for each diplotype were determined by unconditional multivariate logistic regression, using the same covariates as the main effects model but with all other diplotypes as the reference category.

Due to the multiple comparisons inherent in the microarray analysis, adjustments were made to control for false discoveries. A multiple comparisons correction was applied to each observation using the Benjamini-Hochberg method, as previously described (27), to obtain an adjusted P value (B-H P value).

[5] http://www.ncbi.nlm.nih.gov/projects/geo/

Results

Association between CRY2 variants and NHL risk. Compared with controls, NHL cases reported a higher proportion of NHL and other cancers among first-degree relatives. There were no significant differences in age and race between cases and controls (Table 1). Genotypic frequencies were determined at each locus, and no allelic distributions significantly departed from the values expected under Hardy-Weinberg equilibrium among the controls (P < 0.01). Three of the five SNPs, rs11038689, rs7123390, and rs1401417, were found to be significantly associated with case-control status in the overall three-by-two table of allele distribution versus case-control status (Supplementary Table S1), with P values of 0.005, 0.003, and 0.004, respectively, for the 2 degrees of freedom χ^2 test. Each of the three SNPs significantly associated with disease status in the full table was also significant when a recessive model was assumed but not when assuming a dominant model. As such, ORs and CIs were determined by unconditional multivariate logistic regression under the assumption of a recessive model. Variant alleles in the same three SNPs (rs11038689, rs7123390, and rs1401417) that were associated with case-control status in the unadjusted χ^2 analysis were also significantly associated with increased risk of NHL in the adjusted logistic regression model, with ORs (95% CIs) of 2.34 (1.28–4.27; P = 0.006), 2.40 (1.39–4.13; P = 0.002), and 2.97 (1.57–5.63; P = 0.001), respectively (Table 2).

Because NHL is composed of various subtypes with the potential for distinct etiologies, we also performed a stratified analysis including B-cell and T-cell lymphomas only. These results were qualitatively and quantitatively similar to those obtained in the overall analysis with ORs (95% CIs) for homozygous variants of rs11038689, rs7123390, and rs1401417 among B-cell lymphomas only of 2.35 (1.25–4.41; P = 0.008), 2.54 (1.44–4.46; P = 0.001), and 2.87 (1.47–5.58; P = 0.002), respectively. Although similar point estimates for the ORs were observed among T-cell lymphomas only, only rs1401417 reached statistical significance [OR, 4.64 (1.41–15.34; P = 0.012)] due to the small sample size. Further stratification was performed to analyze four common subtypes of B-cell lymphoma (Table 3). Each of the SNPs significantly associated with lymphoma in the full population (rs11038689, rs7123390, and rs1401417) was also significantly associated with follicular lymphoma, with ORs (95% CIs) of 3.17 (1.39–7.19; P = 0.006), 3.67 (1.79–7.56; P < 0.001), and 3.06 (1.24–7.54; P = 0.015), respectively. Two SNPs, rs7123390 and rs1401417, were significantly associated with B-cell chronic lymphocytic leukemia/prolymphocytic leukemia/small lymphocytic lymphoma, with ORs (95% CIs) of 3.38 (1.35–8.45; P = 0.009) and 4.23 (1.53–11.67; P = 0.005), respectively. Only rs1401417 was significantly associated with diffuse large B-cell lymphoma [OR, 2.67 (1.13–6.33; P = 0.026)]. Very few patients were classified as having marginal zone B-cell lymphoma, and no *CRY2* SNPs were significantly associated with this NHL subtype.

Association between CRY2 diplotypes and NHL risk. To further explore the relationship among SNPs, haplotypes were estimated for these 5 SNPs and 13 different haplotypes were identified among all subjects (Supplementary Table S2). Nine haplotypes carried at least one risk allele (i.e., a variant associated with increased risk in the main effects analysis). Because the individual risk estimates indicated a recessive disease model, we used the haplotype information to construct diplotypes for each individual. Figure 1 depicts the location of each marker within the *CRY2* gene, and shows the NHL subtypes that were significantly associated with each homozygous

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Table 2. Association of CRY2 variants with NHL risk

Genotype	All			B cell			T cell		
	Cases *n*	Controls *n*	OR* (95% CI)	Cases *n*	Controls *n*	OR* (95% CI)	Cases *n*	Controls *n*	OR* (95% CI)
rs11038689									
A/A or A/G	408	495	—	326	495	—	29	495	—
G/G	33	17	2.34 (1.28–4.27)	26	17	2.35 (1.25–4.41)	3	17	2.69 (0.74–9.84)
P	—	—	0.006	—	—	0.008	—	—	0.135
rs11605924									
A/A or A/C	327	365	—	263	365	—	25	365	—
C/C	114	151	0.82 (0.62–1.10)	89	151	0.79 (0.58–1.08)	8	151	0.78 (0.34–1.77)
P	—	—	0.182	—	—	0.142	—	—	0.550
rs2292912									
C/C or C/G	418	489	—	324	489	—	32	489	—
G/G	23	29	1.00 (0.55–1.82)	18	29	1.03 (0.54–1.96)	1	29	0.48 (0.06–4.07)
P	—	—	0.990	—	—	0.938	—	—	0.502
rs7123390									
G/G or G/A	396	491	—	315	491	—	29	491	—
A/A	41	21	2.40 (1.39–4.13)	34	21	2.54 (1.44–4.46)	3	21	2.11 (0.59–7.54)
P	—	—	0.002	—	—	0.001	—	—	0.253
rs1401417									
G/G or G/C	409	503	—	331	503	—	28	503	—
C/C	34	14	2.97 (1.57–5.63)	26	14	2.87 (1.47–5.58)	4	14	4.64 (1.41–15.34)
P	—	—	0.001	—	—	0.002	—	—	0.012

*Adjusted for age (continuous), race, and family history of cancer in first- or second-degree relatives.

variant diplotype. Full results are provided in the supplementary material (Supplementary Table S3). ORs (95% CIs) for homozygous variant diplotypes of rs11065924, rs11038689, rs2292912, rs1401417, and rs7123390 using the full population were as follows: 0.81 (0.61–1.08), 2.32 (1.27–4.24), 0.96 (0.53–1.75), 2.81 (1.51–5.23), and 2.36 (1.39–4.02), respectively. The OR associated with having the homozygous variant diplotype at one or more of the risk loci (rs11038689, rs1401417, or rs7123390) was 2.72 (1.61–4.59).

Microarray reveals several cancer- and immune-related genes that are influenced by CRY2. To determine which genes and biological pathways might be influenced by *CRY2*, we silenced the gene *in vitro*, and performed a whole genome array comparing gene expression in cells with reduced *CRY2* to cells with normal *CRY2* levels. Differentially expressed transcripts (734) were identified (B-H $P < 0.01$ in both biological replicates and mean fold change of >|2|), and these genes were further explored through pathway and functional analyses using the IPA software package. Twenty-three networks were identified as significantly associated with the differentially expressed genes at P value of $<1.0E^{-10}$. Among these, seven were associated with Cancer, six networks were important for Immune Response, and four were important for Hematological System Development or Hematological Disease (Supplementary Table S4).

The top network operative in Hematological System Development and Function ($P = 1.0E^{-36}$; Fig. 2) also contained several cancer-related transcripts, including molecules important for DNA repair, cell migration and metastasis, apoptosis, cell proliferation, and angiogenesis. The details of each transcript in the network, including fold changes, adjusted P values, and a short description of function with relevant citations, can be found in the supplementary material (Supplementary Table S5). Briefly, 35 genes were present in the network, 33 of which could be uniquely associated with a single transcript in the microarray. Of these, 27 were significantly up-regulated after *CRY2* silencing, 4 were not significantly altered, and only 2 were significantly down-regulated, which is consistent with the role of CRY2 as a transcriptional repressor. The probability of obtaining at least this many differentially expressed molecules in one network by chance alone is reflected by the P value for the network ($P = 1.0E^{-36}$), indicating that it is very unlikely that the network was obtained randomly and, therefore, *CRY2* is likely to be important for immune response coordination and processes related to hematologic system development. At the center of this network is interleukin (IL)-6, an immunoregulatory cytokine that is important for cell growth. This transcript was strongly up-regulated after *CRY2* silencing, with a mean fold increase of 8.17 (B-H $P = 0$). In addition, a member of the vascular endothelial growth factor family (VEGFC), which is also important for cell growth, as well as angiogenesis, was up-regulated >6-fold (B-H $P = 6.99E^{-4}$). Interestingly, a number of transcripts with proapoptotic, antiproliferative, or other tumor suppressor capabilities were also up-regulated in this network. These include IFNβ1 that was up-regulated >3-fold (B-H $P = 2.96E^{-9}$), thioredoxin-interacting protein, >11-fold up-regulated (B-H $P = 2.47E^{-4}$), retinoic acid receptor responder 3, >2-fold up-regulated (B-H $P = 1.86E^{-4}$), and 2 members of the chemokine (C-X-C motif) ligand family (CXCL9 and CXCL11), >10- and 4-fold up-regulated, respectively (B-H $P = 1.01E^{-5}$ and $= 3.04E^{-6}$).

Apart from the transcripts in this network, several additional genes that may be relevant for lymphomagenesis were identified as significantly altered after *CRY2* silencing. These include three members of the chemokine (C-C motif) ligand family (CCL3, CCL4, and CCL5), which are important for immunoregulation and inflammatory processes, several additional members of the

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interleukin family of cytokines (including IL-11, IL-15, IL-18, IL-28a, IL-28b, and IL-29), two insulin-like growth factor binding proteins (IGFBP3 and IGFBP6), and genes in the MHC, class I (HLA-A, HLA-B, HLA-C, and HLA-E). Of note, *CRY2* itself was significantly down-regulated in both microarray replicates (B-H $P < 0.05$), with exactly the same fold change of −3.17 observed in both samples.

The program also computes a *P* value based on the likelihood of obtaining the observed number of differentially expressed molecules in a given process by chance alone. The top three disease processes associated with the differentially expressed gene set were "Cancer" (B-H $P = 3.75E^{-9}$), "Inflammatory Disease" (B-H $P = 5.19E^{-8}$), and "Hematological Disease" (B-H $P = 8.01E^{-8}$; Fig. 3). In addition, 144 of the differentially expressed transcripts were related to "Tumorigenesis" (B-H $P = 4.57E^{-9}$), 64 molecules were associated with "Immune Response" (B-H $P = 7.03E^{-10}$), and 49 were involved in "Proliferation of Lymphocytes" (B-H $P = 6.76E^{-9}$). The large number of cancer- and immune-related molecules influenced by *CRY2* knockdown provides further evidence suggesting a role for *CRY2* in lymphoproliferative processes.

Discussion

The observed connection between circadian disruption (e.g., shift work) and cancer risk in epidemiologic studies has led to the circadian gene hypothesis, which suggests that genetic variants in genes responsible for maintaining circadian rhythm may affect an individual's susceptibility to human cancers. This hypothesis is supported by results from recent genetic association studies of breast cancer (28, 29), prostate cancer (24), and NHL (5). The findings from the current study of *CRY2* provide more evidence demonstrating a role for the circadian genes in NHL susceptibility.

To the best of our knowledge, genetic variants in *CRY2* have not been previously examined in NHL, and only one of the SNPs genotyped in the case-control portion of this analysis had been studied previously; in a population-based case-control study conducted in China, which showed a significant association between the variant allele of rs1401417 and increased risk of prostate cancer (24). This finding is consistent with the significant association we observed in the NHL population, and further investigations into the nature of these relationships are warranted to determine whether *CRY2* has a global effect on cancer susceptibility.

The observed associations between *CRY2* and NHL risk provide additional molecular epidemiologic evidence supporting the proposed role of circadian genes as tumor suppressors (30). Circadian genes have been shown to affect expression of 2% to 10% of mammalian genes (31) including many cancer-related genes (30). Emerging data from animal models have further shown a substantial effect of circadian genes on tumor-related biological pathways such as cell proliferation, cell cycle control, DNA damage response, and apoptosis (30). Mice with mutations in the circadian gene *PER2* have deficiencies in DNA damage response and are more prone to tumorigenesis (32). Altered expression of circadian genes also occurs in human tumors, as studies have shown that the period (*PER*) genes fail to maintain daily rhythmic expression patterns in cancer cells (33). Although *CRY2* has also been shown to be involved in cell cycle regulation (34), explicit mechanisms for its role in cancer susceptibility, especially NHL tumorigenesis, are currently unknown.

The microarray analysis, which implicated *CRY2* as having the potential to influence gene expression in a number of pathways, including those with relevance for cancer and immune function, provides the first mechanistic evidence suggesting that *CRY2* may

Table 3. Association of CRY2 variants with NHL risk by B-cell subtype

Genotype	DLBCL			FL		CLL/SLL		MZBL	
	Controls *n*	Cases *n*	OR* (95% CI)	Cases *n*	OR* (95% CI)	Cases *n*	OR* (95% CI)	Cases *n*	OR* (95% CI)
rs11038689									
A/A or A/G	495	126	—	90	—	48	—	27	—
G/G	17	6	1.41 (0.54–3.65)	10	3.17 (1.39–7.19)	5	2.75 (0.96–7.86)	2	2.30 (0.50–10.61)
P	—	—	0.484	—	0.006	—	0.060	—	0.285
rs11605924									
A/A or A/C	365	93	—	79	—	31	—	19	—
C/C	151	39	0.98 (0.64–1.50)	21	0.61 (0.36–1.04)	12	0.72 (0.36–1.40)	10	1.21 (0.54–2.70)
P	—	—	0.931	—	0.068	—	0.329	—	0.643
rs2292912									
C/C or C/G	489	127	—	97	—	52	—	27	—
G/G	29	4	0.53 (0.17–1.65)	4	0.95 (0.31–2.93)	1	0.27 (0.03–2.26)	2	1.60 (0.34–7.60)
P	—	—	0.274	—	0.932	—	0.227	—	—
rs7123390									
G/G or G/A	491	124	—	85	—	45	—	27	—
A/A	21	7	1.31 (0.54–3.16)	14	3.67 (1.79–7.56)	7	3.38 (1.35–8.45)	2	1.86 (0.41–8.43)
P	—	—	0.547	—	<0.001	—	0.009	—	0.423
rs1401417									
G/G or G/C	503	122	—	95	—	47	—	29	—
C/C	14	9	2.67 (1.13–6.33)	8	3.06 (1.24–7.54)	6	4.23 (1.53–11.67)	1	1.30 (0.16–10.33)
P	—	—	0.026	—	0.015	—	0.005	—	0.806

*Adjusted for age (continuous), race, and family history of cancer in first- or second-degree relatives.

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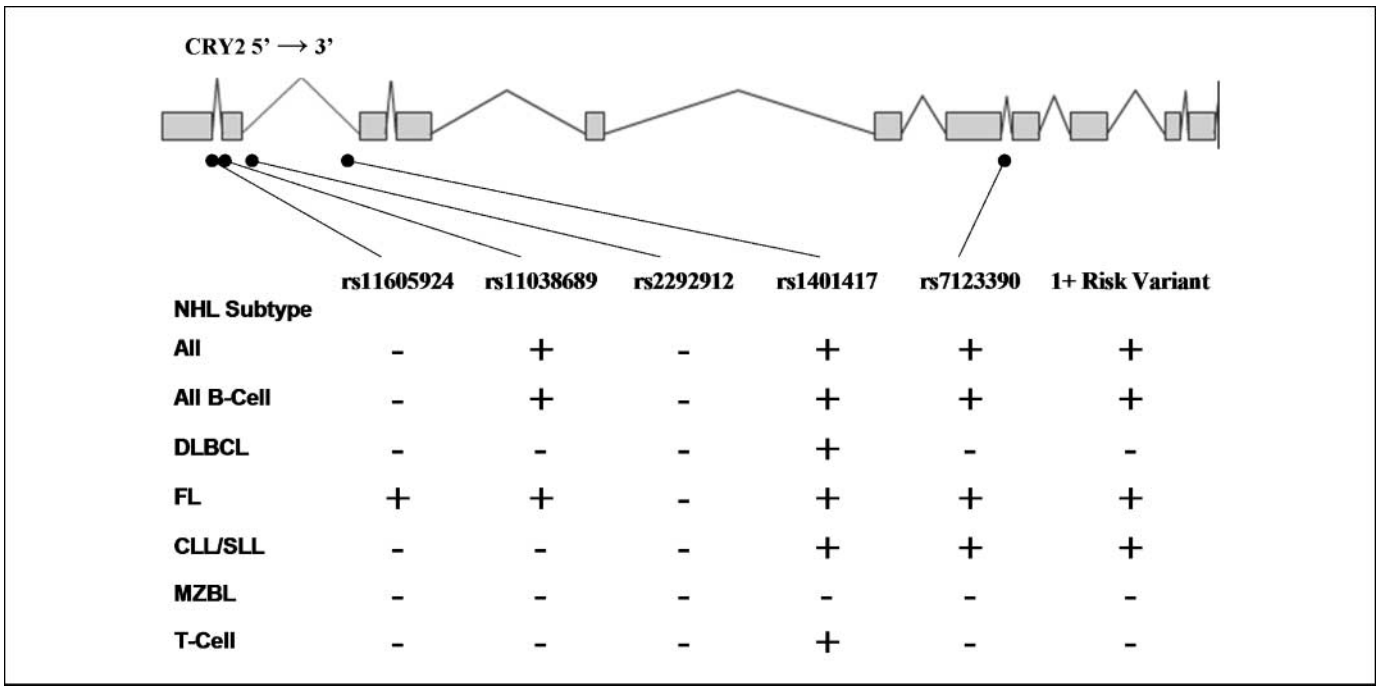

Figure 1. Diplotype analysis and location of CRY2 variants. Haplotypes were estimated for each individual using the PHASE program, and diplotypes were constructed by combining the two haplotype estimates for each individual. Because the main effects associations indicated a recessive disease model, risk estimates were calculated for all combinations of haplotypes resulting in a homozygous variant diplotype at each locus, with all other diplotypes as the reference category. Estimates were obtained for each NHL subtype represented in the population; +, homozygous diplotypes that were significantly associated with each subtype ($P < 0.05$); −, nonsignifcant associations. *1+ risk variant*, any haplotype combination that resulted in a homozygous variant diplotype for at least one of the following makers: rs11038689, rs1401417, or rs7123390. *DLBCL,* diffuse large B-cell lymphoma; *FL,* follicular lymphoma; *CLL/SLL,* B-cell chronic lymphocytic leukemia/prolymphocytic leukemia/small lymphocytic lymphoma; *MZBL,* marginal zone B-cell lymphoma.

be important for NHL susceptibility. The findings relative to IL-6 are of particular interest. It has been shown that IL-6 can inhibit DNA synthesis by preventing cell cycle progression into S phase (35). It has also been fairly well-established that IL-6 plays an important role in B-cell proliferation (36), and antibodies against IL-6 or its receptor have been explored as treatments for B-lymphoproliferative disorder (37) and inflammatory autoimmune diseases (38). In addition, a previous study has shown that mice expressing an IL-6 transgene exhibit lymphoproliferation and plasmacytosis, and nearly a third of these mice developed follicular and diffuse large cell B-cell lymphomas (39). Moreover, serum levels of IL-6 and VEGF have been shown to be of value in predicting clinical outcome in some patients with NHL (40, 41). The observation that silencing of *CRY2* results in >8-fold induction of IL-6, and 6-fold induction of VEGFC, is therefore highly relevant in understanding the etiology of hematologic malignancies, and may have important implications for predicting NHL prognosis. Furthermore, cell signaling by IL-6 is partially mediated by its effects on the Janus-activated kinase (JAK)/signal transducers and activators of transcription 3 (STAT3) pathway (42). Although JAK was unaffected after *CRY2* silencing, STAT3 was up-regulated >2-fold (B-H $P = 7.06E^{-5}$). Because IL-6 has been shown to confer increased survival and chemotherapeutic resistance on lymphoma cells, which is at least partially mediated by STAT3, STAT3 has been proposed as a potential therapeutic target for patients with NHL (43). As such, although the pleiotropic biological effects of CRY2 may make it a poor candidate to be directly targeted by therapeutic agents, its effects on both IL-6 and STAT3 may lead future investigations to consider circadian timing when administering cytokine-targeting chemotherapies.

Apart from IL-6, several other interleukins were also significantly altered in *CRY2* knockdown cells. IL-18, which was up-regulated by >10-fold, may play an important but paradoxical role in cancer risk (44). It has been implicated in cancer protection, through its role in activating immune cells to eliminate sporadic cancers but may also promote tumor progression by encouraging angiogenesis, tumor growth, and local invasion. Interestingly all three members of a newly described class of IFN λs (IL-28a, IL-28b, and IL-29) were significantly up-regulated after *CRY2* silencing. These immunoregulatory cytokines have antiviral and antitumor activity, and may also have potential as therapeutic agents in the treatment of cancer (45).

Three members of the chemokine (C-C motif) ligand family (CCL3, CCL4, and CCL5) were also up-regulated in *CRY2* knockdown cells. These genes are clustered together on the long arm of chromosome 17 and are important for immunoregulation and inflammation (46). CCL3, which was up-regulated >15-fold in *CRY2* knockdown cells, has been shown to be elevated in patients with multiple myeloma and other hematologic cancers compared with healthy controls, and increased serum levels of CCL3 were associated with advanced multiple myeloma stage (47) and poorer prognosis in multiple myeloma (48). Of additional interest was the identification of four genes in the MHC, class I (HLA-A, HLA-B, HLA-C, and HLA-E), which were all significantly up-regulated after *CRY2* silencing. Apart from being central to immunoregulation, these genes have been associated with several cancers, including Hodgkin's disease (49) and non–Hodgkin's lymphoma (50). Taken together, these results suggest that reductions in *CRY2* have the potential to significantly effect processes relevant for lymphomagenesis, and represent a first step in understanding the mechanism

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by which SNPs in the *CRY2* gene might influence NHL susceptibility.

It is important to recognize some of the limitations of the study. First, it is unclear how closely the effect of *CRY2* silencing *in vitro* might mimic the conditions that would arise *in vivo*. At the level of the organism, circadian rhythmicity is made even more complex by environmental cues, such as light exposure, and hormonal pathways, which may influence circadian gene expression. In addition, because one aim of the study was to examine the influence of *CRY2* silencing on immune response pathways, we chose not to use cells derived from lymphoma tissue, which may begin with some level of aberrant immune-related gene expression. As such, it will be necessary for future studies to confirm that the findings we observed are applicable to lymphocytes, and that the changes in gene expression after *CRY2* silencing that we observed at the cellular level are representative of those that would occur at the organismal level.

In summary, the findings from our case-control analysis suggest a novel association between the circadian gene *CRY2* and risk of NHL, supporting the hypothesis that genetic variations in circadian genes may confer inherited susceptibility to NHL. The subsequent loss-of-function analysis and whole genome expression microarray suggest that the observed association could potentially be attributed to the effect of *CRY2* on several genes important for

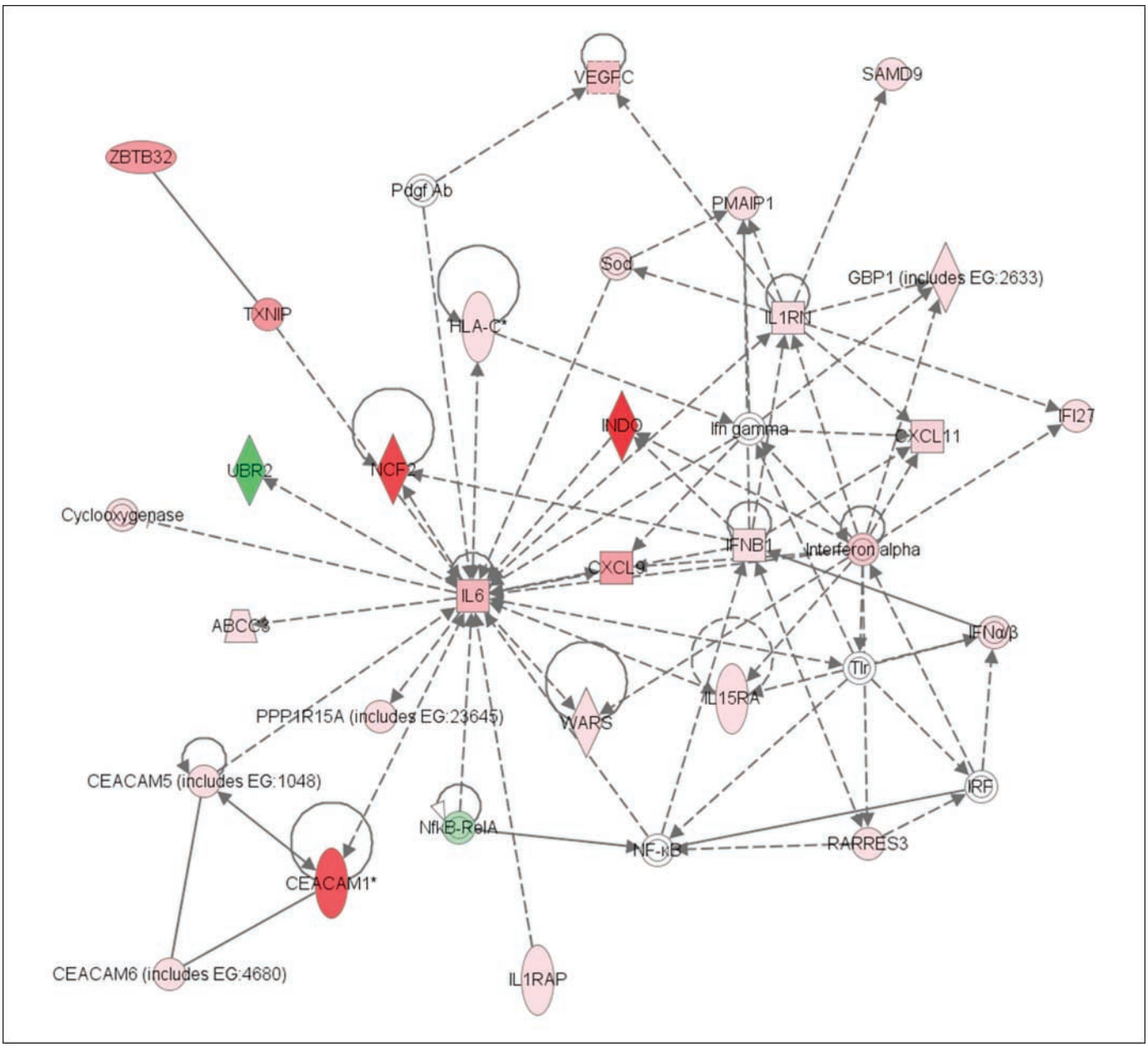

Figure 2. Network of interactions among genes differentially expressed after *CRY2* knockdown. This network was identified as operative in Immune Response, Cell-to-Cell Signaling and Interaction, and Hematological System Development and Function, as determined by Ingenuity Pathway Analysis software. Each shaded transcript was significantly altered after *CRY2* silencing, with a mean fold change of >|2| and B-H *P* value of <0.01 in both biological replicates of the whole genome microarray. *Red,* genes that are up-regulated; *green,* genes that are down-regulated. Higher intensity color corresponds to greater magnitude of the fold change. Of the 29 molecules in the network that were significantly altered, 27 were up-regulated, whereas only 2 were down-regulated, which is consistent with the role of *CRY2* as a transcriptional repressor.

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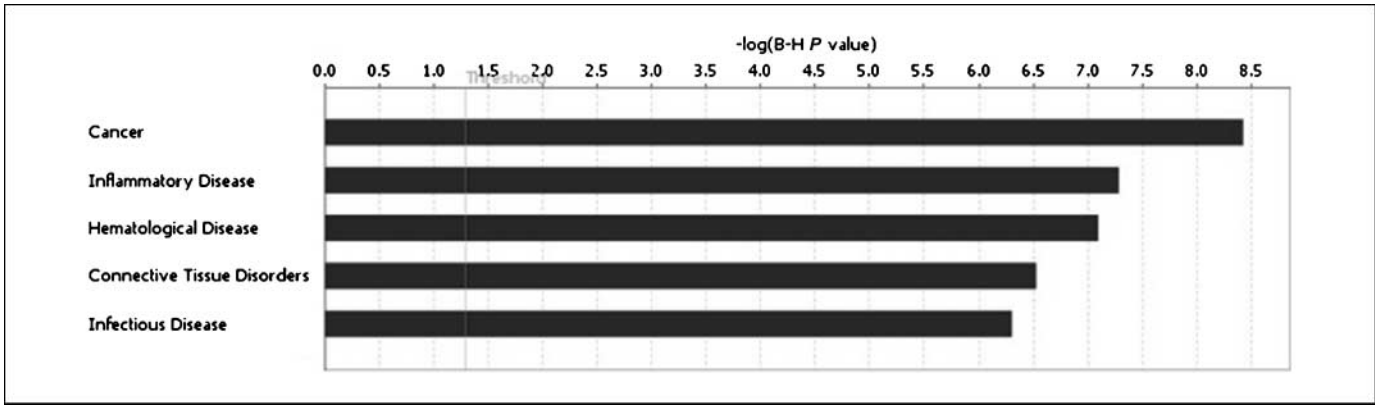

Figure 3. Diseases most strongly associated with the significantly altered genes. The Ingenuity software scans its Knowledge Base of manually curated relationships for instances in the literature that can link a specific molecule to a functional disease process. The software also assigns a *P* value based on the likelihood of obtaining the observed number of disease-related molecules in a given data set by chance alone. Due to the large number of disease processes represented in the database, B-H *P* values are presented to correct for multiple comparisons. *Threshold line,* a B-H *P* value of 0.05.

cancer in general, as well as a number of genes with known relevance for hematologic malignancies. Our findings provide a novel panel of promising biomarkers for NHL risk and prognosis, which warrant further investigation. In addition, because previous studies have suggested that circadian-related environmental exposures, such as light at night or rotating shift work, may influence cancer susceptibility, future investigations into potential interactions between circadian gene variants and environmental exposures may be of interest in developing cancer prevention strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Clock-Cancer Connection in Non–Hodgkin's Lymphoma: A Genetic Association Study and Pathway Analysis of the Circadian Gene Cryptochrome 2

Aaron E. Hoffman, Tongzhang Zheng, Richard G. Stevens, et al.

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