Investigation of Associations between NR1D1, RORA and RORB Genes and Bipolar Disorder

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Abstract

Several genes that are involved in the regulation of circadian rhythms are implicated in the susceptibility to bipolar disorder (BD). The current study aimed to investigate the relationships between genetic variants in NR1D1, RORA, and RORB genes and BD in the Han Chinese population. We conducted a case-control genetic association study with two samples of BD patients and healthy controls. Sample I consisted of 280 BD patients and 200 controls. Sample II consisted of 448 BD patients and 1770 healthy controls. 27 single nucleotide polymorphisms in the NR1D1, RORA, and RORB genes were genotyped using GoldenGate VeraCode assays in sample I, and 492 markers in the three genes were genotyped using Affymetrix Genome-Wide CHB Array in sample II. Single marker and gene-based association analyses were performed using PLINK. A combined p-value for the jointing effects of all markers within a gene was calculated using the rank truncated product method. Multifactor dimensionality reduction (MDR) method was also applied to test gene-gene interactions in sample I. In sample I, the most significant marker in RORA was rs1327836 (OR = 1.53, emp. p-value = 0.024), and in RORB was rs17611535 (OR = 3.15, P = 0.027). In sample II, 45 markers had empirical p-values less than 0.05. The most significant markers in RORA and RORB genes were rs11639084 (OR = 0.69, P = 0.002), and rs17611535 (OR = 3.15, P = 0.027), respectively. Gene-based association was significant for RORA gene (P = 0.0007).
Our results support for the involvement of RORs genes in the risk of developing BD. Investigation of the functional properties of genes in the circadian pathway may further enhance our understanding about the pathogenesis of bipolar illness.

Introduction

Bipolar disorder (BD) that is known as manic-depressive illness, affects approximately 1% of the general population [1] with high heritability [2]. BD patients frequently demonstrate biological rhythm-related symptoms, including diurnal variation of mood and sleep disturbances (e.g., a decreased need for sleep during manic episode, and insomnia or hypersomnia during depressive episode). Moreover, sleep disturbances aggravate emotional dysregulation [3] and are reported to predict relapse of mood episode [4]. BD patients are also characterized by instable social rhythms, such as early awakening, lower activity during depressive episode or higher activity during manic episode. Together with these observations among BD patients, abnormality in circadian function has been postulated to play important roles in the pathophysiology of BD [5].

The circadian system is critical for proper regulation of behavioral and physiological rhythms in human, which mastered by the suprachiasmatic nucleus (SCN) in the hypothalamus. It is regulated by two major transcriptional feedback loops [6]. One positive major transcriptional activator of circadian system is a dimer consisted of products encoded by two genes, the circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein 1 (ARNTL, also known as BMAL1). The other negative feedback loop is regulated by the period (PER) and cryptochrome (CRY) proteins that are translated in the cytoplasm to repress the actions of CLOCK/ARNTL [7]. Recently, an additional negative feedback loop is reported to involve with the two orphan nuclear receptors [8]: nuclear receptor subfamily 1, group D member 1 (NR1D1, also known as rev-erb-α) and retinoic acid–related orphan receptor-A (RORA). The NR1D1 protein enters the nucleus to repress transcription of CLOCK/ARNTL, while RORA protein has opposite transcriptional function to activate ARNTL. Thus, the transcription of ARNTL is the result of competition between NR1D1 and RORA [9].

In the past, several circadian genes have reported to be associated with shorter total sleep time [10], insomnia [11] and daytime fatigue [12] in clinical patients with mood disorders. However, relatively few studies exist to examine the associations of ROR gene family in patients with BD. The ROR family contains 3 different genes: RORA, RORB, and RORC. Among them, only RORC does not express in the SCN [13]. The RORA or RORB deficient mice exhibit aberrant circadian behaviors, whereas no abnormalities in circadian behaviors have been noticed in RORC−/− mice [14, 15]. In human, few studies ever examined the associations between genetic variants in NR1D1, RORA, and RORB genes and BD, which we summarize their results in Table 1. For NR1D1, both positive [16] and negative [17] findings are reported in Caucasian populations. One study conducted in Japanese reported no association for NR1D1 with BD at single marker level, but shows association in one haplotype in the NR1D1 gene with onset age of manic episode [18]. Moreover, there are three studies examined genes in the ROR family. McGrath et al. [19] reported associations for RORB but not RORA with BD subtype I. Using a sample with around 500 cases and 500 controls, Mansour et al. [20] also reported nominal significance for RORB with BD subtype I. On the contrary, Soria et al. [21] found significant associations for RORA with BD. With the few studies conducted so far without consistent findings, we aimed to examine the roles of NR1D1, RORA, and RORB genes in the pathogenesis of BD.
The current study attempted to examine the associations between genetic variants in the \textit{NR1D1}, \textit{RORA}, and \textit{RORB} genes with BD using a culturally and ethnically more homogeneous sample in the Han Chinese population. We also reasoned that genes in the same feedback loop might interact with each other due to the complex nature resides in the biological functions of
the circadian system. Thus, we intended to examine the interaction effects among genetic variants in \textit{NR1D1}, \textit{RORA} and \textit{RORB} genes using multifactor dimension reduction (MDR) approach.

**Materials and Methods**

We conducted a case control genetic association study for the \textit{NR1D1}, \textit{RORA} and \textit{RORB} genes with BD. Two samples were tested for these three genes. Sample I was genotyped in early stage based on selection of candidate genetic variants using a customized array chip, and Sample II was genotyped in 2013 using a genome-wide association (GWA) chip in which we analyzed markers in the three genes.

**Participants**

Clinical patients who met the diagnostic criteria of bipolar I disorder (BD-I) or bipolar II disorder (BD-II) according to the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) were consecutively referred by psychiatrists in Taiwan from 2008 to 2012. Index probands whose age between 18 and 70 years and their biological parents were Han Chinese were recruited. Patients who diagnosed with schizophrenia, schizoaffective or substance-induced mood disorders and whose diagnoses were changed during the data collection period were excluded. Healthy controls were recruited in the community and were screened for mood disturbances and other major psychiatric disorders on the basis of structured interviews. More details of sample recruitment please refer to Tsai et al., [22]. The present study was approved by the Institutional Review Board of National Taiwan University Hospital, National Cheng Kung University Hospital, and Taipei City Hospital. All clinical participants were evaluated by psychiatrists in the participating hospitals for their capacity to sign informed consent. Only patients who had the ability to consent on participation of this study were referred and all our participants provided written informed consent.

Participants were interviewed by well-trained interviewers using the Composite International Diagnostic Interview (CIDI) [23] and the Chinese version of the modified Schedule of Affective Disorder and Schizophrenia-Lifetime (SADS-L) to collect data on demographic and clinical features, and information on major psychiatric disorders (details please refer to Tsai et al., [22]. In addition, blood samples were taken to extract DNA for each individual. Subjects who provided blood samples and completed the interview were included in the case-control association study. For Sample I, we genotyped 280 BD patients (200 BD-I, 80 BD-II), and 200 healthy controls. For Sample II, we genotyped 448 BD patients (262 BD-I, 186 BD-II) and 1770 healthy controls. Control samples in Sample II were community subjects drawn from the Han Chinese Cell and Genome Bank in Taiwan [24]. Subjects were screened for major disease history (including major mental illnesses), however, no detailed interview data were provided.

**Experimental designs and quality control**

DNA was extracted from peripheral white blood cells using commercial kit and stored at $-80^\circ\text{C}$.

We diluted DNA samples to 50ng/$\mu$L; concentration and quality were checked using Quant-iT PicoGreen dsDNA Reagent and Kits. After DNA extraction, we then selected markers in the \textit{NR1D1}, \textit{RORA}, and \textit{RORB} genes for genotyping.

The selection of single nucleotide polymorphism (SNPs) for genotyping in Sample I was based on two criteria. First, we chose SNPs that showed genetic associations with BD in the literature (see the summary Table 1). Second, we searched coding SNPs in the three genes from NCBI dbSNP database (BUILD 37.3). Markers that have been genotyped in HapMap project (http://hapmap.ncbi.nlm.nih.gov/), with minor allele frequency greater than 0.01 in the CHB
samples were considered in the present study. We intended to genotype in total 27 SNPs according to these two selection processes. We used customized Illumina GoldenGate Genotyping Assay with VeraCode Technology to genotype the selected 27 SNPs at the National Taiwan University Center for Biotechnology. Genotyping data showed that two markers (rs1568717 and rs17691363) had low (<5%) minor allele frequency (MAF). Therefore, we discarded these two SNPs in the following analysis. For the remaining 25 SNPs, the call rate was high, ranging from 95.8% to 99.6%, and had p-value greater than 0.05 in the Hardy-Weinberg equilibrium (HWE) test. Three individuals had missing data for all SNPs and were excluded from analysis, including two BD-I and one BD-II patients.

For Sample II, individual genotyping was conducted using the Axiom Genome-Wide CHB Array with 628,132 SNPs in the Genomics Research Center in Academia Sinica. This array is specifically designed for CHB populations with the highest coverage rate of common variants for the Han Chinese populations. Using 20kb upstream and downstream of the gene boundaries, 492 SNPs were mapped into NR1D1, RORA, and RORB protein-coding genes. For Sample II, 18 individuals (3 BD-I and 15 controls) had call rate less than 95% and were dropped from the analysis. To evaluate kinship relationship among samples, we calculated inbreeding coefficient and identity by state (IBS) to eliminate samples with a strong kinship relationship using PLINK (Purcell, http://pngu.mgh.harvard.edu/~purcell/plink/) [25]. Subjects with in-breeding coefficients outside the mean ±3×standard deviation or subjects not in the same cluster with others using the IBS distance value were removed. As a result, 58 individuals (48 controls, 5 BD-I and 5 BD-II) were discarded. Additionally, population stratification was evaluated by multidimensional scaling analysis and 8 controls were removed from analysis. In total, 2134 individuals (254 BD-I, 181 BD-II, and 1699 controls) were retained after quality control procedures.

We then performed quality controls for the 492 markers. We removed SNPs with poor amplification, HWE test p-values less than 0.001, genotype missing rate greater than 5%, MAF smaller than 0.05, and bad calling in clustering. We also compared B allele among two groups (i.e., cases versus whole sample, controls versus whole sample) to remove markers whose difference of B allele frequency greater than 2%. As a result, 429 markers were retained in the following analysis. There were 10 markers genotyped in both samples. We checked the MAF of them in the two samples.

Statistical analysis
Because our patients group consisted of BD-I and BD-II, we first conducted exploratory analysis for the two subtypes to see whether MAF distributions are different in the two subtypes of BD. There were no significant differences between BD-I and BD-II, thus, the two subgroups were combined together into the overall BD group. We performed genetic association analyses using PLINK at single marker and gene-based levels, and odds ratios (OR) were estimated. For single marker analysis, we tested additive, dominant and recessive genetic models, and used a maximum test for associations [26]. Empirical p-values were generated using the max (T) permutation approach for pointwise estimates (EMP1). For gene-based analysis, SNPs were assigned to a gene if they located within the gene region. We adopted the default setting in PLINK to retain SNPs with linkage disequilibrium (LD) lower than 0.5, and had p-value < 0.1. The significance level of each gene was obtained through 10,000 permutations. Because the three genes were chosen with a higher priori to be associated with BD, we also used the rank truncated product method (TPM) to take the issue of multiple testing into account [27]. It is assumed that a set of weakly significant markers may altogether contribute to the risk of developing diseases. Thus, rather than doing association testing for each individual marker, the
TPM calculates a combined p-value for the joining effects of all single markers within a gene. A fixed threshold $\tau$ was chosen to dichotomize SNPs into significant and non-significant marker-set within a gene. We set $\tau = 0.05$ in corresponding to a nominal p-value of 0.05 [27]. All statistical tests were two-sided and the significance level was set at 0.05. Because there were few SNPs genotyped in sample I for each gene, we calculated gene-based associations using sample II. We also performed the MDR analysis [28] to detect gene-gene interactions among 27 SNPs genotyped in Sample I. While many traditional methods are failed or with limited power to characterize epistasis effects, especially with small sample size [29], the MDR analysis can examine gene-gene interaction without main effects. The MDR approach is a model-free and non-parametrical method to identify high dimensional interactions among multiple factors assuming that combinations of genetic factors provide the most information in the classification of high versus low risk groups. It is suggested to be a useful method to identify gene-gene interactions in high dimensional data [30]. There are three steps in conducting MDR analysis. First, we divided samples into training and testing datasets to perform ten-fold cross-validation to avoid the over-fitting problem in the model. Second, a set of $n$ genetic markers were selected by the models to form all possible genotypic combinations. For example, for two loci with three genotypes each, there are nine possible two-locus combinations. The ratio of cases/controls within each combination can then be calculated. Finally, a high or low risk group is assigned for each genotypic combination based on the comparison of case/control ratio. If the case/control ratio of a multifactor combination is higher than that in the sample population, the combination is assigned a high risk group, and vice versa. The best marker combination is selected according to the testing-balanced accuracy and cross-validation consistency.

Results

In Sample I, there was 48.6% and 31.0% males in BD patients (age 34.8 ± 11.5 years), and controls (age 47.3 ± 8.7 years), respectively. In Sample II, there was 45.5% and 46.4% males in BD patients (age 36.4 ± 12.5 years), and controls (age 46.2 ± 14.7 years). The characteristics of demographic variables in the two samples were shown in Table 2.

Table 3 displays the characteristics of associated markers in the two samples. All SNPs were under the HWE ($P > 0.001$) and the MAF was greater than 0.05. In sample I, one marker each in the $RORA$ and $RORB$ genes showed associations with BD ($P < 0.05$). The minor allele of
### Table 3. Single marker associations with bipolar disorders (P < 0.05)

| Gene | Ch | SNP     | Position       | MAF  | Control  | Model | OR  | EMP |
|------|----|---------|----------------|------|----------|-------|-----|-----|
| RORA | 15 | rs8041381 | 60862962       | 0.133| 0.108    | ADD   | 1.31| 0.022|
|      |    | rs11632600 | 60873670       | 0.224| 0.207    | REC   | 1.90| 0.010|
|      |    | rs75981965 | 60918491       | 0.956| 0.707    | DOM   | 1.68| 0.028|
|      |    | rs339998  | 60947763       | 0.435| 0.409    | REC   | 1.90| 0.010|
|      |    | rs2553236 | 60986572       | 0.284| 0.306    | REC   | 1.90| 0.010|
|      |    | rs13329238 | 60998847       | 0.227| 0.210    | DOM   | 1.90| 0.010|
|      |    | rs17237353 | 61021047       | 0.145| 0.178    | DOM   | 1.90| 0.010|
|      |    | rs1020729 | 61045072       | 0.275| 0.343    | ADD   | 1.90| 0.010|
|      |    | rs1020730 | 61048578       | 0.208| 0.244    | ADD   | 2.90| 0.010|
|      |    | rs12900122 | 61055411       | 0.155| 0.186    | DOM   | 1.90| 0.010|
|      |    | rs8025689 | 61059261       | 0.127| 0.165    | ADD   | 2.90| 0.010|
|      |    | rs9302215 | 61060367       | 0.307| 0.344    | ADD   | 2.90| 0.010|
|      |    | rs1482057 | 61064751       | 0.169| 0.139    | ALL   | 1.27| 0.023|
|      |    | rs11639084 | 61066516       | 0.128| 0.169    | ADD   | 2.90| 0.010|
|      |    | rs12594188 | 61067805       | 0.222| 0.260    | ALL   | 1.27| 0.023|
|      |    | rs10519067 | 61068347       | 0.108| 0.134    | ADD   | 2.90| 0.010|
|      |    | rs11071557 | 61068954       | 0.145| 0.186    | ADD   | 2.90| 0.010|
|      |    | rs11071558 | 61069421       | 0.147| 0.187    | ADD   | 2.90| 0.010|
|      |    | rs922781  | 61070344       | 0.252| 0.225    | DOM   | 2.90| 0.010|
|      |    | rs1963497 | 61071791       | 0.111| 0.135    | ADD   | 2.90| 0.010|
|      |    | rs17270446 | 61073802       | 0.139| 0.159    | REC   | 2.90| 0.010|
|      |    | rs877228  | 61076591       | 0.287| 0.406    | REC   | 2.90| 0.010|
|      |    | rs16943117 | 61078836       | 0.167| 0.201    | ADD   | 2.90| 0.010|
|      |    | rs12915776 | 61079377       | 0.154| 0.193    | ADD   | 2.90| 0.010|
|      |    | rs6494227 | 61106118       | 0.087| 0.114    | DOM   | 2.90| 0.010|
|      |    | rs79610262 | 61106460       | 0.086| 0.113    | DOM   | 2.90| 0.010|
|      |    | rs87507043 | 61114212       | 0.449| 0.489    | DOM   | 2.90| 0.010|
|      |    | rs16943172 | 61117865       | 0.267| 0.301    | DOM   | 2.90| 0.010|
|      |    | rs75084363 | 61145871       | 0.141| 0.142    | REC   | 2.90| 0.010|
|      |    | rs8041466 | 61155328       | 0.227| 0.225    | REC   | 2.90| 0.010|
|      |    | rs11631432 | 61174348       | 0.422| 0.437    | REC   | 2.90| 0.010|
|      |    | rs2414686 | 61194350       | 0.505| 0.469    | DOM   | 2.90| 0.010|
|      |    | rs12910281 | 61195749       | 0.485| 0.452    | REC   | 2.90| 0.010|
|      |    | rs2899664 | 61200750       | 0.246| 0.208    | ALL   | 1.25| 0.016|
|      |    | rs11855147 | 61202300       | 0.075| 0.103    | ADD   | 1.25| 0.016|
|      |    | rs79995443 | 61224299       | 0.084| 0.106    | DOM   | 1.25| 0.016|
|      |    | rs72752802 | 61270307       | 0.370| 0.391    | REC   | 1.25| 0.016|
|      |    | rs74687025 | 61324825       | 0.133| 0.151    | REC   | 1.25| 0.016|
|      |    | rs4775350 | 61333253       | 0.202| 0.228    | REC   | 1.25| 0.016|
|      |    | rs782908  | 61378610       | 0.148| 0.148    | REC   | 1.25| 0.016|
|      |    | rs76824799 | 61385004       | 0.103| 0.135    | ADD   | 1.25| 0.016|
|      |    | rs4774388* | 61466998       | 0.383/(0.384) | 0.378/(0.348) | DOM | 1.14/(1.53) | 0.366/(0.024) |
|      |    | rs34720147 | 61498706       | 0.449| 0.408    | ALL   | 1.19| 0.029|
|      |    | rs11631786 | 61512161       | 0.158| 0.189    | ALL   | 0.80| 0.041|
| RORB | 9  | rs17611535 | 77158236       | 0.089| 0.085    | REC   | 3.15| 0.027|

(Continued)
rs4774388 in RORA had an increased risk for BD (OR = 1.53, P = 0.024), and marker rs1327836 in RORB revealed association with BD (OR = 1.75, P = 0.003) in dominant model. In Sample II, there were 45 SNPs (43 SNPs in RORA and 2 SNPs in RORB) showed associations with BD. The most significant marker in each gene was rs11639084 in RORA (OR = 0.69, P = 0.002) and rs17611535 in RORB (OR = 3.15, P = 0.027). One marker reported in sample I (rs4774388) did not show association with BD in Sample II. With a joint analysis to combine results from the two samples using Comprehensive Meta-Analysis Version 2 software [31], the overall p-value of this marker with BD become non-significant (P = 0.19).

For gene-based analysis, there were 47 markers in RORA and 3 in RORB with p-values less than the threshold of 0.05 and were truncated into the significant marker-set, while the remaining 392 (346 in RORA, 43 in RORB, 3 in NR1D1) SNPs were in the ‘non-significant’ marker-set. TPM analysis yielded a p-value of $1.6 \times 10^{-6}$, 0.7, and 1.0 for RORA, RORB and NR1D1, respectively. Notably, the association of RORB with BD (combined p-value = 0.007) was significant using a loose threshold $\tau = 0.1$. These results indicated strong association of RORA, weak association of RORB, and no association of NR1D1 with BD for the main effects. For the MDR analysis, the two- to four-way gene-gene interaction models are listed in Table 4. Consistent with the single marker analysis, the rs1327836 in the RORB gene had the highest testing-balanced accuracy among the 25 SNPs using sample I data. A four-way gene-gene interaction among markers rs2071427 (NR1D1), rs4774388 (RORA), rs3750420 (RORB), and rs11144047

| Gene1 | SNP1 | Gene2 | SNP2 | Gene3 | SNP3 | Gene4 | SNP4 | Training Bal. Acc. (%) | Testing Bal. Acc. (%) | Cross validation consistency |
|-------|------|-------|------|-------|------|-------|------|------------------------|------------------------|--------------------------|
| RORB  | rs1327836 | RORB  | rs12941497 | 0.5778 | 0.5200 | 7/10 |
| RORB  | rs1327836 | NR1D1 | rs12941497 | 0.5971 | 0.5239 | 3/10 |
| RORB  | rs1327836 | RORB  | rs3750420 | RORA  | rs809736 | 0.6355 | 0.4807 | 2/10 |
| RORB  | rs3750420 | RORB  | rs11144047 | RORA  | rs4774388 | NR1D1 | rs2071427 | 0.7016 | 0.5325 | 8/10 |

Training Bal. Acc.: training-balanced accuracy
Testing Bal. Acc.: testing-balanced accuracy
SNP: single-nucleotide polymorphism

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Discussion

Disruption of the circadian clock has been implicated in the etiology of BD in prior behavioral, genetic and biochemical studies. Few genetic data directly addressed the roles of NR1D and ROR gene families in BD, and none examined their interaction effects. We found that several genetic variants in the RORA and RORB genes were associated with BD. In addition, important four-way gene-gene interactions were detected among NR1D1, RORA, and RORB genes. Our findings support the potential roles of these circadian genes in the pathogenesis of BD.

In Table 1, we summarized previous findings of the three genes, and inconsistent results are seen in the NR1D1 and RORA genes with both positive and negative findings. Most of the study samples consisted of a mixture of BD subtype I and II diagnoses (the proportion of BD subtype I ranged from 63–80%). It is unlikely that the mixture of diagnoses can fully explain the inconsistent association findings. One possible explanation is that none of the individual genetic association studies genotyped genetic variants in full coverage for these candidate genes. Thus, negative findings may result from non-genotyped variants. We also noticed that the only significant association in the NR1D1 gene is from a family-based design [16], while all other case-control studies fail to show the main effect of NR1D1 with BD. Lastly, we are interested in exploring interaction effects between NR1D1 and RORs gene family as they are in the same transcriptional feedback loop in circadian pathways. Our gene-gene interaction results support this idea that the best model for BD indeed requires the interactions among RORA, RORB, and NR1D1 genes (Table 4). Thus, the NR1D1 gene may exert its effect on BD through interacting with RORs gene family.

We reported associations of several genetic variants in RORA gene with BD in the present study. In a genetic association study with 19 circadian genes in the Spanish population, Soria et al. [21] reported that nine circadian genes (ADCYAP1, ARNTL, ARNTL2, CLOCK, CRY1, NPAS2, PER3, VIP, and VIPR2) exhibited nominal significance for major depressive disorder (MDD), whereas 3 genes (BHLHB3, CSNK1E, and RORA) were significantly associated with BD. This finding is consistent with ours that RORA demonstrates a significant association with BD in gene-based testing (P = 0.0007). While RORA gene did not show association with MDD in the Spanish samples, positive associations were reported in Finland samples for MDD and the presence of early morning awakening and fatigue [12]. These results may suggest the non-specific roles of RORA gene in mood- and sleep-related phenotypes, but not specific to BD diagnosis.

For the RORB gene, marker rs1327836 showed association with BD (OR = 1.75, P = 0.003) in the present study. Two prior studies also examined the RORB gene with BD (Table 1) and reported associations with different markers in this gene. Mansour et al. [20] studied 21 circadian genes and reported that the RORB gene was associated with both BD subtype I and schizophrenia. Another study found that four SNPs in RORB gene (rs1157358, rs7022435, rs3750420, and rs3903529) demonstrated associations with pediatric BD in Caucasian samples [19]. We noted that our significant marker was also genotyped in McGrath et al.’s [19] study without significant finding; the MAF of rs1327836 was much higher in our BD samples (0.37) than that in Caucasians (0.12). On the other hand, their reported significant markers did not show association with our BD samples either. Although allelic heterogeneity may explain these observations, it is also possible that associated markers reported in these studies merely tagged to other untested genetic variants in the RORB gene. Our significant marker, rs1327836 is located in the intronic region of the gene, which does not code for amino acids. Nevertheless, it is still
possible that intronic markers influence eukaryotic gene expression through different regulation mechanisms, such as miRNA or methylation [32].

No markers in NR1D1 showed main effects with BD in our samples. Results from previous genetic association studies (summarized in Table 1) are inconsistent with predominantly negative findings. Four out of five studies did not report significant associations for NR1D1 with BD. In accordance with our negative findings, this may imply that NR1D1 does not play a major role in the pathophysiology of BD but interact with other genes to exert its effect. Moreover, it is found that lithium, a popular treatment for manic episodes [33], can inhibit glycogen synthase kinase 3β (GSK3β) and facilitate the degradation of NR1D1 [34]. Therefore, NR1D1 may not have direct effect on diagnosis per se, but influences on treatment response in BD. For instance, rs2314339 in NR1D1 is reported to be associated with lithium response [35]. It might be interesting to study the effect of NR1D1 gene with other phenotypes in our BD patients in the future, such as lithium treatment response.

For complex psychiatric disorders, the effect of a single genetic variant can be relatively small and it is assumed that multiple loci would exert their effects jointly or interactively in the pathogenesis of BD. For instance, Shi et al., (2008) conducted an association study of 15 circadian genes in BD family. They found significant multi-locus interactions between BHLHB2, CSNK1E, and CLOCK genes [47]. In the current study, the non-parametric MDR approach allows for interactions testing without the main effects. Combinations of several markers were identified by MDR to be associated with increased risk of BD, including rs2071427 in NR1D1, rs4774388 in RORA, and rs3750420 and rs11144047 in RORB. In the biological loops in circadian rhymes, NR1D1, RORA, and RORB completed ROREs binding in ARNTL. Ueda et al., [36] found that the NR1D1/ROR response element plays an important role in generating circadian night expression in phase with ARNTL gene expression in vitro validation of clock-controlled elements. Thus, the possible mechanisms contributing to such joint actions may include the ARNTL gene and other related circadian genes, which additively or synergistically contribute to increasing the risk for BD. Our findings of potential interaction effects among these genes revealed supporting evidence for the associations between NR1D and ROR families and bipolar illness. Further studies can be expanded to explore all possible interacting effects in circadian pathways for BD. We also noticed that several previous GWA studies of BD have identified few susceptible loci for BD, such as DGKH (diacylglycerol kinase eta), MYO5B (myosin 5B), ANK3 (ankyrin G), SP8, etc [37–41]. Although none of the typical circadian genes reached genome-wide significance level, one GWA study [42] reported that BHLHB3 (a circadian pathway gene) exhibited moderate effect (OR = 1.26, p-value = 5.9×10−5) with BD. Recently, a convergent functional genomics approach is proposed to mine genetic signals from GWAS datasets as well as other resources and individual studies [43]. Interestingly, a few genes in the circadian pathway are ranked on the top for their associations with BD, such as RORA, RORB, and ARNTL genes.

A number of studies have established a close link between nuclear receptor function and expression, circadian regulatory circuitry, and regulation of various physiological processes [14, 44]. Owing to RORA and RORB function as nuclear receptors [45], they exhibit action in core circadian clock genes and in metabolic genes downstream. An animal model of BD reported altered expressions of RORA and RORB in the amygdala and pre-frontal cortex [46]. It is worthwhile to investigate the molecular basis underlying bipolar illness through understanding the functions of the encoded proteins in the circadian pathways.

In conclusion, our results support the involvement of genes in the ROR family in the pathogenesis of bipolar illness. Genetic polymorphisms in the RORA and RORB genes may confer risk for developing BD. The circadian pathway may be an important candidate for further evaluation in studying the pathogenesis of BD. More replication studies and basic research are
needed to investigate the functional properties of these genes, and will contribute on our under-
standing of the etiological mechanisms underlying bipolar illness.

Limitations
There are several limitations that should be noted. First, the power to detect genes with small
effect sizes may be low due to the relatively small number of samples in this study. In addition,
we did not formally correct for multiple testing as the selected markers may have higher priori
to be associated with BD, and using the Bonferroni correction would thus be too stringent.
However, to obtain empirical p-values, we applied permutation tests that assume the associa-
tion effects are under the null. Although permutation tests cannot completely guard against in-
flated type-I errors, we sought for converging evidence using two samples to investigate the
associations between circadian pathway genes and BD. Despite using a GWA chip with a dense
marker set, the genotyped markers in the Affymetrix array are not evenly distributed in the ge-
ome, and there is no guaranteed that the coverage of variations in the three genes is high
enough to capture haplotypic variations in tested genes. In addition, due to small sample size,
we only included markers with a MAF >0.05, and common SNPs usually have small effects.
The current study is not able to identify rare SNPs with large effects, untyped SNPs, and some
structural variations, such as microsatellites, variable number tandem repeats, insertions, dele-
tions, and duplications in the three genes for their associations with BD. Third, some of the as-
sociated markers are intronic markers. Further sequencing and functional studies are required
to confirm whether the disease associations of reported markers are causal.

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Author Contributions
Conceived and designed the experiments: PHK. Performed the experiments: CHC JYW. Ana-
lyzed the data: YCL CFK. Contributed reagents/materials/analysis tools: MLL HCC PYC WWS
RBL. Wrote the paper: YCL PHK.

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