Nuclear Receptor *Rev-Erb*-α Circadian Gene Variants and Lithium Carbonate Prophylaxis in Bipolar Affective Disorder

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Abstract Rev-erb-α is one of the key components of the mammalian circadian mechanism; recently, it was also reported to be involved in the biological action of lithium. We investigated whether polymorphisms in the *Rev-erb*-α gene are associated with the long-term efficacy of lithium carbonate therapy in bipolar affective disorder. Seven single nucleotide polymorphisms (SNPs) were genotyped in a well-characterized sample of patients from Sardinia, Italy, who were followed prospectively for up to 27 years. Genotypic and allelic analysis did not show evidence for association between the polymorphisms and the different levels of lithium response. Further analyses grouping the different levels of response demonstrated that when the patients were separated into groups of nonresponders versus individuals who have had at least a minor or modest improvement in frequency of episodes or admissions, there was a significant increase in the frequency of the T allele in the nonresponder group ($p = 0.0008$). Logistic regression analyses showed that patients carrying at least one copy of the T allele for the rs2314339 marker were shown to be approximately 3.5 times more likely to have no improvement or even a worsening of the illness (odds ratio [OR], 3.56; 95% confidence interval [CI], 1.18-10.76). The results of this study may help to identify potential biological markers that can serve to predict the response of bipolar affective disorder patients to treatment, improving treatment efficacy.

Key words bipolar affective disorder, *Rev-erb*-α gene, lithium carbonate therapy, circadian rhythm, sleep, polymorphism

Bipolar affective disorder (BAD) is a common mental illness, affecting up to 1% of the population worldwide, which is characterized by episodes of mania and depression (Merikangas et al., 2007). Lithium carbonate is considered to be one of the most efficacious drugs for long-term mood stabilization in

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bipolar patients; it is effective in preventing the recurrence of affective symptoms and in reducing the risk of suicidal behavior (Goodwin and Jamison, 1990). Nevertheless, patients with BAD show large interindividual variability in the pharmacological response pattern, and many do not respond to maintenance treatment with this drug (Mamdani et al., 2004).

Clinical evidence and the cyclical nature of BAD itself suggest that affected individuals may have disruption of circadian rhythms (Mansour et al., 2005; Benedetti et al., 2007). Interestingly, *Clock*-d19 mutant mice, a central transcriptional activator in the modulation of circadian rhythms, display behavioral characteristics that resemble human mania, and chronic administration of lithium salts has been shown to restore many of the observed responses to wild-type levels (Roybal et al., 2007). The transcription factor heterodimer BMAL1/CLOCK regulates the transcription of the period and cryptochrome genes. In the nucleus, PER and CRY proteins repress their own production by inhibiting BMAL1/CLOCK, thus forming a core negative feedback loop (King and Takahashi, 2000). The nuclear receptor *Rev-erb-α* gene (*Rev-erb-α*; OMIN: 602408) presumably acts as an additional negative feedback limb by inhibiting *Bmal1* gene transcription. Because *Rev-erb-α* itself is activated by BMAL1-CLOCK, this gene and its protein constitute the molecular connection between the positive and negative loops of the mammalian circadian system (King and Takahashi, 2000; Harms et al., 2004).

Recent studies have implicated the enzyme glycogen synthase kinase-3 (GSK3), which has been shown to be inhibited by lithium, in the stabilization of the Rev-erb-α protein (Yin et al., 2006). Experiments in cultured cells have demonstrated that GSK3β phosphorylates and stabilizes Rev-erb-α and that treatment with lithium salts leads to proteasomal degradation of Rev-erb-α and increased expression of *Bmal1*, suggesting that Rev-erb-α might be a potential candidate for the regulation of the response to lithium therapy (Yin et al., 2006) as well as for the development of mood disorders (Kishi et al., 2008; Kripke et al., 2009; Severino et al., 2009).

Thus, we hypothesize that genetic polymorphisms at the *Rev-erb-α* locus may be associated with long-term efficacy of lithium therapy in bipolar illness. We tested this idea by genotyping 7 single nucleotide polymorphisms (SNPs) located throughout the gene in a sample of bipolar patients with differential responses to lithium prophylaxis.

MATERIALS AND METHODS

The sample included 170 BAD patients on lithium prophylaxis who had been followed prospectively for up to 27 years at the Lucio Bini Center for mood disorders, University of Cagliari, in Sardinia. Sample characteristics and clinical assessment have been described previously (Baldessarini et al., 2003; Silberberg et al., 2008). In brief, most patients were maintained on prophylactic treatment with lithium alone (>96%) or combined with other agents (antipsychotic agent, anticonvulsant or antidepressant). All subjects received regular (at least quarterly) follow-up assessments, including monitoring of serum drug concentrations, and estimates of interval morbidity, as recurrences of DSM-IV episodes of mania, hypomania, mixed states, or major depression. Study subjects were evaluated, treated, and followed up following an average latency of 8.8 ± 9.0 years from the estimated year of illness onset to the start of prophylactic treatment lasting an average of 5.0 ± 5.2 years. Response to lithium was evaluated using prospective data. The following factors were used in the assessment of lithium efficacy: proportion of time of illness before and after going on lithium, number and rate of hospital admissions, and adequate lithium prophylaxis as verified by clinical assessments made by research psychiatrists during follow-up visits at intervals of 2 to 3 months, following semistructured examination protocols and life charts. Treatment response measures were based on percentage of time ill during maintenance treatment as the primary outcome and were divided into 4 categories: 1, absent (no improvement or worsening of illness); 2, poor response (minor or modest improvement in frequency of episodes or admissions; significant morbidity); 3, partially good response (marked improvement but not episode free); and 4, good response (complete remission). It should be noted that the assessment of response was generally very clear for the very good and very bad responders, but the intermediate categories were considerably more uncertain. In these cases, more than one rater was consulted to make a judgment, and unclassifiable cases were excluded from the study. Informed consent was obtained from each participant recruited for the study. Ethical approval for the study was granted by the appropriate local ethical committees and by the IOP/SLAM ethical committee.

A total of 7 SNPs encompassing the *Rev-erb-α* gene (rs4794826, rs2314339, rs2071427, rs2269457, rs12941497, rs939347, and rs2071570) were selected for genotyping. The SNPs were selected according to

the linkage disequilibrium (LD) map of the CEU (white) population in the HapMap database (http://www.hapmap.org/index.html.en). Genotyping was performed under contract by Prevention Genetics (Marshfield, WI).

Hardy-Weinberg equilibrium, genotype, and allele frequencies were compared using a χ^2 test and checked via Monte Carlo simulation. Odds ratios were derived from logistic regression and adjusted for the effect of confounding variables. The analyses were carried out in Statistical Package for Social Sciences (v.15; SPSS, Chicago, IL). The LD and haplotype analysis were performed with HAPLOVIEW software. We used the position weight matrices available in the ESEfinder (2.0) tool (http://rulai.cshl.edu/tools/ESE/) to predict functional exonic splicing enhancers (ESEs) recognized by the SR proteins SF2/ASF, SC35, and SRp40. The default threshold values from the program were selected.

RESULTS

The genotype frequencies of the 7 polymorphisms (rs4794826, rs2314339, rs2071427, rs2269457, rs12941497, rs939347, and rs2071570) were in Hardy-Weinberg equilibrium in our sample set. The mean genotyping success rate was approximately 90% for the 7 SNPs. Genotypic distribution did not provide evidence for an association between markers in the *Rev-erb-α* gene and the 4 levels of lithium response ($p > 0.05$) (Table 1).

Significant LD was found between markers rs2314339 and rs2071427, rs2269457, rs12941497, rs939347, and rs2071570 ($D' > 0.6$; $p < 0.05$) but not between rs2314339 and rs4794826 ($D' = 0.04$; $p > 0.05$) (Fig. 1). Haplotype analyses including all markers generated a total of 12 different haplotypes with a frequency >1%. No significant association with the different levels of lithium response was observed ($p > 0.05$) (data not shown). Based on the LD results and considering $D' > 0.7$, 2 haplotype blocks consisting of markers rs2314339, rs2071427, rs2269457 and rs12941497, rs939347, and rs2071570 could be identified. Association analyses were conducted, and no significant finding was identified ($p > 0.05$).

Additional analyses were conducted comparing the groups of individuals who have had at least a minor or modest improvement in frequency of episodes or admissions (groups 2, 3, and 4) versus the group of nonresponders (group 1). Upon analyses, a significant difference in genotypic distribution of the rs2314339 polymorphism ($p = 0.02$) was observed (Fig. 2). The frequency of the T allele was higher in the nonresponder group ($p = 0.0008$). The last result remained significant even after Bonferroni correction for multiple testing. Power calculation revealed that our sample has 80% of power to detect differences in allelic frequency >13%, assuming the frequency of 6.2% for the T allele in the responder group, as identified in this sample. Following the allelic association test, logistic regression analysis demonstrated that patients carrying at least one copy of the T allele were approximately 3.5 times more likely to demonstrate no improvement or even a worsening of the illness (OR, 3.56; 95% CI, 1.18-10.76). The effect was still evident when correction for confounding variables such as sex was applied (OR, 3.40; 95% CI, 1.11-10.40). Besides the apparent LD that exists between this and the other markers, no significant association was identified for the additional SNPs. Possible changes in ESEs were assessed using the ESE finder tool (http://rulai.cshl.edu/tools/ESE/). According to the prediction program, the base change of C to T in the rs2314339 SNP abolishes a consensus binding site for the SR protein SC35.

DISCUSSION

In this study, we hypothesized that variations in the *Rev-erb-α* sequence could influence the response of BAD patients to long-term lithium treatment. Genotypic, allelic, and haplotypic analyses did not provide evidence for association between the polymorphism and the 4 levels of treatment response among bipolar patients who had been followed prospectively for up to 27 years. However, further analyses grouping the response levels identified that individuals carrying 1 (C/T) or 2 copies (T/T) of the T allele of the rs2314339 polymorphism had a significantly inferior response to lithium prophylaxis than individuals carrying 2 copies of the C allele (C/C). In fact, these individuals were over 3 times more likely to show a poor response or even no improvement of illness after uninterrupted, closely monitored maintenance therapy using lithium as a primary option.

There is compelling evidence that BAD is associated with irregularity in the circadian system (Mansour et al., 2005; Roybal et al., 2007; Benedetti et al., 2007). Lithium lengthens the circadian period, modulating circadian rhythms in a number of

Table 1. Genotypic distribution of 7 single nucleotide polymorphisms located in the *Rev-erb-α* gene and association results in the lithium response groups.

			Lithium Response					
			Absent	Poor	Partially Good	Good	Total	*p* Value
rs4794826	**G G**	*N*	20	18	38	53	129	0.19
		%	15.5	14.0	29.5	41.1		
	A G	*N*	3	0	9	6	18	
		%	16.7	0.0	50.0	33.3		
rs2314339	**C C**	*N*	19	19	44	61	143	0.16
		%	13.3	13.3	30.8	42.7		
	C T	*N*	5	2	5	4	16	
		%	31.3	12.5	31.3	25.0		
	T T	*N*	1	0	0	0	1	
		%	100.0	0.0	0.0	0.0		
rs2071427	**G G**	*N*	13	10	28	36	87	0.71
		%	14.9	11.5	32.2	41.4		
	G A	*N*	10	11	19	20	60	
		%	16.7	18.3	31.7	33.3		
	A A	*N*	1	0	4	2	7	
		%	14.3	0.0	57.1	28.6		
rs2269457	**A A**	*N*	17	14	38	44	113	0.98
		%	15.0	12.4	33.6	38.9		
	A G	*N*	8	6	14	16	44	
		%	18.2	13.6	31.8	36.4		
	G G	*N*	0	0	1	1	2	
		%	0.0	0.0	50.0	50.0		
rs12941497	**G G**	*N*	16	16	32	41	105	0.62
		%	15.2	15.2	30.5	39.0		
	G A	*N*	9	4	16	19	48	
		%	18.8	8.3	33.3	39.6		
	A A	*N*	1	0	1	0	2	
		%	50.0	0.0	50.0	0.0		
rs939347	**G G**	*N*	17	14	39	48	118	0.71
		%	14.4	11.9	33.1	40.7		
	G A	*N*	6	3	10	10	29	
		%	20.7	10.3	34.5	34.5		
	A A	*N*	1	0	1	0	2	
		%	50.0	0.0	50.0	0.0		
rs2071570	**G G**	*N*	13	10	31	39	93	0.82
		%	14.0	10.8	33.3	41.9		
	G T	*N*	9	5	15	14	43	
		%	20.9	11.6	34.9	32.6		
	T T	*N*	1	0	2	1	4	
		%	25.0	0.0	50.0	25.0		

Treatment response: 1, absent (no improvement or worsening of illness); 2, poor response (minor or modest improvement in frequency of episodes or admissions; significant morbidity); 3, partially good response (marked improvement but not episode free); 4, good response (complete remission). Results of association test equivalent to 10,000 Monte Carlo simulations.

organisms, suggesting that such effect may be important for its efficacy in treating mood disorders (Abe et al., 2000). One way that lithium may influence circadian rhythms is through the Rev-erb-α protein. As shown by Yin et al. (2006), lithium treatment of cultured cells results in the degradation of Rev-erb-α and an increased expression of the *Bmal1* gene, which changes the phase of molecular clock machinery.

The positive association between the rs2314339 SNP and the absence of a response to lithium therapy corroborates the hypothesis that genetic variation in this gene contributes to the interindividual variability that has been observed in BAD patients on long-term lithium therapy. Because splicing variants of the *Rev-erb-α* gene have been reported (Triqueneaux et al., 2004) and the positive rs2314339 SNP is located closely to the start of exon 3, we speculated that the

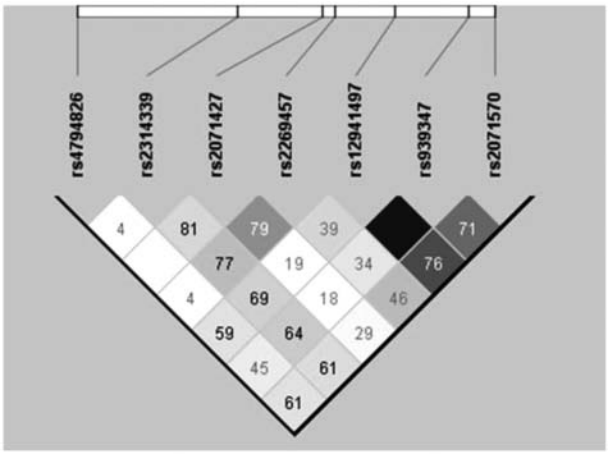

Figure 1. Representation of linkage disequilibrium structure of the genotyped single nucleotide polymorphisms (SNPs) calculated in Haploview. D′ values are given in the cell intersecting for each pair of SNPs. The color code on the Haploview plot is the following: white (|D′| < 1, LOD < 2); gray (|D′| < 1, LOD ≥ 2); light gray (|D′| = 1, LOD < 2); and black (|D′| = 1, LOD ≥ 2) (Gabriel et al., 2002). The numbers in cells are D′ values. D′ values of 1.0 are not shown.

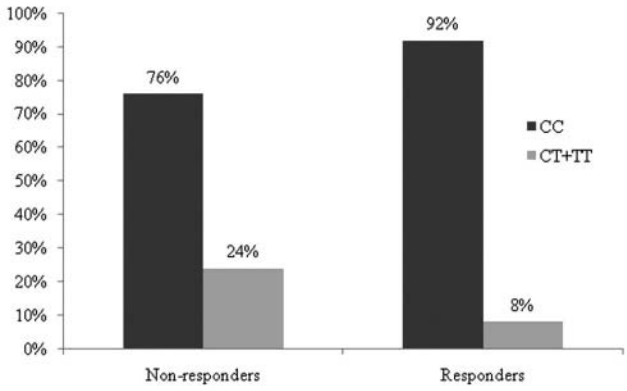

Figure 2. Frequency of the CC and CT + TT genotypes of the rs2314339 in the groups of individuals who have had at least a minor or modest improvement in frequency of episodes or admissions (responders) versus the group of individuals who showed no improvement or worsening of illness (nonresponders) (p = 0.02).

C-T change in the sequence could influence RNA splicing by altering specific short oligonucleotide sequences termed ESEs. The ESEs function as binding sites for members of the serine/arginine-rich proteins, a family of structurally related and highly conserved splicing factors that are also involved in alternative splicing regulation (Meshorer et al., 2002). Indeed, in silico analysis demonstrated that the T allele of the rs2314339 polymorphism abolishes an ESE consensus motif for the SC35 protein. Meshorer et al. (2002) suggested that splicing-related proteins, in particular SC35, may influence mammalian stress responses by controlling and modifying the expression patterns of stress-related proteins or by modifying relevant transcripts to alternate expressed forms. Although this finding suggests a potential function for the rs2314339 SNP, further studies in vitro and in vivo will be necessary to confirm that the predicted changes in the ESEs are relevant for the expression of the *Rev-erb*-α gene.

Besides the fact that most patients were maintained on prophylactic treatment with lithium alone (>96%) and that all subjects received regular (at least quarterly) follow-up assessments, potential limitations of this and other pharmacogenetic studies include possible discontinuation of lithium therapy and the use of additional medications, such as mood stabilizers and antipsychotics that might have influenced the level of treatment response. Nevertheless, the fact that the individuals were followed for a long period of time provides an opportunity for evaluating the importance of genetic factors on such a unique sample. Moreover, the fact that the genotypic association result did not remain significant after Bonferroni correction for multiple testing raises the concern of a false-positive effect. Even so, the strong allelic association and the strong odds ratio estimated by logistic regression as well as the potential change in ESEs identified support the present results. Certainly other studies should be performed in a larger sample set and in different populations to confirm whether this polymorphism can be used as a genetic marker for identifying individuals who may not benefit from lithium treatment.

In summary, we showed that the rs2314339 polymorphism is associated with the response to lithium prophylaxis in a well-characterized sample of Italian bipolar patients. A better understanding of the mechanism of action of lithium will help us develop more personalized treatments for BAD patients.

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