Association between *ARNTL* (BMAL1) rs2278749 polymorphism T >C and susceptibility to Alzheimer disease in a Chinese population

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ABSTRACT. In the present study, we examined whether the *ARNTL* (*BMAL1*) rs2278749 T/C polymorphism was associated with the susceptibility to Alzheimer disease (AD). This case-control study examined the genotypes of apolipoprotein E (*APOE e4*) and *BMAL1* rs2278749 T/C using restriction fragment length polymorphism and the TaqMan assay, respectively. A total of 296 unrelated AD patients and 423 control subjects were included. Both in the entire sample and in *APOE e4* non-carriers, the prevalence of T carriers in *BMAL1* rs2278749 T/C in AD patients was significantly higher than that in control subjects (entire sample: $\chi^2 = 12.950$, $P < 0.0001$; *APOE e4* non-carriers: $\chi^2 = 13.094$, $P < 0.0001$). Both in the entire sample and in *APOE e4* non-carriers, the prevalence of TT genotypes 2278749 in AD patients was also significantly higher than that in control subjects (entire sample: $\chi^2 = 7.765$, $P = 0.024$; *APOE e4* non-carriers: $\chi^2 = 13.062$, $P < 0.0001$). However, among *APOE e4* carriers, the difference

in the prevalence of T carriers or TT genotypes in the *BMAL1* rs2278749 T/C between patients and control subjects presents was not significant (T carriers: χ^2 = 0.078, P = 0.851 or TT genotypes: χ^2 = 2.576, P = 0.325). Among *APOE e4* non-carriers, T carriers in the *BMAL1* rs2278749 T/C were associated with a high susceptibility to AD, but among *APOE e4* carriers, the association between AD and *BMAL1* rs2278749 T/C was not significant.

Key words: Alzheimer Disease; Apolipoprotein E; *BMAL1* rs2278749T/C; Case-control study; Genetic risk factor; Metabolism; Polymorphism; Susceptibility

INTRODUCTION

Behavioral and physiological processes show approximately 24-h oscillations, known as the circadian rhythms (Kovanen et al., 2010; Melo et al., 2013). Circadian rhythms enable organisms to adapt routine environmental changes (Kovanen et al., 2010; Melo et al., 2013). The principal pacemaker of circadian rhythms is located in the suprachiasmatic nuclei of the anterior hypothalamus, which synchronizes to the external 24-h clock based on time-giving cues, primarily daily light-dark transitions. The pacemaker of circadian rhythms coordinates peripheral oscillators that maintain the timing of a range of physiological functions, including cardiovascular function, body temperature, hormone release, and physical activity. Recently, numerous studies have examined the impact of disrupting these rhythms on health (Haus et al., 2012; Vilas et al., 2012; De Giorgi et al., 2013; Melo et al., 2013; Ono et al., 2013).

On the molecular level, a network of proteins control circadian rhythms (Gebicke-Haerter et al., 2013). The clock protein (encoded by *CLOCK*) is the main functional protein that pairs with the aryl hydrocarbon receptor nuclear translocator-like (*ARNTL* or *BMAL1*) protein. Neuronal PAS domain protein 2 (*NPAS2*) can substitute for CLOCK and aryl hydrocarbon receptor nuclear translocator-like 2 (*ARNTL2* or *BMAL2*) for ARNTL. Paired CLOCK/NPAS2-ARNTL/ARNTL2 heterodimers then activate the transcription of their target genes (Reick et al., 2001; Oishi et al., 2003; Sasaki et al., 2009). Genetic variations in circadian rhythms genes have been associated with sleep, mood, and metabolic disorders. Among these disorders, circadian rhythms variants have been linked to diurnal preference, delayed sleep phase syndrome, metabolic syndrome, and obesity, and *ARNTL* gene variants to bipolar disorder, type 2 diabetes, and hypertension (Johansson et al., 2003; Nievergelt et al., 2006; Partonen et al., 2007; Woon et al., 2007; Scott et al., 2008). Many of these diseases have been found to be risk factors for Alzheimer disease (AD).

The association of AD with circadian rhythms is studied by more and more researcher (Reick et al., 2001; Johansson et al., 2003; Oishi et al., 2003; Nievergelt et al., 2006; Partonen et al., 2007; Woon et al., 2007; Scott et al., 2008; Sasaki et al., 2009). Various studies have shown that AD may be associated with circadian rhythms (Gritton et al., 2009; Cochrane et al., 2012). Our previous studies confirmed AD patients exhibit disorders in the circadian rhythm of arterial blood pressure or thyroid-stimulating hormone levels; additionally, polymorphisms in the CLOCK gene, including rs4580704 C/G, rs1554483 G/C, and 3111T/C, were associated with the susceptibility to AD (Chen et al., 2013a,b,c,d; Yang et al., 2013). However, no other polymorphisms in circadian rhythm genes have been associated with AD, and whether other circadian rhythm genes variations are also associated with the susceptibility to AD remains unknown (Chen et al., 2013c,d; Yang et al., 2013). ARNTL or BMAL1 is a key gene regulating circadian rhythm, and a T carrier at BMAL1

rs.2278749 T/C was found to be related to obesity, hypertension, and diabetes, which are risk factors for AD (Kovanen et al., 2010; Hemmeryckx et al., 2011; Milagro et al., 2012; Pappa et al., 2013). Therefore, a T carrier in the BMAL1 rs.2278749 T/C polymorphism may be a candidate gene for AD. Thus, in the present study, we examined the association between the BMAL1 rs2278749 T/C polymorphism and AD susceptibility in a case-control study.patients exhibit disorders in the circadian rhythm of arterial blood pressure or patients exhibit disorders in the circadian rhythm of arterial blood pressure or thyroid-stimulating hormone levels; additionally, polymorphisms in the CLOCK gene, including rs4580704 C/G, rs1554483 G/C, and 3111T/C, were associated with the susceptibility to AD (Chen et al., 2013a,b,c,d; Yang et al., 2013). However, no other polymorphisms in circadian rhythm genes have been associated with AD, and whether other circadian rhythm genes variations are also associated with the susceptibility to AD remains unknown (Chen et al., 2013c,d; Yang et al., 2013). ARNTL or BMAL1 is a key gene regulating circadian rhythm, and a T carrier at BMAL1 rs.2278749 T/C was found to be related to obesity, hypertension, and diabetes, which are risk factors for AD (Kovanen et al., 2010; Hemmeryckx et al., 2011; Milagro et al., 2012; Pappa et al., 2013). Therefore, a T carrier in the BMAL1 rs.2278749 T/C polymorphism may be a candidate gene for AD. Thus, in the present study, we examined the association between the BMAL1 rs2278749 T/C polymorphism and AD susceptibility in a case-control study.thyroid-stimulating hormone levels; additionally, polymorphisms in the CLOCK gene, including rs4580704 C/G, rs1554483 G/C, and 3111T/C, were associated with the susceptibility to AD (Chen et al., 2013a,b,c,d; Yang et al., 2013). However, no other polymorphisms in circadian rhythm genes have been associated with AD, and whether other circadian rhythm genes variations are also associated with the susceptibility to AD remains unknown (Chen et al., 2013c,d; Yang et al., 2013). ARNTL or BMAL1 is a key gene regulating circadian rhythm, and a T carrier at BMAL1 rs.2278749 T/C was found to be related to obesity, hypertension, and diabetes, which are risk factors for AD (Kovanen et al., 2010; Hemmeryckx et al., 2011; Milagro et al., 2012; Pappa et al., 2013). Therefore, a T carrier in the BMAL1 rs.2278749 T/C polymorphism may be a candidate gene for AD. Thus, in the present study, we examined the association between the BMAL1 rs2278749 T/C polymorphism and AD susceptibility in a case-control study.

MATERIAL AND METHODS

Patients

Inclusion criteria of AD patients and controls have been reported previously (Chen et al., 2013c,d; Yang et al., 2013). Briefly, from a primary care setting, AD patients were recruited according to the National Institute of Neurological and Communicative Diseases and Stroke/ Alzheimer's Disease and Related Disorders Association criteria from January 2010 to June 2012. The severity of AD in all patients was classified according to the criteria, and included patients had AD for more than 1 year. To eliminate the influence of drugs used for treating AD, patients receiving drug treatment for AD were excluded. The primary care setting was a section of the Geriatrics Department of a hospital in a city in west-south China that conducts medical examinations for the elderly. Cases included 142 men and 154 women, with a mean age of 77.84 ± 3.97 years and ranging from 65 to 85 years. Control subjects were of similar age, sex, daily activity levels, sleep quality, and health status (except AD). Mini-Mental State Examination (MMSE), a sensitive and Chen-Dureliable questionnaire, was conducted for all participants to detect cognitive function. Daily activities were reported by participants and their family members, and sleep quality was

measured using the Pittsburgh Sleep Quality Index. In addition, the participants' health status was evaluated according to medical records. The control subjects included 423 subjects, with a mean age of 76.92 ± 3.76 years, ranging from 65 to 85 years, including 204 men and 219 women. Patients with prior diagnosis of cardiovascular, cerebrovascular, and peripheral vascular diseases; sleep apnea syndrome; or chronic renal failure were excluded. Informed consent was obtained from all participants (as well as their legal proxies). The research ethics committee of Sichuan University approved the study.

Genotyping

Genomic DNA was isolated from whole blood drawn from the antecubital vein using commercial DNA isolation kits from Qiagen (Hilden, Germany) according to standard procedures (Chen et al., 2013c; Chen et al., 2013d; Yang et al., 2013). We performed genotyping of *BMAL1* polymorphisms using a TaqMan assay with allele-specific probes using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to standardized laboratory protocols. We performed genotyping of the apolipoprotein gene (*APOE*) as previously described (using the restriction fragment length polymorphism method) (Chen et al., 2013c,d; Yang et al., 2013).

Statistical analysis

The allele frequencies and genotype distribution of AD patients and control subjects were determined using the allele-counting method and analyzed using Pearson χ^2 tests using SPSS software (SPSS, Inc., Chicago, IL, USA). Using χ^2 or Fisher exact test (where the expected cell count was < 5) for categorical variables and unpaired Student *t*-test for continuous variables, baseline characteristics were compared between AD patients and control subjects. Power analysis was used to determine the sample size using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/ purcell/gpc/cc2.html) according to a previous study; the results showed a power greater than 0.8. $P < 0.05$ was considered to be statistically significant, and all P values were 2-sided. Because the APOE e4 allele, the only confirmed genetic risk factor for AD, dose-dependently increases the risk of AD and is associated with 40 to 70% of cases, the data were analyzed according to APOE e4 carriers and non-carriers (Chartier-Harlin et al., 1994; Eichner et al., 2002).

RESULTS

Baseline characteristics have been reported previously (Chen et al., 2013c; Chen et al., 2013d; Yang et al., 2013). Among the initially enrolled 719 participants included, there were 296 AD patients and 423 control subjects. Compared with the control subjects, AD patients had significantly lower MMSE scores (29.01 ± 4.73 *vs* 17.67 ± 3.75, $t = 6.165$, $P < 0.0001$) and a higher prevalence of *APOE e4* (27.38 *vs* 21.01%; $\chi^2 = 22.433$, $P < 0.0001$) (Table 1). In MMSE examination, the total score that can be achieved is 30, and higher scores indicate better cognitive function. Subjects without dementia had MMSE scores higher than 27, and those with MMSE scores lower than 24 were typically diagnosed with dementia. In this study, MMSE scores in the control subjects were all higher than 27, and those in AD patients were all lower than 24.

There was a significant difference in the prevalence of genotypes (C/C: 55.41 *vs* 66.43%;

Table 1. Baseline demographics.

Group	AD (N = 296)	Controls (N = 423)	χ^2 or t	P
Age	77.84 ± 3.97	76.92 ± 3.76	0.581	0.935
Women	154 (51.69%)	219 (51.72%)	0.869	0.351
MMSE	17.67 ± 3.75	29.01 ± 4.73	6.165	<0.0001
APOEε4	81 (27.38%)	89 (21.01%)	22.433	<0.0001

APOE ε4: Apolipoprotein E; MMSE: Mini-mental state examination; AD: Alzheimer disease.

C/T: 27.37 *vs* 22.22%; T/T 17.22 *vs* 11.35%, χ^2 = 7.765, P = 0.024). AD patients showed a higher prevalence T carriers in *BMAL1* rs.2278749 T/C (30.91 *vs* 24.82%, χ^2 =12.950, P < 0.0001) than was observed in controls (Table 2).

According to *APOE e4* subgroups, we further compared the difference in the prevalence of genotypes and *BMAL1* rs2278749 T/C between patients and controls. Among *APOE e4* non-carriers, there was a significant difference in the prevalence of genotypes (C/C: 56.28 *vs* 70.66%; C/T: 23.72 *vs* 17.66%; T/T 20.00 *vs* 11.68%; χ^2 = 13.262, P < 0.0001). AD patients showed a higher prevalence of T carriers (31.86 *vs* 20.51%; χ^2 = 13.094, P < 0.0001) than was observed in controls (Table 2).

Table 2. Comparing allelic and genotypic frequencies of BMAL1rs.2278749 T/C polymorphismin AD patients and controls.

Total Group	AD patients (N = 296)	Healthy controls (N = 423)	χ^2	P
Allele				
	C409 (69.09%)	656 (75.18%)		
	T183 (30.91%)	190 (24.82%)	12.950	<0.0001
Genotype				
	C/C164 (55.41%)	281 (66.43%)		
	C/T81 (27.37%)	94 (22.22%)		
	T/T51 (17.22%)	48 (11.35%)	7.765	0.024
	APOEε4 carriers 81 (27.38%)	89 (21.01%)		
Allele				
	C116 (71.60%)	125 (70.22%)		
	T46 (28.40%)	53 (29.78%)	0.0783	0.851
	GenotypeC/C43 (53.08%)	45 (51.02%)		
	C/T30 (37.04%)	35 (39.71%)		
	T/T8 (9.88%)	9 (10.11%)	2.576	0.325
	APOEε4 non-carriers 215 (72.62%)	334 (78.99%)		
Allele				
	C293 (69.14%)	491 (73.49%)		
	T 137 (31.86%)	137 (20.51%)	13.094	<0.0001
Genotype				
	C/C121 (56.28%)	236 (70.66%)		
	C/T51 (23.72%)	59 (17.66%)		
	T/T43 (20.00%)	39 (11.68%)	13.262	<0.0001

APOE ε4: Apolipoprotein E; AD: Alzheimer disease.

However, among *APOE e4* carriers, there was a non-significant difference in the prevalence of genotypes (C/C: 53.08 *vs* 51.02%; C/T: 37.04 *vs* 39.71%; T/T 9.88 *vs* 10.11%; χ^2 = 2.576, P = 0.325) and T carriers (28.40 *vs* 29.78%, χ^2 = 0.0783, P = 0.851) than in controls (Table 2).

DISCUSSION

In the present study, the prevalence of *APOE e4* carriers was significantly higher in AD patients than in controls (27.38 *vs* 21.01%, χ^2 = 22.433, P < 0.0001). There was a difference in the association of AD with *BMAL1* rs2278749 T/C between *APOE e4* non-carriers and carriers. In *APOE e4* non-carriers and in the entire sample, a T carrier in *BMAL1* rs2278749 T/C was

associated with an increased risk of AD. However, in *APOE e4* carriers, there was no association between AD and *BMAL1* rs2278749 T/C.

We examined the association between AD and circadian rhythm gene polymorphisms. The polymorphism in circadian rhythm genes included different alleles in the *CLOCK* and *BMAL1* genes (Chen et al., 2013c,d; Yang et al., 2013). Interestingly, the associations between AD and polymorphisms in circadian rhythm genes were similar. In *APOE e4* non-carriers and in the entire population, circadian rhythm gene polymorphisms were associated with the susceptibility to AD, and subjects with minor genotypes had a higher risk of AD. In *APOE e4* carriers, there was no significant difference in the association between AD and circadian rhythm gene polymorphisms.

In the present study, we found that the associations between AD and circadian rhythm gene polymorphisms were similar. Several loci on circadian rhythm genes have been found to be associated with metabolic syndrome and obesity, which are risk factors for AD (Chen et al., 2013c,d; Yang et al., 2013). AD is also a metabolism-associated disease, and the minor genotypes of circadian rhythm genes may increase the risk of AD through metabolic syndrome (Woon et al., 2007; Scott et al., 2008). However, no direct evidence has demonstrated this association.

The associations between AD and circadian rhythm gene polymorphisms were the same as those between AD and the *APOE e4* allele (Chartier-Harlin et al., 1994; Eichner et al., 2002). In the Chinese population, *APOE e4* was found to be the minor genotype. The *APOE e4* allele dose-dependently increases the risk of AD and was associated with 40 to 70% of cases. The *APOE e4* allele is associated with an increased risk of obesity. The association between *APOE e4* and increased risk of AD may in part share the same mechanism with the minor genotypes in circadian rhythm genes. In the sample, there were 549 *APOE e4* non-carriers, which was more than 3 times the number of *APOE e4* carriers.

Although among the entire sample, *BMAL1* rs2278749 T/C carriers appeared to be associated with an increased risk of AD and showed a strong association with an increased risk of AD in *APOE e4* non-carriers, BMAL1 rs2278749 T/C carriers showed no association with AD in *APOE e4* carriers. The association between BMAL1 rs2278749 T/C carriers and an increased AD risk was only the representation of that in APOE e4 non-carriers. This indicated that the strong association between AD and *BMAL1* rs2278749 T/C among the entire sample agreed with results observed in *APOE e4* carriers.

For the association between the *BMAL1* rs2278749 T/C polymorphism and AD, we observed a difference between *APOE e4* carriers and non-carriers. Among the *APOE e4* carriers, the strong association between *BMAL1* rs2278749 T/C and the increased risk of AD was not observed in *APOE e4* non-carriers. Both *APOE e4* carriers and *BMAL1* rs2278749 T/C carriers were at risk of developing AD. However, when the 2 factors were evaluated together, the risk of AD did not significantly increase compared to the presence of only one factor. This can be explained in 2 ways: 1) the prevalence of AD was high in *APOE e4* carriers and carriers with *BMAL1* rs2278749 T/C; when both factors were present, the increase in the prevalence of AD was not significant; 2) carriers of both *BMAL1* rs2278749 T/C and *APOE e4* were at an increased risk of AD because of the same mechanism, but the increase in AD risk was not significant.

BMAL1 is a key gene regulating circadian rhythm, and *BMAL1* rs2278749 T/C was related to the risk of AD, indicating that circadian rhythm is related to AD. The *BMAL1* polymorphism was related to sleep and circadian disturbance, which are risk factors of AD. We excluded these factors and found that the association between *BMAL1* rs2278749 T/C and AD risk was not related to circadian disturbance habits, but may be related to a metabolic mechanism described above. Circadian rhythm (including sleep/awake, blood pressure rhythm, and thyrotrophic-stimulating

hormone rhythm, among others) disorders are confirmed risk factors of AD, and we found that circadian rhythm was related to AD risk on a gene level, particularly a genetic locus related to AD (Chen et al., 2013a,b).

Our study had some limitations (Chen et al., 2013c,d; Yang et al., 2013). The main limitation was the small sample size. In this study, only 719 subjects, including 296 AD patients and 423 normal controls, were included in the study. Ours was a small sample size, but few AD patients were identified according to National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association criteria. By the end of September 2011, we had only recruited 318 subjects (130 AD patients and 188 normal controls) and analyzed the data obtained until that time. The results were the same as those obtained using the larger sample size described here. Another limitation was that the information regarding metabolic syndrome was not collected in the study, so the hypothesis that metabolic mechanism played an important role in the association between *BMAL1* rs2278749 T/C and AD risk could not be confirmed using the current data.

In summary, we found that *BMAL1* rs2278749 T/C was associated with AD risk, and T carriers in *BMAL1* rs2278749 T/C showed a higher risk of AD than did non-carriers. However, the association differed between APOE e4 non-carriers and carriers, and was only detected in APOE e4 non-carriers.

Conflicts of interest

The authors declare no conflict of interest.

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