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Gene polymorphisms (rs324957, rs324981) in NPSR1 are associated with increased risk of primary insomnia

A cross-sectional study

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Abstract

Neuropeptide S and neuropeptide S receptor (NPSR1) are associated with sleep regulation. Herein, the possible contribution of 6 polymorphisms in NPSR1 on the chromosome to primary insomnia (PI) and objective sleep phenotypes was investigated.

The study included 157 patients with PI and 133 age- and sex-matched controls. All subjects were investigated by polysomnography for 3 consecutive nights. The genotyping of 6 polymorphisms was carried out by polymerase chain reaction-restriction fragment length polymorphism method.

A significant difference was detected for rs324957 and rs324981 between PI and controls. The PI patients had a higher frequency of AA than controls in rs324957 ($P = .02$) and rs324981 ($P = .04$). However, for other single nucleotide polymorphisms (rs323922, rs324377, rs324396, and rs324987), no significant differences were observed between PI patients and controls. There were 2 different allelic combinations that were associated with PI susceptibility (CATGTC, GCCAAT) and its risk factor. A significant difference in sleep latency was observed among 3 genotype carriers of NPSR1 gene polymorphism rs324957 in PI group ($P = .04$), with carriers of the A/A genotype having the longest sleep latency (mean ± SD: 114.80 ± 58.27), followed by the A/G genotype (112.77 ± 46.54) and the G/G genotype (92.12 ± 42.72).

This study provided the evidence that the NPSR1 gene polymorphisms (rs324957, rs324981) might be susceptibility loci for PI. Further studies are needed to explore the role of NPSR1 gene polymorphisms in molecular mechanisms of PI in a larger sample size.

Abbreviations: LD = linkage disequilibrium, NPS = neuropeptide S, NPSR1 = neuropeptide S receptor, PI = primary insomnia, PSG = polysomnogram, SNPs = single nucleotide polymorphisms.

Keywords: neuropeptide S receptor, objective sleep phenotypes, polymorphism, primary insomnia

1. Introduction

Insomnia is one of the most common clinical diseases worldwide. A meta-analysis of 17 studies showed that the prevalence of insomnia was 15.0% in China.[1] Long-term insomnia can lead to physiological and psychological damage, including memory decline, irritability, and depression.[2] Insomnia is classified as primary and secondary insomnia. Epidemiology has identified several risk factors for insomnia, such as drinking, depression, older age, and hyperlipidemia.[3–5] However, these risk factors do not predict the risk of primary insomnia (PI). Accumulating evidence showed that PI is genetically controlled[6,7] by clock circadian regulator,[8] genes encoding the regulatory factor X3,[9] and adenosine triphosphate-binding cassette, and subfamily C member 9,[10] suggesting the critical role of genes in PI.

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Neuropeptide S (NPS) is a novel 20-amino acid peptide, mainly expressed in the endocrine tissues and central nervous system.[11] It specifically binds to a metabotropic G-protein-coupled receptor known as the neuropeptide S receptor (NPSR1).[11] NPS and NPSR1 are newly discovered sleep regulatory systems.[12] In recent years, a large number of NPS precursor mRNAs have been found in the primary sensory trigeminal nucleus and the parabrachial nucleus.[12,13] The trigeminal nucleus is forcefully modulated by the sleep/wake cycle, and the parabrachial nucleus also plays a critical role in the ascending arousal network.[14] Animal studies demonstrated that the NPS-NPSR system promotes arousal, mainly to prolong sleep latency, reduce the number of transitions from awakening to slow-wave sleep, increase the wake time, and shorten the slow-wave sleep and rapid eye movement sleep time.[13,15] However, NPSR1 knockout mice showed increased anxiety-like behavior. The behavioral arousal was reduced, which corresponds to a decrease in the exploration activity and an increase in rest time.[16] Moreover, several animal and preclinical studies have shown that the NPS-NPSR system is involved in memory,[17] fear expression, extinction,[18] and facilitation of olfactory function.[19]

Although the NPS-NPSR plays a major role in sleep regulation, most of the discoveries were made in rodents. Accumulating data have recently begun to emphasize the role of single nucleotide polymorphisms (SNPs) in the NPSR1 gene in human sleep regulation. NPSR1 rs324981 is the A/T mutation in the 107th triplet of the NPSR1 gene on locus p14.3 on chromosome 7. Gottlieb et al[20] showed that the average bedtime was delayed in NPSR1 rs324981 T allele carriers. However, a recent study in the Caucasian population[21] revealed that the NPSR1 rs324981 SNP was not significantly associated with bedtime or sleep latency, but the total sleep time was significantly different, and the sleep time of TT homozygous genotype population was significantly shorter than in the individuals carrying the A genotype. Simultaneously, Guerrini et al[22] showed that the NPS-NPSR system is involved in the regulation of human physiological sleep. These studies suggested that the NPSR1 rs324981 T genotype may lead to prolonged sleep latency and shortened sleep duration, implying that NPSR1 rs324981 SNP is associated with PI, intimating the main role of this gene in PI.

Functional SNP of the NPSR1 gene was selected from the HapMap program and the National Center for Biotechnology Information SNP database. We analyzed 6 variations along the NPSR1 gene in PI patients and controls from China. These SNPs (rs323922, rs324377, rs324396, rs324957, rs324981, and rs324987) are localized at chromosome 7p14.3. In addition to the most studied NPSR1 rs324981 SNP, rs323922, and rs324377 have been previously reported to confer genetic risk for asthma.[23] Also, a number of studies demonstrated that NPSR1 polymorphism rs324987 might be relevant to susceptibility and the clinical manifestations of rheumatoid arthritis.[24,25] However, to the best of our knowledge, the association of the NPSR1 gene polymorphism with PI has not been reported in the medical literature. Herein, we tested the hypothesis that the NPSR1 variant of rs323922, rs324377, rs324396, rs324957, rs324981, and rs324987 might be a susceptibility locus for PI and further hypothesized that these variants were associated with the objective sleep phenotypes in patients with PI.

2. Materials and methods

2.1. Subjects

Initially, a total of 468 patients were admitted for polysomnography at the Sleep Medicine Center in Gansu Provincial Hospital from January 2016 to December 2019. Of these, 311 with other sleep-related disorders were excluded from this study. The following exclusion criteria were applied:

(1) Secondary insomnia due to other mental or sleep disorders and medical problems, such as drugs or alcohol, especially to identify and exclude insomnia caused by anxiety disorders;
(2) Other causes of insomnia, such as those who have suffered from severe psychological stress recently;
(3) Patients with severe physical diseases, such as heart disease, hypertension, diabetes, and abnormal thyroid function;
(4) Polysomnogram (PSG) monitoring results in patients with subjective insomnia;
(5) PSG monitoring results of apnea-hypopnea index >15;
(6) Young and old people who are not suitable for scale assessment. The final sample included 157 patients with PI, including 109 females and 48 males. These eligible patients fulfilled the PI diagnostic criteria provided by the 5th edition of the American Diagnostic and Statistical Manual of Mental Disorders.[26] In addition, 133 age- and sex-matched controls were recruited from the physical examination center of the hospital in the same period without any history of mental illness or major physical illness. All samples collected in this study and the clinical data obtained with the informed consent of the individuals were approved by the Ethics Committee of Gansu Provincial Hospital.

2.2. PSG recordings

Sleep during all nights were monitored by AV-PSG at a room temperature of 20° to 22°C. Subjects entered the monitoring room according to the daily schedule and did not take caffeine, alcohol, tea, or sleep medication during the day. The PSG record was tested overnight for a minimum of 7 hour. All subjects were investigated by PSG for 3 consecutive nights. Sleep data were collected and scored using the Alice 5 Diagnostic Sleep System (Philips Respironics, Bend, OR). The following PSG indexes were assessed:

(1) Total sleep time;
(2) Sleep latency: the time between lights off and the onset of the first frame of sleep;
(3) Percentage of period N1 (N1%), period N2 (N2%), period N3 (N3%), and stage R (R%): the proportion of period N1, period N2, period N3, and period R, respectively, to total sleep time.[27]

2.3. Genotyping

2 mL of elbow venous blood samples were collected in an ethylenediaminetetraacetic acid (EDTA) tube from PI patients and controls. Serum was separated immediately by centrifugation at 3000 rpm for 10 minutes at 4°C, and stored in a freezer at –70°C. DNA were extracted from blood white blood cells by using the TIANamp Blood DNA Kit blood genomic DNA extraction kit (Tiangen Biotechnology, Beijing, China). A

Table 1

Baseline characteristics of patients with primary insomnia and controls.

Characteristics	Patients with primary insomnia (N = 157)	Normal controls (N = 133)	*t* test or χ^2 test	*P*
Gender (male/female)	48/109	43/90	0.10	.75
BMI (kg/m^2)	21.96 ± 1.99	22.43 ± 2.97	−1.54	.13
Age (yr)	44.32 ± 10.42	43.38 ± 9.96	0.78	.44
Education (yr)	12.68 ± 3.30	12.35 ± 3.58	0.50	.62
PSG recordings				
Sleep efficiency (%)	49.55 ± 10.36	91.81 ± 3.99	−47.15	<.001
Total sleep time (min)	267.59 ± 55.94	434.85 ± 40.04	−29.58	<.001
Sleep latency (min)	105.87 ± 49.52	24.33 ± 7.13	20.38	<.001
Wake periods (no.)	11.57 ± 4.89	2.63 ± 1.27	22.05	<.001
N1 (%)	17.63 ± 7.25	9.93 ± 2.95	12.17	<.001
N2 (%)	61.64 ± 8.80	54.88 ± 4.88	8.25	<.001
N3 (%)	6.123 ± 3.61	17.42 ± 3.88	−25.51	<.001
R (%)	14.53 ± 5.15	17.73 ± 3.92	−5.99	<.001

PSG = polysomnography, R = rapid eye movement.

NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) was used to measure the concentration and purity of the extracted peripheral blood genomic DNA. The functional SNP of NPSR1 gene was selected from the HapMap program and the National Center for Biotechnology Information SNP database. Six SNPs (rs323922, rs324377, rs324396, rs324957, rs324981, and rs324987) localized in the NPSR1 gene were selected for the study. Entrust a third party (Biomiao Biotechnology, Beijing, China) to conduct SNP typing testing. The genotypes of NPSR1 gene polymorphisms were determined by the polymerase chain reaction-restriction fragment length polymorphisms method.

2.4. Data analysis

Continuous variables were represented as mean ± SD and categorical variables as percentages. The χ^2 test and 2-tailed independent *t* test were performed on categorical and continuous variables, respectively. The Kolmogorov–Smirnov test was used to verify whether the enumeration data had a normal distribution. The Mann–Whitney *U* test was applied for continuous variables that did not conform to normal distribution. Variance analysis was used to study the effect of genotypes on the objective sleep phenotypes. Statistical analysis was performed by SPSS software (IBM SPSS version 23.0, Chicago, IL) with a significance level of $P < .05$. The Hardy–Weinberg equilibrium was tested using a χ^2 test for the goodness of fit.[28] Haploview and SHEsis were used to analyze the linkage imbalance and haplotype of SNP sites, respectively.

3. Results

3.1. Demographic characteristics

The cohort consisted of 157 patients with PI and 133 controls without any significant difference in the distribution of gender ($\chi^2 = 0.10$, $P = .75$), age ($t = 0.78$, $P = .44$), and education time ($t = 0.50$, $P = .62$). Compared to the control group, the PI group had lower sleep efficiency, longer sleep latency, and shorter total sleep time. Data on the objective sleep phenotypes of PI patients and the control group are summarized in Table 1.

3.2. Genotype and allele frequencies of 6 polymorphisms between PI patients and control group

The genotype distribution of the NPSR1 gene polymorphisms (rs323922, rs324377, rs324396, rs324957, rs324981, and rs324987) fulfilled the conditions of Hardy–Weinberg equilibrium in normal controls ($P > .05$). The allele and genotype distributions of the NPSR1 gene polymorphism in all subjects are presented in Table 2. Compared to the wild type, significant differences were detected in rs324957 and rs324981 between PI and control groups in the heterozygous type. The PI patients had a higher frequency of AA than controls in rs324957 (27.3% vs 16.0%; $\chi^2 = 5.20$, $P = .02$). Similarly, patients with PI had a higher AA genotype in rs324981 (29.9% vs 19.2%; $\chi^2 = 4.26$, $P = .04$) than controls. For rs323922, rs324377, rs324396, and rs324987 polymorphisms, no significant differences were detected in genotype distributions or allele frequencies between PI and controls.

3.3. Haplotype frequencies of 6 polymorphisms between PI patients and controls

We calculated the pairwise LD between the investigated polymorphisms using the SHEsis program. Table 3 shows that rs324377 and rs323922 or rs324396 and rs324957 or rs324396 and rs324981 were in complete linkage imbalance (D' = 1.00), while rs324957 and rs324981 were in strong LD (D' = 0.99), but other LDs composed of other polymorphisms were relatively weak.

3.4. Gene haplotype analysis

Table 4 shows allelic combination tests that analyzed the putative interaction between the SNP of this genomic region. For the haplotype analysis of these 6 SNPs, 14 haplotypes were constructed, of which 10 were < 5% in this sample and excluded from the analysis. Finally, we found 2 different allelic combinations that were associated with PI susceptibility (CATGTC, GCCAAT) and its risk factor. Thus, it was concluded that individuals with this specific haplotype had an increased risk of developing symptoms of the disease as compared to individuals without haplotype.

Table 2

Genotype distributions and allele frequencies of 6 polymorphisms between primary insomnia patients and normal controls.

SNP	Allele/genotype	Patients with primary insomnia	Normal controls	χ^2 test	*P*
rs323922	G	162 (51.9%)	131 (49.6%)	0.30	.58
	C	150 (48.1%)	133 (50.4%)		
	GG	47 (30.1%)	28 (21.2%)	2.95	.09
	GC	68 (43.6%)	75 (56.8%)		
	CC	41 (26.3%)	29 (22.0%)		
rs324377	C	161 (51.9%)	129 (49.2%)	0.41	.52
	A	149 (48.1%)	133 (50.8%)		
	CC	47 (30.3%)	27 (20.6%)	3.49	.06
	CA	67 (43.2%)	75 (57.3%)		
	AA	41 (26.5%)	29 (22.1%)		
rs324396	T	136 (44.2%)	108 (41.9%)	0.30	.58
	C	172 (55.8%)	150 (58.1%)		
	TT	36 (23.4%)	20 (15.5%)	2.74	.10
	TC	64 (41.6%)	68 (52.7%)		
	CC	54 (35.1%)	41 (31.8%)		
rs324957	A	146 (47.4%)	112 (42.7%)	1.24	.27
	G	162 (52.6%)	150 (57.3%)		
	AA	42 (27.3%)	21 (16.0%)	5.20	.02
	AG	62 (40.3%)	70 (53.4%)		
	GG	50 (32.5%)	40 (30.5%)		
rs324981	A	153 (49.7%)	115 (44.2%)	1.68	.20
	T	155 (50.3%)	145 (55.8%)		
	AA	46 (29.9%)	25 (19.2%)	4.26	.04
	AT	61 (39.6%)	65 (50.0%)		
	TT	47 (30.5%)	40 (30.8%)		
rs324987	T	158 (50.6%)	124 (47.0%)	0.77	.38
	C	154 (49.4%)	140 (53.0%)		
	TT	51 (32.7%)	33 (25.0%)	2.05	.15
	TC	56 (35.9%)	58 (43.9%)		
	CC	49 (31.4%)	41 (31.1%)		

Because of genotyping failure, the total number of patients with primary insomnia and control numbers for each single nucleotide polymorphism may be <157 and 133, respectively.
SNP = single nucleotide polymorphism.

3.5. NPSR1 gene polymorphism rs324957 and changes in objective sleep phenotypes

Differences in objective sleep phenotype across rs324957 and rs324981 genotypes in patients with PI were analyzed. There were no significant difference in total sleep time, sleep efficiency and the percentage of sleep stages were observed among the different genotype carriers of NPSR1 gene polymorphism rs324957 ($P > .05$). However, a significant difference in sleep latency was observed among 3 genotype carriers of NPSR1 gene polymorphism rs324957 in PI group ($P = .04$). The A/A genotype carriers had the longest sleep latency (mean ± SD: 114.80 ± 58.27), followed by A/G genotype (112.77 ± 46.54) and G/G genotype (92.12 ± 42.72). There were no significant difference in any objective sleep phenotype were observed among the different genotype carriers of NPSR1 gene polymorphism rs324981 ($P > .05$, data are not shown).

4. Discussion

Presently, there are many studies on the mechanism of PI, but there is no obvious conclusion due to its extreme complexity. In this cross-sectional study, we first demonstrated that there is a significant difference were detected for rs324957 and rs324981 between PI patients and the control group in the mutant type compare to wild type. Second, to further confirm the association between NPSR1 gene polymorphism and PI, we carried out linkage unbalance analysis and haplotype analysis on 6 SNPs of NPSR1 gene. The results showed that rs324957 and rs324981 are in strong LD. Haplotype analysis showed that the haplotypes

Table 3

Linkage disequilibrium.

D'	rs324377	rs324396	rs324957	rs324981	rs324987
rs323922	1.00	0.40	0.34	0.33	0.77
rs324377		0.41	0.35	0.34	0.77
rs324396			1.00	1.00	0.50
rs324957				0.99	0.29
rs324981					0.32

Table 4

Analysis of neuropeptide S receptor gene single nucleotide polymorphism for allelic combination with primary insomnia patients and normal controls.

Allelic combination	Normal controls, n (%)	Patients with primary insomnia, n (%)	*P*	OR (95% CI)
C A C A A C	65 (25.2)	85 (27.7)	.38	1.18 (0.81–1.73)
C A T G T C	21 (8.1)	40 (13.1)	.04	1.77 (1.01–3.09)
G C C A A T	16 (6.2)	40 (12.9)	.01	2.33 (1.27–4.29)
G C T G T T	87 (33.8)	96 (31.1)	.65	0.92 (0.65–1.32)

Order of single nucleotide polymorphisms: rs323922, rs324377, rs324396, rs324957, rs324981, rs324987. Allelic combinations with a frequency $< 5\%$ in patients with primary insomnia and control groups were excluded from the analysis.

CATGTC and GCCAAT on the NPSR1 gene were significantly different between the PI patients and control group and served as risk factors. Thus, it could be concluded that individuals with either of these 2 haplotypes have an increased risk of developing symptoms of PI as compared to individuals without these haplotypes. We further explored the effect of these 2 NPSR1 polymorphisms on the sleep pattern by an objective sleep phenotype. A significant difference in sleep latency was observed among 3 genotype carriers of NPSR1 gene polymorphism rs324957. This study suggested that NPSR1 gene polymorphisms, rs324957, and rs324981, maybe a susceptibility locus for PI and may be involved in the development of the condition.

More and more evidence shows that PI is controlled by genes to a certain extent.[6] Many genes and variants have been identified as promising candidates for increasing the risk of PI, while no consensus on certain variants or genes consistently associated with the risk and development of PI.[7,29] To our knowledge, there are only 2 studies performed the genetic association of subjective and objective sleep phenotypes with a functional polymorphism in the NPSR1 gene. Gottlieb et al[20] reported that there was an association of usual bedtime (obtained from subjective sleep questionnaires) with SNP rs324981 in NPSR1 in 749 subjects from a longitudinal study of the cardiovascular consequences of sleep-disordered breathing in USA. They found that individuals who had a non-synonymous mutation encoding an Asn107→Ile107 substitution (rs324981) showed a delayed bedtime by nearly 15 minutes in heterozygotes and 30 minutes in homozygotes. However, Spata et al[21] conducted another study to evaluate the association of NPSR1 gene polymorphism with sleep related parameters measured by relatively objective body dynamic recorder. In that study, they found that NPSR1 genotype at rs324981 were significantly associated with total sleep time while there were no effects on bedtime or sleep onset in 393 white adults. Compared to A-allele carriers, participants with homozygous for the minor T-allele had a significantly decreased total sleep time. However, PSG is the golden standard to assess the objective sleep parameters. We performed the present study in Chinese PI patients by PSG, which increased the accuracy of the sleep parameters. Inconsistent with Gottlieb et al study,[20] although we did not detected any difference among 3 genotype carriers of NPSR1 gene polymorphism rs324981 and any objective sleep phenotype, we found that a significant difference in sleep latency was observed among 3 genotype carriers of NPSR1 gene polymorphism rs324957 in PI group. Furthermore, our population in our study was differ from those studies performed in USA and Germen. Last but not the least, relatively elder subjects reported in previous studies may partly explain the difference in results as sleep habits changed with aging.[30]

Nevertheless, the present study has several limitations. First, the results may not be consistent over a period due to the cross-sectional design of the study. Second, all the patients were enrolled from a single hospital, which might cause a selection bias. Third, the sample size was not sufficient to detect a small or modest genetic effect. Fourth, only Chinese subjects were included in the study, and other ethnic groups should be included in the study in the future.

In conclusion, the present study found that the NPSR1 gene polymorphisms rs324957 and rs324981 may be susceptibility loci for PI. Due to the lack of effective method in studying histology and molecular level of central nervous system without trauma, the NPSR1 gene polymorphisms through clinical phenotype combined with blood is potentially essential in providing clues of the molecular mechanism in PI. Since the results of this study are preliminary, additional studies focusing on gene-to-gene or gene-to-environment interactions are needed to explore the role of NPSR1 gene polymorphisms in molecular mechanisms of PI in a larger sample size.

Table 5

Changes in objective sleep phenotype across rs324957 genotypes in patients with primary insomnia.

Objective sleep phenotype	A/A	A/G	G/G	F test	*P*
Both genders	42	62	50		
Sleep efficiency (%)	50.10 ± 11.30	49.79 ± 9.57	48.32 ± 10.67	0.41	.67
Total sleep time (min)	270.53 ± 61.00	268.84 ± 51.66	260.92 ± 57.59	0.41	.67
Sleep latency (min)	114.80 ± 58.27	112.77 ± 46.54	92.12 ± 42.72	3.28	.04
Wake periods (no.)	10.96 ± 4.49	11.66 ± 4.75	12.21 ± 5.42	0.74	.48
NI (%)	18.15 ± 7.43	16.71 ± 6.81	18.38 ± 7.80	0.85	.43
N2 (%)	61.13 ± 7.56	63.32 ± 8.99	60.31 ± 9.46	1.76	.18
N3 (%)	6.03 ± 3.46	5.64 ± 3.80	6.61 ± 3.52	1.00	.37
R (%)	14.74 ± 5.08	14.38 ± 5.16	14.35 ± 5.38	0.08	.92

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Author contributions

Conceived and designed the experiments: Yuping Xie, Liya Zhou and Lijun Zhao. Performed the experiments: Yuan Zhao, Jinfeng Wang, Shangli Zhang and Wei Ma. Analyzed the data: Yuan Zhao, Peilin Hui and Jing Wang. Wrote the paper: Yuan Zhao and Liya Zhou. Collected the samples: Xiaoyan Su, Yu Liu, Jie Fan, Jun Yang, Wenjuan Chen and Bin Guo.

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