NEURAL REGENERATION RESEARCH
Volume 7, Issue 25, September 2012
www.nrronline.org

doi:10.3969/j.issn.1673-5374.2012.25.010 [http://www.crter.org/nrr-2012-qkquanwen.html]
Li XR, Sun N, Xu Y, Wang YF, Li SP, Du QR, Peng JY, Luo JX, Zhang KR. The norepinephrine transporter gene is associated with the retardation symptoms of major depressive disorder in the Han Chinese population. Neural Regen Res. 2012;7(25):1985-1991.

The norepinephrine transporter gene is associated with the retardation symptoms of major depressive disorder in the Han Chinese population★

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Abstract
The norepinephrine transporter plays an important role in the pathophysiology and pharmacological treatment of major depressive disorder. Consequently, the norepinephrine transporter gene is an attractive candidate in major depressive disorder research. In the present study, we evaluated the depression symptoms of subjects with major depressive disorder, who were all from the North of China and of Han Chinese origin, using the Hamilton Depression Scale. We examined the relationship between two single nucleotide polymorphisms in the norepinephrine transporter, rs2242446 and rs5569, and the retardation symptoms of major depressive disorder using quantitative trait testing with the UNPHASED program. rs5569 was associated with depressed mood, and the GG genotype may be a risk factor for this; rs2242446 was associated with work and interest, and the TT genotype may be a risk factor for loss of interest. Our findings suggest that rs2242446 and rs5569 in the norepinephrine transporter gene are associated with the retardation symptoms of depression in the Han Chinese population.

Key Words
Norepinephrine transporter; major depressive disorder; quantitative trait locus; gene polymorphism; retardation symptoms; Hamilton Depression Scale; endophenotype; single nucleotide polymorphism; pathogenesis

Research Highlights
We performed a genetic association study of the influence of the norepinephrine transporter gene on the clinical phenotypes of major depressive disorder in the Han Chinese population, and found that the norepinephrine transporter gene has a relationship to retardation symptom of major depressive disorder.

Abbreviations
NET, norepinephrine transporter; SNPs, single nucleotide polymorphisms; HAMD, Hamilton Depression Scale

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Received: 2012-06-05
Accepted: 2012-08-06
(N20120229004/WJ)

INTRODUCTION

Major depressive disorder is characterized clinically by retardation, anxiety, insomnia, and cognitive disturbance[1-4]. Generally, the most serious symptom of major depressive disorder is retardation, which includes depressed mood, loss of interest, psychomotor retardation, and genital symptoms. Until recently, the molecular causes of major depressive disorder have remained unknown. However, its heritability is approximately 60%[5-7], family, twin,

and adoption studies have suggested that genetic factors make a significant contribution to the etiology. Abnormalities in the norepinephrine neurotransmission system are thought to be related to the pathophysiology of major depressive disorder[8-9]. The gene *SLC6A2*, which encodes the norepinephrine transporter (NET), is located on chromosome 16q12.2 and its 14 exons span approximately 45 kb[10]. Recent studies on *NET* gene polymorphisms have focused on two single nucleotide polymorphisms (SNPs). A silent G1287A SNP (rs5569), located in exon 9 of the *NET* gene, is a particularly interesting candidate because it has higher heterozygosity than other markers[11-12]. Zill *et al* [13] identified the T–182C polymorphism (rs2242446) located in the 5'-flanking promoter region of the *NET* gene. Because this region contains several *cis*-elements that play a critical role in transcription regulation[14-15], changes in the DNA structure of this promoter may lead to altered transcriptional activity and be responsible for predisposition to major depression. However, the data from these two SNPs are contradictory. Some studies have demonstrated a positive association between the *NET* gene and major depressive disorder[16-20], while others have refuted it[21-24]. Some studies suggest that different predisposing genes may be involved in the distinct presentations of the clinical symptoms[25-26]. Investigation of the relationship between genetic polymorphisms and specific clinical symptoms may be an effective way to reveal the pathological mechanisms of major depressive disorder.

The present study was designed to examine the relationship between the *NET* gene and the retardation symptoms of major depressive disorder in the Han Chinese population by quantitative trait analysis.

RESULTS

Quantitative analysis of subjects

432 unrelated patients with major depressive disorder were recruited; all were included in the final analysis.

Baseline analysis of subjects

The χ^2 goodness-of-fit test showed that the genotypic distributions of two SNPs, rs2242446 (χ^2 = 0.047, P = 0.829) and rs5569 (χ^2 = 0.390, P = 0.532), were in Hardy-Weinberg equilibrium, suggesting that the groups were representative.

Association between SNPs in the NET gene and the symptoms of major depressive disorder

Among our subjects, the Hamilton Depression Scale (HAMD) [27-28] total score, anxiety/physical symptoms, insomnia symptoms, and retardation symptoms were 21.84 ± 3.33, 5.10 ± 2.11, 2.95 ± 1.86, and 6.99 ± 2.00, respectively (high scores represent severe symptoms). The results of quantitative trait testing for association between two SNPs in the *NET* gene and the retardation symptoms of major depressive disorder are summarized in Tables 1 and 2.

Table 1 Association of *NET* gene alleles and genotypes with HAMD total and itemized scores in 432 patients with major depressive disorder

Marker	*P*			
	rs2242446		rs5569	
	Allele (T/C)	Genotype (TT/TC/CC)	Allele (G/A)	Genotype (GG/GA/AA)
HAMD total score	0.960	0.074	0.084	0.021[a]
Anxiety/physical symptoms	0.406	0.187	0.095	0.071
Insomnia symptoms	0.461	0.092	0.173	0.360
Retardation symptoms	0.801	0.215	0.390	0.070
HAMD 17 items				
Depressed mood	0.721	0.118	0.091	0.020[b]
Feelings of guilt	0.776	0.951	0.251	0.061
Suicide	0.459	0.346	0.932	0.757
Insomnia early	0.834	0.748	0.494	0.191
Insomnia middle	0.396	0.703	0.928	0.802
Insomnia late	0.820	0.943	0.578	0.767
Work and activities	0.096	0.011[c]	0.772	0.396
Retardation: psychomotor	0.237	0.488	0.834	0.320
Agitation	0.947	0.971	0.703	0.520
Anxiety (psychological)	0.523	0.270	0.928	0.036[d]
Anxiety somatic	0.071	0.053	0.060	0.160
Somatic symptoms (gastrointestinal)	0.443	0.329	0.146	0.309
Somatic symptoms general	0.649	0.596	0.097	0.208
Genital symptom	0.643	0.858	0.774	0.773
Hypochondriasis	0.881	0.945	0.070	0.109
Loss of weight	0.622	0.850	0.794	0.062
Insight	0.379	0.552	0.056	0.156

a: one-way analysis of variance, F = 3.742; df = 2; P < 0.05.
b: one-way analysis of variance, F = 7.861; df = 2; P < 0.05.
c: one-way analysis of variance, F = 4.558; df = 2; P < 0.05.
d: one-way analysis of variance, F = 3.270; df = 2; P < 0.05.
HAMD: Hamilton Depression Scale; NET: norepinephrine transporter.

As shown in Table 2, there was a significant genotype association of rs5569 with HAMD total score (χ^2 = 7.72, P = 0.021), depressed mood (χ^2 = 7.86, P = 0.020), and anxiety (psychological; χ^2 = 7.86, P = 0.036), and of rs2242446 with work and activities (χ^2 = 9.06, P = 0.011). The associations of rs5569 with HAMD total score and depressed mood, and of rs2242446 with work and activities all remained statistically sig-

nificant after 10 000 permutations (global P = 0.037, global P = 0.038, global P = 0.014, respectively); however, its association with anxiety (psychological) did not remain significant (global P = 0.074). Because few individuals carried the CC and AA genotypes, we reanalyzed the data after combining genotypes TC/CC and GA/AA. As shown in Table 3, the TT carriers had a higher score for work and activity than the TC/CC carriers did (t = 2.624, P = 0.009), and the GG carriers had a higher HAMD total score (t = 2.338, P = 0.020) and depressed mood (t = 2.471, P = 0.014) than the GA/AA carriers did.

Table 2 Mean total and itemized HAMD scores for *NET* genotypes in patients with major depressive disorder

SNP	HAMD	Genotype HAMD score			χ^2	P	Adjusted P
rs2242446		TT (n = 191)	TC (n = 194)	CC (n = 47)			
	Work and activities	2.3±0.7	2.0±0.8	2.2±0.6	9.1	0.01[a]	0.01
rs5569		GG (n = 222)	GA (n = 179)	AA (n = 31)			
	HAMD total score	20.4±5.4	19.0±5.2	20.3±5.3	7.7	0.02[b]	0.04
	Depressed mood	2.7±0.6	2.5±0.7	2.7±0.6	7.9	0.02[c]	0.04
	Anxiety	1.8±0.8	1.7±0.8	2.0±0.7	6.6	0.04[d]	0.08

a: one-way analysis of variance, F = 4.558; df = 2; P < 0.05.
b: one-way analysis of variance, F = 3.978; df = 2; P < 0.05.
c: one-way analysis of variance, F = 3.978; df = 2; P < 0.05.
d: one-way analysis of variance, F = 3.270; df = 2; P < 0.05.
HAMD: Hamilton Depression Scale; NET: norepinephrine transporter; SNP: single nucleotide polymorphism.

Table 3 Mean total and itemized HAMD scores after combining the *NET* genotypes

SNP	HAMD score	Genotype		t	df	P
		Mean score				
rs2242446		TT (n = 191)	TC/CC (n = 241)			
	Work and activities	2.26±0.70	2.07±0.76[a]	2.624	430	0.009
rs5569		GG (n = 222)	GA/AA (n = 210)			
	HAMD total score	20.44±5.39	19.26±5.07[b]	2.338	430	0.020
	Depressed mood	2.65±0.56	2.51±0.64[b]	2.471	430	0.014

[a]P < 0.05 *vs*. TT; [b]P < 0.05 *vs*. GG. Data are expressed as mean ± SD, n = 432 (independent samples t-test). HAMD: Hamilton Depression Scale; NET: norepinephrine transporter; SNP: single nucleotide polymorphism.

Linkage disequilibrium analysis

The two SNPs were not in linkage disequilibrium with each other (D' = 0.052, r^2 = 0.002). Accordingly, haplotype analyses were not applicable.

Power analysis

In a previous study, we calculated an odds ratio for SNP rs2242446 of 1.33[27-28]. Here, for a main effect of each polymorphism with a relative risk of at least 1.33 in an additive mode, a sample size of 432 would have been sufficient. Assuming a disorder-related gene frequency of 0.50 and a test size of α = 0.05, the power of the study reached 77.5%.

DISCUSSION

We have provided evidence that norepinephrine is very likely to be involved in the pathophysiology of major depressive disorder, as reported by many previous studies[8, 29-30]. Our quantitative trait testing suggested that the *NET* gene may be associated with HAMD total score, depressed mood, and work and activities for major depressive disorder; these findings remained statistically significant after 10 000 permutations. The TT and GG genotypes might be risk factors for work and activities, and for HAMD total score and depressed mood, re-spectively. Depressed mood is the core symptom of major depressive disorder[31], which is believed to be linked to inefficient information processing in the amygdala and ventromedial prefrontal cortex. Reduced, dysfunctional, and/or inefficient noradrenergic functioning in these regions is depicted here as hypoactive. Loss of interest is another key symptom of major depressive disorder[31], which is believed to be linked to the hypothalamic “drive” center and the nucleus accumbens “pleasure” or interest center. Alterations in the *NET* gene may, at least in part, underlie these pathological processes.

Notably, we found a robust positive association between each *NET* SNP and a single different component of the HAMD score. It is possible that, although each item is related to retardation, different clinical phenotypes have different hereditary bases. Second, it is possible that the subjects did not reveal their genital symptoms for cultural reasons. More studies are needed to confirm our results and investigate the etiological independence of the components of the retardation phenotype. Reducing phenotypic heterogeneity will be crucial to identify susceptibility genes for major depressive disorder. For instance, we have previously identified a positive association between SNP rs11568817 of the *HTR1B* gene and suicidal tendency using the same sample set, although

there was no association with major depressive disorder in general. The testing of quantitative traits is the best way to dissect the complex heredity of this disease and to illustrate its pathogenesis[32].

In conclusion, the present study provides preliminary evidence in support of a relationship between the *NET* gene and the retardation symptoms associated with major depressive disorder in the Han Chinese population. Further investigations are needed with a large independent sample to replicate and extend these results and to delineate the clinical phenotypes of major depressive disorder.

SUBJECTS AND METHODS

Design

A cross-sectional genetic study.

Time and setting

The experiments were performed at the Institute of Basic Medical Science, Chinese Academy of Medical Sciences, China from December 2006 to May 2007.

Subjects

432 unrelated patients with major depressive disorder (203 males and 229 females; mean age, 34.10 ± 9.59 years, range 18–64 years) were recruited from the First Hospital of Shanxi Medical University between March 2004 and May 2007. They were all of Han Chinese origin and came from the same geographical area in Northern China.

Inclusion criteria

At least two psychiatrists evaluated the patients and diagnosed them according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria for major depressive disorder[33-34].

The inter-rater reliability kappa value was 0.81 for the Structured Clinical Interview Disorders. The severity of major depressive disorder was assessed with the 17-item Hamilton Rating Scale for Depression[28].

Only subjects with a minimum score of 17 on the HAMD entered the study. Two-thirds (67.2%) of the patients were in the first episode of disease; the other 32.8% had experienced prior episodes (range 2–20, 3.89 ± 2.73).

Exclusion criteria

Patients suffering from any organic brain disorder, or who had a history of alcohol or drug abuse or major neurological disease were excluded from this study.

On the basis of the *Administrative Regulations for Medical Institutions formulated by the State Council of China* [35], informed consent was obtained from all subjects.

Methods

Clinical assessment of depression symptoms

The following clusters of HAMD items were computed for each patient and analyzed separately from the total HAMD score: anxiety/physical (items 10 [anxiety (psychological)], 11 [anxiety somatic], 12 [somatic symptoms (gastrointestinal)], 15 [hypochondriasis], and 17 [insight]), insomnia (items 4 [insomnia early], 5 [insomnia middle], and 6 [insomnia late]), and retardation (items 1 [depressed mood], 7 [loss of interest], 8 [psychomotor retardation], and 14 [genital symptoms]). The severity was scored with from 1 to 5 (absent = 1, mild = 2, moderate = 3, severe = 4, extreme = 5).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes according to the standard phenol/chloroform procedure. Two SNPs, rs2242446 (T–182C) and rs5569 (G1287A), were examined. Primers were designed using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA), and the specificity of each was checked using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

PCR reactions for SNP rs2242446 were performed in a total volume of 25 μL, containing 60 ng of genomic DNA, 200 μM dNTPs, 0.2 μM each primer, 2.5 μL 10 × PCR buffer (Tiangen, Beijing, China), and 1 unit of Taq DNA polymerase (Tiangen). The cycling conditions were an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 63°C for 30 seconds, 72°C for 30 seconds, and a final elongation at 72°C for 10 minutes. PCR reactions for SNP rs5569 were performed in a total volume of 25 μL, containing 60 ng of genomic DNA, 200 μM dNTPs, 0.2 μM each primer, 2.5 μL 10 × PCR buffer (Tiangen), and 1 unit of Taq DNA polymerase (Tiangen). The cycling conditions were an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds, and a final elongation at 72°C for 10 minutes[27, 36].

The PCR products were purified and then sequenced bidirectionally using an ABI 3700 DNA sequencer (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). SNP genotypes were identified using Chromas v2.31 (Technelysium Pty Ltd., Brisbane, Australia). The primer sequences and the length of the PCR products are listed in Table 4. Sample sequencing chromatograms are

shown in Figures 1 and 2.

Table 4 Primer sequences

SNP	rs2242446	rs5569
Gene symbol	*NET*	*NET*
Location	5' promoter	Exon 9
Polymorphism	T/C	G/A
Primer sequence (5' –3')	F: CTG TGG CTG TTG AAG TGT CGC R: GGC TCT GCT TGG ATA AAG GGA AA	F: GGG TTT TGG TGT TTT ACT GCT T R: CTG TGG TGC TGT TGT ATT GAC G
Annealing temperature (°C)	63	59

NET: Norepinephrine transporter; F: forward; R: reverse; SNP: single nucleotide polymorphism.

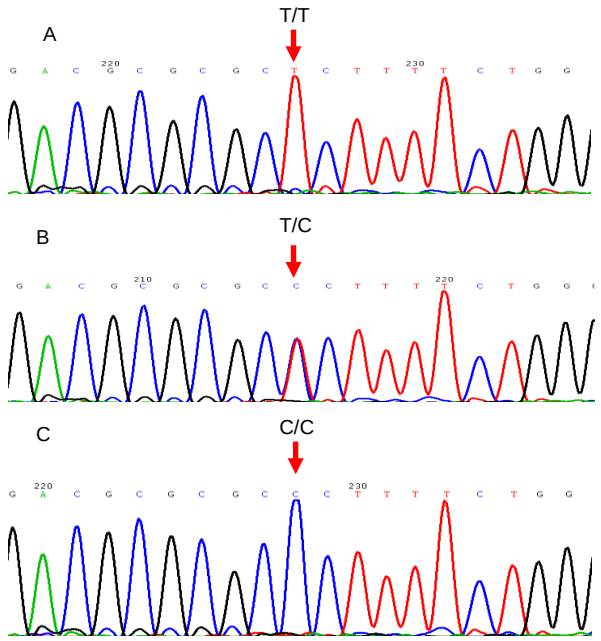

Figure 1 Sequencing chromatograms showing the genotype of the norepinephrine transporter gene single nucleotide polymorphisms T-182C.

The red arrow above each chromatogram shows the homozygote T/T (A), the heterozygote T/C (B), and the homozygote C/C (C) genotype.

Statistical analysis

Statistical analysis was conducted using SPSS for Windows v15.0 (SPSS, Chicago, IL, USA). The Hardy-Weinberg equilibrium for the genotypic distribution of each SNP was tested using the χ^2 goodness-of-fit test. UNPHASED v3.0.12 (Microsoft, Seattle, WA) was applied to analyze the genotyping data and test for linkage disequilibrium[37-38].

The strength of the linkage disequilibrium between the polymorphisms was estimated by calculating *D*' and r^2. The additive value, which represents the change in the expected trait value due to the allele relative to the reference allele, was estimated for association between each scored symptom and the SNPs tested. $P < 0.05$ was considered statistically significant (using the independent samples *t*-test and one-way analysis of variance) and the permutation test was used to correct the global *P*-value.

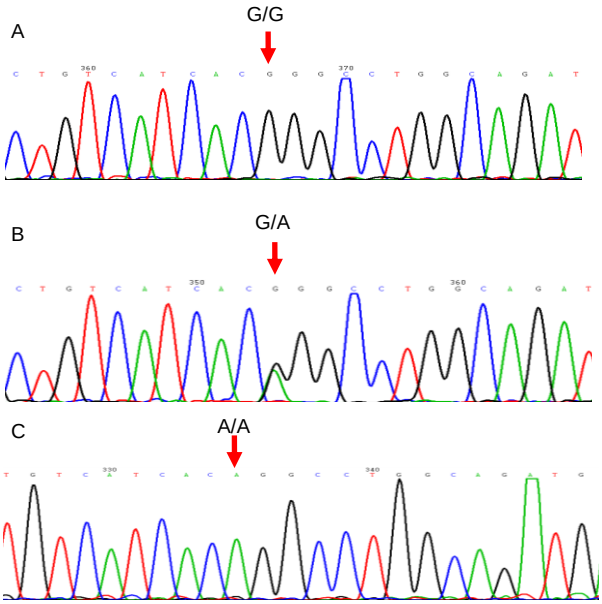

Figure 2 Sequencing chromatograms showing the genotype of the norepinephrine transporter gene single nucleotide polymorphisms G1287A.

The red arrow above each chromatogram shows the homozygote G/G (A), the heterozygote G/A (B), and the homozygote A/A (C) genotype.

Funding: This project was supported by the National Natural Science Foundation of China, No. 30770770, 30971054, and 81171290; Science Foundation for the Youth of Shanxi, No. 2011021036-1.

Author contributions: Xinrong Li wrote the manuscript and conducted the biological experiments. Ning Sun was responsible for data analysis and conducted the biological experiments. Yong Xu, Yanfang Wang, Juyi Peng, and Jinxiu Luo collected the participants. Suping Li and Qiaorong Du assessed the patients' symptoms. Kerang Zhang designed the study and revised the manuscript.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Ethics Committee for Medicine of the First Hospital of Shanxi Medical University, China.

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(Edited by Deng H, Yi ZH/Su LL/Song LP)