

Investigation of the correlation between norepinephrine transporter gene polymorphisms and essential hypertension

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Abstract. Essential hypertension (EH) is an etiological risk factor that poses a serious threat to human health. The aim of this study therefore was to investigate the correlation between the two SNPs (rs1805067 and rs2397771) of the norepinephrine (NE) transporter gene in the sympathetic nervous system and essential hypertension (EH). A total of 96 hypertensive patients (EH group) and 96 normal subjects (control group) were selected by epidemiological investigation and the sequences at rs1805067 and rs2397771 in the two groups were investigated using polymerase chain reaction (PCR) and Sanger sequencing. In the EH group, systolic and diastolic blood pressure, body mass index (BMI), levels of creatinine, low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), and the percentage of drinkers were found to be higher compared with those of the control group ($P < 0.05$). The results of the analysis of the allele frequencies of rs1805067 (A/G) revealed that there was no difference between the two groups ($P > 0.05$). The analysis results of the allele frequencies of rs2397771 (C/G) in the EH group revealed that G accounted for 48.96% and C for 51.04% of the patients, while in the control group, these values were 64.58 and 35.42%, respectively. These results indicated a significant difference between the two groups ($P < 0.05$). In the population studied, the onset of EH may be correlated with BMI, TG, LDL-C and high-density lipoprotein cholesterol (HDL-C). EH may be related to the rs2397771 (C/G) C allele polymorphism of the NE transporter gene but not to rs1805067 (A/G).

Introduction

Essential hypertension (EH) is an etiological risk factor for a variety of cardiovascular and cerebrovascular diseases and is a serious threat to human health (1-3). The disease has become a major public health problem worldwide. EH is a polygenic disease, and the variation sites of each gene almost includes all the introns, exons and regulatory sequences (4).

Currently, over 150 genes that encode proteins have been found to be involved in the regulation of blood pressure via physiological, biochemical and metabolic pathways (5). These genes are termed candidate genes for EH. The genes associated with EH are mostly involved in the renin-angiotensin-aldosterone system (RAAS), sympathetic nervous system, endothelial system and other signaling pathways. The enhanced reactivity of the sympathetic nerve is closely correlated with the onset of hypertension. The correlation between gene polymorphisms in the sympathetic nervous system and hypertension has been the subject of a number of studies (6). The norepinephrine (NE) transporter gene is a member of a large gene family of the sympathetic nervous system. Certain studies (7) have reported the existence of a correlation with hypertension, although such studies have not been performed in China. The aim of this study was to investigate the correlation between the two SNP sites (rs1805067 and rs2397771) of the NE transporter gene in the sympathetic nervous system and EH using polymerase chain reaction (PCR) and the Sanger sequencing method.

Patients and methods

Patients. Patients from the Fuxin City and Liaoning Province areas were selected for this study, based on the recommended diagnosis standard of hypertension in China in 2004. Selection of patients was based on criteria including systolic blood pressure (SBP) ≥ 140 mmHg, diastolic blood pressure (DBP) ≥ 90 mmHg or patients who were ingesting antihypertensive agents. The screened patients, who were aged ≥ 18 years and suffered from hypertension, were selected for an epidemiological survey, which included a general survey, history of cardiovascular disease, family history and living and eating habits. When filling in the questionnaire, the patients were directly questioned face-to-face by way of non-induced

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Table I. Comparison of data obtained by epidemiological survey from the hypertension and control groups.

Variable	EH group (n=96) mean \pm SD	Control group (n=96) mean \pm SD	P-values
Age (years)	38.35 \pm 6.549	67.54 \pm 5.427	<0.001
SBP (mmHg)	150.01 \pm 21.186	126.22 \pm 10.236	<0.001
DBP (mmHg)	98.24 \pm 13.64	76.99 \pm 6.672	<0.001
BMI (kg/m ²)	26.8536 \pm 3.59466	21.8521 \pm 2.78093	<0.001
TC (mmol/l)	5.3340 \pm 0.98605	5.2436 \pm 1.02181	>0.05
HDL-C (mmol/l)	1.3513 \pm 0.36568	1.4978 \pm 0.37207	<0.001
LDL-C (mmol/l)	3.0481 \pm 0.75099	2.8179 \pm 0.74969	>0.05
TG	0.2368 \pm 0.28285	0.1121 \pm 0.22973	<0.001
FPG (mmol/l)	5.6478 \pm 1.33695	5.8758 \pm 1.76878	>0.05
Serum creatinine (μ mol/l)	88.089 \pm 14.1849	53.850 \pm 12.2449	<0.001
Smokers (%)	53.1%	52.4%	>0.05
Drinkers (%)	48.8%	26.4%	<0.001

BMI, body mass index; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride; FPG, fasting plasma glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; EH, essential hypertension.

inquiry. If the elderly patients were not able to answer the questions, the questionnaire was completed by their relatives. The selected patients provided informed consent. In the EH group, 96 patients were enrolled, 73 of whom were male and 23 female. In the control group, samples from non-hypertensive healthy individuals, aged ≥ 60 years with blood pressure $< 140/90$ mmHg, were collected in the Fuxin City area. These healthy individuals completed the same epidemiological questionnaire. A total of 96 control subjects were enrolled, comprising 55 males and 41 females. The study was approved by the Ethics Committee of China Medical University.

Research methods

Physical examination. The blood pressure, height, weight and abdominal circumference of the patients and control subjects were measured.

Laboratory examinations. A 12-h fasting venous blood sample was collected from the subjects. One tube of blood was sent to the Mining Group General Hospital in Fuxin City, China, and examined with biochemical tests, including tests for liver and kidney function, fasting blood glucose, blood ion and blood lipids [total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C)]. A second tube of blood was used for the extraction of genomic DNA.

Genomic DNA extraction. DNA was extracted from the blood samples according to the manufacturer's instructions, and the concentration of the DNA was determined.

PCR assay. The primers were designed using Primer 5.0 primer design software and synthesized by Beijing Luhe Technology Co., Ltd. (Guangzhou, China). The sequences of the primers were: rs1805067 upstream, 5'-AGGATTCTAGGAGACTGGGAGC-3' and downstream, 5'-TG GCGTCACCT TCAGCACTTT-3'; rs2397771 upstream, 5'-CCCCACCCAACCCACTTAG-3' and downstream, 5'-GAGACAGACCAAACGGGAAACA-3'.

A 25 μ l PCR assay system was used in this study, which contained 1 μ l genomic DNA, 2.5 μ l 10X Ex Taq buffer, 2 μ l dNTP (2.5 mmol/l), 0.5 μ l upstream primer (10 mmol/l), 0.5 μ l downstream primer (10 mmol/l), 0.2 μ l Ex Taq and 18.3 μ l H₂O. The conditions of the PCR assay were denaturation at 94°C for 5 min, continued at 94°C for 40 sec, annealing at 54°C for 40 sec and extension at 72°C for 60 sec for 35 cycles, with a final extension at 72°C for 10 min. PCR amplification was detected by agarose gel electrophoresis. Following electrophoresis, the results were observed using a gel imaging analysis system, the image was developed under appropriate exposure conditions and the plastic photographic map was saved. The PCR products were purified and then sequenced using a 5 μ l system of sequencing reaction including 2 μ l template, 1 μ l primer and 2 μ l BD. The reaction system was sequenced using the ABI 3730 sequencer.

Statistical analysis. Results were analyzed using statistical SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) and the continuous data were shown as the mean \pm standard deviation. The comparison of the two independent samples was performed using the t-test. The qualitative data were compared using the χ^2 test. The goodness-of-fit of genetic balance was tested using the Hardy-Weinberg law of genetic equilibrium.

Results

Analysis of epidemiological data. In the EH group, SBP (P<0.001), DBP (P<0.001), body mass index (BMI; P<0.001), creatinine (P<0.001), LDL-C (P<0.05), TG in serum (P<0.001) and the percentage of drinkers (P<0.001) of the patients were found to be higher than those in the control group (Table I). The number of individuals of an advanced age (P<0.001) and the HDL-C levels (P<0.001) in the EH group were lower than those of the control group. No significant difference was found between the total protein, albumin, TC, fasting plasma glucose

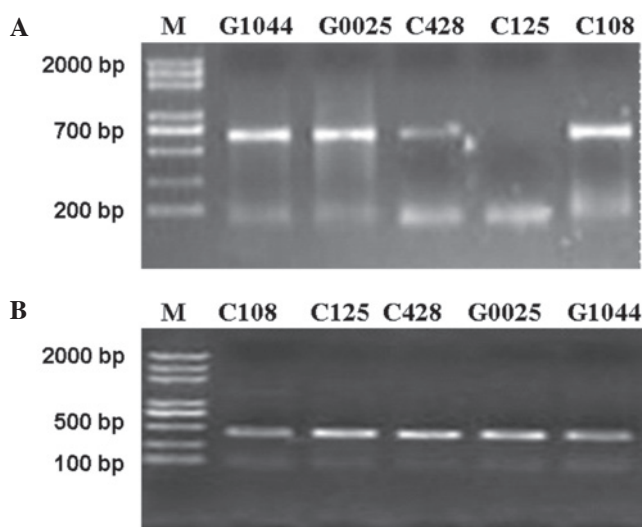


Figure 1. Amplified products of the two sites (rs1805067 and rs2397771) of norepinephrine transporter genes by PCR. The amplified objective fragments of rs1805067 and rs2397771 are (A) 640 bp and (B) 437 bp, respectively. M, marker; C108, C125 and C428 are three samples from the control group; G0025 and G0044 are two samples from the essential hypertension (EH) group.

(FPG) and the percentage of smokers in the EH and control groups ($P>0.05$).

PCR electrophoresis product of the two sites of NE transporter gene rs1805067 and rs2397771. Samples were amplified by PCR with the upstream and downstream primers (Fig. 1). The fragment of the target gene rs1805067 was 640 bp and rs2397771 was 437 bp.

NE transporter gene rs1805067 (A/G). The analysis of the allele frequencies of rs1805067 (A/G) in the EH and control groups revealed that G accounted for 100 and A for 0% of

the subjects. No difference was found between the two groups ($\chi^2=0$, $P>0.05$). The genotypes of the subjects in the two groups were GG homozygous and the polymorphisms were not found. For the control group, the Hardy-Weinberg law of genetic equilibrium was adopted to detect the genotype distribution, but no genetic imbalance was found ($P=0.00$). The sequences of the EH and control groups are shown in Fig. 2A and B, respectively, and the allele frequencies and genotype distribution are shown in Table II.

The NE transporter gene rs2397771 (C/G). The analysis of the allele frequencies of rs2397771 (C/G) in the EH group revealed that G accounted for 48.96% and C for 51.04% of the individuals, whereas in the control group, these values were 64.58 and 35.42%, respectively. A significant difference was observed between the two groups ($\chi^2=4.7751$, $P=0.0289$, $P<0.05$) and the odds ratio (OR) was found to be 1.9011 [95% confidence interval (CI), 1.0657-3.3916]. With regard to genotypes, in the EH group, CC accounted for 12.5, GG for 39.58 and CG for 47.92% of the individuals, while in the control group these values were 8.33, 47.92 and 43.75%, respectively. No significant difference was found between the two groups ($\chi^2=1.7437$, $P=0.4182$, $P>0.05$). For the control group, the Hardy-Weinberg law of genetic equilibrium was adopted to detect the genotype distribution. As $P=0.348$ ($P>0.05$), it reached genetic balance, suggesting the group was representative. The sequences of the EH and control groups are shown in Fig. 2C and D, respectively, and the allele frequencies and genotype distribution are shown in Table III.

Discussion

NE is a catecholamine neurotransmitter, which is found in the postganglionic sympathetic and central nervous systems (8). In noradrenergic neurons, synaptic transmission includes three steps: NE is released from the presynaptic membrane, NE in the synaptic cleft gap acts on postsynaptic membrane

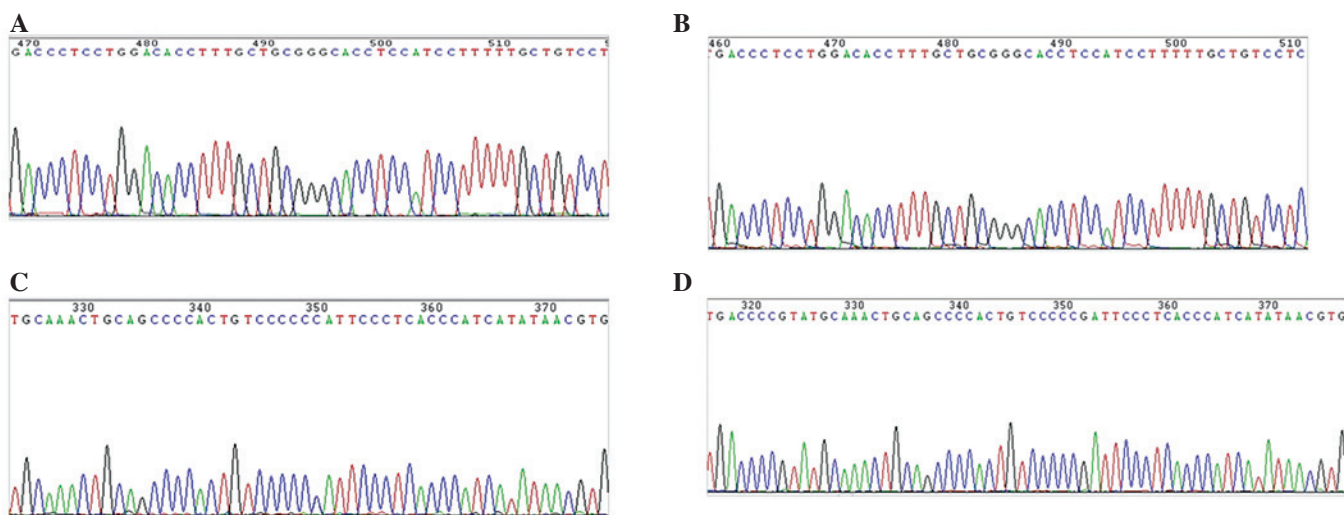


Figure 2. Sequencing figures of the two sites (rs1805067 and rs2397771) of the norepinephrine transporter gene of the patients in the EH and the control groups. The sequencing figure of (A) rs1805067 (A/G) polymorphism in the EH group; (B) rs1805067 (A/G) polymorphism in the control group; (C) rs2397771 (C/G) polymorphism in the EH group and (D) rs2397771 (C/G) polymorphism in the control group. EH, essential hypertension.

Table II. Allele frequency and genotype distribution of rs1805067 (A/G) site, n (%).

Group	Case	Allele A	Allele G	Genotype AA	Genotype GG	Genotype AG
EH group	96	0 (0)	96 (100)	0 (0)	96 (100)	0 (0)
Control group	96	0 (0)	96 (100)	0 (0)	96 (100)	0 (0)
χ^2		0			0	
P-value		>0.05			>0.05	

EH, essential hypertension.

Table III. Allele frequency and genotype contribution of rs2397771 (C/G) site, n (%).

Group	Case	Allele C	Allele G	Genotype CC	Genotype GG	Genotype CG
EH group	96	49 (51.04)	47 (48.96)	12 (12.5)	38 (39.58)	46 (47.92)
Control group	96	34 (35.42)	62 (64.58)	8 (8.33)	46 (47.92)	42 (43.75)
χ^2		4.7751		1.7437		
P-value		0.0289		0.4182		

GG:CC $\chi^2=1.4101$, $P=0.2350$, OR=1.8158 (95% CI, 0.6731-4.8986); CG:CC $\chi^2=0.3914$, $P=0.5316$, OR=1.3696 (95% CI, 0.5101-3.6768). EH, essential hypertension; OR, odds ratio; CI, confidence interval.

receptors and NE is reabsorbed by presynaptic neurons or glial cells. The reuptake process of NE requires the involvement of the sodium-dependent NE transporter (NET). NET is located in the presynaptic adrenergic nerve membrane and belongs to the family of monoamines (including NE, dopamine and serotonin), which are sodium and chlorine-dependent transport proteins. The function of NET is to reuptake NE released from the neuron back to the presynaptic membrane, which is crucial in the regulation of NE concentration in the synaptic cleft, termination of nerve impulse signals and maintenance of the sensitivity of neurotransmitter receptors. NET, as a significant regulator of adrenergic neurotransmitters and the neurotransmitter receptor signaling pathway, is important in regulating the functions of the cardiovascular system (9).

An abnormal increase in sympathetic activity is a significant pathogenesis of hypertension. In a study by Schlaich *et al* (10), electrical impulses from the postganglionic sympathetic sural nerve were recorded using a micro-electrical nerve. NE labeled with ^3H was intravenously injected and the NE kinetics of healthy individuals and patients with hypertension were determined by isotope dilution. The systemic and local (heart and kidney) NE release rates were determined following the administration of the NET inhibitor desipramine. The plasma levels of 3,4-dihydroxyphenylglycol (DHPG) in the NE neurons and 3-methoxy-4-hydroxyphenylglycol (MHPG) outside the NE neurons were simultaneously detected. The results showed that the electrical activity of the sympathetic nerve in hypertensive patients was increased compared with that in healthy individuals and the release of systemic and cardiac NE were also increased. The level of DHPG was reduced, while the level of MHPG was increased. The reduction of NE reuptake and the function of NET reuptake are important in enhancing sympathetic activity, but the mechanism by which this occurs is not clear and the

correlation between the functional decline of the NET gene and polymorphism change of the NET gene was not confirmed (10).

The human NET gene SLC6A2 is located at chromosome 16q12.2 and its length is 45 kb. The gene contains 14 unevenly distributed exons, whose lengths are between 60 and 400 bp and the lengths of the introns vary widely from 0.2 to 13.2 kb. The exon was found to be significantly correlated with the transmembrane functional area of the protein. In the process of transcription, different splicing of the exons results in three variants of protein with different carboxyl terminals, hNET, hNET Ct var1 and hNet Ct var2. Of these variants, hNET and hNET Ct var2 are involved in transcription, although the detailed tissue distribution of the three variants is not yet clear (11). Few studies concerning NET gene polymorphisms have been performed. Rumantir *et al* (12) detected the reduction of NE reuptake in the neurons of a hypertensive population using the radioactive tracing method. Ono *et al* (13) found 13 polymorphism sites in the SLC6A2 gene of a Japanese population comprising 1,950 individuals in 2003. This was the first study to report that the polymorphisms in the promoter 3A/G are correlated with hypertension in the relevant Japanese population and the distribution frequencies of the AG and GG genotypes in the Japanese hypertensive population were higher. In a study by Liu *et al* (14), the results of animal experiments suggested that the abnormal expression of NET may be caused by heart failure based on cardiac sympathetic nerve dysfunction. In 2004, Schlaich *et al* (10) found that the Gly478Ser polymorphism in the NET gene was significantly correlated with hypertension and suggested that the Gly478Ser polymorphism may lead to a decrease in the affinity of NE and NET in the synaptic gap.

No study concerning the correlation between the SLC6A2 polymorphism and hypertension in China has been performed

previously. In this study, rs1805067 and rs2397771, the two SNP sites of the SLC6A2 gene, were amplified by PCR and then adopted with Sanger sequencing to evaluate their correlation with hypertension. The results showed that the rs1805067 site (A/G) had no significant correlation with hypertension. In the Liaoning province of China, this locus may be homozygous and no polymorphism was found, which was inconsistent with the results of studies from other countries. This observation indicates that there are ethnic and regional differences in the polymorphism at this site. Previous studies revealed that the polymorphism of rs2397771 (C/G) may be associated with heart failure and depression, but its correlation with hypertension has not been reported. Our results indicate that the allele frequency of rs2397771 (C/G) C may be associated with hypertension. With regard to genotype distribution, no significant difference was found between the two groups. However, these results require further validation in a clinical trial with a larger sample size.

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