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ENOS-G894T polymorphism is a risk factor for essential hypertension in China

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Vascular endothelial cells produce nitric oxide (NO), which contributes to the regulation of blood pressure and regional blood flow. Polymorphisms of the endothelial nitric oxide synthase (eNOS) gene are associated with coronary artery disease; however, associations between polymorphism (G894T) of the *eNOS* gene and essential hypertension remain unclear. This study was designed to investigate the association between a *eNOS*-G894T polymorphism and essential hypertension (EH). A total of 190 Chinese EH patients (EH group) and 94 healthy participants (control group) were included in the study. *eNOS*-G894T was determined using multi-polymerase chain reaction and polymorphisms in *eNOS*-G894T were genotyped using gene chip technology. Patients carrying eNOS GT + TT genotypes had a higher risk of EH than those carrying the GG genotype (OR = 2.82, 95% CI: 1.05-7.60, P = 0.033). The EH group showed a significantly higher frequency of the T-allele compared with controls (OR = 3.48, 95% CI: 1.34-9.07; P = 0.007). *eNOS*-894T was found to be significantly associated with EH in the dominant genetic model. Thus, the study demonstrated a significant and independent association between a *eNOS*-G894T polymorphism and EH in the Chinese patients. The study also showed that *eNOS*-G894T polymorphism is a risk factor for EH in Chinese patients.

Keywords: Endothelial nitric oxide synthase; Single nucleotide polymorphism; Essential hypertension; Gene chip

Hypertension is a key risk factor for disability and death both in the China and Western countries. It accounts for 13% of global mortality, with more than 90% of patients having essential hypertension (EH). EH is a multi-factor disease caused by the genetic and environmental factors. Genetic factors account for 30%-50% of EH[1]; however, the exact mechanisms of EH remain unclear.

Nitric oxide (NO) is a strong vasodilator and involves in regulation of platelet aggregation, smooth muscle proliferation and leukocyte adhesion[2]. It is continuously produced by the endothelial cells and maintains vascular tone. Reduction of NO release may increase the risk of hypertension, atherosclerosis and vasospasm[3,4]. In endothelial cells, NO is produced by the nitric oxide synthase (eNOS). eNOS is expressed in the normal arteries, but is decreased in fatty streaks and atherosclerotic plaques, suggesting that it plays a key role in the initiation and development of hypertension and atherosclerosis[5].

Animal studies suggest that *eNOS* gene plays a key role in regulating blood pressure[6-8]. The *eNOS* gene, which is located on chromosome 7q35-35, 22 kb contains 26 exons and 25 introns. There are six polymorphisms in the *eNOS* gene and of them, polymorphism G894T, located on exon 7 has been found to be a risk factor for the EH in the Japanese and coronary artery disease in Caucasians[9,10], but not in Tunisians[11] and Australians[12]. In the present study, we have investigated the relationship between *eNOS*-G894T and EH in the Chinese subjects.

Materials and Methods

Subjects

A total of 190 EH (106 males/84 females; mean age 63.52 ± 4.60 yrs) and 94 (37 males/57 females; mean age 63.94 ± 4.84 yrs) healthy control patients was included in this case-control study. These subjects were recruited in Dongtai County, Jiangsu province. All the patients and controls were of the

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Abbreviations: BMI, body mass index; BUN: blood urea nitrogen; Cr: creatinine; DBP, diastolic blood pressure; FBS, fasting blood sugar; LDL, low density cholesterol ; HDL, high density cholesterol; PBS: postprandial blood sugar; SBP, systolic blood pressure; TC: total cholesterol; TG, triglyceride; UA, uric acid; WHR, waist/hip ratio.

Chinese (Han) origin. Hypertension was defined according to the WHO guidelines. Patients with liver or kidney diseases, diabetes, or cardiovascular disease were excluded. The study protocol was approved by the Institutional Review Board of Nanjing Medical University. Informed consent was obtained from each participant of the study.

Inclusion criteria

(i) The patients were diagnosed according to the diagnostic standard of hypertension set by WHO/ISH in 2005 (systolic blood pressure≥140 mmHg and/or diastolic blood pressure ≥90 mm Hg); (ii) Healthy controls were selected from the same population with SBP<140 mm Hg and DBP<90 mm Hg and without hypertension history, and (iii) The enrolled participants completed standard questionnaires by a face-to-face interview. The subjects were of the Han Chinese ethnicity with a long-term local residence rather than immigrants from other areas. All subjects had not been prescribed any antihypertensive drugs.

Exclusion criteria

(i) Participants with secondary hypertension or patients with family history of hypertension were not selected; and (ii) Subjects with chronic liver and kidney diseases and diabetes mellitus were excluded. The study was conducted in accordance with the principle outlined in the Helsinki declaration for the investigation of human subjects.

DNA and primer design

Genomic DNA was isolated from the buffy coat using the Flexi Gene DNA kit (250) (QIAGEN, cat.NO.51206) and stored at -80°C. Software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer 3.cgi) was used to design the primer for G894T. The forward primer was 5'-CAT GAG GCT CAG CCC CAG AC-3', and the reverse primer was 5'-CAG TCA ATC CCT TTG GTG CTC AC -3'.

Multi-polymerase chain reaction (PCR)

The reaction pool included dNTP 0.3 umol/L, Tricine-KOH 40 mmol/L (pH = 8.7), KCl 16 mmol/L, $MgCl_2$ 3.5 mmol/L, BSA 3.74 ug/mL, upper and lower primer 2umol/L, 60 ng DNA and 1.2× titanium Taq DNA polymerase (Clontech Laboratories Inc. USA). The conditions for multi-PCR reaction were as follows: 95°C for 3 min, followed by 40 cycles (each for 30 s at 95°C, 60 s at 66°C, and 90 s at 68°C) and 68°C for 10 min. The PCR product was 206 bp and tested by using a 1.5% agarose gel.

Genotype

The PCR product was purified using the QIA quick PCR Purification Kit (Qiagen, Cat. no. 28106). The purified product was cut-off using DNase I (Fermentas, Lithuania, 0.001 U/μg DNA) and labeled by fluorescein using terminal deoxynucleotidyl transferase. Probes for G894T included two specific ones (eNOS-894T: 5'-CCAGATGATCCCCCAGAA-3'; eNOS-894G: 5'-CCAGATGAGCCCCCAG-3') and one mismatched probe (eNOS-894A: 5'-CCAGATGAACCCCCAGAA-3'). The chips were prepared by OmniGridTM 100 (GeneMachine, San Carlos, CA, USA) and scanned using GenePix 4000B (Axon Instruments, USA). Results were detected by GenePix Pro (Axon instruments, USA) and analyzed using the allelic fraction. Results were confirmed by DNA sequencing with a reproducibility of 100%.

Biochemical tests (FBS, PBS, TC, TG, BUN, Cr, UA, HDL and LDL) were performed by OLYMPUS AU2700 in Dongtai People's Hospital.

Statistical analysis

A student's *t*-test was used to compare means for continuous variables in cases and controls. The genotype and allele frequencies in patients and controls were determined, and populations were tested for conformity to the Hardy-Weinberg equilibrium using χ^2 test between observed and expected numbers. Genotype distributions of these four SNPs between patients and controls were analyzed using multiple logistic regressions by controlling for age, sex, BMI (body mass index), dyslipidemia, glucose and diabetes mellitus. All P-values were two-tailed and P<0.05 was considered statistically significant. Data of the case-control study were analyzed using SPSS 11.5 (SPSS Inc. USA).

Results

There were significant differences in SBP (P<0.05), DBP (P<0.05) and BMI (P<0.05) between cases and controls, while no differences were found in other biochemical parameters (Table 1). Frequencies of the G894T were within the Hardy-Weinberg equilibrium in both patients and controls (P>0.05). No difference in genotype distributions was found between EH patients and controls (Table 2). The T-allele was significantly higher in EH patients than in controls (8.7% vs. 2.7%; OR = 3.48, 95% CI: 1.34-9.07; P = 0.007), while the 894T polymorphism was significantly associated with EH in a dominant genetic model (OR = 2.82, 95% CI: 1.05-7.60,

Table 1—Clinical characteristics of the study population

Variables	EH (n = 190)	Controls (n = 94)
Sex (male, %)	106 (55.8%)*	37 (39.4%)
Age (yr)	63.52 ± 4.60	63.94 ± 4.84
Height (cm)	160.47 ± 7.47	164.19 ± 8.40
BMI	25.37 ± 2.69*	24.34 ± 2.60
WHR	0.89 ± 0.05	0.89 ± 0.05
SBP (mm Hg)	161.63 ± 11.53*	122.54 ± 10.78
DBP (mm Hg)	95.24 ± 8.53*	77.46 ± 7.34
FBS (mmol/L)	4.56 ± 1.02	4.35 ± 1.00
PBS (mmol/L)	6.12 ± 1.32	5.97 ± 1.03
Bun (mmol/L)	5.45 ± 1.20	5.27 ± 1.33
Cr (umol/L)	102.44 ± 9.40	104.62 ± 11.14
UA (umol/L)	290.21 ± 110.0	302.93 ± 112.88
TC (mmol/L)	4.59 ± 0.87	4.56 ± 0.87
TG (mmol/L)	1.56 ± 0.76	1.47 ± 0.68
HDL (mmol/L)	1.55 ± 0.39	1.55 ± 0.55
LDL (mmol/L)	2.71 ± 0.40	2.72 ± 0.49

BMI: body mass index; BUN: blood urea nitrogen; Cr: creatinine; DBP: diastolic blood pressure; FBS: fasting blood sugar; HDL: high density cholesterol; LDL: low density cholesterol; SBP: systolic blood pressure; PBS: postprandial blood sugar; TC: total cholesterol; TG: triglyceride; UA: uric acid; WHR: waist/hip ratio. *: $P<0.05$

Table 2—Genotype distribution and allelic frequencies of the *eNOS*-G894T polymorphism in hypertensive patients and controls

	EH patients	Controls	P	OR	95% CI
Total population	N = 190	N = 94			
Genotypes, n (%)					
GG	164 (86.3)	89 (94.7)	--	--	--
GT + TT	26 (13.7)	5 (5.3)	0.033	2.82	1.05-7.60
Allele frequency (%)					
G	91.3	97.3	--	--	--
T	8.7	2.7	0.007	3.48	1.34-9.07

P = 0.033). Logistic regression showed that age, sex and FBS were the main risk factors for EH (Table 3), however, the presence of the 894G allele decreased the risk of EH.

Table 3—Results of logistic regression

Variables	Beta-coefficient	SE	P	OR	95% CI
Age	-0.03	0.03	0.22	0.96	0.222-1.023
Male	0.90	0.28	0.002	2.46	1.396-4.337
BMI	0.16	0.05	0.003	1.18	1.056-1.315
FBS	0.32	0.14	0.02	1.72	0.545-0.956
eNOS-G894T GG	-1.71	0.56	0.002	0.18	0.059-0.547

SE: standard error; OR: odd ratio; CI: confidence interval.

Discussion

In present study, we performed a community-based case-control study and found that the presence of *eNOS*-894T is a risk factor for EH in the Chinese patients. This association was independent from other factors related to the hypertension.

There are three isoforms of NOS — eNOS, neuronal (nNOS) and inducible (iNOS). NO is synthesized by eNOS in the endothelium. As a rate-limiting enzyme of NO production, eNOS can be inhibited by an analogue of L-arginine. eNOS expression in the endothelium is present in the normal arteries. The chemical and mechanical irritation increases the Ca^{2+} level to active eNOS. In *eNOS*-/- mice, blood pressure has been found to significantly increase. In contrast, in mice with overexpression of eNOS, SBP and DBP are decreased significantly[6-8]. These results indicate that eNOS plays an important role in blood pressure regulation. The production of basal NO is significantly decreased in EH patients as compared to healthy controls[13]. The G894T polymorphism causes a Glu298 change to 298Asp, which alters the structure of eNOS and affects its activity. The production of NO decreases, ultimately increasing the blood pressure[14].

In the Japanese, Shoji *et al*[9] studied 183 cases of hypertension and 193 normal cases comparing subject eNOS and found that the G894T polymorphism was associated with the hypertension. The subjects carrying 894T alleles showed significantly higher diastolic blood pressure and mean arterial pressure. Similar results were observed in two other studies[15,16]. In Asian Indians[17], GT + TT genotypes increased the risk of EH significantly, compared with TT genotypes. In Indian Tamilians[18] and Caucasians[19], 894T was also found to be involved in the hypertension and the T-allele was significantly higher in hypertensive patients than in controls. However, no such association between eNOS G894T polymorphism with EH was found in Australian population[20].

Another study[21] based on the northern Han Chinese population found that genotype distributions and allelic frequencies of the three polymorphisms (T-786C, intron4b/a and G894T) did not differ between the cases and controls (all P>0.05), which suggested that G864T is unlikely to be major genetic susceptibility factors for EH in the northern Han Chinese population. Likewise, the results based on Tunisians[11] , Americans[12,22] and Finnish populations[23] did not confirm the relationship between G894T and EH. Zintzaras *et al.*[24] performed a meta-analysis and found no relationship between G894T and hypertension; however, Wang *et al.*[25] in another meta-analysis study found that 894T was a risk factor for hypertension in the Chinese. Thus, data from the

above reports had shown differences in the relationship of G894T and EH. The possible reasons for the difference were small sample sizes, unmatched age of cases and controls, different allelic frequencies in different ethnic groups, different definitions for case status and the interactions of gene-environmental factors.

Hypertension is a major risk factor both for cardiovascular disease and stroke and *eNOS*-G894T may also increase the risk of these two diseases. G894T was found to be associated with an increased risk of ischemic stroke in Germany[26] and Tunisia[27], but not in Turkey[28] or China[29]. G894T was also shown to be associated with coronary spasms in the Japanese[14]. In eastern Taiwanese populations, 894T was found to be a risk factor for cardiovascular disease[30]. In the Mexican population 894T was found to be a risk factor for myocardial infarction[10]. In our previous study, we also found that 894T was associated with myocardial infarction in the elder Chinese Han population.

In the present study, the cases and controls were all recruited from Dongtai County, (Jiangsu Province) with similar environments, living pressures, and lifestyles between groups. The limitations of the present study were: the small sample size and the results were not confirmed in other independent populations. Thus, further prospective cohort studies with larger sample sizes are needed.

In conclusion, the present case-controlled study found that *eNOS*-G894T is a risk factor for EH in the southern Chinese population.

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