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Endothelial Nitric Oxide Synthase Gene Intron 4, 27 bp Repeat Polymorphism and Essential Hypertension in the Kazakh Chinese Population

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Abstract To investigate the relationship between 27 bp repeat polymorphism in intron 4 in the endothelial nitric oxide synthase (eNOS4) gene and essential hypertension in the Kazakh Chinese population, 151 patients with essential hypertension and 138 healthy people were selected from the Boertonggu countryside of Shawan region in the Xinjiang Uygur Autonomous Region of China in 2006. The polymorphism of *eNOS* in the two groups was detected with polymerase chain reaction assays and the genotype frequencies in each group were calculated following the Hardy-Weinberg law. Four and five tandem 27 bp repeats were designated as "a" and "b", respectively. It was found that the frequencies of b/b, b/a and a/a genotypes of the *eNOS4* gene were 84.06%, 15.22% and 0.72% in the control group, and 81.46%, 15.89% and 2.65% in the hypertension group, respectively. The frequencies of gene "b" and "a" were 91.67% and 8.33% in the control group and 89.40% and 10.60% in the hypertension group, respectively. It was found that plasma eNOS activity was not associated with genotypes and alleles of *eNOS* gene. Plasma eNOS activity in the hypertension group was significantly decreased compared with the control group (P<0.01). The results suggest that *eNOS4* gene polymorphisms are unlikely to be the major genetic susceptibility factors for essential hypertension in the Xinjiang Kazakh population. However, a positive association between plasma eNOS activity and essential hypertension has been revealed.

Key words hypertension; eNOS4; gene polymorphism; Kazakh Chinese

Hypertension is a multifactorial disease with genetic and environmental components. As nitric oxide (NO) is a major regulator in the cardiovascular system [1], it is hypothesized that abnormality in the activity of endothelial nitric oxide synthase (eNOS) might lead to NO deficiency and cause clinical hypertension [2]. The *eNOS* gene was mapped on chromosome 7q36 [3]. It consists of 26 exons spanning approximately 21 kb of genomic DNA and encoding an mRNA of 4052 nucleotides. A 27 bp repeat in intron 4 of *eNOS* (*eNOS4*) was found to be important in the occurrence and severity of essential hypertension, but the results of most associated studies were inconsistent among different ethnic groups. Wang *et al.* [4] reported that *eNOS4* genotype polymorphism is related to the activity of eNOS in European populations. Pulkkinen *et al.* [5] reported that the 4a allele of the *eNOS* gene was associated with elevated diastolic (P=0.032) and mean arterial blood pressure (P=0.030) in the Japanese population, whereas Yokoyama *et al.* [6] found no association between "a" allele gene polymorphism and essential hypertension in Japanese.

eNOS4 gene belongs to nonfunctional variable that does not change the structure of protein product at all; the "a" allele is just a marker which does not take part in hypertension genesis directly. However, some investigators reported that the "a" allele could be a risk factor for essen-

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tial hypertension [7]. Tsukada *et al.* [8] presumed that the polymorphisms of *eNOS* gene affect the splicing of mRNA. So we hypothesize that the "a" allele links disequilibrium and real gene locus associated with hypertension genesis. In order to test this hypothesis, we designed and carried out this study. The incidence of hypertension in the Kazakh Chinese population is 17.36% [9]. Kazakh Chinese localize in relatively isolated places where transport is inconvenient. There was no miscegenation among the Kazakh Chinese who took part in this study.

The specific aim of this study was to investigate the association of the polymorphisms of *eNOS* and its activity with essential hypertension in a relatively large sample of Kazakh Chinese. In addition, we tried to reveal the possible relation between plasma eNOS activity and individual polymorphism in different subjects.

Materials and Methods

Participants and data collection

Kazakh Chinese without miscegenation were selected from the Boertonggu countryside of Shawan region in the Xinjiang Uygur Autonomous Region of China in 2006. All subjects were herders and agreed to take part in the study. Participants' eligibility was confirmed by questionnaire and clinical examinations after enrolment in the study. Our research group comprised of approximately 30 professionals in epidemiology, pathophysiology and hereditism, and clinical/occupational specialists. We travelled to the Boertonggu countryside six times and the local government provided us with details of 800 Kazakhs aged from 20 to 73 years. From the 310 essential hypertensive and 490 healthy subjects in this group we chose 151 essential hypertensive and 138 healthy Kazakhs by random cluster sampling. The comprehensive investigation questionnaire, essential auxiliary examination and clinical diagnosis were completed for each individual. We used the comparison design and analysis. The results showed that there was no age or sex statistical difference between those with or without essential hypertension. The minimal sample size was 127 cases for the *eNOS4* gene in each group, which was calculated using **Equation 1**:

$$n = 2\overline{pq}(U_\alpha + U_\beta)^2 \big/ (p_1 + p_2)^2 \qquad 1$$

in which, p_0=0.15, RR=2.7, p_1=0.32, $\overline{p}$=0.24, $\overline{q}$=0.76, α=0.05, β=0.10, U_α=1.96, U_β=1.28.

A total of 289 participants were enrolled in this study. They included 59 male and 92 female Kazakh Chinese with essential hypertension, aged 20 to 73 years, and 54 male and 84 female healthy Kazakh Chinese, aged 20 to 73 years, who did not have physician-diagnosed hypertension, diabetes, coronary heart disease, stroke, any other life-threatening diseases or any history of drug abuse. The 1999 WHO/ISH Hypertension Guidelines were used to diagnose essential hypertension as systolic pressure=140 mmHg (18.7 kPa) and/or diastolic pressure=90 mmHg (12.0 kPa). Participants' blood pressure was measured after 15 min of rest and repeated three times. A nurse recorded participants' height, weight, waist-to-hip ratio, and blood pressure measurements, and doctors conducted participants' electrocardiographic examination and abdominal ultrasound examination. Overnight fasting blood (15 ml) was drawn and processed (centrifuged, separated, frozen and packaged in a –80 ºC freezer) for plasma eNOS activity quantification. Blood cells were prepared for DNA extraction and detection of gene polymorphism.

Genotype assay of *eNOS4*

Genomic DNA was extracted from 0.2 ml of whole blood sample using an SK1252 DNA extraction kit (Sangon, Shanghai, China). Genotyping of each polymorphism was carried out by polymerase chain reaction amplification from 30 to 50 ng of genomic DNA. For *eNOS4* genotyping, the polymerase chain reaction primers were designed to flank the 27 bp direct repeat. The primer sequences were 5′-AGGCCCTATGGTAGTGCCTT-3′ (forward) and 5′-TCTCTTAGTGCTGTGGTCAC-3′ (reverse). Each assay was conducted in a 25 μl mixture containing 1.2 U *Taq* DNA polymerase (BBI, Markham, Canada), 0.5 μM appropriate primer and 250 μM dNTP. Each reaction was initially denatured for 5 min at 94 ºC, followed by 35 cycles of 94 ºC for 60 s, 60.2 ºC for 60 s, and 72 ºC for 60 s, with a final extension at 72 ºC for 10 min. The smaller allele has four tandem 27 bp repeats and was designated as "a"; the larger allele that has five tandem 27 bp repeats was designated as "b". The *eNOS* alleles "a" and "b" were identified as fragments corresponding to 393 bp and 420 bp respectively, after separation on 3% agarose gel (BBI) stained with ethidium bromide (**Fig. 1**). In addition, the 27 bp repeat elements were verified by DNA sequencing (Shanghai GeneCore BioTechnologies, Shanghai, China).

Quantitation of plasma nitric oxide synthase activity

Quantitation of plasma eNOS activity was determined colorimetrically using an assay kit from Nanjing Jiancheng (Nanjing, China). Absorbance at 530 nm was measured using a Model 680 Microplate Spectrophotometer (Bio-Rad, Hercules, USA). The activity of eNOS (unit per

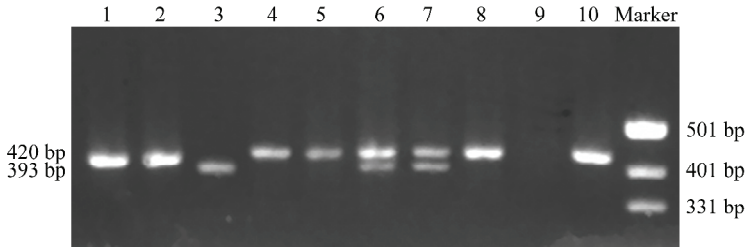

Fig. 1 *eNOS4* genotypes in Kazakh Chinese population by polymerase chain reaction (PCR)

Genomic DNA extracted from whole blood sample was used as a PCR template and PCR products were separated on 3% agarose gel stained with ethidium bromide. The smaller allele with four tandem 27 bp was designated as ''a'' (393 bp) and the larger allele with five tandem 27 bp repeats was designated as ''b'' (420 bp). 1, 2, 4, 5, 8 and 10, b/b genotype; 3, a/a genotype; 6, 7, a/b genotype; 9, negative control.

milliliter) was calculated following the manufacturer's instructions.

Statistical analysis

Continuous variables were presented as mean±SD and differences between two groups were tested using Student's *t*-test and differences of discrete variables were tested using the χ^2-test. The Hardy-Weinberg equilibrium of polymorphism was assessed by the χ^2-test. Differences were considered statistically significant at $P<0.05$.

Results

Clinical characteristics of participants

The clinical characteristics of the 151 hypertensive and 138 normotensive subjects are summarized in **Table 1**. In addition to higher body mass index, systolic blood pressure and diastolic blood pressure, ($P<0.01$), the hypertensive subjects also had a higher plasma total cholesterol level than the controls ($P<0.05$). There was no significant difference between the hypertensive and control groups for smoking status. No differences between the two groups were noted with respect to age, gender, triglyceride, low-density lipoprotein cholesterol or high-density lipoprotein cholesterol ($P>0.05$).

Distribution of selected *eNOS* polymorphisms

The distribution of *eNOS4* polymorphisms in the 289 Chinese Kazakhs did not deviate from the Hardy-Weinberg equilibrium. The genotype and allele frequencies were not significantly different between the two groups ($P>0.05$) (**Table 2**).

eNOS4 genotypes and hypertension multiple logistic regression analysis are summarized in **Table 3**. Body mass index, plasma total cholesterol and high-density lipoprotein cholesterol were significantly higher in the hypertensive group than in the control group ($P<0.05$). No differences between the two groups were noted with respect to *eNOS* genotypes, gender, age, triglyceride, low-density lipoprotein cholesterol or smokers ($P>0.05$).

Table 1 Clinical parameters of essential hypertension and control subjects in Kazakh Chinese population

Parameter	Hypertensive	Control	*P*
Gender (male/female)	59/92	54/84	0.431
Age (years)	47.06±10.65	46.57±10.03	0.400
BMI (kg/m^2)	28.35±5.02	24.71±5.32	<0.001
SBP (mmHg)	157.84±26.21	118.08±11.34	<0.001
DBP (mmHg)	100.97±15.02	76.46±7.23	<0.001
TC (mM)	5.19±1.11	4.90±1.27	0.040
TG (mM)	1.28±0.86	1.12±0.72	0.097
LDL-C (mM)	3.16±0.91	2.95±0.95	0.079
HDL-C (mM)	1.29±0.28	1.37±0.36	0.059
Smokers (yes/no)	116/35	99/39	0.323

Data are presented as mean±SD. BMI, body mass index; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, plasma total cholesterol; TG, triglyceride; SBP, systolic blood pressure; Smokers, consumers who have smoked not less than 100 cigarettes in last 12 months.

Association of *eNOS4* genotype and allele with plasma eNOS activity

Mean values of plasma eNOS activity were not significantly different by genotype or allele. Overall, there was statistically significant difference between control and essential hypertension groups ($P<0.001$) (**Table 4**).

Discussion

Kazakhs represent the major minority ethnic group in

Table 2 Frequency distribution of *eNOS4* genotypes and alleles in Kazakh Chinese healthy and essential hypertension subjects

Group	Hypertensive (n=151)	Control (n=138)	P	OR	95% CI
eNOS4 genotype					
b/b	123 (81.46%)	116 (84.06%)		1.000	–
a/b	24 (15.89%)	21 (15.22%)	0.444	1.078	0.569–2.040
a/a	4 (2.65%)	1 (0.72%)		3.772	0.416–34.248
eNOS4 allele					
b	270 (89.40%)	253 (91.67%)		1.000	–
a	32 (10.60%)	23 (8.33%)	0.354	1.304	0.743–2.288

There were no significant differences in frequencies of genotypes and alleles between hypertensive and control groups (P>0.05). CI, confidence interval; OR, odds ratio. –, not applicable.

Table 3 Multiple logistic regression analysis of Kazakh Chinese healthy and essential hypertension subjects

Group	B	SE	Wald	P	Adjusted OR (95% CI)
eNOS genotype					
b/b	–	–	–	–	–
a/b	–0.306	0.403	0.576	0.448	0.737 (0.334–1.622)
a/a	1.758	1.228	2.049	0.152	5.802 (0.522–64.447)
Gender					
Female	–	–	–	–	–
Male	0.168	0.378	0.197	0.657	1.183 (0.564–2.482)
Age (years)					
<45	–	–	–	–	–
≥45	–0.331	0.301	1.209	0.272	0.718 (0.398–1.296)
BMI					
<24	–	–	–	–	–
≥24	–0.064	0.423	25.18	0.001	5.102 (2.700–9.643)
TC (mM)					
<4.91	–	–	–	–	–
≥4.91	2.082	0.697	8.919	0.003	8.023 (2.046–31.467)
TG (mM)					
<1.2	–	–	–	–	–
≥1.2	–0.064	0.423	0.040	0.842	0.938 (0.498–1.765)
LDL-C (mM)					
<3.0	–	–	–	–	–
≥3.0	–1.558	0.753	0.022	0.882	0.938 (0.405–2.174)
HDL-C (mM)					
≥1.30	–	–	–	–	–
<1.30	1.630	0.425	4.280	0.039	0.210 (0.048–0.921)
Smokers					
No	–	–	–	–	–
Yes	–0.343	0.426	0.651	0.420	0.709 (0.308–1.634)

eNOS4 genotypes and hypertension multiple logistic regression analysis are summarized. Body mass index (BMI), plasma total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) were significantly higher in the hypertensive group than the control group (P<0.05). No differences between the two groups were noted with respect to *eNOS* genotypes, gender, age, triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) or smokers (P>0.05). B, regression coefficent; CI, confidence interval; OR, odds ratio; SE, standard error; Wald, χ^2. –, not applicable.

Table 4 Plasma eNOS activity in *eNOS4* genotypes and alleles of Kazakh Chinese healthy and essential hypertension subjects

Genotype and allele	eNOS activity (U/ml)		*t*	*P*
	Hypertension	Control		
b/b	6.39±3.86	10.69±5.09	7.387	<0.001
a/b	6.36±3.72	9.79±5.33	2.470	0.019
a/a	5.70±2.39 †	9.50±0.00 †	1.427	0.250
total	6.36±1.82	10.54±2.72	7.949	<0.001
b	6.38±3.83	10.61±5.10	10.770	<0.001
a	6.19±3.39 ‡	9.76±5.08 ‡	0.993	0.003
total	6.36±3.78	10.54±5.09	0.160	<0.001

† $P>0.05$, the ANOVA of three genotypes in hypertension and control groups; ‡ $P>0.05$, Student's *t*-test of two alleles in two groups.

Xinjiang Uygur Autonomous Region, and the prevalence of essential hypertension in this population is the highest among all ethnic groups in the region. Kazakhs show a clear nation aggregation. Kazakhs generally live in isolated mountain areas where transportation is inconvenient. They do not have miscegenation. Moreover, they have more salt and fat and fewer vegetables in their diet. These factors contribute to the major environmental factor of essential hypertension in Kazakh populations in the Xinjiang Uygur Autonomous Region. Therefore, the Kazakh Chinese are ideal for essential hypertension research. Our study was to investigate the association of the polymorphisms of *eNOS* and its activity in essential hypertension of Kazakh Chinese. The genotype distributions and allele frequencies of *eNOS* polymorphisms in this study were similar to a previous report on Japanese [10] and Han Chinese [11], but the "a" allele frequency of our study was significantly lower than that published for Caucasians (8.3% versus 15.0%) [12]. These results suggest that there are differences between different ethnic people and the polymorphism of the *eNOS4* gene. Distribution of all polymorphisms is under the Hardy-Weinberg equilibrium, which suggests the results of this study are unlikely to be biased by population stratification or admixture for essential hypertension.

In our study of normotensive and essential hypertensive Chinese Kazakhs, there is no relationship between the selected *eNOS4* polymorphisms and essential hypertension. The data of Yasujima *et al*. and Miyamota *et al.* [13,14] showed 27 bp VNTR (variable number of tandem repeat) polymorphisms of Japanese were not associated with the genesis of essential hypertension. Some investigators have studied three clinically relevant polymorphisms (T786C, intron4b/a and G894T) of the *eNOS* gene with essential hypertension. Their results show that the three *eNOS* gene polymorphisms are unlikely to be major genetic susceptibility factors. Our results suggest that *eNOS4* gene polymorphisms are unlikely to be the major genetic susceptibility factors for essential hypertension in the Xinjiang Kazakh population.

However, our study showed that hypertension is related to plasma eNOS activity. The plasma eNOS activity of the hypertensive group was lower than that of the control group ($P<0.001$), and mean values of plasma eNOS activity were not significantly different by genotype or allele. So there was no relationship between eNOS activity and the *eNOS4* polymorphisms in our study. A previous study by Zhao *et al.* [15] of 207 Han Chinese also indicated that plasma eNOS activity in those with essential hypertension was lower than that of the control group. However, their results indicated that the variations of eNOS4 might be responsible for the decreased activity of eNOS in hypertensive patients, because there was a lower level in individuals with the "a" allele. These conclusions further suggest that there was a difference between different ethnic people and the polymorphism of the eNOS4 gene. There is no more information available from literatures up to now. The possible reasons for the decreased eNOS activity in Kazakh Chinese hypertensive patients might be: (1) an eNOS cofactor (such as folacin) might be decreased in Kazakh Chinese hypertensive patients. Kazakh Chinese have few vegetables in their diet, which results in less folacin uptake; (2) the sequence variation of the regulatory eNOS gene might decrease its transcription; (3) the polymorphisms of eNOS affect the splicing of mRNA, in that changes at the protein level can also influence its activity; (4) the functional impairment of endothelial cells might be a significant factor for the high occurrence of essential hypertension. The results shown in **Table 1** and **Table 3** show that Kazakh hypertensive subjects had a higher

plasma total cholesterol level than the control (P<0.05). Higher cholesterol might lead to endothelium functional impairment [16]; or (5) Xu *et al.* [17] reported that eNOS G894T genotype frequency is higher in male Kazakh Chinese (P=0.019). The relationship between other important polymorphisms, including the promoter polymorphism, tag SNPs (single nucleotide polyporphism), plasma eNOS activity, and the prevalence of essential hypertension should be examined.

In our study, where was only a 0.72% frequency of the a/a genotype in the control group and a 2.65% frequency in the hypertension group, which might explain why there was no difference between these two groups regarding plasma eNOS activity for the a/a genotype. Kazakh Chinese represent a minority ethnic group in the Xinjiang district of China. Tremendous effort was made to recruit so many subjects in the present study, which is essential for revealing any potential association between eNOS4 polymorphism and hypertension. However, our study does not exclude any possible differences in eNOS4 polymorphism among specific nations. Whether the polymorphism is a causative variant or a marker of another functional variant in different races is still not clarified [18]. Further investigation is critically needed to determine the interaction among different situ of the eNOS4 gene, and interaction with other genes affecting the susceptibility of the Kazakh Chinese population to essential hypertension.

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