Endothelial Nitric Oxide Synthase Gene Polymorphisms Associated with Susceptibility to High Altitude Pulmonary Edema in Chinese Railway Construction Workers at Qinghai-Tibet over 4 500 Meters above Sea Level

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Objective To examine whether the polymorphisms of endothelial nitric oxide synthase (eNOS) gene are associated with the susceptibility to high altitude pulmonary edema (HAPE) in Chinese railway construction workers at Qinghai-Tibet where the altitude is over 4 500 m above sea level.

Methods A case-control study was conducted including 149 HAPE patients in the construction workers and 160 healthy controls randomly recruited from their co-workers, matching the patients in ethnicity, age, sex, lifestyle, and working conditions. Three polymorphisms of eNOS gene, T-786C in promoter, 894G/T in exon 7, and 27bp variable number tandem repeat (VNTR) in intron 4, were genotyped using polymerase chain reaction (PCR) and confirmed with DNA sequencing.

Results The frequencies of 894T allele and heterozygous G/T of the 894G/T variant were significantly higher in HAPE patients group than in the control group (P=0.0028 and P=0.0047, respectively). However, the frequencies of the T-786C in promoter and the 27bp VNTR in intron 4 were not significantly different between the two groups. Haplotypic analysis revealed that the frequencies of two haplotypes (H3,
HIGH altitude pulmonary edema (HAPE) is a rare and potentially fatal acute respiratory disease that occurs in healthy people when exposed to altitude exceeding 2 500 m.¹ It is characterized by high pressure in pulmonary arteries, with edema in pulmonary interstitial tissue and alveoli. Although hypoxia is a major trigger factor, some individuals are more susceptible to HAPE than others when exposed to the same hypoxia conditions. The existence of genetic predisposition to HAPE has been suggested as the possible explanation.² Some candidate genes have already been reported,³⁻⁷ but the precise genetic basis of the development of HAPE remains unclear. Therefore, it is particularly important to identify the candidate genes and determine the effects of these genes on HAPE in a large cohort.

Recent experiments and clinical studies have found that nitric oxide (NO) production is reduced in HAPE patients and NO inhalation could improve the conditions.⁸,⁹ These studies have also demonstrated that NO deficiency may involve in the etiology of HAPE. First identified as “the endothelium-derived relaxing factor” in 1987,¹⁰ NO has been implicated in neuronal transmission, immunological response, and vasodilatation.¹¹,¹² NO is synthesized from the precursor L-arginine by NO synthase, which exists in human body in three distinct isoforms: inducible NO synthase (iNOS), constitutive neuronal NO synthase (nNOS), and endothelial NO synthase (eNOS or NOS3).¹³⁻¹⁵ As the name indicates, eNOS is expressed in the endothelium, and is responsible for the most part of NO production in body tissues. The gene coding eNOS is located on chromosome 7 q35-36, containing 26 exons, spanning 21kb, and occurring as a single-copy gene in the haploid human genome.¹⁵

To date, three clinically relevant polymorphisms of eNOS gene have been associated with cardiovascular disease, including T-786C with a mutation in the promoter region, a 27bp variable number tandem repeat (VNTR) polymorphism in intron 4, and 894G/T with a mutation in exon 7, the third one resulting in a Glu-298-Asp amino acid substitution in the mature eNOS protein.¹¹,¹² Studies on eNOS, as a potential candidate gene responsible for susceptibility to HAPE, have come to inconsistent results that are difficult to confirm. A study in a Japanese population concluded that the 894G/T and the 27bp VNTR polymorphisms of eNOS gene have a positive association with HAPE susceptibility.¹⁶ However, a study in a European population and another one in a three-generation Han Chinese family failed to confirm this conclusion.¹⁷,¹⁸ Apart from the different genetic backgrounds of the studied populations, such inconsistencies might stem primarily from experimental designs, including the use of slightly small cohorts, and sampling variability in the case-control studies. To provide more convincing evidence, we conducted a case-control association analysis in a large population consisting of Han Chinese railway construction workers working in Qinghai-Tibet at an altitude over 4 500 m above sea level to determine whether the polymorphisms of eNOS gene play a role in the genetic etiology of HAPE.

SUBJECTS AND METHODS

Study population
Our study protocol was approved by the Ethics Committee of the Institute of Basic Medical Sciences (Chinese Academy of Medical Sciences & Peking Union Medical College). Written informed consents have been obtained from all the individuals enrolled in this study. The subjects were workers participating for the first time in the Qinghai-Tibet railway construction during the period from July 2001 to December 2005. Before entering that construction project, they were born and had been living at low altitude. They all received standard physical and clinical examinations before ascending to the construction sites to exclude those with hypertension, diabetes, cardiopulmonary disease, asthma, inflammation, and any metabolic diseases. Qualified ones then spent 3–6 days in Golmud at 2 860 m above sea level for primary acclimatization to high-altitude environment. After that, the workers were assigned to different sites of construction located at 4 500-5 072 m above sea level, but with the life routine similarly scheduled, including the same food, same living condition (8 workers sharing a 40-m² room), and same working hour. Moreover, they were all exposed to similar environmental
conditions characterized by high altitude, hypoxia, and extreme cold.

**Selection of HAPE patients and healthy controls**
The onset of HAPE is typically present within 1–6 days after ascending to an altitude of 4 500–5 072 m above sea level. The HAPE group consisted of 149 male workers with an average age of 31.3 years (range, 18–48 years), who were diagnosed on the basis of standard diagnostic criteria. All the HAPE patients met specific criteria, that is, onset of typical symptoms at high altitude, including cough and dyspnea at rest, absence of infection, presence of pulmonary rales and cyanosis, and patchy shadows in chest X-ray. By giving oxygen, bed rest, or descending to lower altitudes (<2 000 m), the condition of these patients was reversed after 2–3 days of treatment, with symptoms and signs of HAPE disappeared.

A total of 160 healthy controls were randomly selected from the co-workers of those HAPE patients, matching the patients in age, sex, ethnicity, lifestyle (for instance, smokers or non-smokers), and working conditions. These subjects remained healthy after working at Qinghai-Tibet railway construction sites for at least 3 months, without suffering from HAPE or high altitude cerebral edema.

**Sample collection**
With informed consents from all the subjects, doctors at a hospital near the Qinghai-Tibet construction setting collected 5 mL venous blood samples from each HAPE patient and control. The whole blood was immediately separated to blood cells and plasma by centrifuging at 2 000 rpm (radius not available) for 10 minutes at 4°C, and then stored at −20°C. The blood cells and plasma were transported to Beijing for the following biochemical assays and genetic polymorphism analysis.

**Determination of plasma NO**
Since eNOS is responsible for most of the NO produced in the body tissues, we could determine eNOS activity by monitoring changes in NO concentration. NO concentrations were measured using a colorimetric assay kit (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer’s instructions, calculating with a nitrate standard curve by summing up the total nitrate and nitrite. The NO concentration of each plasma sample was measured twice and the average value was recorded.

**Determination of genotypes**
Genomic DNA was isolated from peripheral white blood cells with phenol-chloroform extraction. Genotyping was performed using polymerase chain reaction (PCR) with a PTC-200TM thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Primers for PCR were designed based on the published sequence of eNOS (http://www.ncbi.nlm.nih.gov/Genbank, accession number X76307.1) (Table 1).

The fragment containing the T-786C variation in the promoter was amplified by PCR using its specific primers (Table 1). The PCR products were digested with MspI enzyme for 3 hours at 37°C. The digested fragments were separated by means of electrophoresis in 10% polyacrylamide gel and were visualized with silver staining. The 894G/T polymorphism in exon 7 was detected as previously described. The 457bp-fragment was generated by PCR amplification using primers specific for the region containing 894G/T variant. The PCR fragments, after being digested with MboI enzyme for 3 hours at 37°C, were electrophoresed on 3% agarose gel and visualized with ethidium bromide staining.

The length polymorphism of the 27bp NVTR in intron 4 of eNOS gene was identified by PCR using the specific primers (Table 1) as previously described. PCR fragments were separated with electrophoresis in 10% polyacrylamide gels and visualized with silver staining. We determined the length of allele c by electrophoresis in 12% polyacrylamide gel, visualized the fragments with silver staining, and confirmed the structure of allele c by means of direct sequencing (Fig.1) (ABI 377 DNA sequencing, PE Applied Biosystems, Carlsbad, CA, USA).

**Table 1.** Primers sequence and PCR amplification conditions used for genotyping of eNOS polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primers sequence</th>
<th>Procedure temperature</th>
<th>Gel electrophoresis</th>
<th>Restriction enzyme</th>
<th>Allele-specific fragments (bp)</th>
</tr>
</thead>
</table>
| T-786C       | Forward:5’-AGTTTTCTTCTAGTCCCCCATGC-3’  
Reverse:5’-CCACCCCCCATGACTAAGT-3’ | 60°C | 10% polyacrylamide gel | PCR-RFLP | T: 140+40  
C: 90+50+40  
457  
320+137  
eNOS a 393  
eNOS b 420  
eNOS c 447 |
| 894G/T       | Forward:5’-TCCCTAGGGAGGGCATAGGCT-3’  
Reverse:5’-TGGGTTGCCAGGGTCT-3’ | 55°C | 3% agarose gel | PCR-RFLP | MspI |
| Intron 4 VNTR| Forward:5’-AGGCCCTATGATGTGCCTG-3’  
Reverse:5’-TCTTCCTAGTTGCTGTGCAC-3’ | 55°C | 10% polyacrylamide gel | RFLP | MboI |

PCR: polymerase chain reaction; eNOS: endothelial nitric oxide synthase; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; VNTR: variable number tandem repeat; a: 4 repeats of 27bp VNTR; b: 5 repeats of VNTR; c: 6 repeats of VNTR.
Statistical analysis
Statistical analyses in the present study were performed using SAS9.1.3 (SAS Institute Inc., Cary, NC, USA). Continuous values are expressed as means±SD. Deviation of genotype frequency from Hardy-Weinberg equilibrium was assessed by chi-square test with 1 degree of freedom. Allele frequencies were calculated based on genotype frequencies in HAPE and control groups, and the inter-group difference was estimated with chi-square test and Fisher's exact test. Haplotype frequencies were calculated using the EH/EH+ program (version 1.20). P values lower than 0.05 were considered statistically significant.

RESULTS

Clinical characteristics
The baseline clinical characteristics of the two groups are summarized in Table 2. We found statistically significant differences between HAPE patients and healthy controls in all the seven recorded items. The levels of blood urea nitrogen (BUN), C-reactive protein (CRP), and hemoglobin were significantly increased in the plasma of HAPE patients compared with the healthy controls, while systolic blood pressure (SBP), diastolic blood pressure (DBP), pulmonary artery pressure (PAP), and creatinine (CRE) were decreased in HAPE subjects compared to controls.

Distribution of the three polymorphisms of eNOS gene
As shown in Table 3, the frequency distribution of the three polymorphisms of eNOS was in Hardy-Weinberg equilibrium in both HAPE and healthy control groups. The frequency of the T allele in the 894G/T polymorphism was significantly higher in the patients with HAPE than in healthy controls (6.71% vs. 1.88%, P=0.0028), while no difference in the frequency of T-786C polymorphism was found between the two groups. There are three alleles of the 27 bp VNTR within intron 4 in the present study, among which allele c composed of six 27 bp repeats was detected only in control subjects (it was calculated together with allele b for its low frequency).

Plasma NO concentration
In order to determine whether the polymorphisms of eNOS influence the activity of eNOS enzyme, we measured and compared the plasma NO level in HAPE patients and healthy controls, also the NO level in the subjects with different genotypes. We did not find a significant difference in NO generation between HAPE group and control group. Although 894G/T polymorphism was found to be associated with HAPE susceptibility (P<0.01), we did not detect any difference in NO level between individual carriers of the T894 or G894 variants in either patient or control groups (both P>0.05, data not shown).

Haplotype analysis
Haplotype analysis revealed a significant association between the haplotypes of eNOS and susceptibility or resistance to HAPE. As shown in Table 4, there are seven possible haplotypes in the Han Chinese in our study groups (the haplotype C-T-b was excluded from this study for its rare occurrence with a frequency less than 0.05%). Three of these haplotypes were found common (>50% in frequency), and also with significant inter-group differences in their frequencies (all P<0.001). Interestingly, although the allele frequencies did not always show significant difference between the two groups, we found significant difference in the overall distribution of eNOS haplotypes. In particular, the H1 (T-G-b) and H2 (T-G-a) haplotypes were found more common in the control group than in the HAPE group (862.20‰ vs. 802.74‰, P<0.001; 37.89‰ vs. 3.47‰, P<0.0001), suggesting that the two haplotypes may provide some protection against the development of HAPE. In contrast, the H3 (T-T-b) and H4 (C-G-a) haplotypes were more common in the HAPE group than in control group (65.17‰ vs. 15.15‰, P<0.0001, odd ratio=4.54; 102.02‰ vs. 46.96‰, P<0.0001, odd ratio=2.31), indicating that H3 and H4 haplotypes possibly increase susceptibility to HAPE. These two haplotypes account for 16.7% of all the detected haplotypes.
**DISCUSSION**

The present case-control study investigated the association of eNOS gene, at the allelic, genotypic, and haplotypic levels, with the susceptibility to HAPE in a large group. The most noteworthy finding of this study is the significantly positive association of the H2 (T-T-b) and H6 (C-G-a) haplotypes of eNOS gene with HAPE in the studied Chinese railway construction workers at Qinghai-Tibet construction sites over 4 500 m above sea level. The data from haplotype frequency analysis may suggest the possibility to screen out high risk population susceptible to HAPE at genetic level.

Association of the polymorphisms of eNOS with susceptibility to HAPE has not been generally acknowledged, well illustrated by the contradictory findings about the association of 894G/T substitution and 27bp VNTR with HAPE from three studies with cohorts of three different nationalities. The conflict among these studies may be attributed to various factors, the most important of which was genetic background, because the distribution of the 894G/T allele, 27bp VNTR, and T-786C allele of eNOS gene in normal control population varies among different reports involving different populations.

In the present study, the HAPE patients and healthy controls match in demographical characteristics, lifestyle, and working conditions, allowing an unbiased estimation of the relative risk parameters. In addition, the size of the cohort avoids some limitations encountered in studies in small groups of subjects. These designs add more reliability to our findings on association of eNOS alleles and haplotypes with susceptibility or resistance to HAPE.

Of the three polymorphisms studied, only the 894G/T polymorphism was found statistically associated with susceptibility to HAPE, implying that this missense muta-
tion might either affect the function of the eNOS enzyme, or be closely linked to some undetected locus that affects HAPE susceptibility. Since the 894G/T variant is located in exon 7 of the eNOS gene, it may induce changes in eNOS activity, leading to an increase or decrease in NO production. However, no significant difference associated with G894 or T894 variants was found in NO level, leaving the involvement of 894G/T polymorphism in eNOS regulatory activity an open possibility. It is also likely that there is a polymorphism in another gene in linkage disequilibrium with the 894G/T variant. Nagasaki et al. once reported that eNOS genotype and allele have no effect on circulating NO levels in healthy males, hence the 894G/T polymorphism is probably only a genetic marker associated with some undetected locus. On the other hand, the positive association of the two eNOS haplotypes with HAPE susceptibility emphasizes the possibility that interaction of multiple genetic markers in a haplotype may be a major determinant of HAPE susceptibility. Therefore, we postulate that 894G/T polymorphism is a genetic marker associated with an unidentified functional variant. Further studies are necessary to confirm this postulation.

In conclusion, our findings suggest that the haplotypes H1 (T-G-b) and H2 (T-G-a) in eNOS gene might have a protective effect against the development of HAPE, while the haplotypes H3 (T-T-b) and H4 (C-G-a) might increase susceptibility to HAPE. These findings may provide helpful information for singling out individuals susceptible to HAPE.

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REFERENCES
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