Positive Association of the Endothelial Nitric Oxide Synthase Gene Polymorphisms With High-Altitude Pulmonary Edema

Yunden Droma, MD; Masayuki Hanaoka, MD; Masao Ota, PhD; Yoshihiko Katsuyama, PhD; Tomonobu Koizumi, MD; Keisaku Fujimoto, MD; Toshio Kobayashi, MD; Keishi Kubo, MD

Background—A defect of nitric oxide (NO) synthesis in the lung in high-altitude pulmonary edema (HAPE) has been suggested to contribute to its exaggerated pulmonary hypertension. Several polymorphisms have been identified in the gene encoding endothelial nitric oxide synthase (eNOS), which is a key enzyme responsible for NO synthesis, some of which were reported to be associated with vascular disorders.

Methods and Results—We studied 41 HAPE-susceptible subjects (HAPE-s) and 51 healthy climbers (control group) in a Japanese population. We examined 2 polymorphisms of the eNOS gene, including the Glu298Asp variant and 27-base pair (bp) variable numbers of tandem repeats using polymerase chain reaction followed by restriction fragment length polymorphism. The Asp allelic frequency of the Glu298Asp variant was 25.6% in the HAPE-s and 9.8% in the controls, which was significantly different between the two groups (P=0.0044). The eNOS4a allelic frequency of 27-bp variable numbers of tandem repeats was 23.2% in the HAPE-s, significantly higher than that of 6.9% in the controls (P=0.0016). In HAPE-s group, 11 of 41 (26.8%) subjects possessed simultaneously both of the two significant alleles, but among the controls, none did, which showed a high statistical difference between the two groups (P=0.000059).

Conclusions—Both polymorphisms of the eNOS gene were significantly associated with HAPE. A genetic background may underlie the impaired NO synthesis in the pulmonary circulation of HAPE-s. These polymorphisms could be genetic markers for predicting the susceptibility to HAPE. (Circulation. 2002;106:826-830.)

Key Words: hypoxia • hypertension, pulmonary • genes • nitric oxide synthase

High-altitude pulmonary edema (HAPE) is a rare life-threatening condition that occurs in healthy persons after rapid exposure to altitudes in excess of 2500 meters above sea level.1,2 The exact mechanism underlying the development of HAPE remains unclear, although exaggerated pulmonary hypertension has been suggested to play a crucial important role.3–4 Recent studies revealed that the exhaled NO from the respiratory tract of HAPE-susceptible subjects (HAPE-s) was lower than that from controls both at altitude and at sea level exposed to hypoxic breathing, with an inverse relationship to pulmonary artery pressure (PAP).5,6 Alternatively, the inhalation of NO in the patients with HAPE resulted in decreasing the marked PAP and improving the ventilation-perfusion mismatch.7,8 Therefore, a defect in NO synthesis in the lung is considered to contribute to enhance the hypoxic pulmonary vasoconstriction in HAPE.

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NO, an essential endogenous vasodilator, regulates pulmonary vascular tone and maintains physiological low pulmonary vascular resistance (PVR).9 It is synthesized locally in vascular endothelial cells by endothelial nitric oxide synthase (eNOS), which is a key catalytic enzyme responsible for providing basal pulmonary NO release.10 The gene encoding eNOS is assigned on chromosome 7q35-36 and comprised of 26 exons and 25 introns distributed over 21 kb as a single copy in the haploid human genome.11 Several polymorphisms have been identified in the eNOS gene, some of which are reported to be associated with vascular disorders.12–15 With regards to the Glu298Asp polymorphism, a guanine (G) to thymine (T) nucleotide substitution at the open reading frame of the eNOS gene in exon 7 causes amino acid substitution of glutamic acid (Glu) by aspartic acid (Asp) at the 298th position, which is positively associated with essential hypertension.12,13 In the case of 27-base pair (bp) variable numbers of tandem repeats (VNTRs), it presents another class of DNA polymorphism of the eNOS gene within intron 4, in which a 27 nucleotide unit is repeated.14,15 Studies have suggested that the 27-bp VNTR is positively associated with essential hypertension14 and smoking-associated coronary heart disease.15 Recently, animal modal studies also demonstrated that the mice with targeted disruption of the eNOS gene showed pulmonary vasoconstriction and hypertension16 and that the rats transferred with the human eNOS gene in their lungs by

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adenovirus reduced the hypoxic pulmonary vasoconstriction.\textsuperscript{17} Considering the crucial role of pulmonary hypertension in the development of HAPE, we hypothesized that the eNOS gene mutation might be involved in the pathogenesis of HAPE. To decipher the genetic background regarding the pulmonary hypertension in HAPE, we examined the two polymorphisms of the eNOS gene in HAPE-s and healthy climbers.

**Methods**

**Study Population**

We collected venous blood samples from 41 subjects with a history of HAPE (HAPE-s group) and 51 healthy volunteers without a history of HAPE (control group). All subjects were unrelated natives of Japan and were born and resided at altitudes less than that of our institute at Matsumoto City (610 m above sea level). Written informed consent was obtained from each subject after a full explanation of the study, which was approved by the Ethics Committee of Shinshu University.

The subjects with a history of HAPE consisted of 39 males and 2 females, ranging in age from 15 to 75 years with an average age of 31.3 years. They were all healthy athletic persons at sea level. They had experienced at least one episode of HAPE requiring hospitalization while climbing in the Japan Alps. The altitudes at onset of HAPE ranged from 2758 to 3190 m above sea level. We diagnosed HAPE on the basis of the standard diagnostic criteria.\textsuperscript{18} All subjects with HAPE met criteria at the onset of the disorder and recovered promptly and well with hospitalization. The routine examinations and related cardiovascular tests were conducted in-hospital after their recovery to exclude any cardiopulmonary diseases.

We recruited control subjects from the Mountaineering Association of Nagano Prefecture and the Alpine Club of Shinshu University. Matching with the HAPE-s group, the controls consisted of 43 males and 8 females, ranging in age from 18 to 62 years with an average age of 38.6 years. All were elite mountain climbers with a mountaineering average of >2800 m above sea level of >100 times. None reported any history of medical problems related to altitudes or cardiopulmonary disorders in a questionnaire that was answered during recruitment.

**Pulmonary Hemodynamics**

Right cardiac catheterization during room air breathing was performed in 26 of the 41 HAPE-s within 6 hours after they were admitted to our institute and on the 4th through 7th day of recovery as well. PAP, pulmonary artery wedge pressure (PAWP), and cardiac output (CO) were measured by the method described previously.\textsuperscript{19} PVR was calculated by subtracting PAWP from mean PAP and dividing by CO. PVR index and cardiac index were also calculated.

**Identification of the Glu298Asp Variant and the 27-bp VNTR in the eNOS Gene**

Genomic DNA was extracted from venous blood by phenol extraction of sodium dodecyl sulfate (SDS)-lysed and proteinase K–treated cells. The genotype of Glu298Asp was identified with a polymerase chain reaction (PCR) followed by restriction fragment length polymorphism method using the restriction enzymes MboI and BanII to digest mutation and wild alleles, respectively. PCR primers\textsuperscript{15} were generated to amplify the 182-bp fragment encompassing the missense Glu298Asp variant. The reaction was performed in a volume of 25 \(\mu\)L containing 0.2 \(\mu\)g of genomic DNA, 0.4 \(\mu\)mol/L of each primer, 2.0 \(\mu\)mol/L of dNTP, and 0.2 U of Taq polymerase (TaKaRa) according to the following protocol: initial denaturation at 95°C for 2 minutes; 30 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 2 minutes; and a final extension at 72°C for 5 minutes (GeneAmp PCR System 9700, PE Applied Biosystems). A guanine (G) at nucleotide position 894 results in a Glu at amino acid position 298, and a BanII restriction enzyme produces two fragments of 94 and 88 bp in length.

<table>
<thead>
<tr>
<th>TABLE 1. Pulmonary Hemodynamics in Subjects With HAPE-s (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameters</strong></td>
</tr>
<tr>
<td>Mean PAP, mm Hg</td>
</tr>
<tr>
<td>PAWP, mm Hg</td>
</tr>
<tr>
<td>Cardiac index, L \cdot min(^{-1}) \cdot m(^{-2})</td>
</tr>
<tr>
<td>PVR, mm Hg \cdot L(^{-1}) \cdot min(^{-1})</td>
</tr>
<tr>
<td>PVR index, mm Hg \cdot L(^{-1}) \cdot min(^{-1}) \cdot m(^{-2})</td>
</tr>
</tbody>
</table>

*P<0.005 compared with the recovery value by the paired Student’s t test.

A thymine (T) at nucleotide position 894 results in an Asp at amino acid position 298, and a MboI restriction enzyme produces two fragments of 99 and 83 bp in length. The restriction digest products were separated by electrophoresis on 12% polyacrylamide gel at 150 volts and visualized using ethidium bromide staining.

The genotype of 27-bp VNTR within intron 4 was identified with PCR followed by vertical electrophoresis on 12% polyacrylamide gel at 150 volts and visualized using ethidium bromide staining. The forward and reverse primers were accorded by Wang et al.\textsuperscript{15} A 25-\(\mu\)L volume mixture including 0.5 \(\mu\)g of genomic DNA, 0.4 \(\mu\)mol/L of each primer, 2.0 \(\mu\)mol/L of dNTP, and 0.2 U of Taq polymerase was used for PCR amplification.\textsuperscript{15} All alleles were assigned by one single observer.

**Statistical Analysis**

The values of the pulmonary hemodynamics in HAPE-s were expressed as mean±SEM, which were compared by the paired Student’s t test between admission and recovery. Allelic frequency was expressed in percentage. The exact test of Hardy-Weinberg equilibrium (HWE) for multiple alleles was performed by the Markov chain method within the GENEPOP software package.\textsuperscript{20} The Markov chain method has the advantage of obtaining a complete enumeration for testing HWE in cases where the number of alleles and the sample size are small. The significant difference of the genotypic distribution between HAPE-s and controls was compared by the \(\chi^2\) analysis; meanwhile, the allelic positivity and frequency between the two groups were compared by the \(\chi^2\) analysis (2\(\times\)2 contingency table). The positivity was defined as the frequency of individuals having one or two of the particular alleles. The difference for frequency of combining the significant alleles between the two groups was tested by Fisher’s exact probability test. The odds ratio was calculated as the cross-product ratio of a particular allele in HAPE-s group compared with that in control group. An \(\approx\)95% confidence interval (CI) of the odds ratio was given by Woolf’s method.\textsuperscript{21} P<0.05 was considered statistically significant for the Student’s t test, \(\chi^2\), and Fisher’s exact probability test.

**Results**

**Pulmonary Hemodynamics in HAPE-s**

Pulmonary hemodynamic data of the 26 HAPE-s during hospitalization are shown in Table 1. The mean PAP, PVR, and PVR index, but not PAWP and cardiac index, were significantly increased at admission compared with those at recovery (\(P<0.005\)).

The Glu298Asp Variant and the 27-bp VNTR in the eNOS Gene in Control and HAPE-s Subjects

The genotypic distribution as well as the allelic positivity and frequency concerning the Glu298Asp variant and the 27-bp VNTR in the eNOS gene in each group are summarized in Tables 2 and 3, respectively.
As shown in Table 2, the genotypic frequencies for Glu298Glu, Glu298Asp, and Asp298Asp were 0.843 (n=43), 0.118 (n=6), and 0.039 (n=2), respectively, in the control group, and 0.488 (n=20), 0.512 (n=21), and 0 (n=0), respectively, in the HAPE-s group. The genotypic distribution in each group was in HWE (controls, \(P=0.057; \text{HAPE-s, } P=0.119\)). However, the genotypic frequencies showed significant different distribution between the two groups \((P=0.00013)\). The positivity for Asp allele was significantly more increased in HAPE-s than controls \((P=0.00027)\), indicating a significant association of the Glu298Asp genotype with HAPE-s. The frequency of Glu allele was 90.2% in controls and 74.4% in HAPE-s, whereas the frequency of Asp allele was 9.8% in controls but high up to 25.6% in HAPE-s.

There was a significant difference of the Asp allelic frequency between the two groups \((P=0.0044)\). The odds ratio for the Glu298Asp genotype was 7.88, with 95% CI from 2.94 to 21.8 \((\chi^2=17.06, P=0.000036, \text{calculated by } \chi^2 \text{ test } 2\times2 \text{ contingency table (df=1)})\).

Three alleles of 27-bp VNTR within intron 4 were detected. As denoted by Wang et al., eNOS4a is a rare small allele with 4 tandem 27-bp repeats and eNOS4b is a common large allele with 5 tandem 27-bp repeats. In one control individual, we found a new third allele and denoted it as eNOS4c. The eNOS4c was shown to be composed of 6 tandem 27-bp repeats by direct sequencing of its PCR product (ABI 377 DNA sequencer, PE Applied Biosystems).

### TABLE 2. The Glu298Asp Polymorphism of eNOS Gene in Control and HAPE-s Subjects

<table>
<thead>
<tr>
<th>Allele</th>
<th>Control Subjects ((%) (n=51))</th>
<th>HAPE-s Subjects ((%) (n=41))</th>
<th>(\chi^2)</th>
<th>(P)</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu298Glu</td>
<td>43 (84.3)</td>
<td>20 (48.8)</td>
<td>17.85</td>
<td>0.00013</td>
<td>...</td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>6 (11.8)</td>
<td>21 (51.2)</td>
<td>...</td>
<td>...</td>
<td>7.88 (2.94–21.8)</td>
</tr>
<tr>
<td>Asp298Asp</td>
<td>2 (3.9)</td>
<td>0 (0)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Allelic positivity
- Glu: 49 (96.1) vs 41 (100)
- Asp: 8 (15.7) vs 21 (51.2)

### TABLE 3. The eNOS4b/a Polymorphism of eNOS Gene in Control and HAPE-s Subjects

<table>
<thead>
<tr>
<th>Allele</th>
<th>Control Subjects ((%) (n=51))</th>
<th>HAPE-s Subjects ((%) (n=41))</th>
<th>(\chi^2)</th>
<th>(P)</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS4b/b</td>
<td>45 (88.2)</td>
<td>24 (58.5)</td>
<td>13.16</td>
<td>0.0043</td>
<td>...</td>
</tr>
<tr>
<td>eNOS4b/a</td>
<td>4 (7.8)</td>
<td>15 (36.6)</td>
<td>...</td>
<td>...</td>
<td>6.78 (2.22–20.66)</td>
</tr>
<tr>
<td>eNOS4a/a</td>
<td>1 (2.0)</td>
<td>2 (4.9)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>eNOS4a/c</td>
<td>1 (2.0)</td>
<td>0 (0)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Allelic positivity
- eNOS4b: 49 (96.1) vs 39 (95.1)
- eNOS4a: 6 (11.8) vs 17 (41.5)

Allelic frequency
- eNOS4b: 94 (92.2) vs 63 (76.8)
- eNOS4a: 7 (6.9) vs 19 (23.2)

a indicates four 27-bp repeats of VNTR; b, 5 repeats of VNTR; and c, 6 repeats of VNTR.

\(\*P\) was calculated by \(\chi^2\) test 4\times2 \text{ contingency table (degree of freedom \([df]=3\)}\).

\(\ddagger P\) was calculated by Fisher’s exact test.

\(\ddagger\ddagger P\) was calculated by \(\chi^2\) test 2\times2 \text{ contingency table (df=1).}
The genotypic frequencies for eNOS4b/b, eNOS4b/a, eNOS4a/a, and eNOS4a/c were 0.882 (n = 45), 0.078 (n = 4), 0.020 (n = 1), and 0.020 (n = 1), respectively, in the control group and 0.585 (n = 24), 0.366 (n = 15), 0.049 (n = 2), and 0 (n = 0), respectively, in the HAPE-s group. The genotypic frequencies showed significantly different distribution between the two groups ($P = 0.0043$). The positivity for the eNOS4a allele was significantly more increased in HAPE-s than controls ($P = 0.0011$), indicating a significant association of the eNOS4a/b genotype with HAPE-s. The frequency of eNOS4b allele was 92.2% in controls and 76.8% in HAPE-s, whereas the frequency of eNOS4a allele was 6.9% in controls but up to 23.2% in HAPE-s. There was a significant difference in the eNOS4a allele frequency between the two groups ($P = 0.0016$). The odds ratio of the eNOS4b/a genotype was 6.78, with 95% CI from 2.22 to 20.66 ($P = 0.0008$, calculated by Fisher’s exact test).

Because both of the two polymorphisms showed significant difference, we combined the Asp allele in Glu298Asp variant with the eNOS4a allele in 27-bp VNTR to test the combining allelic significance between the two groups (Table 4). The carriers who possessed both the two alleles simultaneously were 11 of 41 (26.8%) in HAPE-s but none in controls, which showed an extremely statistical difference between the two groups ($P = 0.000059$). The odds ratio was up to 20.13, with 95% CI from 4.08 to 99.35.

### Discussion

The most noteworthy finding of this study was a significant positive association of the Glu298Asp variant and 27-bp VNTR polymorphism of the eNOS gene with HAPE-s. Moreover, the potential risk of developing HAPE might be greater in individuals possessing both the Glu298Asp and 27-bp VNTR polymorphisms simultaneously than in those possessing one polymorphism alone. This is the first report of a positive association of the eNOS gene polymorphisms with HAPE.

The distributions of the Glu298Asp and the eNOS4b/a polymorphisms of the eNOS gene in the normal control population have varied greatly among different reports. In our study, the frequency of Glu allele was 90.2%, which was almost equal to that identified in other areas in Japan at around 90%. In contrast, the identification in France by Lacolley et al yielded an exceedingly low frequency of 56%. A similar situation was presented regarding the distribution of the eNOS4b/a polymorphism in different ethnicities. Our study and other identifications in Japanese population showed that the eNOS4b allelic frequency was around 90%. However, Wang et al showed that the frequency of eNOS4b was 83% in Australia. Thus, the two polymorphisms of Glu298Asp and 27-bp VNTR of eNOS gene in Japanese are more uniform than other races in terms of genetic background. Since the Japanese are thought to be racially homogenous, association studies are nevertheless liable to be biased because of uncontrolled stratification. The present study is considered to be valid, although the sample size is small because of the rareness of the disease.

In the present study, the HAPE-s demonstrated increasing PAP and PVR, implying that they were characterized by enhanced hypoxic pulmonary vasoconstriction. Stress failure in pulmonary capillaries caused by the elevated PAP has been shown to play a crucial role in the progression of HAPE. Recently, a defect of pulmonary NO synthesis in HAPE-s and patients was demonstrated, which was hypothesized to contribute to the exaggerated pulmonary hypertension. However, human studies could not distinguish whether the impaired synthesis of NO was the cause or the effect of HAPE. The mice with targeted disruption of the eNOS gene were characterized by hypoxic pulmonary vasoconstriction and hypertension and lacked endothelium-derived relaxing activity. Alternatively, the rats transferred with human eNOS gene in their lungs by adenovirus reduced the elevated hypoxic PAP and PVR without affecting systemic blood pressure. These findings have revealed that the eNOS gene is an essential genetic background responsible for local vascular NO homeostasis to maintain low PVR. Our data do provide a genetic basis for the defected NO-generating system in HAPE. Taking this information together, we postulate that the potentiality of producing NO in the pulmonary vascular bed, which possibly is triggered by shear stress, might be impaired in HAPE-s because of an insufficient background of the eNOS gene. Additional studies are necessary to clarify this proposition.

In the present case, a nucleotide substitution at the open reading frame causes amino acid substitution of glutamic acid to aspartic acid at a codon in the 298th position. Unfortunately, we have only limited information about whether this missense mutation gives rise to functional alteration of eNOS enzymatic activity or is only a genetic marker associated with some causal loci. The Glu298Asp site was shown to have neither a functional domain nor a cis element activity on eNOS gene expression, but the computer analysis revealed that the Glu298Asp mutation resulted in a conformation change in the eNOS protein from helix to tight turn. Regarding the significant association of the VNTR polymorphism of the eNOS gene with HAPE, the present results suggest that this polymorphism may be one of the actual genetic causes that lead to the impairment of synthesis of NO in the pulmonary circulation of HAPE. Another possibility is

### Table 4. The Distribution of the Combining Asp and eNOS4a Alleles of eNOS Gene in Control and HAPE-s Subjects

<table>
<thead>
<tr>
<th>Allelic positivity</th>
<th>Control Subjects (% (n = 51)</th>
<th>HAPE-s Subjects (% (n = 41)</th>
<th>$\chi^2$</th>
<th>$P$</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp-eNOS4a alleles</td>
<td>0 (0)</td>
<td>11 (26.8)</td>
<td>15.54</td>
<td>0.000059*</td>
<td>20.13 (4.08--99.35)</td>
</tr>
</tbody>
</table>

Asp indicates aspartic acid allele; a, four 27-bp repeats of VNTR.

* $P$ was calculated by Fisher’s exact test.
that this polymorphism might have a link with other unknown functional loci.

An obvious constitutional susceptibility has been evidenced in HAPE by the occurrence of repeated episodes in the same individual.\(^1,2,18\) The individual susceptibility has been suggested to be associated with enhanced pulmonary vascular responses to hypoxia, hypobaria, and exercise\(^26\) or with blunted hypoxic ventilatory response.\(^27\) Previously, measuring these physiological variables was a traditional means offering some indicators to the susceptibility.\(^26,27\) Such tests, however, lacked sufficient reliability to gain widespread acceptance. In this study, we used the novel missense variants of the eNOS gene to describe the association of the eNOS gene with HAPE for the first time. The present results provide considerable direct evidence in demonstrating the HAPE susceptibility with genetic involvement. The eNOS gene polymorphisms identified in HAPE-s may possibly be candidate genetic markers for predicting the susceptibility to HAPE.

In conclusion, this study demonstrated that the polymorphisms of Glu298Asp in exon 7 and 27-bp VNTR within intron 4 of the eNOS gene were significantly positively associated with HAPE and that the individuals with both Glu298Asp and eNOS48a a polymorphism simultaneously might have a greater susceptible risk to HAPE. We document considerable direct evidence in demonstrating the HAPE gene with HAPE for the first time. The present results provide overwhelming evidence for a role against inflammation. In this study, we used the novel missense variants of the eNOS gene to describe the association of the eNOS gene with HAPE for the first time. The present results provide considerable direct evidence in demonstrating the HAPE susceptibility with genetic involvement. The eNOS gene polymorphisms identified in HAPE-s may possibly be candidate genetic markers for predicting the susceptibility to HAPE.

**Acknowledgments**

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