# Polymorphisms of human vascular endothelial growth factor gene in high-altitude pulmonary edema susceptible subjects

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**Authors' Contributions**

Hanaoka M is the principal investigator of the study, and he obtained fund and supervised the study and helped to draft the manuscript. Droma Y contributed to the laboratory work and drafted the manuscript. Ota M supervised the study and helped to the statistical analyses. Ito M helped the laboratory work. Katsuyama Y helped the laboratory work and statistical analyses. Kubo K supervised the study. All authors read and approved the final manuscript.

**Summary at a Glance**

The single nucleotide polymorphisms (SNPs) of the vascular endothelial growth factor (*VEGF*) gene, namely C-2578A, G-1154A and T-460C in the promoter, G+405C in the 5'-untranslated region and C936T in the 3'-untranslated region of *VEGF*, were examined in 53 high-altitude pulmonary edema (HAPE) susceptible subjects (HAPE-s) and 69 HAPE resistant mountaineer controls (HAPE-r). This case-control study regarding the association of *VEGF* gene polymorphisms with the susceptibility to HAPE is expected to provide genetic evidence to shed light on the debate concerning the biological role of VEGF in the pathogenesis of HAPE.

## ABSTRACT

**Background and objective:** Based on the reported biological property and function of vascular endothelial growth factor (VEGF) during hypoxic conditions, many investigations have studied the role of VEGF in the pathogenesis of high altitude sicknesses, including high-altitude pulmonary edema (HAPE). Unfortunately, no consistent findings have been demonstrated. We analyzed the association of *VEGF* gene polymorphisms with susceptibility to HAPE in order to shed light on the debate concerning the biological role of VEGF in the pathogenesis of HAPE.

**Methods:** The study included 53 HAPE susceptible subjects (HAPE-s) and 69 HAPE resistant mountaineer controls (HAPE-r). Subjects in both groups were Japanese and comparable in terms of age and sex ratios. The single nucleotide polymorphisms (SNPs) of the *VEGF* gene, namely C-2578A, G-1154A and T-460C in the promoter, G+405C in the 5'-untranslated region and C936T in the 3'-untranslated region of *VEGF*, were examined by allele discrimination experiments. In addition, arterial oxygen tension (PaO$_2$) and pulmonary hemodynamic data were available for 21 of the 53 HAPE-s subjects.

**Results:** No statistically significant differences were found in the allele frequencies, genotype distributions and haplotype frequencies of the examined SNPs of the *VEGF*

gene between the HAPE-s and HAPE-r groups. Furthermore, neither the PaO$_2$ nor the

pulmonary hemodynamics was associated with the examined SNPs in the 21 HAPE-s

subjects.

**Conclusions:** The current genetic study could not evidence that the functional SNPs of

the *VEGF* gene are not associated with susceptibility to HAPE in a Japanese population.

**Key words:** high-altitude pulmonary edema, gene, permeability, polymorphism,

vascular endothelial growth factor.

**Short title:** Polymorphisms of human *VEGF* gene in HAPE

## INTRODUCTION

High-altitude pulmonary edema (HAPE) is a non-cardiogenic pulmonary edema that occurs in non-acclimatized, previously healthy, and often young individuals within 2-4 days of rapid ascent above altitudes of 2,500 meters (m) above sea level.[1] Although the mechanisms underlying the pathogenesis of HAPE are complex, the generally accepted paradigm of the pathogenesis of HAPE is related to the sequential process of high-altitude hypoxia-induced pulmonary hypertension, increased capillary permeability and compromise of the alveolar epithelial barrier, resulting in a high permeability pulmonary edema.[2,3] Recurrent episodes are often reported, suggesting that individuals who have previously developed HAPE are more likely to experience future episodes than the general population.[4] Thus, it has been speculated that a constitutional susceptibility determined by genetic background might underlie the development of this disease.[5]

Vascular endothelial growth factor (VEGF) is shown to be significantly upregulated in the presence of hypoxia in rat[6,7] and human[8] lungs. As well as a powerful angiogenic and endothelial-cell-specific mitogen, VEGF is a potent endothelial permeability factor expressed in most tissues, including lungs.[9] Based on the reported biological property and function of VEGF under hypoxic conditions,[10,11] many studies

have analyzed the function of VEGF in the pathogenesis of high altitude sicknesses, including high-altitude cerebral edema (HACE),[12] HAPE,[13,14] and acute mountain sickness (AMS).[15-18] However, high altitude studies regarding the altered systemic levels of VEGF in humans exposed to high altitude remain inconclusive; some authors have reported enhancing effects of hypoxic condition on VEGF blood concentrations,[16,17] whereas others either found a lack of change[13,15] or a decrease.[14]

The human *VEGF* gene is located on chromosome 6p21.3 and consists of eight exons that are alternatively spliced to generate isoenzymes of the VEGF protein.[19] There is a considerable variation of correlation of the polymorphisms within the *VEGF* gene with the VEGF protein production, and analysis of the promoter region of *VEGF* gene has revealed the presence of many polymorphisms.[20] Haplotype analyses of single nucleotide polymorphisms (SNPs) in the promoter and 5'-untranslated regions showed that the -460C and +405C alleles significantly alter *VEGF* promoter activity and responsiveness to biological stimuli.[21] In addition, Prior et al. showed that the *VEGF* haplotype carrying the C-2578A, G-1154A, and G+405C SNPs impacted the enhancement of *VEGF* gene expression in human myoblasts under hypoxic conditions *in vitro*, which showed an association with maximal oxygen consumption in individuals before and after a standardized program of aerobic exercise training.[22] Meanwhile,

Renner et al. found that carriers of the 936T allele of the C936T SNP in the 3'-untranslated region of *VEGF* gene had significantly lower VEGF plasma levels than noncarriers.[23] Therefore, we propose that analyzing the association of *VEGF* gene polymorphisms with susceptibility to HAPE could shed light on the debate regarding the biological role of VEGF in the pathogenesis of HAPE as described above. We hypothesized that the polymorphisms of *VEGF* gene might be associated with the susceptibility to HAPE. To address this association, five SNPs, including C-2578A, G-1154A and T-460C in the promoter, G+405C in the 5'-untranslated region and C936T in the 3'-untranslated region of *VEGF*, were examined in 53 HAPE susceptible subjects (HAPE-s) and 69 HAPE resistant subjects (HAPE-r) to investigate the association of these five SNPs with susceptibility to HAPE in a Japanese population.

## METHODS

### Study subjects

The case group consisted of 53 HAPE-s subjects, which included 46 males and 7 females with an average age of 34.2 years. All subjects had been hospitalized patients in the Shinshu University Hospital between July 1979 and September 2006 due to the onset of HAPE while climbing the Japan Alps (2,758 to 3,190 m). The diagnosis of

HAPE was based upon the criteria described previously.[24] All subjects met the criteria of HAPE diagnosis at the onset of the disorder and recovered promptly with hospitalization. Examinations and cardiovascular tests were conducted in-hospital after their recovery to exclude any preexisting cardiopulmonary diseases.

The control group consisted of 69 elite mountaineers, including 59 males and 10 females, with an average age of 38.7 years. We defined these subjects as HAPE resistant subjects (HAPE-r) as they did not develop HAPE despite their exposure to a high-altitude environment over 3000 m. All were elite mountaineers in the Mountaineering Association of Nagano Prefecture and the Alpine Club of Shinshu University, Japan. Subjects were given a questionnaire sheet that contained the components of Lake Louise Score[25] during the recruitment, and no subject reported cardiopulmonary disorders and altitude-related problems while climbing mountains.

The HAPE-s and HAPE-r subjects were unrelated Japanese, born and residing in locations at heights of near sea level. HAPE-s and HAPE-r groups were comparable in terms of age, gender, ethnicity, and high-altitude exposure. This study and its investigational protocol were approved by the institutional ethics review board of Shinshu University for human study, and written informed consent was obtained from each case and control subject after a full explanation of the study. The procedures used

in this human study were in accordance with the recommendations found in the Helsinki

Declaration.[26]

**DNA extraction and genotyping assays**

Genomic DNA was extracted from venous blood by phenol extraction of sodium

dodecyl sulfate lysed and proteinase K treated cells according to standard procedures.

The final concentration of genomic DNA was prepared at 10-15 ng/µl for the TaqMan

SNP genotyping assays. The allelic discrimination of the *VEGF* gene polymorphisms

was assessed with the Applied Biosystems 7500 Fast Real-time PCR System (Applied

Biosystems, Foster City, CA, USA) using the fluorogenic 5' nuclease assay with

TaqMan probes. SNP Genotyping Assay Mix for C-2578A (rs699947), G-1154A

(rs1570360) and T-460C (rs833061), G+405C (rs2010963) and C936T (rs3025039) of

*VEGF* gene (Figure 1) were obtained from Applied Biosystems (Tokyo, Japan).

**$PaO_2$ and pulmonary hemodynamics in patients with HAPE**

The $PaO_2$ was measured by standard laboratory methods on admission prior to oxygen

therapy and right cardiac catheterization during breathing of room air was performed

within 6 hours after admission to Shinshu University Hospital. A thermodilution

Swan-Ganz catheter was introduced percutaneously into the pulmonary artery via the

right internal jugular vein. The pulmonary artery pressure (PAP), pulmonary artery

wedge pressure (PAWP), and cardiac output (CO) were measured. Pulmonary vascular

resistance (PVR) was calculated by subtracting PAWP from PAP and dividing by the

CO. The PVR was further corrected per square meter of body surface area and

expressed as PVR index (PVRI).

**Statistical analysis**

Numerical values are presented as means ± SD and categorical values are expressed as

the number (n) and percentage (%). Deviations from Hardy-Weinberg equilibrium were

examined using the observed genotype frequency by the $\chi^2$ test. We assessed differences

in the distributions of alleles and genotypes between the HAPE-s and HAPE-r groups

by using the $\chi^2$ test. Haplotypes were inferred using the R package 'haplo.stats' in the R

Project for Statistical Computing (http://www.r-project.org/). A haplotype was

considered rare when its frequency was 1% or less. The differences in distributions of

the detected haplotypes between the case and control groups were examined by the $\chi^2$

test. The sample size was estimated to be statistically sufficient to detect an association

if the relative risk factor was 5 or greater with 80% power at the 5% significance level.

In addition, the differences of the $PaO_2$, mean PAP and PVRI in patients at the early

stage of HAPE were analyzed by one-way analysis of variance (ANOVA) on variances

of genotype and allele type. The criterion for statistical significance was $P < 0.05$.

## RESULTS

**Genotypes of the *VEGF* gene in study subjects**

Hardy-Weinberg equilibrium was observed for all of the five examined SNPs in both cases and controls ($P > 0.05$). There were no significant differences in distributions of the genotypes and allele types for each of the SNPs of the *VEGF* gene between the HAPE-r and HAPE-s groups ($P > 0.05$, Table 1). The allele frequencies of the five *VEGF* SNPs in both the cases and controls were similar to those previously reported in a Japanese group[27] and another Asian population.[28] Further comparisons of the frequencies of *VEGF* haplotypes for the five SNPs between the HAPE-r and HAPE-s are shown in Table 2. Nine patterns of haplotypes tagged by the five SNPs were identified in the HAPE-r group, and seven patterns were identified in the HAPE-s group. No statically significant differences were found in the frequencies of the *VEGF* haplotypes between cases and controls ($P > 0.05$, Table 2).

**Phenotype of the $PaO_2$ and pulmonary hemodynamics in HAPE-s subjects**

The data for $PaO_2$ and pulmonary hemodynamics at the early stage of disease were available for 21 of the 53 HAPE-s subjects because the examination of pulmonary hemodynamics was not performed in every patient with HAPE. The averages $PaO_2$,

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mean PAP and PVRI were 40.9 ± 9.6 Torr, 28.4 ± 8.1 mmHg and 4.7 ± 2.5 mmHg·L$^{-1}$·min$^{-1}$·m$^{-2}$, respectively, which were abnormal compared to the standard normal values of > 60 Torr, < 25 mmHg and < 2.5 mmHg·L$^{-1}$·min$^{-1}$·m$^{-2}$, respectively, in our hospital. The PaO$_2$, mean PAP, and PVRI at early stage of disease did not show any significant differences between subgroups divided by the allele type of the tested SNPs in *VEGF* gene (Table 3).

## DISCUSSION

We examined five SNPs of the *VEGF* gene that were suggested in previous studies to be functional in regulating VEGF mRNA in lung tissue and modifying VEGF concentrations in systemic circulation.[20-23, 27,28] We did not observe association of any of the five SNPs of *VEGF* with HAPE susceptibility in either genotypes or allele types. Correspondingly, no association between the identified haplotypes and susceptibility to HAPE was found. Moreover, the PaO$_2$ and pulmonary hemodynamics data at the onset of HAPE were not associated with any of the examined SNPs in HAPE-s subjects.

The subjects in both the case and control groups were all Japanese, thus believed to be a homogenous population without population stratification. The allele frequencies of the five *VEGF* SNPs in both groups in the current investigation were similar to those

reported previously in another Japanese group.[27] In addition, the present study used the allelic discrimination assay to demonstrate allelic discrimination with a computerized system. This genotyping system permits the analysis of DNA samples with high sample-to-sample reproducibility. The confidence level of the automated allele calls was reported to be 99.7%. Therefore, we consider that the experimental results are reliable and valid.

Acute hypoxia increases gene expressions of VEGF and its receptor in lung tissue.[6,7,10,29] Hypoxia promotes oxidative base modifications in the promoter of *VEGF* gene in pulmonary artery endothelial cells.[30] However, a recent human study revealed that, in contrary to *in vitro* studies, hypoxia decreases plasma VEGF concentrations in healthy humans,[31] suggesting that systemic VEGF concentration might be regulated differently than its expression on the cellular basis. Although various *in vitro* studies have verified the impact of hypoxia on *VEGF* gene expression, no such hypoxic impact on circulating VEGF levels in humans has been found. High altitude studies regarding the altered systemic levels of VEGF in humans exposed to high altitude remain inconclusive; some authors have reported enhancing effects of hypoxic condition on VEGF blood concentrations,[16,17] whereas others either found a lack of change[13,15] or a decrease.[14,31] In addition, Dorward, et al performed serial measurements of plasma

VEGF levels in 38 healthy lowlanders at 3,650 m after 4-5 days of acclimatization and then 6 hours, 3 and 7 days after arriving at 5,200 m, and found an increase in plasma VEGF levels during acclimatization to high altitude.[18] Their results did not support the conventional hypothesis that circulating unbound VEGF is an important component of the pathogenesis of acute mountain sickness, but rather suggested that VEGF is involved in acclimatization to high altitude.[18] In our previous study, we also reported that the levels of VEGF in both the venous serum and bronchoalveolar lavage fluid of patients with HAPE were significantly higher during the recovery stage than upon admission to the hospital.[14] We suspected that VEGF was probably destroyed in the lung of HAPE patients and it appeared less likely to have a critical function in the pathogenesis of HAPE, but rather an important role in the repair process for the impaired cell layer due to its biological functions in mitogen and angiogenesis.[14] The current study provides genetic evidence in agreement with our previous proposal that VEGF does not have a pathological role in the pathogenesis of HAPE, but probably plays a part in recovering of HAPE or acclimatization to high altitude.

Evidence is accumulating to prove that HAPE is a pressure-induced leak pulmonary edema.[32] HAPE is thought to be caused by exaggerated hypoxic pulmonary vasoconstriction associated with decreased bioavailability of nitric oxide in the lungs.[33]

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The inhomogenous exaggerated hypoxic pulmonary vasoconstriction[34] can cause elevated pulmonary capillary pressure,[35] which leads to high pressure and flow in affected areas, consequent mechanical over-distention of pulmonary capillaries, and subsequent injury of the blood-gas barriers.[36] This pathogenesis causes extravasation of fluid, plasma proteins, and blood cells into the interstitial and alveolar spaces.[37] All these studies[32-37] strongly suggest that the hypoxic pulmonary vasoconstriction other than capillary permeability is the critical primary contributor to the pathogenesis of HAPE, although the capillary permeability may exacerbate the pathological process of pulmonary edema.[38] Whilst the biological function of VEGF has little involvement with pulmonary vasoconstriction but mainly plays a role in powerful angiogenic mitogen and endothelial permeability. Additionally, in our study the examined SNPs of *VEGF* gene were not associated with the $PaO_2$, mean PAP and PVRI in the 21 patients with HAPE. In view of these considerations, it is not surprise that our results did not evidence any associations of the *VEGF* gene polymorphisms with the susceptibility to HAPE.

The genomic DNA was extracted from the whole blood in all of our subjects; however, the plasma was only collected in our recent subjects so that the plasma VEGF concentration was not available for all subjects in this study. So we were unable to examine the association of the *VEGF* gene SNPs with systemic circulating VEGF

concentrations. Another limitation is that the present sample size is relatively small for a genetic case-control association study compared to other case-control studies for common diseases such as type 2 diabetes[27] and breast cancer.[28] However, HAPE is a rare disease that occurs only in susceptible people who are rapidly ascending to high altitudes. Recently, effective mountaineering education in Japan has assisted in alerting susceptible individuals to the early symptoms of HAPE (by AMS score[25]) and enabling them to decide to descend immediately if risky symptoms appear during mountaineering. The present sample size is the most we could obtain in our hospital at present time. We expect to extend the present study to examine the association of the SNPs of *VEGF* gene with systemic VEGF levels to confirm the results in a larger sample size in future study.

In conclusion, we found no evidence that these functional SNPs of the *VEGF* gene are associated with susceptibility to HAPE in a Japanese population. In addition, these SNPs of the *VEGF* gene are not associated with the decreased $PaO_2$ and increased mean PAP and PVRI levels observed in patients with HAPE at the early stages of the disease. Although no relationship between the SNPs of *VEGF* gene and the susceptibility to HAPE was found, we could not exclude the possibility that VEGF plays an important role in the recovering of HAPE or the acclimatization to high altitude.

## ACKNOWLEDGMENTS

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**FIGURE LEGEND**

**Figure 1** Structure of *VEGF* gene. The human *VEGF* gene is located on chromosome 6p21.3 and consists of eight exons. The arrow indicates the position of the single nucleotide polymorphism (SNP) examined in the current study. The solid box indicates exon and the opened box indicates intron, with the numeral indicating the length in base pair (bp). The numeral on dot-line indicates the length (bp) between two tested SNPs.

**Table 1**  Distributions of the allele types and genotypes of the single nucleotide polymorphisms of *VEGF* gene in HAPE-r and HAPE-s subjects

| Genotype or Allele type | HAPE-r (n = 69) | HAPE-s (n = 53) | *P* value* |
|---|---|---|---|
| rs699947, promoter | | | |
| -2578CC | 30 (43.5%) | 27 (50.9%) | 0.686 |
| -2578CA | 34 (49.3%) | 22 (41.5%) | |
| -2578AA | 5 (7.2%) | 4 (7.6%) | |
| -2578C | 94 (68.1%) | 76 (71.7%) | 0.546 |
| -2578A | 44 (31.9%) | 30 (28.3%) | |
| rs1570360, promoter | | | |
| -1154GG | 50 (72.5%) | 39 (73.6%) | 0.939 |
| -1154GA | 17 (24.6%) | 12 (22.6%) | |
| -1154AA | 2 (2.9%) | 2 (3.8%) | |
| -1154G | 117 (84.8%) | 90 (84.9%) | 0.979 |
| -1154A | 21 (15.2%) | 16 (15.1%) | |
| rs833061, promoter | | | |
| -460TT | 30 (43.5%) | 27 (50.9%) | 0.686 |
| -460TC | 34 (49.3%) | 22 (41.5%) | |

| | | | |
|---|---|---|---|
| -460CC | 5 (7.2%) | 4 (7.6%) | |
| -460T | 94 (68.1%) | 76 (71.7%) | 0.546 |
| -460C | 44 (31.9%) | 30 (28.3%) | |
| rs2010963, 5'-UTR | | | |
| +405GG | 24 (34.8%) | 13 (24.5%) | 0.198 |
| +405GC | 36 (52.2%) | 27 (51.0%) | |
| +405CC | 9 (13.0%) | 13 (24.5%) | |
| +405G | 84 (60.9%) | 53 (50%) | 0.089 |
| +405C | 54 (39.1%) | 53 (50%) | |
| rs3025039, 3'-UTR | | | |
| 936CC | 42 (60.9%) | 35 (66.0%) | 0.770 |
| 936CT | 25 (36.2%) | 16 (30.2%) | |
| 936TT | 2 (2.9%) | 2 (3.8%) | |
| 936C | 109 (79.0%) | 86 (81.1%) | 0.678 |
| 936T | 29 (21.0%) | 20 (18.9%) | |

HAPE-r, high-altitude pulmonary edema resistant subjects; HAPE-s, HAPE susceptible

subjects; UTR, untranslated region; VEGF, vascular endothelial growth factor.

*$P$ values (two-tailed) were calculated using $\chi^2$ test with 3 x 2 contingency table

(genotype: df = 2) or 2 x 2 contingency table (allele type: df = 1).

**Table 2**　Frequencies of haplotypes tagged by the five polymorphisms of *VEGF* gene in HAPE-r and HAPE-s subjects

| No. | rs699947 | rs1570360 | rs833061 | rs2010963 | rs3025039 | Frequency | | *P* |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | -2578C>A | -1154G>A | -460T>C | +405G>C | 936C>T | HAPE-r | HAPE-s | value* |
| #1 | C | G | T | C | C | 0.294 | 0.395 | 0.098 |
| #2 | C | G | T | G | C | 0.265 | 0.217 | 0.387 |
| #3 | A | G | C | G | C | 0.138 | 0.090 | 0.248 |
| #4 | C | G | T | C | T | 0.098 | 0.105 | 0.857 |
| #5 | A | A | C | G | C | 0.085 | 0.110 | 0.511 |
| #6 | A | A | C | G | T | 0.058 | 0.041 | 0.548 |
| #7 | A | G | C | G | T | 0.038 | 0.042 | 0.874 |
| #8 | C | G | T | G | T | 0.016 | 0 | 0.191 |
| #9 | C | A | T | G | C | 0.009 | 0 | 0.327 |

HAPE-r, high-altitude pulmonary edema resistant subjects; HAPE-s, HAPE susceptible subjects; VEGF, vascular endothelial growth factor.

*$P$ values (two-tailed) were calculated using $\chi^2$ test with 2 x 2 contingency table (df = 1).

**Table 3** Associations of the PaO$_2$, mean PAP and PVRI at the early stage of HAPE with the allele types of the SNPs of *VEGF* gene in 21 HAPE-s subjects

| Allele type | PaO$_2$ (Torr) | Mean PAP (mmHg) | PVRI ( mmHg· L$^{-1}$· min$^{-1}$· m$^{-2}$ ) |
|---|---|---|---|
| rs699947, promoter | | | |
| -2578C | 42.17 ±10.46 | 27.1 ± 4.3 | 4.19 ± 2.08 |
| -2578A | 41.79 ± 12.30 | 28.4 ± 3.97 | 4.90 ± 2.18 |
| *P* value* | 0.93 | 0.43 | 0.42 |
| rs1570360, promoter | | | |
| -1154G | 42.13 ± 10.52 | 27.2 ± 4.3 | 4.13 ± 2.07 |
| -1154A | 37.88 ± 10.84 | 27.4 ± 3.2 | 4.53 ± 1.66 |
| *P* value* | 0.36 | 0.89 | 0.41 |
| rs833061, promoter | | | |
| -460T | 42.17 ± 10.46 | 27.1 ± 4.3 | 4.19 ± 2.08 |
| -460C | 41.21 ± 12.79 | 28.4 ± 3.9 | 4.90 ± 2.18 |
| *P* value* | 0.83 | 0.43 | 0.42 |
| rs2010963, 5'-UTR | | | |
| +405G | 40.60 ± 11.13 | 27.3 ± 4.3 | 4.15 ± 2.01 |

| | | | |
|---|---|---|---|
| +405C | 43.51 ± 10.25 | 25.9 ± 3.3 | 3.73 ± 1.78 |
| *P* value* | 0.44 | 0.43 | 0.53 |
| rs3025039, 3'-UTR | | | |
| 936C | 41.42 ± 10.38 | 27.2 ± 4.2 | 4.15 ± 2.01 |
| 936T | 43.73 ± 9.12 | 28.2 ± 4.9 | 5.16 ± 2.79 |
| *P* value* | 0.57 | 0.69 | 0.48 |

HAPE, high-altitude pulmonary edema; HAPE-s, HAPE susceptible subjects; $PaO_2$, partial pressure of oxygen in arterial blood; PAP, pulmonary artery pressure; PVRI, pulmonary vascular resistance index; SNPs, single nucleotide polymorphisms; UTR, untranslated region; VEGF, vascular endothelial growth factor.

*$P$ values (two-tailed) were calculated using analysis of variance (ANOVA).