A Genome Wide DNA Microsatellite Association Study and Association of TIMP3 gene Polymorphism in Japanese Patients with High Altitude Pulmonary Edema

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Background

・High altitude pulmonary edema (HAPE) is a non- cardiogenic pulmonary edema that develops in susceptible people who ascend quickly to high altitude.
・The pathogenesis remains to be conclusively elucidated and genetic polymorphisms were highly proposed to be associated with HAPE.There have been many reports about gene polymorphisms in associations with pathogenesis of HAPE, but few of them suggested clear-cut HAPE genes.
・The aim of this study is to attempt to identify the locations of the candidate human genes those might be associated with susceptibility or resistance to HAPE by genomewide study and single nucleotide polymorphisms (SNPs) genotyping within candidate genes.

Methods -Microsatellite study-

Subjects

HAPE-susceptible subjects (HAPE-s): 53 Japanese
All of them had previously developed HAPE in their mountaineering histories of climbing Japan North Alps higher than 2500m.

HAPE-resistant subjects (HAPE-r): 67 Japanese
All of them usually climb mountains vigorously over 3,000 m who never developed acute mountain sickness including HAPE during their mountaineering histories.

Preparation of genomic DNA
Genomic DNA from all subjects were extracted from venous blood by phenol extraction of sodium dodecyl sulfate (SDS) - lysed and proteinase K-treated cells

Microsatellite typing

・A case-control association genome study was performed using 400 polymorphic microsatellite markers (ABI PRISM® Linkage Mapping Set v2.5-MD10 kit)
・The markers were amplified by polymerase chain reaction (PCR) according to the manufacturer's protocols
・The PCR-amplified products were sequenced automatically by using ABI 3130 DNA Analyzer
・The significance of the difference in the distributions of alleles between HAPE-s and HAPE-r was determined by a chi square test.
・*P* values were corrected by multiplying the number of alleles observed at each locus (*Pc*).

Candidate Genes

・The National Center for Biotechnology Information (NCBI) Map Viewer, National Library of Medicine, National Institute of Health were managed to select candidate genes within 100-kb region around 9 significant microsatellite marker to further deeply investigate the SNPs of the candidate genes in association with HAPE. (Table 2)

Result -Microsatellite study-

Table1 Statistically significant microsatellite markers by association of HAPE

Chromosome	locus	allele	HAPE-s (%) N=106 n (%)	HAPE-r (%) N=134 n (%)	OR	P*	Pc†
1	D1S2697‡	284	11 (10.4)	2 (1.5)	7.64	0.0025	0.013
	D1S230#	156	18 (17.0)	46 (34.3)	0.39	0.0025	0.020
5	D5S424‡	212	47 (44.3)	36 (26.9)	2.17	0.0047	0.030
6	D6S257‡	179	26 (24.5)	13 (9.7)	3.03	0.0020	0.030
12	D12S368#	202	43 (40.6)	81 (60.4)	0.45	0.002	0.015
14	D14S283#	139	24 (22.6)	54 (40.3)	0.43	0.00373	0.045
16	D16S3103	323	10 (9.4)	0 (0.0)	29.27	0.0003	0.003
21	D21S263‡	216	7 (6.6)	0 (0.0)	20.28	0.003	0.035
22	D22S280#	221	9 (8.7)	32 (23.9)	0.30	0.0020	0.020

‡ = susceptible markes, # = resistant markers
N = Total number of chromosome, n = Number of positive alleles Allele frequencies
*P value was calculated by Chi-square test (2 × 2 contingency table) for each allele
†Pc = corrected P, which was calculated by muitiplying numbers of alleles in the given locus

・Five markers were identified to be the candidate regions associated with susceptibility, and four markers were identified to be candidate regions associated with resistance by microsatellite study.

Table 2 Candidate genes around 100-kb of each significant markers

Chromosome	locus	Symbols
1	D1S2697	*SPEN, ZBTB17, C1orf64, HSPB7, CLCNKA,CLCNKB, FAM31C*
	D1S230	*INADL*
5	D5S424	*F2R, F2RL1, S100Z, CHRBP*
6	D6S257	*COL21A1*
12	D12S368	Keratin (*KRT*) genes
14	D14S283	T cell recepror alpha variable (*TRAV*) genes
16	D16S3103	*XYLT1*
21	D21S263	Keratin associated protein (*KRTAP*) genes
22	D22S280	*TIMP3, SYN3*

・Candidate gene within 100-kb region around 9 significant microsatellite markers
・D22S280 is located in intron of *TIMP3*

Methods -*TIMP3* SNP genotyping-

・The gene encoding tissue inhibitor of metalloproteinase 3 (*TIMP3*) was selected as candidate susceptibility gene for the case-control study of investigating the associations of SNPs
・Six SNPs distributing within *TIMP3* were applied to the genotyping. The selection were based on the following information from NCBI dbSNP database (build 37.3), the HapMap database, and the SNP database of Applied Biosystems.
・All 6 SNPs were genotyped by Taqman® SNP Genotyping Assays (Applied Biosystems, Foster City, CA) using the Applied Biosystems 7500 Real-time PCR system, according to manufacturer's instructions.
・The measurement (D') of pair-wise linkage disequilibrium (LD) of the six SNPs were examined with Haploview software which was then partitioned into block structures using common approaches to block definition of Solid Spine of LD (*JC Barrett, et al., Bioinformatics, 2005, 21, 263–265*). P and Pc values less than 0.05 indicated statistical significance.

Results -*TIMP3* SNP genotyping-

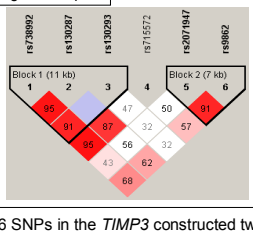
Table 3 Association of 6 SNPs in TIMP3 gene with HAPE

SNP	Observed Allele (1/2)	Frequency Allele1 HAPE-s	Frequency Allele1 HAPE-r	OR (95%CI) (1/2)	*P* value (1/2)
rs738992	C/T	0.481	0.515	0.87 (0.52-1.46)	0.60
rs130287	A/G	0.736	0.776	0.80 (0.44-1.45)	0.47
rs130293	C/T	0.057	0.216	0.22 (0.09-0.55)	0.00049
rs715572	G/A	0.721	0.746	0.88 (0.49-1.57)	0.66
rs2071947	C/T	0.577	0.627	0.81 (0.48-1.37)	0.43
rs9862	C/T	0.660	0.627	0.98 (0.57-1.68)	0.95

95% CI: 95% confidence interval; OR: odds ratio.
P value was calculated by Chi-square test (2 × 2 contingency table) for each allele

・rs130293 showed statistically significant association with HAPE.
・The allele C in *rs130293* showed a significant association with the resistance to HAPE

Figure1 LD plot



・6 SNPs in the *TIMP3* constructed two haplotype blocks that spans 11 kb and 7kb of the *TIMP3* gene.

Table 4 The frequencies of the most four common haplotypes

Number of haplotype#	SNPs rs738992	SNPs rs130287	SNPs rs130293	Frequency HAPE-s	Frequency HAPE-r	p*
1	T	A	T	0.514	0.477	0.565
2	C	G	T	0.254	0.224	0.584
3	C	A	C	0.056	0.208	0.0008
4	C	A	T	0.165	0.083	0.0511

Abbrebiations: HAPE-s= subjects susceptible to high-altitude pulmonary edema; HAPE-r = subjects resistant to high-altitude pulmonary edema; SNPs = single nucleotide polymorphisms; A = adenine; C = sytosine; G = guanine; T = thimine
Number of haplotype was defined in this study
*P valus were calculated by Chi-square test

・The frequency of haplotype CAC was significantly lower in HAPE-s than the HAPE-r for the strength of association of the haplotype 3 with the HAPE-s group

Discussion

・This is the first case-control genome-wide association study aimed at identifying candidate genes for HAPE pathogenesis.
・TIMP-3 is one of four tissue inhibitor of matrix metalloproteinases (TIMPs). TIMP-3 was already reported to function as of pro-MMP2 and pro-MMP9. Both are activated to MMP-2 and to MMP-9. Pirrone et al suggested that hypoxia stimulation induced significant higher activation of MMP-2, and hisotological analysis revealed alveolar emphysema, interstitial edema and a moderate inflammation. *F. Pirrone, et al., Vet Res Commun 2009; 33 (Suppl 1):S121–S124*
・TIMP-3 has also demonstrated as an inhibitor of a disintegrin and metalloproteinase 17 (ADAM17). ADAM17 was reported to modulates the alveolar epithelial barrier through neuregulin-1 and human epidermal growth factor receptor-2 in the pathophysiology of acute lung injury. *James H. F, et al, J. Biol. Chem., 2011, 286: 10660-10670*
・In this study, the rs130293 SNP is located in intron-1 of the TIMP3 gene, this SNP wouldn 't have any direct influence on the conformation of the TIMP3 protein However, it remains possibility that this SNP may have an effect on mRNA stability and transcription and/or translation efficiency. This might cause some influences to function of the *TIMP3* molecule and interfere its regulation of MMP2, MMP-9 and ADAM17, thus might induce inflammation, high permeability in pulmonary artery, modulates the alveolar epithelial barrier, consequently correspond to development of HAPE

Conclusion

・Microsatellite association study revealed 5 markers associated with susceptible to HAPE and 4 markers associated with resistant to HAPE
・This study also demonstrated that the *rs130293* (C/T) SNP in the *TIMP3* gene were likely associated with developing HAPE

Pulmonary artery pressure and serum biomarkers in high-altitude pulmonary edema susceptible subjects during acute hypoxic exposure

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Introduction

High-altitude pulmonary edema (HAPE) is a life-threatening form of noncardiogenic edema characterized by exaggerated hypoxic pulmonary hypertension. Decreased vasodilator nitro oxide (NO) and increased vasoconstrictors peptide endothelin-1 (ET-1) , angiotensin-converting enzyme (ACE)) and an altered vascular endothelial growth factor (VEGF) were thought to play important roles in the exaggerated pulmonary hypertension in HAPE susceptible subjects (HAPE-s) at high altitude. It is not yet clear the overall status of the vasoconstrictors and vasodilators in HAPE-s under hypoxic exposure.

We simultaneously measured such factors in HAPE susceptible subjects (HAPE-s) and HAPE resistant subjects (HAPE-r) before and after hypoxic exposure in order to identify the status of such vasodilator and vasoconstrictors in HAPE-s under hypoxic environment.

Materials and Methods

HAPE-s: 17 Japanese who had previously developed at least one episode of HAPE while climbing the Japan Alps of height from 2,758 to 3,190m above sea level.

HAPE-r: 10 Japanese who usually climb mountains vigorously over 3,000 m. None of them reported history of HAPE or acute mountain sickness and other cardiopulmonary disorders according to their answers to the questionnaire worksheet of Lake Louise Score.

The hypoxic gas (mixed by O2, CO2 and N2; $PETO_2$ = 60 mmHg) was supplied to the subjects for 30 minutes in the oxygen saturation around 75-80% corresponding to altitude about 4,000 m.

Systolic pulmonary artery pressure (aPAP) was measured by doppler echocardiography and venous blood were collected before (normoxia) and after hypoxic breathing (htpoxia). The serum levels of vasoconstrictors and vasodilators were measured ; NO was measured by detecting the colorimetric Griess color reaction, ET-1 was measured by radioimmunoassay (RIA), ACE was measured by colorimetrics, and VEGF was measured by quantitative sandwich enzyme-linked immune-sorbent assay (ELISA). Paired Student's t-test was applied to compare the measurements before and after hypoxic exposure in each group. Unpaired Student 's t-test was used to compare the measurements between the HAPE-s and HAPE-r groups in each experimental condition.

Table.1 Background of the subjects

	HAPE-s	HEPE-r
Numbers	17	10
Ratio of male to female	13:4	9:1
Mean age, yr (Range)	50 (27-64)	51 (25-67)

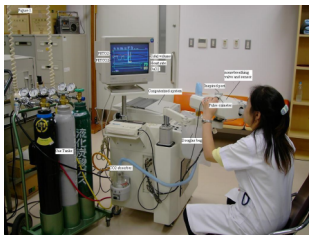

Figure.1 Breathing hypoxic gas

Results

Table.2 Systolic PAP and levels of ET-1, ACE, NOx and VEGF

	Normoxic condition	Hypoxic condition	P value*
HAPE-s (n=17)			
sPAP (mmHg)	24.2±8.91	33.5±11.5	0.001
ET-1 (pg/mL)	1.9±0.5 (#P=0.02)	2.5±0.8 (#P=0.04)	0.03
ACE (IU/L, 3Nox	12.1±4.6	11.3±4.2 (#P=0.02)	n.s.
NOx (µmol/L)	60.8±35.3	41.7±26.2 (#P=0.006)	0.009
VEGF (pg/mL)	392.5±184.3 (#P=0.02)	327.1±258.8	n.s.
HEPE-r (n=10)			
sPAP (mmHg)	25.8±8.49	32.3±6.14	0.02
ET-1 (pg/mL)	1.5±0.4	1.8±0.8	n.s.
ACE (IU/L, 37°C)	16.9±7.5	16.8±7.6	n.s
NOx (µmol/L)	87.3±34.7	72.7±25.2	0.002
VEGF (pg/mL)	216.5±143.6	211.4±136.9	n.s.

Data are expressed as means±SD. * Indiates normoxia vs hypoxia, # indicates HAPE-s vs. HAPE-r

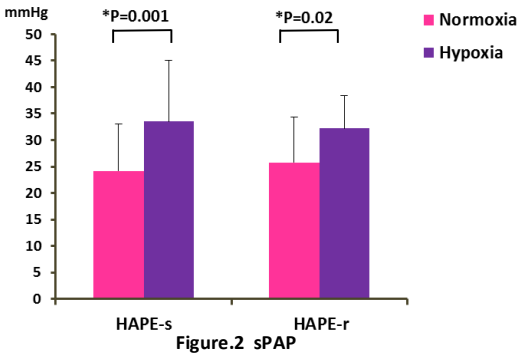

Figure.2 sPAP

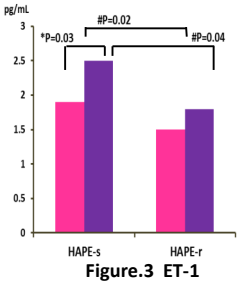

Figure.3 ET-1

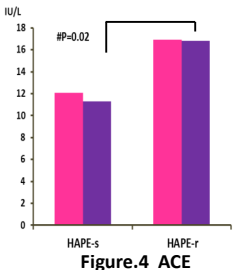

Figure.4 ACE

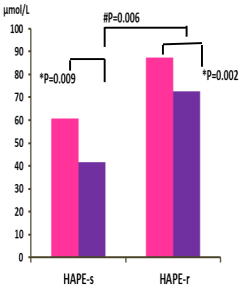

Figure.4 NOx

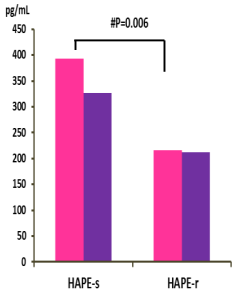

Figure.5 VEGF

Discussion

. The sPAP was significantly increased in both the HAPE-s (p = 0.001) and HAPE-r (p = 0.02) subjects after hypoxic breathing. However, it seems that the sPAP in HAPE-s was more sensitive in response to hypoxia than that in HAPE-r group.

The HAPE-s showed significantly decrease of NO (p = 0.009) and increase of ET-1 (p = 0.03) after hypoxic exposure compared to those of HAPE-r. The change as well as the percent change of NO from the normoxic to hypoxic conditions were in negative correlations with the hypoxia-induced elevated sPAP in the HAPE-s (r = 0.53 and 0.57, respectively). However, the change and the percent change of ET-1 did not show any correlations with the hypoxia-induced elevated sPAP in the HAPE-s. In contrast, We did not find any correlations of sPAP with serum levels of either NO or ET-1 in the control group of HAPE-r subjects. There were no significant changes of ACE activity and VEGF. This might be due to the distinction between their situations in pulmonary circulation and those in systemic circulation.

This study demonstrated that a coexistence of an increase of ET-1 and a decrease of NO concentrations induced by hypoxia was more vital than either of the single factor in the development of HAPE. The increased ET-1 might enhance the effect of vasoconstriction caused by the deficient NO, reversely, the reduced NO might amplify the vasoconstriction due to the increased ET-1. The conjugative effect of the decreased NO and increased ET-1 intensively activates pulmonary vasoconstriction, contributing to elevation in pulmonary artery pressure, eventually, leading to pulmonary edema in HAPE-s at high altitude.

Conclusion

The simultaneously reduced NO level with an augmented ET-1 level during acute hypoxic exposure may determine the pulmonary vascular status that eventually contributes to the increased sPAP in HAPE-s at high altitude by a predominant effect of NO.