ORIGINAL RESEARCH

EPAS1 Gene Polymorphisms Are Associated With High Altitude Polycythemia in Tibetans at the Qinghai-Tibetan Plateau

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Objective.—To test the hypothesis that the polymorphisms in the *EPAS1* gene are associated with the susceptibility to high altitude polycythemia (HAPC) in Tibetans at the Qinghai-Tibetan Plateau.

Methods.—We enrolled 63 Tibetan HAPC patients and 131 matched healthy Tibetans as a control group, from the Yushu area in Qinghai where the altitude is greater than 3500 m. Eight single-nucleotide polymorphisms (SNPs) of the *EPAS1* gene, including rs12619696, rs13420857, rs2881504, rs4953388, rs13419896, rs4953354, rs10187368, and rs7587138, were genotyped by the Sequenom MassARRAY SNP assay.

Results.—The frequencies of the G allele of *EPAS1* SNP rs13419896 were significantly higher in the HAPC group than in the control group ($P < .05$). Moreover, the A alleles of rs12619696 and rs4953354 were prevalent in the HAPC group, and their counterpart homozygotes were prevalent in the normal Tibetan group ($P < .05$).

Conclusions.—Compared with normal Tibetans, Tibetans with HAPC are maladapted and have a different haplotype in *EPAS1* SNPs rs12619696, rs13419896, and rs4953354.

Key words: *EPAS1*, polymorphism, high altitude polycythemia, Tibetans

Introduction

Increased numbers of circulating erythrocytes develop in high altitude dwellers to compensate for the hypoxia associated with high altitude. This phenomenon is termed high altitude polycythemia (HAPC), which is characterized by excessive erythrocytosis (males, hemoglobin [Hb] ≥ 21 g/dL; females, Hb ≥ 19 g/dL). HAPC is prevalent in 5% to 18% of the population on the Qinghai-Tibetan Plateau.^{1,2} HAPC leads to significant increase in blood viscosity, microcirculation disturbance, or even extensive organ damage.^{3,4} Although hypobaric hypoxia is likely to be a cause of HAPC at high altitude, the precise mechanisms underlying the pathogenesis of HAPC are not well understood.

The Tibetans on the Qinghai-Tibetan plateau live permanently at an altitude up to 3000 to 4500 m; they possess heritable adaptations to the hypoxic

environment, as indicated by lower hemoglobin levels, lower hematocrits, higher oxygen saturation of blood in infants, and high work performance.^{5–7} However, some Tibetans living at high altitude still show elevated hemoglobin concentration and even exhibit HAPC.^{8–11}

Growing evidence suggests that the hypoxia-inducible factor (HIF) oxygen-signaling pathway plays an important role in the adaptation of Tibetans.^{12–17} The human *EPAS1* gene is located on chromosome 2p21–p16 and encodes the oxygen-sensitive alpha subunit of HIF-2, which is a key regulator of chronic hypoxia by regulating a large number of genes involved in the cellular and systemic responses to hypoxia. These responses include erythropoiesis, iron homeostasis, pulmonary hypertension and remodeling, vascular permeability, and lung and placental development.¹⁸ Our recent study showed that the polymorphisms in the *EPAS1* gene are associated with the susceptibility to high altitude pulmonary edema (HAPE) in the Han Chinese.¹⁹ However, the association of *EPAS1* gene polymorphisms with HAPC in the Tibetan population remains unclear.

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Up to now, 8 *EPAS1* single-nucleotide polymorphisms (SNPs), including rs12619696, rs13420857, rs2881504, rs4953388, rs13419896, rs4953354, rs10187368, and rs7587138, have been shown to be related to the adaptation to high altitude.^{13,20,21} To explore the potential role of *EPAS1* polymorphisms in the pathogenesis of HAPC in Tibetans, we examined these 8 SNPs of the *EPAS1* gene in 63 subjects with HAPC and 129 healthy controls, all Tibetans from the Yushu area in Qinghai province, where the altitude is greater than 3500 m above sea level.

Methods

SUBJECTS

A total of 63 patients with HAPC (mean age, 45.51 ± 10.07 years) and 131 control subjects (mean age, 45.14 ± 11.78 years) participated in this study. All participants lived in the Yushu area in southwest Qinghai province (altitude 3760 m), and they were all Kangba Tibetans. All HAPC patients were diagnosed at Yushu People's Hospital between March 2011 and June 2013. The inclusion criteria were 1) an Hb concentration of at least 21 g/dL for men and at least 19 g/dL for women, and 2) that HAPC patients were local Tibetans normally living at 3600 to 4400 m. Patients with other diseases having similar clinical manifestations were excluded. Healthy Tibetans matching the patients in age, sex, and working conditions were randomly selected from a physical examination at an outpatient clinic to serve as control subjects. None of the participants had a history of respiratory or cardiovascular disease, such as chronic obstructive pulmonary disease, pulmonary infection, asthma, shunt, valvular disease, congenital heart disease, or hypertensive heart disease. The research protocol was approved by the ethics committee at the Qinghai University School of Medicine (Xining, China). All participants in this study signed informed consent. Hemoglobin concentration and hematocrit (HCT) were determined from venous blood samples using the Mindray Hematology Analyzer (BC-2300; Mindray, Shenzhen, China), and oxygen saturation (Sao₂) levels were tested by pulse oximeter (Ohmeda 3700 Pulse Oximeter; Datex-Ohmeda, Boulder, CO, USA).

DNA EXTRACTION AND GENOTYPING ASSAYS

Genomic DNA was extracted from venous blood by Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to standard procedures. All selected SNPs were genotyped by the Sequenom MassARRAY SNP assay (Capital Bio Corporation, Beijing, China). SNP loci-tested polymerase chain reaction (PCR) primers and single base extension primers were designed by using the Sequenom MassARRAY Assay Design Genotyping Software and Tools

(Sequenom, San Diego, CA, USA). The PCR reaction was performed under the following thermal cycling conditions: 94°C for 4 minutes, then 94°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute for 45 cycles, and 72°C for 4 minutes. PCR products were treated with shrimp alkaline phosphatase to remove free deoxyribonucleoside triphosphates, and single base extension reaction was performed, which consisted of 2.0 µL of EXTEND MIX, 0.619 µL of ddH₂O, 0.94 µL of Extend primer mix, 0.2 µL of iPLEX buffer plus, 0.2 µL of iPLEX terminator, and 0.041 µL of iPLEX enzyme (Sequenom, San Diego, CA, USA). The thermal cycling conditions were as follows: 94°C for 30 seconds, then 94°C for 5 seconds, 52°C for 5 seconds, and 80°C for 5 seconds for 40 cycles, and 72°C for 3 minutes. The MassARRAY Nanodispenser RS1000 (Capital Bio Corporation, Beijing, China) was used for dispensing the purified extension products onto a 384-element SpectroCHIP bioarray (Sequenom, San Diego, CA, USA), and mass spectrometric analysis was performed using the MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) (Sequenom, San Diego, CA, USA). The results were analyzed using TYPER 4.0 software (Sequenom, San Diego, CA, USA).

STATISTICAL ANALYSIS

SPSS software (version 17.0; SPSS, Inc, Chicago, IL, USA) was used for statistical analysis. Allele frequencies were calculated based on genotype frequencies in HAPC and control groups, and the intergroup difference was estimated with the χ^2 test. A probability value of less than .05 was considered significant. Deviations from the Hardy-Weinberg equilibrium (HWE) were assessed by processing the χ^2 test for genotype frequency. Population genetic data were analyzed using Arlequin (version 2.000) software (<http://cmpg.unibe.ch/software/arlequin35/>). Genetic distances were used to measure the divergence between study groups. The genetic distance ranged between 0 and 1, where 1 was complete divergence and 0 indicated no divergence. The smaller the genetic distance, the closer the genetic relationship. A variable used in association with genetic distance to express the proportion of genetic diversity attributable to genotype frequency differences among study groups is F_{ST}.²² The odds ratio (OR), CIs, and probability values between the HAPC groups and control groups were calculated for alleles of polymorphic loci.

Results

CHARACTERISTICS OF SUBJECTS

The data for the sex, average age, Sao₂, Hb, and HCT for the HAPC and control groups are reported in Table 1.

Table 1. Characteristics of the study groups

Study group	N	Sex	Age (year)	Sao ₂ (%)	Hb (g/dL)	HCT (%)
HAPC	35	Male	47.7 ± 1.7	83.4 ± 0.9 ^a	23.0 ± 0.3 ^a	66.6 ± 2.1 ^a
	28	Female	42.8 ± 1.8	85.1 ± 0.9 ^a	20.1 ± 0.2 ^a	59.9 ± 0.8 ^a
Control	74	Male	46.4 ± 1.5	88.8 ± 0.4	16.2 ± 0.1	46.6 ± 0.6
	55	Female	43.2 ± 1.4	87.3 ± 0.6	15.8 ± 0.2	41.6 ± 2.1

The data represent the mean and SE of HAPC patients and healthy control subjects.

HAPC, high altitude polycythemia; Sao₂, arterial oxygen saturation; Hb, hemoglobin; HCT, hematocrit.

^a $P < .05$ vs control group.

The incidence of HAPC was much higher in men than in women, consistent with the preponderance of men with HAPC. The Sao₂ was significantly lower, whereas Hb and HCT were significantly higher in the HAPC group compared with the control group ($P < .05$).

GENOTYPE AND ALLELE DISTRIBUTION

The genotypic distributions and allelic frequencies of 8 *EPAS1* SNPs (rs12619696, rs13420857, rs2881504, rs4953388, rs113419896, rs4953354, rs10187368, and rs7587138) in HAPC and control study groups are shown in Tables 2 and 3.

All these polymorphisms were found to be in HWE in both study groups (Table 3). The AA genotypes of rs12619696 and rs4953354 were significantly more prevalent among the HAPC group (8.2% and 12.9%) than the control group (2.3% and 3.9%) with an OR of 0.227 (95% CI, 0.052 to 1.001; $P = .035$) and 0.199 (95% CI, 0.060 to 0.658; $P = .004$), respectively. Furthermore, the genotype GG rs13419896 differed significantly between HAPC and control groups with an OR of 0.062 (95% CI, 0.007 to 0.530; $P = .001$; Table 2).

Furthermore, we found that there were significant differences in the allele frequency of the rs12619696 SNP between the 2 groups ($P = .014$; Table 2); the A allele was much more prevalent among the HAPC group (24.6%) than the control group (14.3%), with an OR of 0.513 (95% CI, 0.299 to 0.881). The difference was significant when applying the F_{ST} statistics on the genetic distance (.121; Table 3). The G allele for the rs13419896 SNP was significantly more prevalent among the HAPC group (27%) than the control group (14.7%; $P = .004$; Table 2), with an OR of 0.466 (95% CI, 0.275 to 0.789), and the difference was significant when applying the F_{ST} statistics on the genetic distance (.203; Table 3). We observed a significantly higher incidence of the A allele of the rs4953354 SNP in the HAPC group (34.7%) than in the control group (19.0%), with an OR of 0.442 (95% CI, 0.272 to 0.716; $P = .001$;

Table 2), and the difference was significant when applying the F_{ST} statistics on the genetic distance (.410; Table 3). We also found a significant difference for the rs2881504 SNP (A/G) between the HAPC and control groups ($P < .001$; Table 2).

We showed that the rs12619696 SNP was significantly associated with HAPC risk under the dominant model of inheritance (OR, 0.515; 95% CI, 0.271 to 0.981; $P = .042$). The rs13419896 SNP was significantly associated with HAPC risk under the dominant model (OR, 0.506; 95% CI, 0.269 to 0.964; $P = .034$) as well as the recessive model of inheritance (OR, 13.964; 95% CI, 1.642 to 118.737; $P = .002$; Table 2). In addition, the rs4953354 SNP was significantly associated with HAPC risk both under the dominant (OR, 0.399; 95% CI, 0.215 to 0.742; $P = .003$) and recessive models of inheritance (OR, 3.674; 95% CI, 1.149 to 11.745; $P = .020$; Table 2).

Discussion

In a previous study we genotyped 207 SNPs of the *EPAS1* gene in Chr2: 46304028–46851921 in a sample of 31 healthy Tibetans. The results suggested that the polymorphism in the *EPAS1* gene is associated with adaptation to high altitude in Tibetans.¹⁵ Thus we hypothesized that Tibetans with HAPC may carry a different genotype and alleles, and we genotyped 8 SNPs of the *EPAS1* gene, 7 of them located in the same region of Chr2: 46304028–46851921. We genotyped 8 SNPs of the *EPAS1* gene by the SNP assay and analyzed the haplotypes in HAPC and control groups. Collectively, our results indicate that there are significant differences in 3 SNPs (rs12619696, rs13419896, and rs4953354) between the 2 groups.

A previous study reported that rs12619696 was associated with different patterns of adaptation to high altitude between Tibetans and Andeans.²¹ In this study, we found that both the AA genotype and A allele of rs12619696 were significantly more prevalent among the HAPC group (8.2% and 24.6%) than the control group

Table 2. Comparison of genotype distributions, allele frequencies, and association with HAPC risk in HAPC and control groups

SNP	Genotype or allele associated with SNP	HAPC (n %)	Control (n %)	OR (95% CI)	P value
rs12619696	Genotype				
	GG	36 (59.0)	95 (73.6)	1	
	AG	20 (32.8)	31 (24.0)	0.587 (0.297–1.160)	.123
	AA	5 (8.2)	3 (2.3)	0.227 (0.052–1.001)	.035 ^a
	Allele				
	G	92 (75.4)	221 (85.7)	1	
	A	30 (24.6)	37 (14.3)	0.513 (0.299–0.881)	.014 ^a
	Dominant model				
	GG	36 (59.0)	95 (73.6)	1	
Recessive model	AG+AA	25 (41.0)	34 (26.4)	0.515 (0.271–0.981)	.042 ^a
	AA	5 (8.2)	3 (2.3)	1	
	AG+GG	56 (91.8)	126 (97.7)	3.750 (0.886–16.237)	.060
rs13420857	Genotype				
	GG	1 (1.7)	1 (0.8)	1	
	CG	8 (13.3)	21 (16.3)	2.625 (0.146–47.183)	.499
	CC	51 (85.0)	107 (82.9)	2.098 (0.129–34.220)	.595
	Allele				
	G	10 (8.3)	23 (8.9)	1	
	C	110 (91.7)	235 (91.1)	0.929 (0.427–2.019)	.852
	Dominant model				
	GG	1 (1.7)	1 (0.8)	1	
Recessive model	GC+CC	59 (98.3)	128 (99.2)	2.169 (0.133–35.283)	.577
	CC	51 (85.0)	107 (82.9)	1	
	GC+GG	9 (15.0)	22 (17.1)	1.165 (0.501–2.710)	.723
rs2881504	Genotype				
	GG	3 (4.8)	7 (5.4)	1	
	AG	18 (28.6)	44 (34.1)	1.048 (0.243–4.509)	.950
	AA	42 (66.7)	78 (60.5)	0.796 (0.196–3.239)	.749
	Allele				
	G	84 (19.0)	58 (22.5)	1	
	A	102 (81)	200 (77.5)	2.840 (1.883–4.283)	.000 ^a
	Dominant model				
	GG	3 (4.8)	7 (5.4)	1	
Recessive model	AG+AA	60 (95.2)	122 (94.6)	0.871 (0.218–3.489)	.846
	AA	42 (66.7)	78 (60.5)	1	
	AG+GG	21 (33.3)	51 (39.5)	1.308 (0.695–2.459)	.405
rs4953388	Genotype				
	AA	21 (34.4)	57 (44.2)	1	
	AG	29 (47.5)	60 (46.5)	0.762 (0.391–1.487)	.425
	GG	11 (18.0)	12 (9.3)	0.402 (0.154–1.049)	.058
	Allele				
	A	71 (58.2)	174 (67.4)	1	
	G	51 (41.8)	84 (32.6)	0.672 (0.431–1.048)	.079
	Dominant model				
	AA	21 (34.4)	57 (44.2)	1	
Recessive model	AG+GG	40 (65.6)	72 (55.8)	0.663 (0.352–1.248)	.202
	GG	11 (18.0)	12 (9.3)	1	
	AG+AA	50 (92.0)	117 (90.7)	2.145 (0.887–5.185)	.085
rs13419696	Genotype				
	AA	34 (55.7)	92 (71.3)	1	
	AG	21 (34.4)	36 (27.9)	0.634 (0.325–1.234)	.178
	GG	6 (9.8)	1 (0.8)	0.062 (0.007–0.530)	.001 ^a
	Allele				
	A	89 (73.0)	220 (85.3)	1	
	G	33 (27.0)	38 (14.7)	0.466 (0.275–0.789)	.004 ^a
	Dominant model				
	AA	34 (55.7)	92 (71.3)	1	
Recessive model	AG+GG	27 (44.3)	37 (28.7)	0.506 (0.269–0.954)	.034 ^a
	GG	6 (9.8)	1 (0.8)	1	
	AG+AA	55 (90.2)	128 (99.2)	13.964 (1.642–118.737)	.002 ^a
rs4953354	Genotype				
	GG	27 (43.5)	85 (65.9)	1	
	AG	27 (43.5)	39 (30.2)	0.459 (0.238–0.883)	.019 ^a

Table 2 (continued)

SNP	Genotype or allele associated with SNP	HAPC (n %)	Control (n %)	OR (95% CI)	P value
rs10187368	AA	8 (12.9)	5 (3.9)	0.199 (0.060–0.658)	.004 ^a
	Allele G	81 (65.3)	209 (81.0)	1	
	A	43 (34.7)	49 (19.0)	0.442 (0.272–0.716)	.001 ^a
	Dominant model GG	27 (43.5)	85 (65.9)	1	
	AG+AA	35 (56.5)	44 (34.1)	0.399 (0.215–0.742)	.003 ^a
rs7587138	AA	8 (12.9)	5 (3.9)	1	
	AG+GG	35 (87.1)	124 (96.1)	3.674 (1.149–11.745)	.020 ^a
	Genotype GG	55 (91.7)	124 (96.1)	1	
	AG	5 (8.3)	5 (3.9)	0.444 (0.123–1.595)	.203
	Allele AA	0 (0.0)	0 (0.0)		
rs12619696	G	115 (95.8)	253 (98.1)	1	
	A	5 (4.2)	5 (1.9)	0.455 (0.129–1.601)	.209
	Dominant model GG	55 (91.7)	124 (96.1)	1	
	AG+AA	5 (8.3)	5 (3.9)	0.444 (0.123–1.595)	.203
	Recessive model AA	0 (0.0)	0 (0.0)	1	
rs4953354	AG+GG	60 (100)	129 (100)		
	Genotype GG	1 (1.7)	5 (3.9)	1	
	AG	16 (26.7)	31 (24.0)	0.388 (0.042–3.604)	.391
	Allele AA	43 (71.7)	93 (72.1)	0.433 (0.049–3.816)	.438
	G	18 (15.0)	41 (15.9)	1	
rs13419896	A	102 (85.0)	217 (84.1)	0.934 (0.512–1.705)	.824
	Dominant model GG	1 (1.7)	5 (3.9)	1	
	AG+AA	59 (98.3)	124 (96.1)	0.420 (0.048–3.679)	.420
	Recessive model AA	43 (71.7)	93 (72.1)	1	
	AG+GG	17 (28.3)	36 (27.9)	0.979 (0.496–1.934)	.952

HAPC, high altitude polycythemia; OR, odds ratio, SNP, single-nucleotide polymorphism.

^a $P < .05$ vs control.

(2.3% and 14.3%), with an OR of 0.227 (95% CI, 0.052 to 1.001; $P < .05$) and 0.513 (95% CI, 0.299 to 0.881; $P < .05$). In addition, rs12619696 was significantly associated with HAPC risk under the dominant model of inheritance (OR, 0.515; 95% CI, 0.271 to 0.981; $P < .05$).

For the SNP rs13419896, the A allele is proposed as being advantageous for Tibetans.^{14,22} In this study, the A allele was reported as 73% in Tibetans with HAPC and 85.3% in a control group who were Tibetans adapted to high altitude. The genotype AA differed significantly between the HAPC (55.7%) and control groups (71.3%). Therefore, the A allele of rs13419896 is the allele that is advantageous for Tibetans to adapt to hypoxia at high altitude. A high incidence of the G allele is associated with HAPC in Tibetans. Indeed, the G allele of rs13419896 was significantly more prevalent among the HAPC group (27%) than the control group (14.7%; $P < .05$). The SNP rs13419896 was also significantly

associated with HAPC risk under the dominant as well as the recessive model of inheritance.

For the SNP rs4953354, the G allele had a high incidence in healthy Tibetans.^{14,22} Interestingly, we observed a significantly lower incidence of the G allele in the HAPC group (65.3%) compared with the control group (81.0%). However, the AA genotype was significantly more prevalent among the HAPC group (12.9%) than the control group (3.9%). The rs13419896 SNP was significantly associated with HAPC risk both under the dominant and recessive models of inheritance. Therefore, our results indicated that a high incidence of the A allele and AA genotype of rs4953354 is associated with the risk of HAPC in Tibetans.

In addition, we found significant differences between 2 groups as measured by F_{ST} on the genetic distances for the 3 SNPs: rs12619696, rs13419896, and rs4953354. The divergences resulted from different genotypes of these SNPs in the 2 groups.

Table 3. Comparison of Hardy-Weinberg Equilibrium (HWE) and genetic distance in HAPC and control groups

SNP	Study group	N	HWE P value	Genetic distance		
				SG	1	2
rs12619696	HAPC	61	.365	1	.511	.500
	Control	129	.802	2	.121 ^a	.247
rs13420857	HAPC	60	.324	1		
	Control	129	.802	2		
rs2881504	HAPC	63	.559	1		
	Control	129	.808	2		
rs4953388	HAPC	61	.858	1		
	Control	129	.502	2		
rs13419896	HAPC	61	.319	1	.488	.573
	Control	129	.207	2	.203 ^a	.252
rs4953354	HAPC	62	.760	1	.284	.707
	Control	129	.843	2	.410 ^a	.309
rs10187368	HAPC	60	.736	1		
	Control	129	.822	2		
rs7587138	HAPC	60	.723	1		
	Control	129	.251	2		

HAPC, high altitude polycythemia; SNP, single-nucleotide polymorphism; SG, study group; F_{ST} , proportion of genetic diversity attributable to genotype frequency differences among study groups.

^a $P < .05$ computed from F_{ST} statistics.

However, we should note several limitations of this study. First, we did not measure other clinical parameters of the subjects, such as systolic blood pressure, diastolic blood pressure, mean arterial pressure, and pulmonary artery systolic pressure, which would provide more information on the significance of *EPAS1* SNPs in Tibetans with HAPC. Second, we only focused on 8 SNPs of *EPAS1* and did not screen other genes. Third, we examined a modest number of subjects in the study. More subjects will be included in the 2 groups to increase the power to detect the association of *EPAS1* SNPs with HAPC in Tibetans.

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