Genetic association analysis of chronic mountain sickness in an Andean high-altitude population

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Background and Objectives. Millions of people live above an altitude of 2,500 m and are at risk of chronic mountain sickness (CMS), a disorder of excessive red cell and hemoglobin production. Preferential ethnic backgrounds, familial character, and heritability studies have suggested that genetic factors make a major contribution to the pathogenesis of CMS, thus our goals were to exploit a probable founder or population admixture effect in the Andean population to determine the genetic determinants of the extreme erythropoietic responses and CMS.

Design and Methods. The association of functional candidate genes with severe polycythemia was studied in Andean subjects from Cerro de Pasco, Peru (altitude 4,438 m). We used microsatellites linked to candidate genes known to be involved in hypoxia sensing and erythropoiesis: erythropoietin, erythropoietin-receptor, hypoxia-inducible factor-1a, von Hippel-Lindau, prolyl hydroxylase domain (*PHD*) containing 1, 2, 3, and phosphatase and tensin homolog deleted on chromosome ten (*PTEN*). Analysis of co-variance (ANCOVA) was used to test the effect of genotypes on hemoglobin values.

Results. Case-control comparisons revealed no significant difference in genotype and allele frequencies at any marker. Initial analysis, with age as a covariate, showed a possible association between *PHD3* (marker *D14S1049*) and severe polycythemia (p=0.05). After the inclusion of alternative co-variates and after adjusting for multiple comparisons, no p values could be considered statistically significant.

Interpretation and Conclusions. Our study does not find evidence of associations between the polymorphisms linked to the candidate genes and severe polycythemia; this does not, however, exclude that variations in these genes contribute to polycythemia and possibly CMS.

Key words: high altitude, polycythemia, Monge's disease, microsatellites.

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The pathologic loss of adaptation to high altitude was first described by C. Monge in 1925 and is called chronic mountain sickness (CMS) or Monge's disease.[1] CMS is manifested by hematologic, neurologic, cardiac and respiratory symptoms, and its severity increases with advancing age. A severe polycythemia, far in excess of the physiologic normal for a particular altitude, is the main characteristic and primary diagnostic sign of this condition. The most common symptoms are headaches, dizziness, dyspnea, insomnia, tinnitus, mental fatigue, loss of appetite, and bone and muscle pain. The most common signs are an intermittent or permanent cyanosis and venous dilatation in hands and feet. CMS has been described in different high-altitude regions of the world including Peru, Chile, Bolivia, The United States, China and Tibet.[1-5] There is a great variability in susceptibility to disease between and within populations; the prevalence is estimated at 1.21% in Tibetans, 5.59% in Han Chinese and 15% in Quechuan Andeans at similar altitudes.[5-7] It has been observed that this disease affects not only humans but also domestic animals introduced into the mountains in historically recent times.[8] Because of its prevalence and chronic nature, CMS and other diseases related to high altitude are now considered as public health problems potentially affecting millions of people in many mountainous areas of the world.

Studies on populations living at high altitudes are limited to a few locations in the world. The Andes has the advantage of containing large populations living at more than 3,500 m, as well as people from an ethnic group (Quechuas). The Quechuan people in Peru are descendents from popu-

lations who have resided at high altitude for thousands of years, raising the possibility that the Quechua were genetically adapted to hypobaric hypoxia; however, it appears that there is no distinct high-altitude adapted phenotype in the Andean high-altitude dwellers. The Andean region has a history of population contact between European (Spanish), Native American, and to a lesser extent West African groups. Monge noted that, in the Peruvian Andes, CMS had an impressive familial character and was more frequent in men of European descent.[1] It was also noted in Bolivian Andeans that CMS was predominant in males of a mixed or entirely European ethnic background.[3] Quantitative genetic analysis revealed that the proportion of phenotypic variance in hemoglobin concentration attributable to genetic factors (i.e. the heritability of hemoglobin concentration) was 86% in Tibetans and 87% in Andeans.[9] Although these data indicate that genetic factors account for a large proportion of phenotypic variance in hemoglobin concentration, they do not identify the specific genetic factors underlying intrapopulation or interpopulation variation in response to altitude or provide insight into the mechanism.

Erythropoietin (Epo) is the principal hormone regulating red cell production or erythropoiesis. This hormone interacts on the surface of erythroid progenitor cells with its specific receptor, erythropoietin receptor (EpoR), thus initiating signal transduction pathways that lead to the production of red blood cells. There is marked variability in Epo and erythrocytic response to high altitude among mountain dwellers; however, there is no correlation between serum Epo levels in Andean natives with severe polycythemia and CMS and those without these pathologic conditions.[10] Epo-mediated control of red blood cell mass involves the transcription factor hypoxia-inducible factor-1 (HIF-1), which has a critical role in regulating cellular and systemic oxygen response.[11] HIF-1 is a dimer composed of an oxygen-regulated subunit (HIF-1α) and an oxygen-independent subunit (HIF-1β).[12] HIF can associate with the highly conserved hypoxia response elements within regulatory sequences of hypoxia responsive genes (e.g. *EPO*) and, in conjunction with other factors, induce their expression. The level of the α subunit is remarkably high during hypoxia and is maintained at low levels under normal oxygen tensions by the von Hippel-Lindau protein (pVHL)-mediated ubiquitin-proteasome pathway.[13] All pVHL mutants causing the von Hippel-Lindau disease are unable to target HIF-1α for degradation, causing constitutive expression of HIF-1α and its target genes[14] and specific mutations have been linked to a primary polycythemia known as Chuvash polycythemia.[15,16] The recognition of HIF-1α by pVHL depends on the enzymatic hydroxylation of specific prolyl residues on HIF-1α,[17,18] a reaction that is catalysed by prolyl hydroxylase domain (PHD) enzymes, PHD1, PHD2, PHD3.[19] Although HIF-1 is regulated mainly by oxygen tension, other factors also modulate HIF-1, such as PTEN (phosphatase and tensin homolog deleted on chromosome ten), which suppresses HIF-1α stabilization in hypoxia.[20]

Thus, based on pathways and processes strongly implicated in known-oxygen sensing mechanisms and erythropoiesis regulation, we considered *EPO*, *EPOR, HIF1A, PHD1, PHD2, PHD3, VHL, PTEN*, as good functional candidate genes for the altered erythropoietic response observed in CMS subjects. Suspecting a probable admixture or founder effect in the Andean population living over 4,000 m above sea level, we hypothesized that variation in one or more genes could affect the prevalence and/or severity of severe polycythemia and likely CMS. To test this hypothesis we used microsatellite polymorphisms located within and in close proximity to the candidate genes as genetic markers. Dinucleotide markers were selected because of their high polymorphism rate, which decreases the probability of identity by state or allele sharing without a common founder.

Design and Methods

Subjects

One hundred and four male Peruvian Quechua natives from Cerro de Pasco city, located at an altitude of 4,438 m in the Andean highlands, participated in the study. All subjects were screened via a brief clinical history and physical examination. Subjects were diagnosed as having CMS (cases) by a CMS score ≥12 and Hb ≥213 g/L (mean±20 SD of the normal distribution of the male population).[6,21] Individuals not meeting these criteria were utilized as the control population. The CMS score is a standard measure based on the 10 most frequent symptoms and signs found in CMS.[22,23] A value of zero was assigned to negative answers. Positive answers were divided as *occasional* (≤2 signs per month) and frequent (≥3 signs per month) and had values of two (or three) and four (or six), respectively. The sum of these assigned values constituted the CMS score.

To achieve a more uniform study population, factors that could obscure or exacerbate the excess polycythemia phenotype, including female sex (high prevalence of iron deficiency), smoking more than 5 cigarettes daily, phlebotomy within the past year, and chronic obstructive respiratory, cardiovascular and renal diseases, constituted exclusion criteria. Each subject gave written, informed consent according to guidelines approved by the Institutional Review Board at the Universidad Peruana Cayetano Heredia, Lima, Peru. At screening, a venous blood sample was obtained and

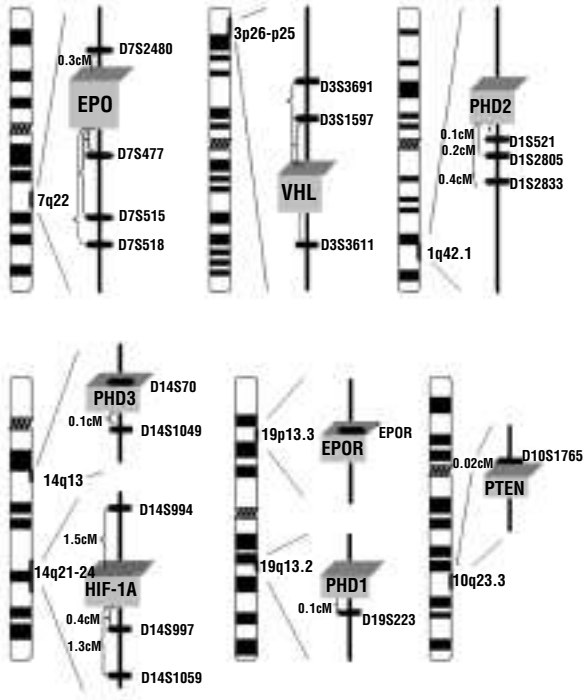

Figure 1. Schematic representation of the chromosomal locations of the candidate genes, indicating the relative positions of the microsatellite markers and their distances from genes.

analyzed with a Hemocue Blood Hb Analyzer (Angelholm, Sweden) in order to determine the hemoglobin concentration.

Candidate genes and microsatellite markers

Functional candidate genes were selected because no linkage or other localization data are available. Eight genes with well established roles in the oxygen sensing and erythropoietic response pathway were selected. Polymorphic dinucleotide markers, linked to candidate gene loci (Figure 1), were selected from published sources. The eighteen polymorphic microsatellite markers located in close proximity (0.02 to 1.5 cM) to the eight candidate genes were: *D7S515, D7S518, D7S2480, D7S477* for *EPO; D14S997, D14S994, D14S1059* for *HIF-1A*; *D3S1597, D3S3691, D3S3611* for *VHL*; *D19S223* for *PHD1*; *D1S251, D1S2833, D1S2805* for *PHD2*; *D14S70, D14S1049* for *PHD3*; *D10S1765* for *PTEN*; and the dinucleotide $(GA)_n$ repeat from the 5' untranslated region of *EPOR*.

Genotyping

DNA was extracted from peripheral blood leukocytes using a QIAamp DNA Blood Mini kit from QIAGEN® and target DNA was amplified by polymerase chain reaction (PCR) using appropriate primers and optimal conditions as stated below. Each reaction was carried out in a total reaction volume of

Table 1. Primer sequences for the 18 markers.

Gene	*Marker*	*Primer sequence*
EPO	*D7S477*	F: 5'TTGCACCACTGTCTCCAGTC3'
		R: 5'TTTGGGTATCCCCTGTTCAC3'
	D7S2480	F: 5'ACCTTGACTGTGGTAGTTGG3'
		R: 3'GTTCACCTCATAGGAAAATCTTG3'
	D7S515	F: 5'GGGAGTTACTACCCTCACTTAATG3'
		R: 5'GGACTGGGCAGCAAAG3'
	D7S518	F: 5'CAGTAGGCAGGGGTGG3'
		R: 5'GGGTGTGTCTGTGTGACAAC3'
HIF-1A	*D14S1059*	F: 5'GGTGAGGGCAGGTTGTC3'
		R: 5'CCATTTGCGTTATCAGGC3'
	D14S994	F: 5'GGCAGACAGGGCTAGAA3'
		R: 5'CGTTATCAGATGTAAGAGACTCCAG3'
	D14S997	F: 5'TGGTCTTGGCAACCCTATAAAAATC3'
		R: 5'TTGAGAGTCCAGAAATAAACCACA3'
VHL	*D3S3611*	F: 5'GCTACCTCTGCTGAGCAT3'
		R: 5'TCATCAATAAAGTGGGGGTGA3'
	D3S3691	F: 5'TCTCAGCAATAGCAAACATCAGG3'
		R: 5'TTGAAACCAGGGTGACAAATACATC3'
	D3S1597	F: 5'AAATTCTGGGATGAGAAGGTACA3'
		R: 5'GCAAATCGTTCATTGCT3'
PHD1	*D19S223*	F: 5'CAAAATCGAGGTGCATAGAA3'
		R: 5'ACCATGACTGGCTAATTGTG3'
PHD2	*D1S2805*	F: 5'GCACCAACCCTCATGCTATT3'
		R: 5'ATTACAAAGAGGCGCAGGAA3'
	D1S2833	F: 5'AGAAAACTCAGGAGGATGCTTT3'
		R: 5'GCTGTTAGTTTGGGGGTAATTT3'
	D1S251	F: 5'GTAATCCTGTGAGCCAATTC3'
		R: 5'GACCAAGCAACTTCACTCC3'
PHD3	*D14S70*	F: 5'AAGAGACACATTTCCTATCCACACA3'
		R: 5'AATTCCTTGTTCCACATTTAGGG3'
	D14S1049	F: 5'GGAAAACACTGGCACCTT3'
		R: 5'TTTGAGGAGCAGGCAAT3'
PTEN	*D10S1765*	F: 5'CCAGGCTTGTCCTAAGTGATTTT3'
		R: 5'GGCCTATCAGTGGGTTTTTAT3'
EPOR	*EPOR*	F: 5' AGGAAGGAAGGAAGGAAGGAAA3'
		R: 5'GCACATAGCGAACATTCCAA3'

7.5 μL, containing 20 ng of genomic DNA, 0.5 μM of each primer (Table 1) and ABI PRISM® TrueAllele™ PCR Premix. Forward PCR primers were fluorescently labeled with either 6-FAM, HEX or TET fluorescent dyes attached to the 5' end of the primer (Integrated DNA Technologies). Reverse primers were tailed in the 5' end with the sequence GTTTCTT to facilitate accurate genotyping.[24] Denaturation at 95°C for 12 min was followed by 10 cycles of denaturation at 94°C for 20 s, primer-specific annealing for 20 s and extension at 72°C for 35 s, then 25 cycles of denaturation at 89°C for 20 s, primer-specific annealing for 20 s and extension at 72°C for 35 s, and a final extension step at 72°C for

Table 2. Characteristics of the case-control study population.

	Controls n=56	*CMS n=48*	*p*
Age (years)	38.4±9.94	45.4±11.81	0.0014
Height (m)	1.62±0.06	1.62±0.06	NS
Weight (kg)	61.1±7.28	65.2±8.86	0.017
BMI (kg/m^2)	23.2±0.02	24.7±0.03	0.0097
Hemoglobin (g/L)	187±12.2	229±11.8	< 0.001
Oxygen Saturation (%)	84.6±3.4	81.8±3.09	0.0084
CMS score	6.2±5.17	12.8±4.94	<0.0001

Data are expressed as mean ± SD. Student's t test was used to compare the mean values for CMS subjects and controls.CMS: chronic mountain sickness; BMI: body mass index.

30 min. The annealing temperatures of individual primer pairs were: 55°C for *D1S2805, D7S477, D3S3691, D7S2480, D7S515, D19S223, D7S518, D14S1049, D14S1059, D14S994, D14S70*, and *EPOR*; 57.2°C for *D3S3611* and *D1S2833*; 62°C for *D14S997*; 46.5°C for *D1S251*; 54°C for *D3S1597*; and 60.9°C for *D10S1765*. PCR products were pooled prior to electrophoresis according to the dye label and expected allele sizes. PCR from a DNA reference sample was included on every gel as a control. Electrophoresis was performed on 4% polyacrylamide gels using Run Module GS Run36C-2400 in an ABI PRISM 377 DNA Sequencer. The GeneScan® Analysis software Version 3.1 (Applied Biosystems) was used to analyze the data collected by the ABI Prism 377 Sequencer to size the PCR products using GeneScan-500 TAMRA as a size standard. The Genotyper® Software Version 2.5 (Applied Biosystems) was used to convert GeneScan fragment data to called genotypes. All sample identities were masked and were run twice for verification.

Statistical analysis

The exact *p* value test of Hardy-Weinberg proportions for multiple alleles was simulated by the Markov chain method with the GENEPOP software package.[25] The exact *p* value was estimated by simulations under the following parameters: the dememorization number = 1000, the number of batches = 500 and the iterations per batch = 8000. A power analysis based on a power of 0.80 at a 0.05 level of significance determined that a minimum sample of 48 subjects per group would be required to detect a hypothesized effect size of 0.35 (considering no previous data on allele frequencies). The frequencies of the alleles were determined by genotype counting and compared with the values predicted on the basis of the assumption of Hardy-Weinberg equilibrium. Contingency tables containing genotype and allele frequencies for each marker were generated and evaluated by the χ^2 test. To test for the association between hemoglobin values and genotypes per locus, analysis of co-variance (ANCOVA) was performed. Age, weight and height were co-variates used in the analysis to model the effect of genotype on hemoglobin better.

Results

Characteristics of the study population

The main characteristics of the case subjects and controls are summarized in Table 2. Subjects ranged in age from 20 to 67 years old and their hemoglobin values ranged from 160 to 260 g/L. The hemoglobin values and the CMS scores were significantly higher in CMS subjects than in controls. CMS subjects were significantly older, heavier and had lower arterial oxygen saturation (SaO_2) values than did controls.

Hardy-Weinberg equilibrium, allele frequencies and heterozygosity

Divergence from Hardy-Weinberg equilibrium was tested for and markers that initially were not in equilibrium were re-genotyped with the same or, when necessary, redesigned PCR primers. The *p* values obtained for each marker (Table 3) were higher than the expected 5% false-positive error ($p>0.05$), meaning that the observed genotype frequencies are not significantly different from those predicted for a population in Hardy-Weinberg equilibrium. The studied microsatellite loci were moderately to highly polymorphic with 5-18 alleles per locus and heterozygosity values of 0.651 to 0.855 (Table 3). Two of the eighteen studied loci (*D7S515* and *D3S3611*) displayed high variation in allele length (124 to 180 and 139 to 179 base pairs, respectively). The observed heterozygosity for each marker is given in Table 3.

Association analysis of EPO, EPOR, HIF1A, PHD1, PHD2, PHD3, VHL, and PTEN

ANCOVA analysis was performed to test the effect of the genotypes at each locus on hemoglobin values. The analyses used age, weight and height as co-variates. Initial analysis, with age as the co-variate, suggested a possible association between marker *D14S1049*, linked to *PHD3*, and severe polycythemia ($p=0.05$). The *p* values after other co-variates were included were all >0.05 indicating no deviations greater than those expected by chance and therefore no association between hemoglobin values and the genotypes for each marker. Analysis of contingency tables for the case-control comparisons showed no significant difference in genotype and allele frequencies at any marker.

Table 3. Observed heterozygosity and *p* values of Hardy-Weinberg equilibrium (HWE) for all markers.

Marker	*Heterozygosity*	*HWE*
D7S477	0.476	0.405
D7S2480	0.656	0.253
D7S515	0.810	0.740
D7S518	0.855	0.752
D14S1059	0.581	0.269
D14S994	0.329	0.507
D14S997	0.511	0.328
D3S3611	0.834	0.191
D3S3691	0.763	0.768
D3S1597	0.716	0.622
D19S223	0.651	0.397
D1S2805	0.173	0.422
D1S2833	0.813	0.267
D1S251	0.781	0.080
D14S70	0.645	0.180
D14S1049	0.775	0.169
D10S1765	0.775	0.137
EPOR	0.677	0.083

Discussion

CMS is an example of adaptive failure at high altitude affecting millions of highlanders around the world, and is particularly common among Andean natives. People who die from CMS often suffer right heart failure and strokes. Although evidence is still controversial, a decreased ventilatory drive was proposed as a significant risk factor for CMS, with an important contribution from age.[26,27] Another potential contributing factor is cobalt, known to stimulate erythropoiesis, but its possible role requires further definition since data from similar groups of subjects from the same city are contradictory.[28,29] Heritability studies, preferential occurrence in certain ethnic groups and familial character have suggested a role for genetic factors in the pathogenesis of CMS,[1,3,9] although monogenic Mendelian segregation within families is not evident. We, therefore, tried to establish the existence of an association between candidate genes selected based on known-oxygen sensing mechanisms, and the excessive red cell and hemoglobin production in response to chronic hypoxia. CMS was considered an almost exclusively male condition since pre-menopausal women are thought to be protected from CMS by female hormones that increase alveolar ventilation and the hypoxic ventilatory response.[30] Furthermore, the high prevalence of iron deficiency resulting from the blood and iron loss of menstruation, pregnancies and lactation hinders erythropoiesis, protecting against the development of severe polycythemia. Thus, women develop CMS abruptly after menopause[31] whereas men can develop CMS in early adulthood as well as at a later age. To minimize variability and risk of misclassification, only male subjects were enrolled in the current study. Our data shows that CMS subjects are significantly older and have a higher weight for height (p=0.0014 and p=0.017, respectively) than do individuals without CMS. Age has been reported to be a risk factor for the development of CMS[6,26,32] and our data confirm those results. Elevated weight was observed in polycythemic subpopulations in a hematologic survey in La Paz, Bolivia (3700 m)[33] and in Cerro de Pasco,[6] and our data also confirm those results. The cause or effect of weight in CMS and how it is associated with severe polycythemia is not known. Possibly, extreme weight affects breathing mechanisms, limiting respiration, and leading to lower blood oxygen saturation which activates erythropoiesis.[33] CMS subjects also had significantly lower SaO_2 levels. It was recently found that Andean natives, with or without severe polycythemia, had a significantly lower mean SaO_2 during the night, showing values below 80% mainly in CMS subjects.[34] An SaO_2 of 80% has previously been identified as a threshold for the stimulation of erythropoiesis,[35] but there is no known link between these changes in nocturnal SaO_2 and severe polycythemia. No association was found between hemoglobin values and the genotypes for any studied marker, except for marker *D14S1049* that showed a p value of 0.05. The *D14S1049* marker is closely linked to *PHD3*, which is strikingly sensitive to hypoxia, and thus provides a suggestion that this gene could influence the altered erythropoietic response to chronic hypoxia. However, after the inclusion of alternative co-variates in the analysis and after adjusting for multiple comparisons, no p values could be considered statistically significant. Additional analysis performed after excluding rare genotypes also showed no significant association. The high heterozygosity of most of the markers increased the number of comparisons for evaluating the dataset, and therefore decreased the threshold for significance because of multiple testing, possibly masking real associations. The divergent frequencies observed highlight the need for studies on larger population samples.

Microsatellites are primarily in non-coding regions and their polymorphisms rarely have functional consequences; however they are used as markers to identify particular genomic regions that possibly contribute to a disease state. Using this approach we did not detect any difference in the distribution of alleles nor did we find evidence of a major monogenetic contribution of

the loci tested on the degree of polycythemia. Even though multi-allelic microsatellite analyses are more informative due to high heterozygosity and enable the use of fewer markers, more common genetic variations, such as single nucleotide polymorphisms (SNP), could be alternatively used. Individual SNP are less informative than microsatellites, but they are more abundant, stable, and can be used for performing haplotype frequency estimations over several SNP from a locus. A *post hoc* power analysis indicated that the presence of rare alleles led to a fall in power for most of the markers. The non-significant *p* values we obtained could reflect the fact that there is no genetic association but also that the study did not reach sufficient power to assess a possible influence of a locus on polycythemia. In conclusion, our data provide no evidence supporting an association between the individual candidate genes tested and severe polycythemia in the Andean high-altitude sample population; however they do not exclude the possibility that variations in the studied genes contribute to the risk to polycythemia and CMS. More studies in larger population samples or with other kinds of polymorphisms are needed to determine the role of the candidate genes in the risk of developing CMS.

OM contributed to the design of the study, collection of samples and data, was responsible for the laboratory data, analysis and interpretation of data and for drafting the manuscript. JTP contributed to the design of the study, collection of samples and revised the article critically. FLV contributed to the collection of data and samples, provided supervision in the field work and revised the article critically. AH contributed to the collection of samples and revised the article critically. DWS contributed to the design of the study, collection, analysis and interpretation of data, provided supervision of the study and revised the article critically. All authors approved the final version of the manuscript. The authors declare that they have no potential conflicts of interest.

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