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## Search for genetic determinants of individual variability of the erythropoietin response to high altitude

Katerina Jedlickova,<sup>a</sup> David W. Stockton,<sup>a</sup> Hua Chen,<sup>a</sup> James Stray-Gundersen,<sup>b</sup>  
Sarah Witkowski,<sup>c</sup> Ge Ri-Li,<sup>c</sup> Jaroslav Jelinek,<sup>a</sup>  
Benjamin D. Levine,<sup>c,\*</sup><sup>1</sup> and Josef T. Prchal<sup>a,1</sup>

<sup>a</sup> Baylor College of Medicine, Texas Medical Center, Houston, TX, USA

<sup>b</sup> Norwegian Institute for Sport and Physical Education, Oslo, Norway

<sup>c</sup> Institute for Exercise and Environmental Medicine, Presbyterian Hospital of Dallas and University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75231, USA

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### Abstract

There is marked variability in the erythropoietin (Epo) and erythrocytic response to extreme high altitude among mountain dwellers, as well as to hypoxic training among athletes, at least in part because of the variation in the erythropoietic response to hypoxia. We hypothesized that this may be genetically determined. Forty-eight athletes were exposed to 24 h of simulated altitude to 2800 m in a hypobaric chamber. Serum Epo concentrations were determined at baseline and after 24 h. The Epo responses ranged from –41 to 433% of baseline values after 24 h at simulated altitude. The association of the Epo response to hypoxia with the *EPO* gene and eight genes involved in Epo regulation utilizing 16 polymorphic dinucleotide repeats was examined. Initial analysis showed a possible association between the *EPO* gene (marker D7S477) and the increase of the Epo level ( $P = 0.018$ ). We then tested the possibility that sequence abnormalities in the 3' and 5' hypoxia response elements (3' *HRE*) and (5' *HRE*) of the *EPO* gene could explain the differences in Epo response. We found a 3434 C → T polymorphism in the 3' *HRE* sequence. However, this polymorphism showed no correlation with the differences in Epo levels. Further, when we analyzed two additional markers flanking the *EPO* gene by less than 0.3 cM, we found no association of the allelic variants at these loci with the Epo hypoxic response. In conclusion, we could find not convincing association between markers tightly linked to *EPO* or eight genes involved in Epo regulation and Epo differential responses to hypoxia.

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### Introduction

Acclimatization to moderately high altitudes, when combined with low-altitude training (so called “living high–training low”), improves sea-level performance in endurance-trained athletes, in part because of the erythropoietic effect of altitude exposure [1–4]. However there is substantial individual variability in the response to all forms of

altitude training [2,3]. A large component of this individual variability appears to be related to variability in the peak and rate of decay of the increase in erythropoietin (Epo) in response to altitude exposure [4]. Animal models involving different strains of Sprague–Dawley rats that have either a normal or an exaggerated erythropoietic response to hypoxia have suggested that such differences are transcriptionally regulated through physiological factors regulating oxygen availability in the kidney (i.e., hypoxic ventilatory response, pulmonary diffusing capacity, p50 of oxyhemoglobin, renal oxygen delivery, and renal oxygen consumption) [5]. However, for humans, even the same degree of renal tissue hypoxia may induce substantial differences in Epo production in response to high altitudes [6]. We hy-

\* Corresponding author. Institute for Exercise and Environmental Medicine, Presbyterian Hospital of Dallas, 7232 Greenville Ave., Suite 435, Dallas, TX 75231 Fax: +1-214-345-4618.

E-mail address: [benjaminlevine@texashealth.org](mailto:benjaminlevine@texashealth.org) (B.D. Levine).

<sup>1</sup> Both authors should be considered senior authors.

pothesized that this individual variability is a function of genetic differences in hypoxia-sensing and hypoxia-responsive pathways.

The oxygen-sensing pathway regulates a number of genes that determine the response to hypoxia. It includes genes that control erythropoiesis, angiogenesis, energy metabolism, carcinogenesis, and apoptosis. A decrease in oxygen concentration in the kidneys is the main regulator of Epo production [7–9]. The response to hypoxia is mediated by a conserved hypoxia-sensing regulatory mechanism [7,8]. Central to this pathway is the hypoxia-inducible transcription factor 1 (HIF-1). The heterodimer HIF-1 recognizes pentanucleotide (5'-RCGTG-3') HIF-responsive elements (HREs) within the promoters of hypoxia-responsive target genes, including *EPO* [10,11]. The 3'HRE of *EPO* has been well characterized, but only recently the identical regulatory sequence flanking the 5' end of the gene was identified [12]. HIF-1 is a heterodimer composed of two subunits: the hypoxia-regulated HIF-1 $\alpha$  subunit and the oxygen-insensitive HIF-1 $\beta$  subunit. Under hypoxic conditions, degradation of the HIF-1 $\alpha$  subunit is blocked, allowing HIF-1 $\alpha$  to dimerize with HIF-1 $\beta$  [7]. Under normal oxygen concentration the HIF-1 $\alpha$  subunit is rapidly degraded. The degradation of HIF-1 $\alpha$  by ubiquitination is triggered by hydroxylation of conserved proline residues in the so-called oxygen degradation domain (ODD) [13,15]. The tumor suppressor von Hippel–Lindau protein (pVHL), a component of an ubiquitin E3 ligase complex, tags the hydroxylated HIF-1 $\alpha$  subunit for degradation mediated in the proteasome following its ubiquitination [14–16]. This critical regulatory event is carried out by a family of iron (II)-dependent prolyl hydroxylase enzymes that use O<sub>2</sub> as a substrate to catalyze hydroxylation. Oxygen appears to be the rate-limiting step for prolyl hydroxylase activity [17,18]. The activation of the serine-threonine kinase (AKT) leads to HIF-1 $\alpha$  stabilization, whereas the tumor suppressor gene *PTEN* attenuates hypoxia mediated HIF-1 $\alpha$  stabilization by regulation of AKT activity. During tumor expansion the loss of *PTEN* causes deregulation of AKT activity and alters the expression of HIF-1 [19,20].

The interaction of Epo with its receptor (EpoR) leads to homodimerization and activation of a signal transduction pathway. This results in (a) stimulation of the mitotic activity, (b) inhibition of apoptosis, and (c) differentiation of specific erythroid progenitor cells, leading to the production of more erythrocytes [21,22].

Aerobic organisms generate reactive oxygen species (ROS) during respiration as a product of specific oxidases. Recent studies implicate the redox state as a determinant of many cellular decisions. Renal NADPH oxidase (*RENOX*) is highly expressed at the site of Epo production in the renal cortex as shown by in situ RNA hybridization [23,24].

There is a substantial individual variability in the Epo response to hypoxia, making the ultimate outcome of altitude training uncertain. To determine whether there are genetic mechanisms that cause this variability, we studied a

group of competitive runners at simulated high altitude. We hypothesized that the association of microsatellite markers tightly linked to the functionally important genes in hypoxia sensing, described above, as follows: *EPO*, *HIF-1A*, *EPOR*, *VHL*, prolyl hydroxylase genes (*PHD1*, *PHD2*, and *PHD3*), *RENOX*, and *PTEN*, with an Epo hypoxic response phenotype would allow us identify the gene (or genes) responsible for individual variability of the Epo response to hypoxia.

## Material and methods

### Subjects

Forty-eight young, healthy subjects (32 men and 16 women, 21  $\pm$  2.5 years of age) volunteered to take part in the study. All subjects received written and verbal explanations of the experiment before giving consent. The Institutional Review Boards of the University of Texas Southwestern Medical School and Presbyterian Hospital of Dallas approved the study.

### Epo concentration

Epo concentration was measured at sea level (before decompression) and after 6 and 24 h at simulated altitude. For logistical reasons, in half of the subjects, Epo was measured in plasma by radioimmunoassay (Ramco, Houston, TX), and in the other half, it was determined in serum with an enzyme-linked immunosorbent assay kit (Human Epo Quantikine IVD, R&D Systems) [4,25]. The plasma and serum Epo values were tightly correlated with a regression coefficient of 0.96, a slope of 1.0, and an intercept of 2.7. To minimize any influence of this difference between the two methods for calculating percentage changes in Epo, all serum values were adjusted by this offset (2.7) to make them equivalent to the plasma measurements.

### Polymorphic markers

Polymerase chain reaction (PCR) was used to amplify 16 polymorphic markers located in close proximity to 9 different genes. All the markers were dinucleotide (CA)<sub>n</sub> and (GA)<sub>n</sub> repeats. All reactions were carried out within a volume of 20  $\mu$ l in the presence of 0.1 mM dNTPs, 2.0 mM MgCl<sub>2</sub>, and 0.3 units of *Taq* DNA polymerase (Qiagen). For each of the polymorphic repeats, 100 ng DNA, obtained from peripheral blood, was amplified using 0.3  $\mu$ mol/liter of forward primer and 0.3  $\mu$ mol/liter of reverse primer (Table 1). Denaturation 15 min at 95°C, amplification for 14 cycles of 20 s at 94°C, 1 min at 63°C (–0.5°C per cycle), 90 s at 72°C; and 20 cycles of 1 min at 56°C, 90 s at 72°C. All forward primers were labeled with HEX, FAM, or TET fluorescent dyes (Integrated DNA Technologies).

A mixture containing 1  $\mu$ l formamide, 0.5  $\mu$ l loading buffer (50 mg/ml Blue Dextran, 25 mM EDTA), 0.5  $\mu$ l

Table 1  
The sequences of the primers for 16 dinucleotide polymorphic markers

Gene	Marker	Primer sequence
<i>PHD2</i>	D1S2805	<i>D1S2805F</i> :5'-GCACCAACCCTCATGCTATT-3' <i>D1S2805R</i> :5'-ATTACAAAGAGGCGCAGGAA-3'
	D1S251	<i>D1S251F</i> :5'-GTAATCCTGTGAGCCAATTC-3' <i>D1S251R</i> :5'-CACAAGGAGATAAGCCAAAAG-3'
<i>VHL</i>	D3S3691	<i>D3S3691F</i> :5'-TTCTGGCACTGTAGACT-3' <i>D3S3691R</i> :5'-ACATTGTGTTGAGGATAGTAGGG-3'
	D3S1597	<i>D3S1597F</i> :5'-AGTACAAATACACACAAATGTCTC-3' <i>D3S1597R</i> :5'-GCAAATCGTTCATTGCT-3'
	D3S3611	<i>D3S3611F</i> :5'-GCTACCTCTGCTGAGCAT-3' <i>D3S3611R</i> :5'-TAGCAAGACTGTTGGGG-3'
	D7S2480	<i>D7S2480F</i> :5'-TTGACTGTGGTAGTTGG-3' <i>D7S2480R</i> :5'-GTTACCTCATAGGAAAATCTTG-3'
<i>EPO</i>	D7S477	<i>D7S477-F1</i> :5'-TTGCACCACTGTCTCCAGTC-3' <i>D7S477R1</i> :5'-TTGGGTATCCCCTGTTCCAC-3' <i>D7S477-F2</i> :5'-AGTCTGTGTGACAGTGAGA-3' <i>D7S477R2</i> :5'-CTTCTCTTCTGCCAATTCAG-3'
	D7S2498	<i>D7S2498-F</i> :5'-TTTAAGGTAGCACCCCTCTCC-3' <i>D7S2498-R</i> :5'-ACTACTTAGTTTAGCCTTCAGAACTCG-3'
	D7S662	<i>D7S662-F</i> :5'-GTTGACAGACAAGCACAGAC-3' <i>D7S662-R</i> :5'-AGCTGTTTCCCATTTC-3'
	D10S1765	<i>D10S1765F</i> :5'-ACACTTACATAGTGCTTTCTGCG-3' <i>D10S1765R</i> :5'-GTTTCTTCAGCCTCCCAAAGTTGC-3'
	D11S931	<i>D11S931F</i> :5'-GATTGCTTGAGCCAG-3' <i>D11S931R</i> :5'-GTTTCTTGAGAAAATAGTATGTGTTGCC-3'
	D14S70	<i>D14S70F</i> :5'-ATCAATTTGCTAGTTTGGCA-3' <i>D14S70R</i> :5'-AGCTAATGACTTAGACACGTTGTAG-3'
<i>PTEN</i>	D14S1049	<i>D14S1049F</i> :5'-GGAAAACACTGGCACCTT-3' <i>D14S1049R</i> :5'-TTTGAGGAGCAGGCAAT-3'
	D14S997	<i>D14S997F</i> :5'-TGGTCTTGGCAACCCTATAAAAAATC-3' <i>D14S997R</i> :5'-CAAAGGAGCATGTTTCCATAGC-3'
<i>RENOX</i>	D19S223	<i>EPORF</i> :5'-GGTGACAGAGCAACACCCTG-3' <i>EPORR</i> :5'-GTTTCTTATCAGCATCTCTTCCAGCC-3'
<i>PHD3</i>	D19S223	<i>D19S223F</i> :5'-CAAAAATCGAGGTGCATAGAA-3' <i>D19S223R</i> :5'-ACCATGACTGGCTAATTGTG-3'

commercial size standard Genescan500 (Applied Biosystems), and 1.5  $\mu$ l diluted PCR product was prepared, incubated at 96°C for 4 min, and cooled on ice. One and a half microliters the mixture was loaded in a 4.5% denaturing polyacrylamide gel and electrophoresed for 3 h on an ABI PRISM 377 DNA analyzer. The GENESCAN computer program was utilized to analyze the raw data in order to identify and to determine the size of each DNA fragment on the gel. The GENOTYPER software was used for analysis of the experimental fragments at each locus for every sample (Applied Biosystems).

If the allele to genotype relationship was found not to be in Hardy–Weinberg equilibrium for a subset of the markers, these were reassayed from independent aliquots of the DNA to verify the original results. If genotyping errors were not detected to correct the deviation from Hardy–Weinberg equilibrium, new pairs of PCR primers were designed that did not overlap the original pairs to minimize the possibility of nonamplification because of PCR primer site polymorphisms.

#### *Evaluation of the 5' hypoxia response element and evaluation of the 3' hypoxia response element of EPO gene*

The upstream hypoxia-inducible enhancer, the 368-nt fragment flanking the 5' of the *EPO* gene, and the 256-nt *EPO* gene 3' flanking sequence element were amplified by PCR. Reactions were carried out in a volume of 20  $\mu$ l in the presence of 0.1 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, and 0.3 units of *Taq* DNA polymerase (Invitrogen Corp.). One hundred nanograms of template DNA were amplified using 0.5  $\mu$ mol/liter of forward primer and 0.5  $\mu$ mol/liter of reverse primer (Table 2). Amplification was done by denaturation for 15 min at 95°C and amplification for 35 cycles of 40 s at 95°C, 40 s at 55°C, and 40 s at 72°C. The sequences of PCR products were determined by labeling with multiple fluorescent dyes using the BigDye Primer Kit (Applied Biosystems). A mixture containing 4  $\mu$ l formamide, 1  $\mu$ l loading buffer (50 mg/ml Blue Dextran, 25 mM EDTA), and 1.5  $\mu$ l diluted PCR product was prepared and incubated

Table 2  
The sequences of the primers for hypoxia response elements of the *EPO* gene

Region	Primer sequence
5'HRE of <i>EPO</i>	<i>EPO</i> -L:5'-AGTAGATGCAGGAGCCTGGT-3' <i>EPO</i> -R:5'-TATCAGGCCACATCCTTCC-3'
3'HRE of <i>EPO</i>	<i>DEPO2</i> -F:5'-CCACCAATATGACTCTTGGCTT-3' <i>DEPO2</i> -R:5'-GTGAGGCCTTGAATGGAGAC-3'
C3434T polymorphism of 3'HRE of <i>EPO</i>	<i>EPOF</i> :5'-AGCAGTGCAGCAGGTCCA-3' <i>EPOR</i> :5'-GGCTGTTGAGACAGCACGTA-3' <i>6FAM</i> :5'-ACCCCTCgTTCC-3' <i>VIC</i> :5'-ACCCCTCaTTCC-3'

at 96°C for 5 min and cooled on ice; 1.5 µl of this mixture was loaded in a 4.5% denaturing polyacrylamide gel and electrophoresed for 4 h on the ABIPRISM 377 DNA Sequencer. The sequence was analyzed using Sequencing Analysis version 3.3 (Applied Biosystems).

#### Evaluation of the 3434 C → T polymorphism in the 3' HRE of *EPO* gene

The 3434 C → T single nucleotide polymorphism was detected by multicolor allelic discrimination assay performed on ABI Prism 7000 Sequence Detection System (Applied Biosystems). The forward and reverse primers (Table 2) were selected to amplify a DNA fragment from the 3' HRE of *EPO* gene overlapping the polymorphic site. The probes specific for the polymorphic site were labeled with FAM (detecting the 3434C allele) and VIC (detecting the 3434T allele) fluorescent dyes. The optimization of the primer/probe concentrations gave the following assay conditions: 20–100 ng DNA, Universal Master Mix, 0.1 µmol/liter of each probe, and 0.9 µmol/liter of forward and reverse primers. PCR amplification was achieved under the following conditions: 10 min at 95°C and then 40 cycles of 92°C for 15 s and 60°C for 60 s. The ABI PRISM 7000 Sequence Detection software was used for allele discrimination based on the fluorescence reading after the completion of PCR.

#### Statistical analysis

The studied subjects were divided into three groups according to their Epo response to hypoxia at simulated altitude of 2800 m after 24 h. The cutoff points were determined from natural breaks in the distribution of Epo response. The subjects with the highest increase of Epo were designated as “high responders” ( $n = 8$ , 17%), the group with the lowest increase of Epo were labeled as “low responders” ( $n = 11$ , 23%), and the third group as “intermediate responders” ( $n = 29$ , 60%).

The allele and genotype frequencies, the observed marker heterozygosity and polymorphism information con-

tent (PIC) were calculated for each locus. Additionally, we tested the deviation from Hardy–Weinberg equilibrium using chi-square testing, the exact methods for loci with four or less alleles and Markov chain methods to generate an unbiased estimate for loci with a larger number of alleles using the GENEPOP computer program (version 3.3) [26]. Deviations from Hardy–Weinberg equilibrium were further evaluated by additional genotyping with redesigned primers and using the *U* test (tests for heterozygote excess and deficiency) as implemented in the GENEPOP computer program [26].

To test for association between the erythropoietic response to hypoxia and the polymorphic loci, distributions of allele frequencies were compared between high and low responders using chi-square and Fisher's exact tests. The studied subjects were sorted by the presence or the absence of the “putative allele” and the unpaired *t* test done on the change in Epo levels between groups.

## Results

### Epo levels

The physiological study and subject characteristics were previously published [6]. The hypoxic Epo responses are shown below. Serum Epo was  $14.3 \pm 0.8$  mU/ml at sea level; it increased significantly to  $20.9 \pm 12.6$  mU/ml after 6 h at simulated altitude of 2800 m. Mean Epo concentration continued to increase significantly after 24 h at 2800 m to  $27.2 \pm 5.3$  mU/ml. There was a marked individual variability in Epo release, but responses were generally consistent among individuals. The hypoxic Epo responses ranged from –41 to 433% of baseline values after 24 h at simulated altitude of 2800 m. The coefficient of variation from baseline after 24 h at 2800 m was 0.83. The subjects were designated as high responders (17%) (the Epo response ranged from 158 to 433%), low responders (23%) (the Epo response ranged from –41 to 35%), and intermediate responders (60%) (the Epo response ranged from 40 to 145%).

### Allele frequencies, heterozygosity, PIC index, and Hardy–Weinberg equilibrium

The allele frequencies of the 16 examined microsatellite loci, the repeat lengths, observed heterozygosity, and PIC index values are shown in Table 3. The number of alleles observed per locus ranged from 5 (*DI4S70*) to 16 (*EPOR* marker). The overall mean heterozygosity of all markers was 0.772 and the overall mean PIC index was 0.741.

Departures from Hardy–Weinberg equilibrium were not statistically significant, with only one exception at *D7S477* ( $P = 0.0$ ). After these data were verified by repeating all typing on independent aliquots of the samples, a new pair of PCR primers for *D7S477* marker was designed and the

Table 3  
Allele frequencies at 16 polymorphic markers

Allele <sup>a</sup>	D1S2805	D1S251	D3S3691	D3S1597	D3S3611	D7S2480	D7S477	D7S2498	D7S662	D10S1765	D11S931	D14S70	D14S1049	D14S997	EPOR	D19S223
5																
6																
7																
8				0.074									0.114			
9			0.01										0.083		0.011	
10				0.032									0.073			
11				0.032									0.146			
12				0.265									0.01			
13	0.01			0.021									0.375			
14	0.021												0.146			
15	0.074	0.083		0.309				0.01	0.011			0.302	0.146			
16	0.128		0.01	0.148		0.15		0.228				0.188	0.042	0.33		0.011
17	0.521	0.219	0.174	0.117		0.0125	0.011					0.25	0.01	0.042		0.149
18	0.021	0.094	0.054	0.021	0.344		0.271	0.01	0.467			0.229		0.021	0.021	0.223
19		0.146	0.14		0.011		0.177		0.01			0.031		0.031	0.032	0.181
20		0.042	0.56		0.056		0.26		0.022	0.106				0.219	0.032	0.064
21	0.032	0.052	0.01		0.089		0.031		0.087*	0.043				0.229	0.14	0.032
22	0.074	0.021	0.022		0.244	0.125	0.198		0.01	0.085				0.01	0.074	0.096
23	0.021	0.27	0.01		0.078	0.138	0.052	0.01		0.117				0.021	0.202	0.149
24	0.032			0.1	0.288			0.01	0.033	0.128					0.095	0.043
25	0.021	0.021		0.033	0.175			0.01	0.01	0.043					0.138	
26	0.021	0.063		0.011	0.063			0.43	0.033	0.23					0.011	
27	0.032			0.05			0.022		0.17					0.095		
28	0.01			0.011				0.067	0.043	0.053					0.011	
29								0.033	0.022	0.011					0.032	
30				0.022				0.39	0.01						0.011	
31								0.022							0.011	
32											0.198					
33																
34																
35										0.01						
36										0.094						
37																
38										0.24						
39										0.438						
40										0.01						
41										0.01						
Heterozygosity	0.696	0.833	0.608	0.79	0.819	0.823	0.785	0.656	0.717	0.863	0.702	0.758	0.789	0.778	0.887	0.854
PIC	0.677	0.817	0.59	0.764	0.775	0.79	0.735	0.593	0.686	0.846	0.656	0.715	0.766	0.743	0.876	0.837
HWE	$P = 0.999$	$P = 0.169$	$P = 0.916$	$P = 0.925$	$P = 0.993$	$P = 0.959$	$P = 0.0002$	$P = 0.99$	$P = 0.14$	$P = 0.209$	$P = 0.985$	$P = 0.931$	$P = 0.534$	$P = 0.138$	$P = 0.325$	$P = 0.709$

<sup>a</sup> Allelic nomenclature is based on the number of repeat units.

samples were reanalyzed with these primers. The results of the analysis were still in disagreement with the Hardy–Weinberg equilibrium. Using the *U* test for heterozygote excess [26] found no deviation from the Hardy–Weinberg equilibrium ( $P = 0.999$ ) but the *U* test for heterozygote deficiency was not in agreement with the Hardy–Weinberg equilibrium. ( $P = 0.001$ ). Analysis of the data, split into high responder, intermediate responder, and low responder groups, showed departure for the Hardy–Weinberg equilibrium in high and low responder groups ( $P = 0.021$  and  $P = 0.001$ ).

#### Association analysis of *PHD1*, *PHD2*, *PHD3*, *EPOR*, *VHL*, *HIF1-A*, *RENOX*, and *PTEN* genes

A total of 12 dinucleotide markers located in close proximity (0.4 to 0.9 cM) to eight genes involved in the regulation of Epo were analyzed for any evidence of association between the polymorphic markers and the Epo hypoxic response. No significant evidence of association has been found in any of these dinucleotide repeats in the group of 48 studied subjects.

#### Association analysis of the *EPO* gene

Two dinucleotide markers tightly linked to *EPO* were analyzed (the distance between these two markers is 0.53 cM and the *EPO* is between them). One of the markers, *D7S477* (0.70 Mbp 3' from the *EPO* gene), showed significant nonrandom allelic association with the Epo hypoxic response phenotype (Tables 4, 5, and 6). However, marker *D7S2480* (0.26 Mbp 5' from the *EPO* gene) showed no allelic association with the Epo hypoxic response phenotype. Significant differences were found in the frequency of the alleles for the marker *D7S477* between high responders and low responders using chi-square test ( $\chi^2 = 11.94$ ;  $df = 5$ ,  $P = 0.036$ ) (Table 4). The 185-bp allele was the most

Table 4  
The association analysis of *D7S477* marker<sup>a</sup>

Alleles (bp)	181	183	185	187	189	191	Total
High responders	3	0	9	0	2	2	16
Low responders	8	7	4	1	1	1	22

<sup>a</sup> Chi-square test,  $\chi^2 = 11.94$ ,  $df = 5$ ,  $P = 0.036$ .

Table 5  
The frequency of the 185-bp allele in high responders and low responders<sup>a</sup>

185-bp allele	Present	Absent	Total
High responders	9	7	16
Low responders	4	18	22

<sup>a</sup> Fisher exact test,  $P = 0.015$ .

common allele in the group of high responders. The difference in alleles between these two groups was more pronounced after inspection for “standout” polymorphism of the 185-bp allele of the *D7S477* marker when tested with the Fisher exact test ( $P = 0.015$ ) (Table 5). The 185-bp allele showed a significant correlation to the Epo hypoxic response. When all subjects were considered, if the 185-bp allele was present the increase of Epo level was  $134.78 \pm 17.86\%$  versus  $78.02 \pm 13.61\%$  when the 185-bp allele was absent ( $P = 0.018$ ) (Table 6).

These data were followed by a search for a functional basis for this association. Direct sequencing of two regions of the *EPO* gene was performed in the eight high responders, the 11 low responders, the 29 intermediate responders, and five controls. Sequencing of the 3' *HRE* of *EPO* gene revealed a polymorphic site C3434T (Gene Bank M11319). The sequence between nucleotide 3333 and nucleotide 3599 was amplified and sequenced in the forward and reverse directions. The 3434 C → T polymorphism was 19 nt upstream of the HIF-1 binding site of the 3' *HRE* element. This single nucleotide polymorphism was not described in the NCBI SNP database. Based on the fact that the allelic discrimination analysis (SNPs analysis) is more accurate and efficient method for mutation detection, we performed it on the samples from the 48 athletes and 244 controls (Table 7). The aim of SNPs analysis was to distinguish homozygotes C/C, homozygotes T/T, and heterozygotes C/T. Only two of the athletes (4%) were heterozygotes C/T; both were Caucasians. One of them belonged to the high responders and the other to intermediate responders. The rest of the athletes (96%) were homozygotes C/C. The group of studied athletes were Hispanic (10%), African-American (4%), and Caucasian (86%). The controls were separated according to ethnicity (Hispanics, 54; African-Americans, 60; Asians, 55; and Caucasians, 75). In the groups of African-Americans and Asians only homozygotes C/C (100%) were detected. Hispanics showed homozygotes C/C (98%) and heterozygotes C/T (2%). The Caucasians revealed homozygotes C/C (89%), heterozygotes C/T (8%), and homozygotes T/T (3%). In the second sequenced region, 5' *HRE* of *EPO* gene, no sequence abnormalities were found in the athletes or controls.

We then identified and evaluated other *EPO*-linked polymorphic markers. We analyzed 5' *EPO*-linked *D7S2498* and 3' *EPO*-linked *D7S662* polymorphic repeats. No allelic association was detected between these markers and the Epo hypoxic response.

## Discussion

This study represents the first systematic search for genetic determinants of the erythropoietic response to high altitude, shown in previous studies to be one of the key features responsible for the marked individual variability in the response to altitude training [2,3]. The search for specific candidate genes was comprehensive and based on known oxygen-sensing mechanisms. We examined a robust, and well-characterized phenotype that exhibited marked individual variability, namely the increase in Epo concentrations after 24 h of exposure to the 2800-m simulated high altitude.

Epo production is regulated by hypoxia. HIF-1 is a transcriptional factor that plays a central role in the regulation of gene expression by oxygen. In oxygenated and iron repleted cells, the HIF-1 $\alpha$  subunit is rapidly destroyed by a mechanism that involves ubiquitination of the protein followed by the degradation in the proteasome. The interaction between human pVHL and a specific oxygen degradation domain (ODD) of the HIF-1 $\alpha$  subunit is regulated through hydroxylation of a proline residue (HIF-1 $\alpha$  Pro<sup>564</sup>) by unique prolyl hydroxylases (*PHD1*, *PHD2*, and *PHD3*). The absolute requirement of oxygen as a cosubstrate and iron as cofactor suggests that HIF-PHD functions directly as a cellular oxygen sensor [16–18,27]. The *PTEN* gene attenuates hypoxia-mediated HIF-1 $\alpha$  stabilization. Cells lacking functional *PTEN* ablate hypoxia induction of HIF-1-regulated genes [19,20]. *RENOX* is highly expressed at the site of Epo production in the renal cortex as revealed *in situ* RNA hybridization [24]. In previous studies *RENOX*, as a renal source of reactive oxygen species, was considered a likely candidate for the oxygen sensor function regulating oxygen-dependent gene expression; however, this has been questioned by recent studies [23,24].

The allele and genotype frequencies are expected to be in Hardy–Weinberg equilibrium in a randomly selected sample from an outbred population if there is no functional selection at the locus begin evaluated. Our data indicate substantial congruence with Hardy–Weinberg equilibrium with the exception of the *D7S477* marker ( $P = 0.0$ ). Our data provide indirect support for the claim that the deviation from Hardy–Weinberg equilibrium in *D7S477* is presumably caused by the selection for or against this locus. This could lead to an excess of homozygotes because homozygosity for a susceptibility of protective allele would have a greater effect than heterozygosity for that allele. Another

Table 6  
The level of Epo in athletes with and without 185bp allele<sup>a</sup>

185-bp allele	<i>N</i>	Mean of the level of Epo after 24 h in hypoxia (%)
Athletes with allele 185	15	$134.78 \pm 17.86$
Athletes without allele 185	33	$78.02 \pm 13.61$

<sup>a</sup> *t* test,  $P = 0.018$ .

Table 7  
The 3434 C → T polymorphism of the 3' HRE of the EPO gene

Alleles	Athletes		Controls				Total	(%)
		(%)	African-Americans	Asians	Hispanics	Caucasians		
C/C	46	96	60	55	53	67	235	96
C/T	2	4	0	0	1	6	7	3
T/T	0	0	0	0	0	2	2	1

possible explanation is that there is not true excess of homozygosity, but instead there are missing alleles (so called “null alleles”) [28] for the marker making heterozygotes look like homozygotes. This case may be the result of polymorphism within the binding site of one of the PCR primers, which prevents the amplification on some chromosomes or a small genomic deletion or rearrangement. The use of two different pairs of PCR primers for *D7S477* that lead us to identical results decreases the likelihood of the former.

We found significant evidence for association between the marker *D7S477*, flanking the 3' end of the *EPO* gene, and hypoxic Epo phenotype ( $P = 0.018$ ). This result was obtained from analysis of the entire group of 48 athletes. This finding led us to directly sequence the 5' HRE and 3' HRE sequences of *EPO* gene for the group of 48 athletes. We examined the possibility that sequence abnormalities in the 5' HRE or 3' HRE sequences of the *EPO* gene could explain the differences of Epo values in response to hypoxia. We found a novel single nucleotide polymorphism 19 nucleotides upstream of the HIF-1 binding site of the 3' HRE of the *EPO* gene. This 3434 C → T had not been previously reported. This suggested that this single nucleotide polymorphism could influence the binding of HIF-1 to the 3' HRE of the *EPO* gene. Two of the athletes were heterozygotes C/T (4%), both were Caucasians. One belonged to the high responders: He was heterozygous for the presence of the 185-bp allele of *D7S477* marker. The other was a member of the group of intermediate responders and the 185-bp allele was not present in the athlete's genotype. The remainder were homozygotes C/C (96%). In the SNP analysis we used 244 controls. In the African-Americans and the Asians only homozygotes C/C were detected; in the Hispanic group analysis revealed homozygotes C/C (98%) and heterozygotes C/T (2%). Caucasians were the only ethnic group that exhibited all three genotypes. Thus the 3434 C → T polymorphic site cannot explain the differential Epo responses of studied athletes. The possible functional impact of the 3434 C → T polymorphic site on Epo regulation was studied no further. Based on these findings we analyzed two additional polymorphic markers tightly linked to *EPO*. We found no association between these and the Epo hypoxic response, which is consistent with our initial data obtained in the analysis with the other *EPO* linked marker *D7S2480*.

There are several possible causes for the inability to

identify an association between the observed physiological variation and the genetic loci tested. A limited sample size was tested and if there is genetic locus heterogeneity responsible it may be missed because too small a subset of the individuals may be associated with each locus. If the adaptation to altitude is ancient, the relative instability of the microsatellite polymorphisms may have erased any evidence of linkage disequilibrium. Last, if the genetic variations occurred multiple times on different genetic backgrounds no common haplotype will be detectable even if a more stable polymorphism type is used such as an indel.

In conclusion, we could find no consistent allelic association among polymorphic markers in the vicinity of *EPO*, *PHD1*, *PHD2*, *PHD3*, *EPOR*, *VHL*, *HIF-1A*, *RENOX*, and *PTEN* genes and the Epo response to hypoxia, except one of five loci tested that were linked to *EPO*. It is possible, that our conclusions are limited by the number of studied subjects. We cannot rule out the possibility that a larger number of enrolled athletes would increase the power to detect association between a potential susceptibility locus and Epo phenotype. Although we did not detect the association of other *EPO* linked markers and Epo response to hypoxia, we cannot rule out the possibility that the association detected at the *D7S477* locus by our analysis was genuine and due to another gene linked to this locus.

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