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Detecting natural selection in high-altitude human populations

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Abstract

High-altitude natives have distinctive biological characteristics that appear to offset the stress of hypoxia. Evolutionary theory reasons that they reflect genetic adaptations resulting from natural selection on traits with heritable variation. Furthermore, high-altitude natives of the Andean and Tibetan Plateaus differ from one another, perhaps resulting from different evolutionary histories. Three approaches have developed a case for the possibility of population genetic differences: comparing means of classical physiological traits measured in samples of natives and migrants between altitudes, estimating genetic variance using statistical genetics techniques, and comparing features of species with different evolutionary histories. Tibetans have an inferred autosomal dominant major gene for high oxygen saturation that is associated with higher offspring survival, a strong indicator of ongoing natural selection. New approaches use candidate gene and genomic analyses. Conclusive evidence about population genetic differences and associations with phenotypes remains to be discovered.

Keywords: High altitude; Human; Andes; Tibet; Natural selection

1. Introduction

During the past 100–200,000 years (Trinkaus, 2005) humans have expanded our niche to an enormous range of environments including extremes of heat, cold and ultraviolet radiation to which we have adapted by behavioral buffers and biological responses. High-altitude environments occupied in South America, Asia and Africa were uniquely stressful because traditional societies had no technology to create non-hypoxic microclimates and therefore relied solely on physiological responses. Theoretically, high-altitude environments provide informative natural experiments to test hypotheses about the evolution of adaptations because potentially confounding individual factors influencing exposure are absent. Everyone at a given altitude is exposed to the same, constant, ambient hypoxia regardless of individual characteristics, such as age or socioeconomic status. Two natural experiments have been evaluated—one on the Andean and one on the Tibetan Plateau (a third on the East African Plateau has not been thoroughly investigated). Research with the populations that have inhabited the Andean and Tibetan Plateaus for millennia has identified distinctive morphological and physiological characteristics thought to offset the stress of high-altitude hypoxia, such as the relative hypoventilation (Brutsaert et al., 2005) of Andean high-altitude natives or the elevated exhaled nitric oxide of Tibetans (Beall et al., 2001). Evolutionary theory reasons that characteristics distinguishing high-altitude natives from low-altitude natives or one high-altitude native group from another may be adaptations resulting from natural selection. Andean and Tibetan high-altitude natives differ from each other in the mean values of many characteristics related to oxygen delivery and offer evidence that evolutionary processes have resulted in different adaptive outcomes in these two independent natural experiments. Thorough tests of these hypotheses have been challenging because the genetic bases of the traits remain unknown. This paper briefly describes research that has contributed to the effort to detect natural selection in indigenous high-altitude human populations and then describes a small, scattered body of recent research incorporating developments in molecular biology and genomics that offers enormous new opportunities to detect natural selection.

2. Discussion

2.1. Evolution by natural selection at high altitude

The elements of the process of evolution by natural selection include an environment that stresses function or performance,

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genetic variation that enables organisms with some variants to have a better chance to contribute to the next generation with the result that over generations a higher proportion of individuals (perhaps all) have the adaptive trait and the population gene pool and biological characteristics have changed from the ancestral state. Natural selection may be ongoing at the time of the study or it may have occurred previously. Those two situations may produce different signatures of natural selection and complicate efforts at detection. A complete case for natural selection will include identification of the environmental stress, genetic variation causing biological variation in the population under study, performance variation among genotypes and their associated phenotypes, and the average phenotype.

The environmental challenge is clear at high-altitude: starting with a smaller supply of oxygen in every breath of air, an uninterrupted supply must be maintained at sites where oxygen-using processes occur, especially in the mitochondria for energy production. The element of the process of evolution by natural selection that has posed a particular problem for researchers has been identifying heritable adaptive variation and change from a non-adapted ancestral population. People have lived on the Andean Plateau for ∼11,000 years (∼550 generations of 20 years each) and on the Tibetan Plateau for ∼22,000 years (∼1100 generations) (Aldenderfer, 2003) and have been exposed to the opportunity for natural selection for traits to improve oxygen delivery or use.[1] Theoretically, natural selection has had plenty of time to increase the frequency of adaptive alleles or new mutations. Adaptations to other environmental features, such as falciparum malaria or a diet containing milk, have occurred in that length of time (Wiesenfeld, 1967; Tishkoff et al., 2007). However, oxygen delivery is conceptualized as an integrated process involving many physiological systems including the pulmonary, hematological, and cardiovascular. Thus, choosing the appropriate physiological trait or set of traits to investigate for natural selection is not straightforward. In addition, the classical physiological measures of oxygen-dependent characteristics are expressed quantitatively rather than categorically as in the case of hemoglobin or lactase gene variants. Few analytical tools are available to discover the genes for quantitative traits. As a result, studies designed to identify the *possibility* of genetic variation have dominated research. Determining whether the distinctive characteristics (phenotypes) of high-altitude natives are caused by distinctive genotypes has been the focus of considerable effort for more than 40 years. Most of the research has involved comparing population means of physiological traits. The present review provides illustrative examples. Comprehensive reviews have appeared periodically recently (Hochachka, 1998; Hochachka et al., 1998, 1999a,b; Moore et al., 1998, 2004; Beall, 2000a,b, 2001, 2003, 2006; Hochachka and Monge, 2000; Ward et al., 2000; Moore, 2001; Rupert and Hochachka, 2001; Marconi et al., 2006; Wu and Kayser, 2006).

2.2. Population comparisons using migration and admixture models

The most common strategy to identify the possibility of genetic adaptations begins by considering whether acclimatization or developmental adaptation accounts for the distinctive physiological characteristic (phenotype) of the high-altitude population. If those processes are ruled out, that is, if homeostatic mechanisms common to all people or achievable by any who grow up under the stress do not produce the high-altitude phenotype, then the inference is that the high-altitude gene pool has changed from the ancestral state and the feature has a genetic basis unique to the high-altitude population. This approach is based on a conceptual framework published in 1966. It compares two or more populations (indigenous to high and to low altitude) and two residential altitudes (high and low) (Harrison, 1966). Sometimes called the 'migration model', it compares samples of residents and migrants from one altitude to another. Resident–migrant similarities are interpreted as evidence against a unique high-altitude population genetic basis for the adaptive response and as evidence for the expression of the human homeostatic 'norm of reaction' (range of phenotypes expressed in a range of environments). The model cannot determine whether similar phenotypes in residents and migrants are caused by the same genotype. Resident–migrant differences in the mean value of some trait thought to offset high-altitude hypoxia or to reflect better function are interpreted as evidence of a genetic basis for the trait in the high-altitude population.

A response within the common human homeostatic capacity is the elevated hemoglobin concentration of Andean high-altitude natives and European residents at altitudes of about 1500 m and higher that is reversible upon return to sea level (Okin et al., 1966; Faura et al., 1969; Hochachka et al., 1996b; Ward et al., 2000; Beall, 2001). In contrast, a relatively low hemoglobin concentration of Tibetan and Ethiopian high-altitude natives as compared with Andean or European populations at the same high altitudes may reflect homeostatic regulation around a different mean. This implies higher survival and function at the 'new' mean.

Monge and colleagues used mathematical modeling to analyze the benefits of the elevated hemoglobin concentration of Andean highlanders and concluded that the optimal hemoglobin concentration at high-altitude is the normal sea-level range rather than the normal, elevated Andean high-altitude range (Villafuerte et al., 2004). This implies better function for those with relatively low hemoglobin concentrations at high altitude. Some studies reported that reducing hemoglobin concentration improves exercise capacity (Winslow et al., 1985; Villafuerte et al., 2004). However, another reported a comparison of samples of Andean men at 3700 m with normal and low (for Andean highlanders) hemoglobin concentrations revealing that the men with

[1] For this review, studies of Andean populations will include those reporting on the Quechua and Aymara populations and studies of Tibetans will include those reporting on Tibetans, Sherpas, Ladakhis and Bods, who are all members of the same ethnic group (Sherpas migrated from Tibet to Nepal about 500 years ago; Ladakhis, also known as Bods, were part of the Western Tibetan kingdom for centuries.). Reports that do not clearly identify the study population are not included.

low hemoglobin concentrations also had low exercise capacity (Tufts et al., 1985). More research relating hemoglobin concentration with function is required.

The population differences in mean hemoglobin concentration influence other traits, such as the arterial oxygen content (a function of hemoglobin concentration and oxygen saturation of hemoglobin). Andean, Tibetan, and Ethiopian high-altitude natives have, respectively, higher, lower, and the same average arterial oxygen content as sea-level natives at sea-level (Beall et al., 2002). Perhaps the trait subject to natural selection was hemoglobin concentration, perhaps oxygen saturation of hemoglobin, perhaps arterial oxygen content or some other unmeasured feature of oxygen transport that has not been considered. Analytical tools to answer such questions have not been available.

The recently introduced 'admixture model' elaborates on the migration model. It reasons that the population of the Andean region has both high and low-altitude ancestry (indigenous Andean and immigrant Spanish) and that an individual's ancestry is quantifiable as a proportion of Native American ancestry ranging from 0 to 100%. A panel of ancestry-informative genetic markers quantifies ancestry. It reasons that if a response to hypoxia has a genetic basis then individuals with a higher proportion of Native American ancestry will respond more adaptively to high altitude. For example, compared with lowlanders at high altitude, Andean highlanders have relatively low hypoxic ventilatory response (HVR, the increase in ventilation when the arterial partial pressure of oxygen or percent oxygen saturation of hemoglobin is lowered) and low ventilation during exercise. One study quantified the association of Native American ancestry with these traits by evaluating a sample of low-altitude Peruvian natives of mixed Native American and Spanish ancestry at sea level and after 10–12 h at 4338 m (Brutsaert et al., 2005). The average estimated "Native American Ancestry Proportion" (NAAP) was 85%. Higher NAAP correlated with lower HVR after ten minutes of experimental hypoxia at high altitude and with ventilation during exercise ($R^2 \sim 25\%$). These results were interpreted as "the first direct evidence that ventilatory traits, probably unique to Andeans, have a population genetic basis. Our quantification of ancestry as an independent variable has led us to infer both a genetic mechanism and an evolutionary origin for these traits" (Brutsaert et al., 2005, p. R232). That is, those results imply that natural selection produced a distinctive 'Andean' gene pool at unknown loci regulating these traits.

Population comparison studies, such as these have presented evidence for the possibility of unique genetic variation in high-altitude populations for some oxygen transport traits including oxygen saturation of hemoglobin, hemoglobin concentration, resting ventilation, hypoxic ventilatory response, exhaled nitric oxide concentration, and birthweight (refer to the reviews mentioned in the introduction), but cannot identify the genetic loci or alleles involved or give clues about their nature (for example, are they structural or regulatory, are they fixed or polymorphic in the population). Furthermore, these studies compared population means, while natural selection requires population genetic variation.

2.3. Quantitative genetics models

An approach to measuring genetic variation associated with phenotypes in high-altitude populations has been to quantify genetic variance with a measure called heritability. Collecting phenotypic data from large samples of biological relatives allows calculating heritability (h^2), the proportion of variance in a quantitative trait that is attributable to genetic relationships. Heritability can range from a theoretical low of 0 (no genetic variance and no opportunity for natural selection) to a high of 1 (all the variance is attributable to genetic factors and maximal opportunity for natural selection). Heritability estimates pertain to the population providing the data and cannot be generalized to others because the particular combination of genes and relevant environment is probably unique to each population. However, estimating h^2 of high-altitude populations enables comparison of the opportunities for natural selection. A non-significant h^2 could mean that the population never had any genetic variance, that natural selection has already acted on the population to bring a new allele to fixation and there is no longer any variance, or that environmental factors (including other genes) prevent the expression of variance.

Heritability varies among oxygen-delivery traits and between high-altitude populations, implying varying opportunity for ongoing natural selection. For example, Tibetan populations have significant h^2 for hemoglobin concentration, percent of oxygen saturation of hemoglobin, resting ventilation and hypoxic ventilatory response while Andean populations have significant h^2 in hemoglobin concentration and hypoxic ventilatory response but not resting ventilation or percent of oxygen saturation of hemoglobin. The h^2 are in the range of 0.20–0.40 and Tibetan values are higher than those of Andean highlanders, implying greater opportunity for natural selection (Beall et al., 1997a,b, 1999). The exception is hemoglobin concentration, for which both populations have very high h^2 and the Andean value of 0.89 is higher than the Tibetan value of 0.65 (Beall et al., 1998). Such studies can detect whether genetic variation in a particular physiological trait exists in a population and guide the choice of traits or populations for further study, but cannot identify the genes involved or give clues about function.

An analytic approach to identifying the type of genetic variation associated with an oxygen transport trait is segregation analysis to test the hypothesis that there is a major gene, an inferred allele with a large quantitative effect, rather than multiple genes with small quantitative effects. A major gene, with an autosomal dominant mode of inheritance associated with 6–10% higher oxygen saturation of hemoglobin, has been detected in three different areas of the Tibet Autonomous Region at altitudes of 3800–5450 m, with an allele frequency of 0.55–0.78 (Beall et al., 1994, 1997b, 2004). That is, despite uniform ambient hypoxic stress at any given high altitude, Tibetans vary widely in the level of physiological hypoxia and this variation is partly accounted for by the inferred major gene. Furthermore, it has important consequences with direct relevance to the hypothesis of natural selection. In one large sample of Tibetans, the estimated genotypic mean for the high oxygen saturation genotypes was 89.4% and for the low oxygen saturation genotypes was

79.5%. Tibetan women highly likely to have genotypes for high oxygen saturation (by statistical genetics estimation) had significantly lower infant mortality and more surviving offspring than women estimated to be homozygous for low oxygen saturation. Women with all three estimated genotypes reported an average of 4.6–4.9 pregnancies and 4.5–4.8 live births suggesting similar fecundity and ability to carry a fetus to term. However, women estimated to have the high oxygen saturation genotypes had an average of 3.6–3.8 living children as compared with just 1.6 for women with the low oxygen saturation genotype (Beall et al., 2004). The difference was due primarily to higher infant mortality of children born to the low oxygen saturation genotypes. The analysis of fertility used data provided by nearly 700 women and roughly 3000 people provided phenotypes for the quantitative genetics analysis. These results demonstrate that oxygen saturation genotypes have different capacities to contribute to future generations on the Tibetan Plateau, that is, that natural selection is ongoing.

A measure of the strength of natural selection is Darwinian fitness, "*w*", the relative reproductive success of genotypes. It can be calculated as a ratio of the number of living offspring of the least and most reproductively successful genotypes. In this case, the ratio of the number of living children among women estimated to have the lower saturation genotype as compared with those estimated to have a high saturation genotype, yielded a "*w*" value of only 0.44. For comparison, in the case of the classic example of natural selection in human populations, the balanced polymorphism for hemoglobins S and A in populations exposed to falciparum malaria, "*w*" can be calculated as the number of living children of hemoglobin AA homozygotes (who are more likely to have severe cases of falciparum malaria) to AS heterozygotes. The "*w*" value was 0.66 (Firschein, 1961). The inference is that high-altitude hypoxia exerts a stronger force of natural selection on the Tibetan population than the most thoroughly understood existing example of natural selection in humans. If the current fitness level reflects past levels, then the mutation for high oxygen saturation occurred recently – within the past 1000 years– and has been rapidly increasing in frequency due to strong natural selection. This is strong evidence for natural selection on one trait in one population, however the analysis cannot identify the genes involved. Nor can it determine whether a similar process occurred in the past in the Andean population that presently does not have genetic variance in oxygen saturation.

Taken together, research using these approaches has established a good case for the presence of genetic variation in oxygen transport traits of high-altitude populations and the likelihood of population differences in genetic variation and of the opportunity for natural selection. It has also been informative about the range and nature of variation in oxygen-dependent traits within and among high-altitude populations and provided new knowledge about human physiology. However, a major limitation to detecting natural selection has been the inability to identify the genetic locus or loci involved and to determine the biological pathway from the gene to the whole organism and how one DNA sequence causes an organism with one variant to be more fit in the Darwinian sense (better function or performance that enables higher survival or reproduction rate) than another at high altitude. That limitation is surmountable, in principle, by applying new concepts, methods and knowledge of molecular biology and genomics to address questions about the evolution of physiological adaptation to high-altitude hypoxia. These have revealed new details of physiology and new traits, such as nitric oxide metabolism and the hypoxia inducible factor 1 transcription factor, and provided new tools for discovery of the genetic bases of the quantitative traits associated with oxygen delivery and adaptation to high-altitude hypoxia. A small body of work using these approaches illustrates some of the challenges and opportunities for future studies of natural selection and evolution in high-altitude populations.

2.4. Molecular approaches

The new approaches turn attention to the fundamentals of oxygen homeostasis. Oxygen is consumed by metabolism in the mitochondria, the final destination for oxygen delivery, and by more than 1000 oxygen-dependent enzymatic reactions (Falkowski, 2006; Raymond and Segre, 2006). However, "O_2 concentration ranges found *in vivo* are typically low and vary from near zero to ~10 μMol. . ." (Hochachka and Rupert, 2003, p. 518). That is, human mitochondria operate normally at very low oxygen levels despite tens of millions of years of essentially modern levels of atmospheric oxygen (Brocks et al., 2005) and the evolution of oxygen-dependent systems.[2] Indeed, cellular oxygen levels have remained low and unchanged for nearly 2 billion years and "the basic cellular machinery has been established since the early days of evolution. . ." (Massabuau, 2003, p. 857). A central component of this machinery is hypoxia inducible factor 1 (HIF1), found in all multicellular animals investigated so far (Webster, 2003). HIF1 has been called the 'master regulator' of cellular and systemic oxygen homeostasis because levels increase virtually instantaneously in response to falls in cellular oxygen levels and initiate the transcription of more than seventy genes whose products directly or indirectly offset the lower oxygen levels by increasing oxygen delivery or modifying metabolism (Semenza, 2000, 2001, 2002, 2004). HIF1 target genes include erythropoietin (EPO stimulates proliferation and differentiation of red blood cell precursor cells and is an antioxidant), vascular endothelial growth factor (VEGF stimulates the development of new blood vessels and increases blood vessel permeability), endothelin (ET is a vasoconstrictor produced by endothelial cells in the blood vessels), and many others. These 'new' (to science) gene products are quantitative phenotypes

[2] A frequently repeated assertion that requires updating is a statement that early hominid evolution took place under mild high-altitude hypoxia in East Africa (Hochachka, 1998; Hochachka et al., 1998, 1999a,b; Hochachka and Monge, 2000). Evidence from multiple sources in an area of East Africa that has yielded many important finds of early hominids spanning millions of years indicates that the sites were at about 500–600 m altitude (Redfield et al., 2003; Bonnefille et al., 2004; Quade et al., 2004). Thus, it is reasonable to assume that hominid evolution took place at sea level and that occupation of high-altitude areas entailed moving into a new environment.

intermediate between the DNA sequence and the whole organism.

HIF1 and its target genes are obviously important genes to evaluate for involvement in high-altitude adaptation and natural selection, perhaps by increasing the frequency of an existing variant or a new variant. HIF1α, the oxygen-dependent subunit of HIF1, is both synthesized and degraded continuously when oxygen levels are normal. Hypoxia stabilizes HIF1α and it combines with the continuously present HIF1β subunit to form HIF1. When oxygen levels fall, HIF1 levels increase and target gene transcription into messenger RNA (mRNA) increases. Therefore, the gene for HIF1α has been the focus of investigation to determine whether there has been natural selection for high-altitude population specific DNA sequences or unusual allele frequencies of known variants that may be associated with different cellular and phenotypic responses to fluctuations in oxygen level.

HIF1α DNA sequences were determined in two small studies of high-altitude natives. Finding that the HIF1α sequence of three Andean high-altitude natives was the same as reported for non-Andean samples (Hochachka and Rupert, 2003) caused the authors to suggest that hypoxia sensing was more likely than HIF1 mediated response to be the locus of selection. Alternatively, purifying selection may have been acting to maintain the usual DNA sequence by removing new variants or the small sample size precluded identifying new variants. In contrast, a sample of 20 Tibetans (Sherpa) had a newly discovered sequence and the allele frequencies of known variants differed from low-altitude native Japanese controls ($n = 30$) (Suzuki et al., 2003). These findings raise the interesting possibility of population differences in genes coding for the key cellular regulator of oxygen homeostasis. The samples were small, however, and no phenotypic data were reported, no tests of the functional significance of the variants were reported, and no demographic or genomic analyses of natural selection were undertaken. These studies are not directly comparable because of the different sample sizes and control populations. Yet they illustrate the potential to analyze and compare DNA sequences of candidate genes, the need for careful selection of controls, adequate sample sizes and the need to associate genotype with phenotype and function.

Another level of genetic analysis quantifies the level of gene expression by quantifying cellular mRNA (gene transcription) or gene product (gene translation). Gene expression can vary depending upon regulation by other genes including transcription factors or factors in the nucleus, the cytoplasm or in other cells. Genes and regulatory factors in turn may be influenced by environmental features other than hypoxia. For instance, both cobalt and heat stress induce HIF1 stabilization (Jefferson et al., 2002; Maloyan et al., 2005). The gene expression level of analysis provides intermediate phenotypes that link genotypes to oxygen-delivery phenotypes and function of high-altitude natives.

HIF1α mRNA levels in samples of white blood cells of ten Andean highlanders at altitude and after 1 h at sea level were compared with those of 10 U.S. controls tested at their usual residence of 1500 m (Appenzeller et al., 2006). HIF1α mRNA levels were quantified in the vastus lateralis muscle of three Tibetans after an average of 17 days at 1300 m and compared with three Nepali lowland controls from the same altitude (Gelfi et al., 2004). The graph in the Andean study (no quantitative data were presented) indicates that Andean high-altitude natives at altitude expressed about three times as much HIF1α mRNA as U.S. controls at 1500 m and that their HIF1α levels plummeted to control level after 1 h at sea level. This suggests chronic up regulation of HIF1 at altitude and perhaps up regulation of HIF1 target genes among Andean high-altitude natives. In contrast, HIF1α mRNA levels of high-altitude Tibetans at low altitude were about two-third lower than the low-altitude controls (Gelfi et al., 2004). It is difficult to interpret these findings without a high-altitude baseline. The results of these studies are not directly comparable because they differ in the tissues sampled, altitude of measurement, and details of the process used to semiquantify mRNA levels. However the studies illustrate the potential for relating HIF1α sequence and its variants with mRNA expression levels in appropriate tissues of larger samples of high-altitude natives in order to ask questions about evolution of the genes fundamental to oxygen homeostasis.

2.5. Genomic approaches

Researchers have taken two approaches to selecting HIF1 target genes to investigate for DNA sequences and possible new variants, allele frequency differences between indigenous high-altitude and low-altitude control samples, and measures of gene expression. The most common has been selection of biologically plausible candidate genes, such as those coding for a protein product known to influence a relevant physiological trait, such as erythropoietin and hemoglobin concentration, or one in a metabolic pathway, such as the nitric oxide synthases.

The gene for EPO has been sequenced, based on the reasoning that it could account for the elevated Andean hematocrit relative to high-altitude Tibetans and sea-level natives. The EPO DNA sequence (including known regulatory domains) of three Andean highlanders did not differ from published sequences of normal EPO (Hochachka and Rupert, 2003) suggesting, as for HIF1, that there has been no natural selection for unique variants, that natural selection has acted to maintain the sequence unchanged or the sample size was too small to detect sample variation. Combining these findings to speculate about physiological implications, the elevated HIF1α mRNA level of Andean natives at altitude is consistent with a report that Andean highlanders have slightly higher EPO levels than Tibetans when matched for hematocrit level (Winslow et al., 1989).

Candidate genes for pulmonary vasodilators induced by HIF1 have been examined, based on the reasoning that they could improve blood flow and oxygen diffusion in the lung. A study of the endothelial nitric oxide synthase gene (eNOS, also called NOS3, is one of three NOS catalyzing the synthesis of nitric oxide) of Tibetans (Sherpas) found a higher frequency of 'wild type' alleles than among lowland Nepali controls at 1300 m ($n = 105$ Tibetans and 111 controls) (Droma et al., 2006). This study reported no association between eNOS genotype and phenotype as measured by serum levels of nitric oxide metabolites,

perhaps because of NO synthesis by the other two NOS. Further complicating the interpretation is the finding that genetic background may influence the association of eNOS genotype with levels of serum nitric oxide metabolites. The association is different in samples of Australians as compared with Japanese (Tsukada et al., 1998; Bekker et al., 2004) and perhaps in samples of Tibetans, too. Another study reported no difference in eNOS mRNA expression levels in muscle cells of three Tibetans at low altitude for an average of 17 days and lowland Nepali controls while reporting a doubling of nNOS levels (nNOS is sensitive to hypoxia, but is not a HIF1 target) (Gelfi et al., 2004). Genotypes or nitric oxide-related phenotypes were not reported. These studies illustrate the need to consider the multiple genetic networks and metabolic pathways that may connect a genotype at one locus with intermediate phenotypes, and the potential influence of genetic background on the association of genotype with phenotype.

The expression of HIF1 target candidate genes that could increase oxygen diffusion or decrease oxygen consumption in the mitochondria has been examined. mRNA levels of vascular endothelial growth factor (VEGF induces the growth of new blood vessels and increases blood vessel permeability), and phosphoglycerate kinase 1 (PGK1 is an enzyme in the anaerobic glycolytic pathway) have been quantified in white blood cells of Andean high-altitude natives. VEGF and PGK1 mRNA levels were more than doubled at altitude and fell to U.S. 1500 m control levels within 1 h of arrival at sea level (Appenzeller et al., 2006). This is consistent with the reported chronic upregulation of HIF1α at altitude and down regulation at sea level. In a sample of six Tibetans at low altitude, PGK protein levels were undetectable in muscle cells. Protein levels of several other enzymes in the glycolytic pathway and involved in mitochondrial metabolism led to a conclusion that there is a partial blockade of the glycolytic pathway in the muscle cells of Tibetans (Gelfi et al., 2004). No genotypes or phenotypes were reported.

Another plausible candidate, not known to be a HIF1 target gene, is the gene for myoglobin, a protein that contributes to oxygen storage and diffusion in skeletal and cardiac muscle. A screen of the myoglobin gene exon 2 for novel variants or deviations from Hardy–Weinberg equilibrium in a sample of 146 Tibetans did not find any distinctive sequences (Moore et al., 2002). Myoglobin mRNA levels in muscle cells did not differ between samples from high-altitude native Tibetans at low altitude and lowland Nepali controls. However, myoglobin protein levels were doubled in a sample of six Tibetans from high altitude and elevated 30% in those from low altitude as compared with six lowland Nepali controls (Gelfi et al., 2004).

Other genes not known to be induced by HIF1 have been examined in Andean samples for distinctive allele frequencies or novel alleles. These include genes for hypercoagulability ($n = 63$ people), folate metabolism ($n = 69$ people), the renin-angiotensin system and the β2 adrenergic receptors that regulate blood pressure ($n = 110$ people) (Rupert et al., 1999a,b, 2000, 2003a,b; Monsalve et al., 2003). The low-altitude controls have included American Indians from the west coast of Canada, Central American and South America. None of the studies reported phenotypic data. There was no evidence for natural selection on the DNA sequences.

Variation in the angiotensin converting enzyme (ACE) gene illustrates an important point about strategies for identifying informative genetic loci. ACE influences levels of some vasodilators and vasoconstrictors by converting a precursor to a vasoconstrictor (angiotensin II) and by breaking down a vasodilator (bradykinin). ACE has two common variants called the insertion (I) and deletion (D) alleles because a short sequence of bases is present (I) or deleted (D). The insertion (I) allele is associated with low serum levels of ACE, low levels of the vasoconstrictor angiotensin II, and longer half-life of the vasodilator bradykinin and thus could allow an increased blood flow. The ACE insertion/deletion (I/D) polymorphism has been investigated for association with a phenotypic marker of successful adaptation to hypoxia, pulmonary artery pressure. Low-altitude native ACE II homozygotes who were acutely exposed to a 30-day stay at 3500 m had lower pulmonary artery pressure and a lower incidence of pulmonary hypertension as compared with other genotypes (Kumar et al., 2003), consistent with expectation of less vasoconstriction based on the biology of ACE at low altitude. In contrast, in a sample of high-altitude natives (Kyrghyz from central Asia), ACE II homozygotes were more likely to have pulmonary hypertension and to have experienced an increase in pulmonary artery systolic pressure 10 years later (Morrell et al., 1999). Consistent with those findings, there was no excess of the I allele among high-altitude native Tibetans (Sherpas) (Suzuki et al., 2003), Indian Ladakhis (Kumar et al., 2003) or Peruvians (Rupert et al., 1999a) as might be expected if it were advantageous in those populations.

The contrast between the association of the I allele with a lower risk of pulmonary hypertension among acutely hypoxic low-altitude natives and its association with a greater risk of pulmonary hypertension among chronically hypoxic high-altitude natives is instructive for designing future studies. Genetic background may influence the expression of the I allele or perhaps the I allele is linked to the causal polymorphism (Morrell et al., 1999). From the perspective of developing and testing hypothesis about natural selection, those based on genotype–phenotype associations derived from low-altitude populations may be misleading.

So far the 'plausible candidate gene' approach using contemporary molecular and genetics techniques has been interesting but has not yielded evidence for natural selection. It does illustrate the need for samples large enough to make powerful statistical inferences, for making measurements in the same tissue under the same conditions, for an effective strategy to identify appropriate candidate genes, and for integrating measures of gene sequences, gene expression and phenotype.

A very different genomic approach to identifying informative candidate genes for study proceeds deductively by scanning for single nucleotide polymorphisms (SNPs) throughout the genome of high and low-altitude natives to identify large differences in allele frequency that might reflect natural selection to increase the frequency of adaptive alleles (Moore et al., 2004, 2006; Shriver et al., 2006). Comparison of high-altitude Andean natives with a low-altitude Central American population and a

low-altitude East Asian population identified four loci with a nearby SNP frequency that was highly likely to be different from that of the two low-altitude populations. Three were HIF1 target genes (inducible nitric oxide synthesis (iNOS orNOS2), endothelin1 (ET1 is a vasoconstrictor), and the α1 adrenergic receptor (it binds epinephrine or norepinephrine) and one was an oxygen-dependent enzyme that regulates the continuous destruction of HIF1α depending upon the level of cellular oxygen (PHD3, prolyl hydroxylase 3). These loci, selected without prior biological information and from the entire genome, are biologically plausible. Investigating whether genetic variants at these loci influence survival and reproduction in Andean populations seems likely to be more productive than simply selecting candidates from a list based on their potential involvement. Several pieces of evidence support their involvement, including their relationship with HIF1, some phenotypes, and distinctive SNP frequencies. There is a report of normal sea-level values of ET1 levels among Andean high-altitude native women although ET1 genotypes were not provided (Moore et al., 2004). This is likely to be a very useful and more efficient strategy to identify candidate genes. Significantly, it employs newly available techniques to systematically evaluate the entire genome and choose genes for study.

A related genomic approach also analyzes SNPs throughout the genome. It searches for shared homozygous alleles among individuals in a single adapted population. Comparison of SNPS found that eight of nine Tibetans (Sherpa) were homozygous for the same allele at three polymorphic loci. The interpretation was "that the chromosomal segments detected by such DNA markers may include genes involved in adaptation to hypobaric hypoxia" (Malacrida et al., 2007, p. 1). No phenotypes were reported.

These new techniques offer great potential for substantial progress in discovering the genetic bases underlying oxygen homeostasis of high-altitude populations. Natural selection acts on phenotypes: progress in phenotyping needs to keep pace with that in genotyping and genetic analyses. Technologies must be developed to enable collecting and integrating data for sufficiently large samples to apply meaningful statistical analyses. For example, gene expression of muscle cells can be informative, yet muscle biopsies of sufficient numbers of people with a variety of genotypes over a range of ages are probably not realistically feasible. Thorough analyses of natural selection must integrate information on genotypic variation with various molecular and physiological measures and with information on how such variation influences function, performance, survival or reproduction at high altitude. These require large-scale studies because the outcome variables are subject to many other influences.

An important outcome variable is birthweight because it is related to infant survival at high and low altitudes (Beall, 1981; Moore et al., 2004). Average birthweight decreases with increasing altitude, however Andean and Tibetan newborns weigh more at a given altitude than those born to mothers of low-altitude ancestry who are residing at high altitude. Maternal factors associated with heavier birthweight and thus higher likelihood of survival include hypoxic ventilatory response among Andean women and intrauterine blood flow among Tibetan women (Moore et al., 1986, 2001a). This important topic has been recently reviewed (Moore et al., 2004).

Integrating multiple sources of information, such as those described here will enable modeling the mechanisms and processes whereby one DNA sequence enables better performance at high-altitude and how performance differences change high-altitude gene pools and physiology.

2.6. Comparative physiology approach

Carlos Monge and his colleagues used a comparative physiology approach to identify genotypically adapted animals "native to the mountains" (Monge and Leon-Velarde, 1991, p. 1137) and phenotypically adapted animals "introduced in the mountains in recent times" (Monge and Leon-Velarde, 1991, p. 1137). Llamas and yaks are examples of genotypically adapted species whose normal physiology reduces hypoxic stress while sheep and cows are examples of phenotypically adapted animals whose physiology is changed by hypoxia. Writing in 1991 when data on Andean high-altitude populations were more abundant than those on Tibetans, Monge and Leon-Velarde considered that "High-altitude humans belong to the group of phenotypically adapted mammals" (Monge and Leon-Velarde, 1991, p. 1159). Later, when more data on Tibetans had become available, Monge and co-authors wrote that "From an evolutionary physiology point of view, natural selection does not seem to have acted on Andean humans as much as on other high-altitude species" (Villafuerte et al., 2004, p. 1586).

Monge and Leon-Velarde identified a set of features common to genotypically adapted species (Table 1) (Monge and Leon-Velarde, 1991). Comparing these features in the Andean and Tibetan populations, although a within species comparison, is another way to address the question of the genetic bases of their adaptations. One feature of genotypically adapted species is a high hemoglobin affinity for oxygen, measured as a low value of p50 (the arterial oxygen tension at which 50% of hemoglobin is saturated with oxygen). At high altitude where arterial oxygen tension is low, a low p50 would load more oxygen onto hemoglobin in the lung. Genetic changes in hemoglobin and its structure lower p50 in several species including chinchillas and guinea pigs native to the Andes, chickens introduced to the Andes in the past 500 years, and high-altitude populations of deer-mice in the Rocky Mountains (Velarde et al., 1991; Ostojic, 2002; Storz et al., 2007). Low intracellular levels of 2,3 bisphosphoglycerate mutase (BPGM, also known as 2,3 DPG) lower p50 and are found among pika and yak on the Tibetan Plateau (Adams et al., 1975; Ge et al., 1998). In contrast, both Andean and Tibetan high-altitude natives have normal p50 that is achieved by a combination of elevated 2, 3 BPGM that lowers oxygen affinity and lower arterial partial pressure of CO_2 that raises oxygen affinity (Winslow and Monge, 1987; Winslow et al., 1988, 1989). There are no reports of hemoglobin variants with high or low oxygen affinity and both are reported to have normal hemoglobin A (Adams and Strang, 1975; Larrick and Topgyal, 1985; Beall et al., 1998). On this measure, neither human high-altitude population displays the features of a genotypically adapted population.

Table 1
Characteristics of genotypically and phenotypically adapted high-altitude animals (Monge and Leon-Velarde, 1991), Tibetan and Andean high-altitude natives

Trait	Genotypically adapted high-altitude animals	Tibetan high-altitude natives	Andean high-altitude natives	Phenotypically adapted high-altitude animals
High hemoglobin affinity (low p50)	Present	Absent	Absent	Absent
Moderate or absent polycythemia	Present	Present	Absent	Absent
Low venous pO_2	Present	No data	Present	Absent
Thin-walled pulmonary vascular tree that responds moderately to hypoxia	Present	Present	Absent	Absent
Absence of chronic mountain sickness (CMS)	Present	Present	Absent	Absent

Another feature of genotypically adapted animals is moderate or absent polycythemia (Table 1). Initially, knowledge based on acutely exposed lowlanders and Andean highlanders led to the conclusion that more red blood cells and consequently more hemoglobin per unit blood was a beneficial adaptation to high-altitude hypoxia. Polycythemia could restore arterial oxygen content when oxygen saturation of hemoglobin is unavoidably low due to altitude. Andean highlanders exhibit increased hemoglobin concentration as low as about 1500 m (Cosio, 1972) and the response increases with altitude. More recent data on Tibetans reveal moderate to absent polycythemia as indicated by little or no elevation of hemoglobin concentration at altitudes as high as 4000 m. For example, a comparison of 53 published samples of men at altitudes above 3000 m reported an estimated hemoglobin concentration of 16.9 g/dL for Tibetans as compared with 18.1 g/dL for Andean highlanders (Beall, 2001, 2006). In addition, Tibetans have generally lower hemoglobin concentration than Han Chinese measured at the same altitude as adults and as children (Garruto et al., 2003; Wu et al., 2005). Tibetan, but not Andean, high-altitude natives, have the moderate to absent polycythemia characteristic of genotypically adapted populations.

A low venous pO_2, an indicator of tissue oxygenation, is another feature of genotypically adapted animals according to the Monge and Leon-Velarde model described in Table 1. They reasoned that the high affinity of hemoglobin and moderate or absent polycythemia do not favor the release of oxygen to the tissues and will result in low venous pO_2 and the need for tissue level adaptations. Andean highlanders have venous pO_2 about 10–18% lower than sea level (Table 1 in Villafuerte et al., 2004). There appear to be no reports of venous pO_2 for Tibetans. Indirectly, however, Monge and Leon-Velarde note that phenotypically adapted organisms may maintain tissue oxygenation by increasing blood flow. Tibetans' high rates of blood flow, as compared with acclimatized lowlanders, when stressed by temporary occlusion, exercise or pregnancy (Huang et al., 1992; Moore et al., 2001b; Schneider et al., 2001) may be indirect evidence that tissue hypoxia is not a feature of the Tibetan suite of adaptive traits. Direct measurements of this feature remain to be collected.

Thin-walled pulmonary vasculature that responds moderately to hypoxia is also characteristic of genotypically adapted organisms (Table 1). Underlying this feature is a mild or absent hypoxic pulmonary vasoconstriction reflex. Chronic vasoconstriction under chronic, global hypoxia at high altitude causes hypertrophy of the smooth muscles of the small pulmonary arteries. The combination of vasoconstriction and hypertrophy elevate pulmonary artery pressure (Rhodes, 2005). However, high-altitude species, such as the llama, yak and pika have little or no pulmonary hypertension or hypertrophy (Ge et al., 1998; Rhodes, 2005). Tibetans have 'minimal' elevation of pulmonary artery pressure, respond little to further hypoxia, and have thin-walled pulmonary vasculature (Gupta et al., 1992; Groves et al., 1993; Halperin et al., 1998; Hoit et al., 2006). In contrast, Andean high-altitude natives display hypoxic pulmonary vasoconstriction, elevated pulmonary artery pressures and muscular pulmonary artery trees (Rotta et al., 1956; Groves et al., 1993). Two years at sea-level reverses these traits, indicating that they were indeed phenotypic adaptations (Sime et al., 1971). Tibetan, but not Andean, high-altitude natives have this feature of genotypic adaptation.

Finally, genotypically adapted species do not exhibit chronic mountain sickness, an illness attributed to the loss of adaptation. The characteristic features are excessive polycythemia, hypoventilation, and elevated pulmonary artery pressure (Monge and Leon-Velarde, 1991). Domestic species introduced to high-altitude including cattle and chickens are at risk of chronic mountain sickness. Andean highlanders are at high risk of chronic mountain sickness, but Tibetans are not. For example, at 4300 m about 16% of Andean highlanders have chronic mountain sickness as compared with less than 1% of Tibetans (Wu, 2005). Tibetan, but not Andean, high-altitude natives express this feature of genotypic adaptation.

This comparative physiology analysis reveals that Tibetans display three of the five features characteristic of genotypically adapted species, do not display one, and there are no data on another. A different comparative physiology model focuses on metabolic and biochemical features of anoxia tolerant vertebrates, such as diving seals and turtles. It provides a second set of features for evaluating the genetic bases of the traits of high-altitude native human populations. Anoxia tolerant vertebrates have very low metabolic rates in the brain and preferentially use glucose as a metabolic fuel in heart muscle (Holden et al., 1995; Hochachka et al., 1996a,b,c). Those biochemical features are adaptive when there is little available oxygen because they reflect a switch to anaerobic metabolism in the brain and an increased use of fuel with a higher yield of energy per unit of oxygen consumed. Samples of Andean (Quechua) and Tibetan (Sherpa) highlanders were tested for these features immediately upon arrival at sea level and 3–4 weeks later. The logic was that

features caused by acclimatization would still be in effect at the first testing while features caused by genetic adaptation would remain in effect at the second testing. The Andean highlanders had low brain metabolic rates at both times while the Tibetans had normal sea-level brain metabolic rates at both times and when exposed to experimental hypoxia at sea level (Hochachka et al., 1996c). The interpretation was that other "whole body physiological mechanisms" deliver enough oxygen to obviate the need for biochemical adaptations in the brain. Both Andean and Tibetan highlanders had biochemical indicators of heart metabolism favoring the use of glucose in heart muscle (Holden et al., 1995; Hochachka et al., 1996b). These indicators were generally stable relative to lowlanders throughout the 2–3 weeks at low altitude and were interpreted as true biochemical adaptations that offset limited oxygen supply. Thus, Tibetans had one of the biochemical characteristics of anoxia tolerant species while Andean highlanders had both.

The two comparative approaches identify seven features of physiology and metabolism found in non-human species exposed chronically or intermittently to hypoxia. Tibetans share four, Andean highlanders share three. Perhaps both high-altitude human populations have undergone natural selection for improved function at high altitude.

3. Summary and conclusion

High-altitude natives have distinctive biological characteristics that appear to offset the stress of hypoxia, such as the elevated hemoglobin concentration of Andean high-altitude natives or the elevated resting ventilation of Tibetans. Evolutionary theory reasons that they reflect genetic adaptations resulting from natural selection favoring more effective adaptive responses. Because natural selection operates on heritable variation, a major research focus has been determining the genetic basis of these traits. This has been difficult for several reasons including the difficulty of deciding which components of the integrated oxygen-dependent system to study and the few tools available to detect the genetic basis of quantitative traits. The most common research design compared classical physiological traits measured in samples of natives and migrants between altitudes. If acclimatization or developmental adaptation could not account for the distinctive high-altitude physiology, a genetic basis unique to the high-altitude populations was inferred. That is, the conclusion was based on exclusion of alternatives rather than demonstration of an association between a gene or genes and the biological characteristic.

Statistical genetics techniques to quantify heritable variation and to estimate the opportunity for natural selection found that Tibetans generally have higher genetic variance and variance in more traits than Andean highlanders, indicating greater potential for natural selection. Taken together, this work made a solid case for the possibility of genetic variation within and among high-altitude populations and for the opportunity for natural selection. Tibetans have an autosomal dominant major gene for high oxygen saturation associated with higher offspring survival, suggesting ongoing natural selection. However, these approaches could not identify the genes involved or determine how they caused the phenotype. Nor could they detect past natural selection.

New work on the cellular machinery of oxygen homeostasis uses molecular and genomic approaches to study the hypoxia inducible factor 1α gene, HIF1 target genes and a few other candidate genes. DNA sequences and gene expression studies using small samples of Andean and Tibetan high-altitude natives have not yet yielded any conclusive evidence about molecular population differences.

However, these studies can guide the design of future work. For example, large-scale research will probably be required to detect natural selection in high-altitude populations. Many factors regulate a genetic locus and must be taken into account when analyzing associations between genotypes and phenotypes. Genetic background and environmental context may modify gene expression. The study of natural selection at high altitude is entering an era of linking genomics, genetics, molecular biology and physiology to understand what makes an organism better able to function, survive and reproduce – fit in the Darwinian sense – under the chronic lifelong stress of high-altitude hypoxia.

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