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Congenital and evolutionary modulations of hypoxia sensing and their erythroid phenotype

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Exposure to hypoxia elicits a myriad of adaptive responses that are mediated by hypoxia inducible factors (HIFs). An essential physiologic response to hypoxia is increase of production of erythrocytes necessary to maintain adequate oxygen delivery to the tissues; it has also been reported that the survival of erythrocyte may increase. Congenital defects of upregulation of the hypoxia-sensing pathway result in inappropriately elevated erythrocyte concentration. Evolutionarily, after many generations of living at high altitude, high altitude dwellers such as Tibetans, Andeans, and Ethiopians have selected genomic signatures, some in HIF pathway, enabling them to prosper in high altitude hypoxic environment. However, the entire diverse complex of molecular mechanisms of high-altitude adaptation is still largely unknown.

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Introduction

Oxygen is vital for energy metabolism; its delivery is executed by erythrocytes that transport oxygen bound to hemoglobin. In hypoxia, erythropoiesis is augmented, and the increased erythrocyte survival may further increase the red cell mass [1]. The response to hypoxia is controlled by hypoxia inducible factors (HIFs) [2] that also activate glycolysis and reduce the formation of reactive oxygen species [3]. HIFs (HIF-1, HIF-2, HIF-3) are heterodimeric transcription factors composed of a highly regulated α and a constitutively expressed β subunits. In normoxia, HIFα subunits are hydroxylated by prolyl hydroxylases (PHD), and are rapidly degraded by the von Hippel-Lindau (VHL) protein-ubiquitin-proteasome pathway. The targeting of HIFα subunits requires oxygen, PHD, α-ketoglutarate and iron, and VHL; this complex constitutes the oxygen sensor [4,5]. HIF-2 is the principal regulator of erythropoietin (EPO) [6] — the hormone that drives erythropoiesis. In hypoxia, HIFα subunit escape degradation, translocate into the nucleus, dimerize with its β subunit, and bind to hypoxia response elements (HRE), leading to increased transcription of HIF target genes [2]. Mutations in the genes in this oxygen-sensing pathway can result in augmented erythropoiesis. The germline mutations of VHL, EGLN1 (encoding PHD2), EPAS1 (encoding HIF-2α) and EPO receptor (EPOR) genes have all been known to lead to polycythemia [7].

High altitude dwellers, who have been living at high altitude for many generations, have undergone genetic adaptations to overcome the challenges of hypoxic environment. Thus Tibetans, who have been living on the Himalayan plateau for more than 20 000 years, have been largely protected from polycythemia and chronic mountain sickness (CMS). Their strongest selection signals are *EPAS1* and *EGLN1* [8–11] that are virtually Tibetan-specific; no such unique, population-specific haplotype has been found in Andean and Ethiopian native highlanders so far. The Tibetan *EPAS1* haplotype is found to be introgressed from the Denisovan genome [12], and we reported the Tibetan-specific combined variant of $EGLN1^{12C>G,380G>C}$ that is gain-of-function in hypoxia and blunts the hypoxic response [13]; other functional effects of this haplotype have also be reported [14].

In this review, we will discuss germline mutations in the modulations of hypoxia sensing pathway, and contrast it with the evolutionarily selected genomic signatures of highlanders.

Congenital conditions modulating hypoxia pathway

In germline mutations in hypoxia signaling pathway, the resulting polycythemia is invariably inappropriate as there is no increased physiologic demand for tissue oxygenation.

VHL mutations

Chuvash polycythemia, the first described congenital disorder of hypoxia sensing, is an autosomal recessive polycythemia resulting from a missense mutation of $VHL^{598C>T}$(VHL^{R200W}) [15], endemic in the Chuvash Republic of Russia and on the Italian island of Ischia. This loss-of-function mutation causes reduced affinity of VHL for HIFα subunits, leading to delayed proteasomal

degradation and increased transcription of HIFs target genes, including EPO. The individuals with Chuvash polycythemia have increased concentration of EPO due to augmented HIF signaling, but their erythroid progenitors *in vitro* assays are also hypersensitive to EPO, a characteristic of primary polycythemia [15]. Affected subjects have varicose veins, pulmonary hypertension and high risk of arterial and venous thromboses; their risk of thrombosis is inversely related to hematocrit [16]. Other homozygous as well as compound heterozygous VHL mutations have been reported with polycythemic phenotype but not tumors [17–20]. This is in contrast to VHL tumor predisposition syndrome (TPS) wherein heterozygous germline loss-of-function VHL mutations are followed by somatic VHL mutations in trans leading to hemangioblastomas, pheochromocytomas/paragangliomas, pancreatic endocrine and endolymphatic sac tumors [7] but not polycythemia [19]. The genetic basis for this difference is still unclear, and may be attributed to multifunctional facet of the VHL protein [17], and certain VHL mutations are shown to have age-related variable penetrance of tumor development [21]. A VHL germline mutation in intron 1 or a synonymous coding sequence mutation in exon 2 causes alternative VHL splicing, which leads to either congenital polycythemia or TPS phenotypes [22].

PHD2 mutations

Loss-of-function mutations of EGLN1 leading to polycythemia were described; first were $EGLN1^{950C>G}$ ($PHD2^{P317R}$) and $EGLN1^{1112G>A}$ ($PHD2^{P371H}$), where one individual had sagittal sinus thrombosis [23,24]. Both these mutations were heterozygous, indicating that a partial loss of function is sufficient to induce polycythemia, although their polycythemia was mild and EPO was normal. Another heterozygous $EGLN1^{1121A>G}$ ($PHD2^{H374R}$), associated with recurrent paraganglioma [25], located in a conserved sequence that affected Fe^{2+} binding. In the tumor, there was loss-of-heterozygosity, that is mutation of both alleles, suggesting EGLN1 is a tumor suppressor gene. The $EGLN1^{1096T>C}$, found in the patient and a relative, had no history of thrombosis [26]; a homozygous missense $EGLN1^{124T>C}$ mutation in two siblings caused polycythemia that was not present in heterozygous parents [27]. Over 20 other frameshift and nonsense EGLN1 mutations described earlier, all were leading to familial polycythemia [28].

Germline EPAS1(HIF-2α) mutations

These $EPAS1^{1609G>T}$, $EPAS1^{1604T>C}$, and $EPAS1^{1620C>G}$ are gain-of-function polycythemic mutations [29,30]. All are heterozygous, with alteration close to the hydroxylation site, thereby preventing HIF from hydroxylation and subsequent proteasomal degradation.

Mosaicism of EPAS1(HIF-2α) mutations

Another group of EPAS1 mutations leads to polycythemia as well as TPS. $EPAS1^{1588G>A}$, $EPAS1^{1589C>T}$ and $EPAS1^{1121T>A}$ were described in patients with congenital polycythemia and recurrent paragangliomas and other tumors. In this unique syndrome, these causative mutations are associated with mosaicism of wild type and mutated cells, found in high frequency in the tumor organ and only in only low proportion, almost undetectable in the blood [31,32]. Family involvement of this syndrome may be present but generally not seen; however, it remains to be determined whether these mutations are germline or there is genetic predisposition to mosaicism.

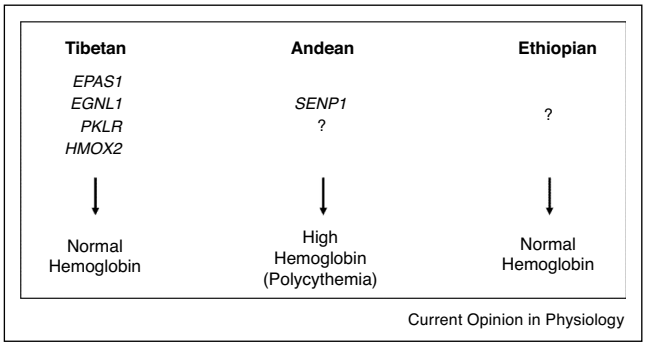
Tibetan evolutionary adaptation to hypoxia

Tibetans have been living at high altitude for ~25 000 years, selecting evolutionary advantageous haplotypes that are beneficial for their existence [33]. While hypoxia augments erythropoiesis, majority but not all Tibetans living at altitudes up to 4000 m, have normal range of hemoglobin and hematocrit comparable to those at sea level, while the those living >4000 m have increased prevalence of higher hemoglobin [34]. Arterial oxygen saturation of newborn Tibetan babies is higher than Han Chinese at high altitude, protecting them from infantile mountain sickness [35]; they also have larger nasal cavity facilitating greater oxygen uptake [36].

EAPS1 (HIF-2α)

Strongest Tibetan natural selection signal is *EPAS1* haplotype [11,37,38]. It introgressed from Denisovan genome, is Tibetan-unique, and contains both Denisovan and non-Denisovan variants, suggesting a slow decay of this ancestral haplotype [12]; it is associated with lower hemoglobin [8,37] (Figure 1). A 3.4 kb deletion at 80 kb downstream of *EPAS1* is in linkage disequilibrium with *EPAS1* variants associated with polycythemia protection, but this deletion is not of Denisovan origin [39•]. It has

Figure 1



Known genetic variants affect hemoglobin concentration at high altitude.

been reported that *EPAS1 rs149594770* in intron 1 weakens promoter activity, resulting in decreased HIF-2α expression in human umbilical endothelial cells and placentas, contributing to Tibetans' lower hemoglobin [40]. In contrast, SNP *(rs56721780:G>C)* and indel (−742 nt) mutation in promotor region of *EPAS1* induces *EPAS1* transcription via changing binding affinity of transcriptional factors including IKAROS family zinc finger 1 (*IKZF1*) and Sp1 [41]. This may be associated with Tibetans' differences in amnion and birth weight via high expression of lysyl oxidase (*LOX* — one of HIF-2 target gene) [41]. In contrast, three *EPAS1* SNPs (*rs13419896*, *rs12619696*, *and rs4953354*) are associated with high altitude polycythemia in Tibetans [42]. It was also reported that Tibetan enriched SNPs of *CYP17A1* and *CYP2E1* genes are involved in modulation of polycythemia, while Tibetan variants of *ITGA6* and *ERBB4* reduce the risk of polycythemia while *ERBB4* SNP *rs6710946* is associated with a high risk of polycythemia [42,43].

EGLN1 (PHD2)

Two Tibetan-selected *EGLN1* missense mutations have been evolutionarily co-adapted and are always present in *cis* in a single haplotype $EGLN1^{12C>G,380G>C}$ ($PHD2^{D4E:C127S}$). This haplotype is a gain-of-function mutation in hypoxia, resulting in increased degradation of HIFα subunits in hypoxia. Thus, erythroid progenitors with $EGLN1^{12C>G,380G>C}$ have ameliorated proliferation in hypoxia [13]. $EGLN1^{12C>G,380G>C}$ is associated with lower hemoglobin in Tibetan males [44] but not in females [45] (Figure 1).

This haplotype is not only involved in lower hemoglobin concentration but also associated with increased rate of having lung cancer in Nepalese [46]. However, the role of the haplotype in development, progression, or metastasis of lung cancer is unknown.

We also showed that this haplotype is associated with age-dependent lower hemoglobin, even at low altitudes, and together in those with *EPAS1 rs142764723* (C/C) also decreases hemoglobin at high altitude; however, these two genetic determinants do not fully account for entire Tibetan protection from polycythemia. This indicates that other genetic factors than $EGLN1^{12C>G,380G>C}$ and *EPAS1* variants must contribute to lower hemoglobin in Tibetans [47•].

Other Tibetan genetic variants associated with hypoxic response

PKLR is one of Tibetan enriched haplotypes, and it encodes liver and erythrocyte pyruvate kinase enzyme [11,37,48]. This enzyme is in the terminal portion of the glycolysis pathway, and its decreased activity leads to accumulation of proximal intermediates, including 2,3 diphosphoglycerate (2,3-DPG). 2,3-DPG shifts the hemoglobin dissociation curve to right, allowing hemoglobin to readily release oxygen to the tissues [49]. While this may be beneficial in hypoxic environment, this haplotype is not unique to Tibetans; however, it has the highest frequency in Tibetans (89%), followed by Chinese and Mongolians (77%) and only 11% in Caucasians. It may contribute to the erythroid heterogeneity of hypoxic responses in human populations [50]. In reticulocytes, the *PKLR* transcript progressively decreases with increasing altitudes and Tibetans with enriched *PKLR* haplotype have lower *PKLR* transcript than those without it [50]. The Chuvash polycythemia patients having constitutive high HIFs by a hypomorphic $VHL^{598C>T}$ mutation have lower *PKLR* transcript, and this along with effect of altitude on *PKLR* transcript, suggest that HIF negatively regulates *PKLR* transcription [50]. However, hemoglobin-oxygen affinity in Tibetan and Han Chinese at high altitude is still within broad range of normal [51,52].

Another hypoxic response gene, *HMOX2* (Heme oxygenase 2), is also positively selected in Tibetans [37]. Tibetan males with C allele of *rs4786504:T>C* have lower hemoglobin than those with T allele. Tibetans with C allele express higher *HMOX2*, leading to increased degradation of heme [53] perhaps contributing to their lower hemoglobin (Figure 1). *EDAR* (ectodysplasin A receptor), transmembrane protein receptor of ectodysplasin A (EDA), has also been reported as being selected in Tibetans and associated with higher oxygen saturation (*rs10865026*) and platelet counts (*rs3749110*) [54].

Evolutionary high-altitude adaptation in Andeans and Ethiopians

Andeans

Among the high altitude population, Andeans have highest hemoglobin, associated with high incidence of CMS [55]. Like Tibetans, their birth weight is higher than lowlanders and Ethiopians [56].

Whole genome sequencing (WGS) of Quechuas, one of the two indigenous Andean highlanders, reported two selected genes (SENP1 and ANP32D) and their association with protection from CMS [57]. These two genes were reported to be upregulated in CMS and suppressed in those without CMS, and overexpression of SENP1 was reported to induce erythrocytosis via upregulating GATA1 expression [58] (Figure 1).

In Aymaras, the other indigenous Andean highlanders, WGS showed strong selection signals in BRNIP3, NOS2, and TBX5 which are related to cardiovascular function and development [59•]. Previous study based on SNP array suggested selection in HIF-pathway related genes [60] but by WGS analysis, only ELGN1 had low selection signal [59•]. Quechua's selected SENP1 and ANP23D and Tibetan selected ELGN1

and EPAS1 variants were not found in Aymara [61]. Aymaras with CMS have inappropriately low-normal serum EPO and have endogenous erythroid colonies, feature of primary polycythemia [62].

Ethiopians

Compared to Tibetans and Andeans, Ethiopian highlanders have the shortest history of living at high altitude for ~5000 years [33]. They have similar hemoglobin level to the Tibetans and lowlanders but higher oxygen saturation than Tibetans [63]. SNP arrays of Ethiopian Amhara population reported selection of genes involved in high altitude adaptation including CBARA1, VAV3, ARNT2, THRB, and BHLHE41 [64,65]; however, while the hypoxic response related genes (*CUL3*, *ADRBK1*, *CORO1B*, *MAPKAPK2*, and *UTRN) were selected in Ethiopian highlanders but none of them were associated with hemoglobin level* [66]. WGS of Ethiopians reveal selection of CIC, LIPE, and *PAFAH1B3* genes involved in hypoxia tolerance [67]. Ethiopian Wolaita population living in mid and high altitude of southern Ethiopia had selection signals for SNPs in *PPARA*, *CDKAL1*, *NEGR1*, and *SLC24A5* [68]. These genomic analyses reveal that some of selected genes in Ethiopians are involved in hypoxic responses but the genes associated with low hemoglobin level have not yet been identified (Figure 1).

Conclusion

Mutations in HIF signaling pathway cause inappropriate polycythemia independent of physiological needs. These mutations may also be associated with thrombosis, but some may also be associated with TPS, such as certain mutations of VHL, EPAS1 and EGLN1.

However, high altitude dwellers have acquired unique genetic adaptation to thrive in hypoxic environment, and HIF pathway is one of the important cellular processes that has endured evolutionary pressure. Although protection from polycythemia in Tibetans and Ethiopians and polycythemia in Andeans may confer some physiologic advantage, it is likely that evolutionarily selected HIFs' modulation at high altitude likely has other beneficial effects than erythroid phenotype that are yet to be identified.

Conflict of interest statement

Nothing declared.

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