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Grasping the genetic determinants of human adaptations: the "Kings of the Mountains" (Sherpa) case study

Marco Sazzini

Department of Biological, Geological and Environmental Sciences (BiGeA) Laboratory of Molecular Anthropology & Centre for Genome Biology, University of Bologna, Italy e-mail: marco.sazzini2@unibo.it



Considerable biological and cultural adaptabilities represent some of the main strengths of the *Homo sapiens* species and probably the cornerstone on which our amazing evolutionary success is based. These characteristics have provided key prerequisites for anatomically modern humans to spread all over the world and to effectively colonize a variety of environmental and ecological contexts, which triggered diversified metabolic, immune and physiological changes in the human body (Hancock *et al.*, 2011; Brown, 2012; Jeong & Di Rienzo, 2014).

Having been forced to face multiple and severe selective pressures during their relatively recent evolutionary history, populations that

have settled at high altitude can be viewed as some of the most iconic examples of human biological adaptability. Interestingly, among the stresses that most challenge human life at altitude (e.g. cold temperatures, poor nutritional resources, high ultraviolet radiation), the decrease in oxygen pressure as elevation increases (i.e. hypobaric hypoxia) and the consequent difficulty to make it available for tissues and cells is the sole constraint that cannot be mitigated by means of cultural or technological improvements. This means that present-day high-altitude populations are subjected to exactly the same intensity of the hypoxia-related selective pressure that was experienced by their ancestors thousands of years ago when they first occupied this extreme environment. Therefore, studying their biological adaptations to hypobaric hypoxia represents an invaluable opportunity to investigate in *real time* how natural selection is acting on human populations and how it is still shaping their patterns of genomic and phenotypic variation.

Among human groups that permanently reside at altitude, Tibetans and Sherpa from the Himalayas (i.e. who live on the Tibetan Plateau and/or in Nepal on the southern slopes of the Himalayan mountain range) have undoubtedly evolved the most effective physiological adjustments in response to hypoxia. In particular, Sherpa people were well known for their elite performance on the highest peaks of the world and this has guaranteed them the nickname of *Kings of the Mountains*. This is mainly due to the fact that the ancestors of modern Tibetans and Sherpa moved from low-altitude Chinese regions to the Tibetan Plateau probably more than 20,000 years ago (Su *et al.*, 2000; Zhao *et al.*, 2009; Aldenderfer, 2011; Rhode, 2016), definitively settling there after the Last Glacial Maximum (LGM) (Qin *et al.*, 2010). Subsequently, migrations of populations speaking proto-Tibeto-Burman languages from Western China occurred during the Neolithic and further boosted the occupation of the plateau thanks to the admixture of incoming farmers with

the small local groups that had survived to the LGM (Qi *et al.*, 2013; Lu *et al.*, 2016). Therefore, high-altitude populations from the Himalayas spent an appreciable fraction of their recent evolutionary history in a particularly challenging environment. Conversely, other populations, such as those from the Andes, Ethiopia or Central Asia, colonized high-altitude territories too recently (i.e. from few thousand years ago in the case of Andeans to some hundred years ago in the case of African Oromo) to have had enough time to evolve full genetic adaptations by means of natural selection (Beall *et al.*, 2007; Alkorta-Aranburu *et al.*, 2012; Cilli *et al.*, 2019). Accordingly, Tibetan and Sherpa people have developed cellular and physiological characteristics that help them cope with low oxygen availability. These changes are unique among modern humans. The features include a reduction in the number of mitochondria in muscle cells and the absence of erythropoietic response to hypoxia, as well as an increase in resting pulmonary ventilation, concentration of nitric oxide in the lungs, capillary distribution and overall blood flow (Kayser *et al.*, 1991; Beall *et al.*, 2001; Hoppeler *et al.*, 2003; Erzurum *et al.*, 2007; Moore *et al.*, 2011; Vitzthum, 2013; Gilbert-Kawai *et al.*, 2014).

However, although the biology and the evolutionary history of Tibetans and Sherpa have been extensively studied, when we launched our research project in 2015, the genetic bases of most of their adaptive traits were far from being elucidated. Indeed, for at least two decades, the successful adaptation of Tibetans to high altitude was associated mainly to their lower hemoglobin concentration compared to lowlanders who are exposed to hypotaric hypoxia (Zhuang et al., 1996). In fact, this protects them against the long-term detrimental effects of polycythemia (i.e. the increase in the number of red blood cells aimed at providing more hemoglobin to capture the little oxygen available at altitude), which arises due to physiological acclimatization to hypoxia and eventually leads to chronic mountain sickness (CMS) (Winslow & Monge Cassinelli, 1987; Vargas & Spielvogel, 2006). Strong positive selection on a couple of genes (i.e. hard selective sweeps on *EPAS1* and *EGLN1*) was then inferred as the main factor that significantly increased the frequency of the genetic determinants of this favorable trait in Tibetan and Sherpa populations (Beall et al., 2010; Simonson et al., 2010; Yi et al., 2010; Lorenzo et al., 2014; Peng, 2017). Moreover, a considerable quantity of research that relies on genome-wide data has been conducted to search for other loci which show signatures of adaptive evolution in response to high-altitude selective pressures, but no genes other than EPAS1 and EGLN1 have been successfully replicated in multiple studies (Bigham et al., 2010; Xu et al., 2011; Jeong et al., 2014; Hu et al., 2017; Yang et al., 2017).

Despite the fact this knowledge regarding Tibetan and Sherpa adaptation to high-altitude appeared to be a closed chapter according to the picture described above, we strongly believed that a smooth erythropoietic response to hypoxia represents only some kind of indirect adaptive trait that decreases susceptibility to CMS, but which could not explain how Himalayan people are able to exploit the low oxygen levels available for cells and tissues at altitude to the point of performing so well in such a challenging environment. In particular, we hypothesized that the multifaceted adaptive phenotype observable in these populations cannot be the result of a limited number of selective sweeps, but rather it was achieved through a weak but pervasive natural selection on many genes that interact with each other and contribute to the same biological function. This model of *polygenic adaptation* was proposed by some scholars as the main mode of action of natural selection on human populations (Pritchard & Di Rienzo, 2010; Pritchard *et al.*,

2010; Hernandez *et al.* 2011; Schrider & Kern 2017), but unfortunately until very recent times, no statistical approaches were available to draw inferences about it using genetic data. This was due to the fact that each genetic variant subjected to natural selection is expected by such a model to individually exert a limited effect on the phenotype, being thus hardly detectable by means of traditional selection scans designed according to the *hard sweep* model (Jeong & Di Rienzo, 2014).

Therefore, at the very beginning of our project, we formulated a scientifically sound hypothesis, but we were lacking both data and methods to properly test it. In fact, genome-wide SNP array data for Tibetan and Sherpa populations were already published in 2015, but they implemented mainly moderate-to-high frequency genetic variants, which unlikely play a substantial role in polygenic adaptive mechanisms. We thus planned to collect new DNA samples to be submitted to whole genome sequencing in order to obtain information also concerning low-frequency and small-effect variants that presumably constitute the adaptive haplotypes when polygenic adaptation occurs. Moreover, we aimed to focus our research on Sherpa people from Nepal rather than on populations from the Tibetan Plateau because Prof. Anna di Rienzo and Dr. Choongwon Jeong from the University of Chicago informed us that they were collecting evidence for a certain degree of admixture between Tibetan and low-altitude Chinese groups (Jeong et al., 2017). Instead, in our study, we preferred to avoid as much as possible the confounding effects of gene flow from populations that are not adapted to high altitude, which may be a relevant issue when searching for low-frequency variants that combine diversely in differing adaptive haplotypes as assumed by a model of polygenic adaptation. To this end, we contacted Mr. Davide Peluzzi and Mr. Giorgio Marinelli, the founders of the ExPlora Nunaat International nonprofit organization, a group of Italian mountaineers and volunteers that since 2011 have been organizing humanitarian expeditions in the Rolwaling Himal, a remote high-altitude valley located in the Nepalese Gaurishankar Conservation Area and separated from the Tibetan Plateau by Himalayan peaks of around 7,000 m above sea level (a.s.l.). At that time, the ExPlora team had already established a successful collaboration with the Nepalese organization Mount Everest Summitter's Club, and especially with Mr. Phurba Tenjing Sherpa, one of the most renowned professional mountaineers and Himalayan Guides of Nepal, who was native from the Rolwaling Himal.

In the autumn of 2015, I thus had a first opportunity to join a one-month expedition led by the ExPlora team and by covering a trekking route of more than 170 km we succeeded in collecting DNA samples and physiological parameters from the Tamang and Sherpa communities who inhabit the Rolwaling valley respectively at low/medium altitude in the jungle (no more than 2,000 m a.s.l.) and at the high-altitude villages of Beding (3,690 m a.s.l.) and Na (4,180 m a.s.l.). We then generated mitochondrial DNA, Y-chromosome and genome-wide SNP array data from these samples to draw preliminary inferences about the demographic history of these human groups. This laid the foundation for the PhD project of Dr. Guido Alberto Gnecchi-Ruscone, which was carried out at the University of Bologna under my supervision, and for an explorative study aimed at testing the hypothesis of appreciable genetic isolation experienced by the Rolwaling Sherpa people. In fact, conversely to what had been previously observed for Sherpa from the well-known Nepalese region of Khumbu (i.e. the valleys that rise up to Mt. Everest), we provided evidence supporting a very limited gene flow from outside the Rolwaling Himal, which distributes along a low- to high-altitude cline of geographical isolation (Gnecchi-Ruscone *et al.*, 2017). In detail, we

demonstrated that compared to Tibetans, Rolwaling Sherpa have been subjected to long-term isolation and show a reduced effective population size. Moreover, we detected genetic signatures of historical admixture with groups of South Asian ancestry and, in particular, we found a close affinity to the low-altitude Tibeto-Burman tribe of Nagas from Northeastern India only in Tamangs, but not in the Sherpa. This suggested that despite having originated from the same proto-Tibeto-Burman ancestors, these populations diverged anciently, likely before the occupation of high-altitude environments by Tibetans and Sherpa (Gnecchi-Ruscone *et al.*, 2017).

These findings were thus pivotal in guiding the continuation of our project. On the one hand, they corroborated our belief that Rolwaling Sherpa represent an ideal case study for investigating the occurrence of adaptive events during the evolutionary history of high-altitude Himalayan populations because they embody the best present-day proxy of the ancestral gene pool that early experienced altitude-related selective pressures. On the other hand, they pointed to Tamangs as an invaluable *negative* (i.e. not adapted to altitude) control population that could be included in our study due to their common ancestry shared with Tibetans and Sherpa but coupled with long-term occupation of low-altitude environments. According to this rationale, we conceived an experimental design based on the comparison between genomic profiles of high-altitude Himalayan people and of closely related low-altitude East Asian populations (i.e. Tamangs and Han Chinese), which was specifically designed to test the hypothesis of polygenic adaptation in response to hypobaric hypoxia in the former but not in the latter groups (Gnecchi-Ruscone et al., 2018). For this purpose, in 2017, I joined a second expedition organized by the ExPlora team in the Rolwaling Himal to collect additional DNA samples. Due to the budget constraints of the project, few of these samples were used to generate whole genome sequence data, but which when combined with those made available in the meantime in literature were essential to properly perform haplotype-based selection scans. The remaining samples were instead used to generate SNP array data, which enabled us to enlarge our already available dataset with the aim of validating the results obtained by the analysis of a dozen genome sequences on a considerably larger sample size.

However, assembling these *discovery* and *replication* datasets would not have been enough to draw inferences about potential polygenic adaptive mechanisms had we limited our study to the application of traditional neutrality tests. Luckily, a new analytical method that well suited our purposes was developed in 2017 just a few months before the launch of our sampling campaign. This approach relies on scores computed for multiple neutrality statistics to reconstruct networks of genes simultaneously targeted by natural selection and it can be used to explicitly test whether selective pressures have acted in a population on specific subparts of a biological pathway (thus modifying a given function) rather than on single genes that are not functionally related to each other (Gouy *et al.*, 2017). This was exactly what we needed to escape from the classical viewpoint that centered on the concepts of hard/soft selective sweeps, which characterized the studies that indicated *EPAS1* and *EGLN1* as the sole reliable high-altitude adaptive genes. In fact, by implementing this analysis, we succeeded in disclosing previously undetected signatures of natural selection at multiple loci belonging to the interconnected Integrin in angiogenesis, b-1 integrin, and a6-b4 integrin functional pathways. Interestingly, these gene networks were found to have been pervasively subjected to natural selection in high-altitude Himalayan people, but not in the closely related low-altitude control populations (Gnecchi-Ruscone et al., 2018). All the genes constituting

the identified networks were also known to be involved in promoting angiogenetic functions, thus plausibly playing a role in the modulation of some of the Tibetan and Sherpa physiological adjustments evolved along the oxygen transport cascade, such as increased blood flow and capillary distribution, which result in their augmented tissue blood perfusion in response to hypoxic stress (Gilbert-Kawai *et al.*, 2014).

That being so, we believe that our studies on the high-altitude adaptation of Himalayan populations have provided an unexpected contribution to the identification of the hidden genetic determinants underlying their complex adaptive phenotype. More importantly, we hope this could serve as a successful example of a research project that tried to address apparently well-known and long-studied themes by exploring neglected hypotheses and by implementing innovative methods. In our opinion, this highlights once more the importance of ideas, curiosity and creativeness as the pillars of scientific research and of how formulating scientifically sound research questions and conceiving accurate experimental designs is still an effective course of action, even in an era in which studies are weighed mainly in terms of sample size and amount of generated data.

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