

Genetic convergence in the adaptation of dogs and humans to the high altitude environment of the Tibetan plateau

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Abstract

The high-altitude hypoxic environment represents one of the most extreme challenges for mammals. Previous studies of humans on the Tibetan plateau and in the Andes Mountains have identified statistical signatures of selection in different sets of loci. Here, we first measured the hemoglobin levels in village dogs from Tibet and those from Chinese low lands. We found that the hemoglobin levels are very similar between the two groups, suggesting that Tibetan dogs might share similar adaptive strategies as the Tibetan people. Through a whole-genome sequencing approach, we have identified *EPAS1* and *HBB* as candidate genes for the hypoxic adaptation on the Tibetan plateau. The population genetic analysis shows a significant convergence between humans and dogs in Tibet. The similarities in the sets of loci that exhibit putative signatures of selection and the hemoglobin levels between humans and dogs of the same environment, but not between human populations in different regions, suggests an extraordinary landscape of convergent evolution between human beings and their best friend on the Tibetan plateau.

Introduction

High-altitude plateaus (>2,500m) are one of the most extreme living conditions on earth (Li and Zhang 2012; Zhao, et al. 2009). A formidable physiological challenge for animals is the low oxygen level. Genome-wide scans for detecting molecular signatures of high altitude adaptation have been conducted across a wide range of geographic locations (Beall, et al. 2010; Bigham, et al. 2010; Ji, et al. 2012; Peng, et al. 2011; Simonson, et al. 2010a; Xu, et al. 2011; Yi, et al. 2010). Interestingly, studies of signatures of selection among Tibetan, Andean, and high-altitude Ethiopian human populations have revealed distinct genetic components and different physical characteristics for hypoxia adaptation (Alkorta-Aranburu, et al. 2012; Bigham, et al. 2010; Scheinfeldt, et al. 2012). For example, Tibetan people are found to have similar hemoglobin levels (Hb) to people from lower areas, while people from Andes or Ethiopia have much elevated Hb levels (Alkorta-Aranburu, et al. 2012; Beall 2007; Scheinfeldt, et al. 2012), suggesting that diverse strategies that can be employed for high altitude adaptation.

As the first domesticated animal and man's closest friend, domestic dog (*canis familiaris*) has followed human beings and travelled across a wide variety of ecological niches with us (Clutton-Brock 1995; Pang, et al. 2009; Savolainen, et al. 2002). Previous research found that convergent evolution driven by similar environments seems to have shaped both of our genomes at multiple phenotypes (Wang, et al. 2013). How the two species response to the high altitude environment poses an interesting and important question.

Using a SNP genotyping strategy, one previous research provided the first glimpse on the genetic mechanisms for high-altitude adaptation of Tibetan Mastiffs. (Li, et al. 2014). However, considering the sparse spacing in the canid SNP array (average distance is 70 kb), the survey of the adaptive mechanisms might be quite incomplete. In the present study, we want to take a whole genome sequencing approach and wish to learn the adaptive strategy of Tibetan village dogs at both the phenotypic and genic level and to compare the high-altitude adaptation between humans and dogs.

Results

Hemoglobin concentrations.

Firstly, we measured the hemoglobin level in the peripheral blood of 141 dogs from two high-altitude dog populations: Tibetan dogs in Lijiang (~2700-m altitude) and Yushu (~3500-m altitude), and two low-altitude dog populations: breed dogs in Beijing (~50-m altitude) and Guangzhou (~50-m altitude). Statistical analysis reveals that the hemoglobin levels of Tibetan dogs from high-altitude areas are similar to breed dogs from low-altitude area (Fig. 1). The average hemoglobin concentrations are 160 and 161 g/L for Tibetan dogs and breed dogs, respectively. These concentrations are quite close to the canine hematology reference value (137.7-203.8) (Moritz, et al. 2004). The observation suggests Tibetan dogs appear to share the same strategy as Tibetan people.

Population sampling and sequencing

In order to identify possible signatures of positive selection in the genomes of high-altitude dogs, we carried out whole-genome sequencing on 10 individuals from four different populations of dogs. They are 1) two Tibetan indigenous dog populations (TID1 and TID2), both being from the Tibetan plateau and living in an elevations of more than 3,500 meters; 2) Chinese indigenous dogs (CID), and 3) a mixture of modern dog breeds (DB); the latter two being from provinces across China at altitudes of below 2000 meters (Supplementary Materials and Methods). Genomic DNA samples of each population were pooled equally and sequenced with Illumina GAII platform, resulting in 248 to 317 million raw reads for each population. After mapping the short reads to the reference genome (version CanFam2, May 2005) (Lindblad-Toh and Wade 2005), we were able to cover the dog genome for about 8.9 to 15.9-fold per population (Table 1). By a set of stringent criteria, 2.4 million high quality single nucleotide polymorphisms (SNPs) were extracted. Adjacent SNPs were separated by a median distance of 272 bp (mean distance of 907 bp) that facilitates evolutionary analyses. In order to measure the accuracy of site frequency on the pooled strategy, 39 SNPs were selected and individually sequenced using Sanger technology in all 40 individuals (Tables S1 and Tables S2). Correlation analysis found

that allele frequencies calculated from the pooled sample match with the results from the individual sequencing (Figures S1). The significant correlation observed in the individual and pooled sequencing suggests that pooled strategy provides a cost-effective approach for extracting information about allele frequencies from a population (Supplementary Notes 1).

Genomic regions with high population differentiation

We employed the F statistic (F_{st}) to look for genomic regions with high population differentiation (Akey, et al. 2002; Weir and Cockerham 1984). While population demographic histories affect gene frequencies at all loci similarly, positive selection on the other hand will increase allele frequency at some loci in some populations only, leading to much elevated F_{st} value (Barreiro, et al. 2008). Using allele frequencies extracted from sequence reads, we calculated F_{st} values in all four high-low population comparisons (Akey, et al. 2002; Weir and Cockerham 1984). $F_{st}(TID1, CID)$, $F_{st}(TID1, DB)$, $F_{st}(TID2, CID)$, and $F_{st}(TID2, DB)$ across all 2.4 million SNPs distributed over 38 autosomes were calculated using the Weir and Cockerham (1984) method (Figures S2)^{13,14}. Chromosome X was not included because of a different effective population size (Akey, et al. 2002). In order to combine multiple values of F_{st} , we calculated the root mean square of F_{st} : $F_{st}(rms)$ based on all four comparisons. Using a stringent cutoff of 0.8, we identified 437 high F_{st} SNPs (~0.018%, Table S3), of which 257 of them located in genic regions over a total of 20 genes (Fig. 2A and Tables S4).

In an alternative measure, we performed a Fisher's exact test, as a second empirical outlier analysis, combining the Tibetan dogs (TID1+TID2) as the case population and treating the other lower altitude dogs (CID+DB) as a control group. This provides a complementary measure of allele frequency differentiation between the two phenotypically distinct groups. Using the approach, we identified 469 polymorphic sites showing statistical evidence of positive selection (Table S5). Among them, 277 were found in genic regions distributed over 34 genes (Fig. 2B and Tables S6).

In summary, 14 genes in 12 genomic regions pass the threshold using both the F_{st} value and Fisher's exact test. Among them, endothelial PAS domain protein 1 (*EPAS1*) and hemoglobin beta (*HBB*) harbor non-synonymous mutations as shown in Tables S7.

The most prominent one is *EPASI*, with 225 SNPs in a region of 145 kb that significantly differentiate Tibetan and low land dogs. Four nonsynonymous mutations were founded on *EPASI*: G305S ($F_{st}(rms) = 0.95$; $P_{FET} = 4.47E-07$), D494E ($F_{st}(rms) = 0.93$; $P_{FET} = 8.83E-11$), V500M ($F_{st}(rms) = 0.95$; $P_{FET} = 1.06E-09$) and P750S ($F_{st}(rms) = 0.92$; $P_{FET} = 2.77E-07$). We chose G305S for a more extensive survey on a larger sample of 61 Tibetan dogs from Tibet and 149 indigenous dogs from low-altitude areas. The frequency of the mutant allele at the *EPASI* locus is 95% in Tibet but only 6% among the low-land dogs ($P = 1.14E-70$). A second noteworthy gene, *HBB*, is the hemoglobin beta gene, which functions in the oxygen transport from the lung to various peripheral tissues (Mairbaurl and Weber 2012; Storz and Moriyama 2008; Wang 1961). Two nonsynonymous mutations were found in *HBB*: G14S ($F_{st}(rms) = 0.87$; $P_{FET} = 3.19E-08$) and L15M ($F_{st}(rms) = 0.92$; $P_{FET} = 1.94E-11$), which was also strongly altitude-dependent by the extensive survey (86.4 % in Tibet vs 17.5% on the low lands, $P = 3.27E-41$). Interestingly, the L15M mutation is predicted to be probably damaging with a score of 0.996 based on polyphen-2 (Adzhubei, et al. 2010), which could influence Hb-O₂ affinity (Natarajan, et al. 2013).

The *EPASI* gene and its convergent evolution between humans and dogs

Several earlier studies have identified noncoding SNPs near *EPASI* as extreme outlier loci in genome scans in the high-altitude adaptation in Tibetan human populations (Beall, et al. 2010; Peng, et al. 2011; Xu, et al. 2011; Yi, et al. 2010). This gene is a member of the basic-helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription factors and play a crucial role in systemic responses to hypoxia (Lee and Percy 2011). It encodes a transcription factor involved in the induction of genes regulated by oxygen, which is induced as oxygen levels fall. Sequence comparisons found that the glycine at codon 305 is conserved across all known vertebrates as shown in Fig. 3A. Interestingly, the G305S, indicated by a red arrow in Fig. 3B, is in the G β -sheet of the PAS-B domain (Key, et al. 2009; Scheuermann, et al. 2009). This domain is located in a preformed solvent-inaccessible cavity and is the major functional domain binding to its ligands (Key, et al. 2009). The G305S mutation with a predicted functional change in the binding domain (a score of 0.998 based on polyphen-2 (Adzhubei, et al. 2010)) might be the major contributing factor for the high-altitude hypoxic adaptation.

The observation that *EPAS1* emerged as a F_{st} outlier of Tibetan dogs is surprising because human adaptation to the Andes does not appear to involve this locus. Note that the mutation frequencies in *EPAS1* are comparable in humans and dogs. The mutant allele in Tibet is 94% for dogs and 87% in humans. In low lands, the mutant frequency is low in dogs and humans, at 4.9% and 8.75%, respectively (Yi, et al. 2010). The population genetic signature shows a strong convergence between humans and dogs in Tibet as well. Figure 3C and 3D depict the regions of hitchhiking that differentiate Tibetans from lowlanders. Both regions in two species center around the *EPAS1* locus. Although the sizes differ somewhat in the two species (145 kb in dogs and 220 kb in humans) and level of frequency difference is higher in dogs and than in humans, the modest differences probably reflect the general differences in the genomes of the two species. For example, the haplotype blocks in dogs are typically much lower than in humans (<10kb for indigenous dogs (Wang, et al. 2013), ~ 60 kb in non-Africans humans (Reich, et al. 2001)). Statistical analysis revealed that the overlapping of selected segments between dogs and humans was significantly higher than expected ($P < 10E-7$, chi-square test). Meanwhile, when we compared our list of selected genes in dogs with that from humans presented in Yi et al, both the *EPAS1* and *HBB* exist in this overlapping set between two species. This level of overlapping is highly significant ($P = 4E-4$, chi-square test). This is also true when we try to overlap our list of selected genes with Simonson et al (*EPAS1* exists in both sets with a p value of 0.009) (Supplementary Notes 2). Taken together, the data present a strongly convergent pattern between humans and dogs in their adaptation to the Tibetan environment.

Discussion

Whole genome resequencing strategy offers more dense genetic markers for the population genetic analysis compared to the microarray approach (Li, et al. 2014). The Tibetan village dogs are characterized by a rapid rate of decay of linkage disequilibrium as a function of physical distance, which allowed us to perform genome-wide scans for recent positive selection for traits that are adaptive at high altitude (Karlsson, et al. 2007; Lindblad-Toh and Wade 2005). Using more than 2.4 million SNPs extracted from populations of village dogs that both lived at high

altitude on the Plateau and from lower altitudes, we successfully mapped the two most evident genes, *EPASI* and *HBB*, that could have contributed to high-altitude hypoxic adaptation in dogs. Interestingly, a recent work also indentified these two genes as candidates showing signs of high elevation adaptation based on a set of independent populations and approaches (Gou, et al. 2014), which also supports the conclusion of the present study.

Furthermore, we found that a nonsynonymous mutation on *EPASI*, G305S, might be the major contributing factor for the high-altitude hypoxic adaptation, as the codon 305 is not only conserved across all known vertebrates but also located in the PAS-B domain that is the major functional domain binding to its ligands (Key, et al. 2009). Meanwhile, *HBB* also carries a nonsynonymous mutation, L15M, that could potentially contribute to the hypoxic adaptation. Several previous studies in other high-altitude species showed that amino acid mutations in the alpha- and/or beta-globin genes can alter Hb-O₂ affinity, for example Andean hummingbirds (Projecto-Garcia, et al. 2013) and deer mice ([Natarajan, et al. 2013](#); [Storz, et al. 2010](#); [Storz, et al. 2009](#)). If we intersected the results of Fst analysis and Fisher's exact test approaches, the *EDNRA* gene (Endothelin-1 receptor Precursor) shows up as an another candidate selected gene involved in the HIF pathway and is also found in Tibetans presented in Simonson et al. (2010).

Interestingly, several previous studies suggested that *EPASI* aided hypoxic adaptation in Tibetan human populations (Beall, et al. 2010; Simonson, et al. 2010b). Surprisingly, studies looking at high altitude adaptation in people living around the Andes mountains revealed rather different genetic components in the hypoxia response (Bigham, et al. 2010) with several differences in physiological traits from the Tibetan regions (Beall 2007). These conclusions collectively seem to suggest that humans and dogs in the Tibetan plateau have adapted to the high altitude environment by modifying a set of similar genes and pathways. Through evolutionary analysis, we found that there are significant convergence between human and dogs at genetic level on the Tibetan plateau. The convergent adaptive evolution between dogs and humans in the same environment in the absence of such convergence between different human populations in different regions suggests that the shared environment may have played a pivotal role (Wang, et al. 2013). For example, sharing much of the diet and

complex interactions between human and dogs might have shaped both species adapting to the common environment. Similarly, the differences between Andeans and Tibetans might be due to dissimilarities between local ecological pressures in their respective geographic locations. Differences in genetic background in the two human populations might have also contributed to this observed disparity. As we look further into many organisms living in Tibetan regions and other high altitude environments, we might unveil a very intriguing array of successful adaptations with diverse mechanisms as well as a new understanding of the convergent evolution between human beings and their closest animal companions. These results shed new light on the field of convergent evolution and offers opportunities to gain new insights into high altitude settlement shared by dogs and humans.

Materials and Methods

Measuring hemoglobin level

The peripheral bloods were collected for measuring hemoglobin level using in four populations at the stated elevations: 9 Tibetan dogs in Yushu (~3500 meters altitude), 56 Tibetan dogs in Lijiang (~2700 meters altitude), 52 breeds dogs in Beijing (~50 meters altitude), and 24 breed dogs in Guangzhou (~50 meters altitude), respectively. The dog populations that measured hemoglobin level are different from those used in the population genomic survey.

Populations and resequencing

Four dog populations were collected for this study: 1) Tibetan dogs living in Tibet and Qinghai province at elevations of about 3,500 meters; 2) population of indigenous dogs gathered from other provinces of China with elevations lower than 2000 meters; and 3) a population of modern dog breeds mixed by Chow Chow, Rottweiler, Chihuahua, and Saint Bernard. The genomic DNA was extracted using the Phenol/Chloroform method from peripheral venous blood. For each population, genomic DNA for 10 individuals was pooled together equally. Note that 3 chow chows, 3 Rottweilers, 3 Chihuahuas and 1 Saint Bernard were mixed as one population to represent modern dog breeds. The Illumina Genome Analyzer GAII was used to generate paired-end reads for each population pool according to standard protocols specified by the manufacturer. The read length of raw sequence data was

shown on Table 1.

Reads Mapping and SNP calling

After trimming low-quality bases, paired-end reads were aligned to the dog genome assembly (CanFam2)(Lindblad-Toh and Wade 2005) using Burrows-Wheeler algorithm implemented in BWA-short module with default parameters (Li and Durbin 2009). SAMtools was subsequently used to call raw SNPs (Li, et al. 2009) with these four conditions: 1) The base quality is of no less than 20; 2) the mapping quality is no less than 20; and 3) the coverage is no less than 8 , 4) the coverage is no more than 100.

Population differentiation

We estimated the allele frequency of each population by counts of different alleles in the sequencing pool. F_{st} is calculated using the method from Weir and Cockerham (1984)(Akey, et al. 2002; Weir and Cockerham 1984). The root mean square (RMS) of F_{st} was calculated as

$$F_{st}(rms) = \sqrt{\frac{1}{4} (F_{st}(TID1, CID)^2 + F_{st}(TID1, DB)^2 + F_{st}(TID2, CID)^2 + F_{st}(TID2, DB)^2)}$$

Fisher's exact test was carried out by testing each position against their population grouping (high or low elevation). Since the number of founding chromosomes in each pool in the association studies is 40 (20 diploid genomes), in order to avoid the situation where positions with very high coverage will generate too small p-values, we used the hypergeometric distribution to project down the total coverage to 40. The average p values across multiple replicates (1000) were used as the p value for that site.

Genotyping candidate SNPs on large population

PCR primers were designed for sequencing the three SNPs in the *EPAS1* and *HBB* showing the most significant frequency differences between dogs groups. 3 regions surrounding *EPAS1* and 6 regions surrounding *HBB* were selected to measure the accuracy of site frequencies surveyed using the pooled strategy. After PCR amplification, Sanger sequence technology was employed to sequence the target regions in two population samples. The PCR primers were given in the Table S1.

Enrichment analysis

Gene orthologous relationship between human and dog was downloaded from Ensembl database (www.ensembl.org). Whole genome alignment between human and dogs was downloaded from UCSC (genome.ucsc.edu). The proportions of positively selected candidates (>100kb) in two species were both calculated. The p value was calculated as the proportion of simulated datasets that have equal or higher number of overlapped genes and segments than the observed count (1,000,000 replications).

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Figure Legends

Fig. 1. Hemoglobin levels over different *HBB* genotypes in different dog populations. Boxplot of hemoglobin levels measured in the Tibetan dogs in Yushu (~3500-m altitude) and Lijiang (~2700-m altitude), and breed dogs in Beijing (~50-m altitude) and Guangzhou (~50-m altitude).

Fig. 2. Genome-wide association mapping of high-altitude adaptation in dogs. (A) The distribution of Root Mean Square (RMS) of F_{st} along 38 autosomes. The horizontal dashed line indicates the threshold of $F_{st}(rms)$ (0.8). (B) The distribution of $-\log(P \text{ value})$ from the Fisher's exact tests after a Bonferroni correction procedure along 38 autosomes. The dashed black line indicates the cutoff significance level (0.1%).

Fig. 3. Population differentiation at the *EPAS1* gene together with its evolution and structure. (A) Sequence alignment across vertebrates surrounding the G305S mutation at the *EPAS1* protein. The two black boxes indicate two conserved β sheet regions in the PAS-B domain. (B) Structural model of the C-Terminal PAS-B domain of *EPAS1* (PDB ID: 1P97) plotted using the Cn3D software (Wang, et al. 2000). The PAS-B domain (Erbel, et al. 2003) adopts a typical α/β PAS domain fold with several α -helices flanking a five-stranded anticonvergent β sheet (Erbel, et al. 2003). Circles indicate the locations of the ligand-binding sites (Erbel, et al. 2003; Scheuermann, et al. 2009) and the mutation G305S is marked with a red tick. (C) Genetic association across a 800-kb region surrounding the *EPAS1* locus in Tibetan dogs. (D) Genetic association for a 800-kb region surrounding the *EPAS1* locus in Tibetan people from human genotype data (Beall, et al. 2010).

Tables

Table 1. Whole-genome resequencing results for four dog populations.

Populations	Reads Length	Number of Reads (M)	Total Bases	Matching Reads (M)	Matching Bps	Effective depth (fold)
Tibetan dog population 1 (TID1)	171	317.26	54.25	292.89	38.01	15.94
Tibetan dog population 2 (TID2)	125	361.45	45.18	321.83	36.76	15.41
Indigenous dog from low-altitude area (CID)	121	248.52	30.07	209.27	21.27	8.92
Breed dogs (DB)	125	296.68	37.08	269.25	36.62	15.35

Figure 1

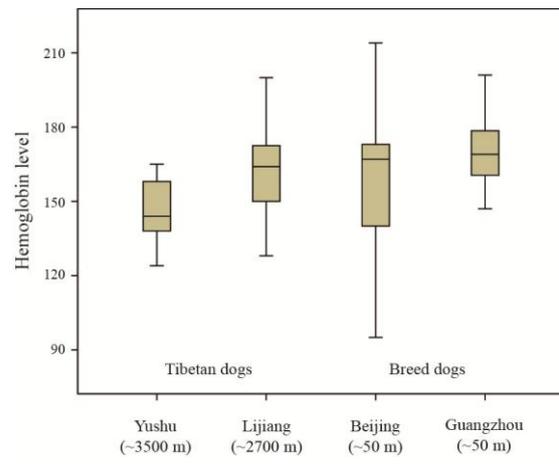


Figure 2.

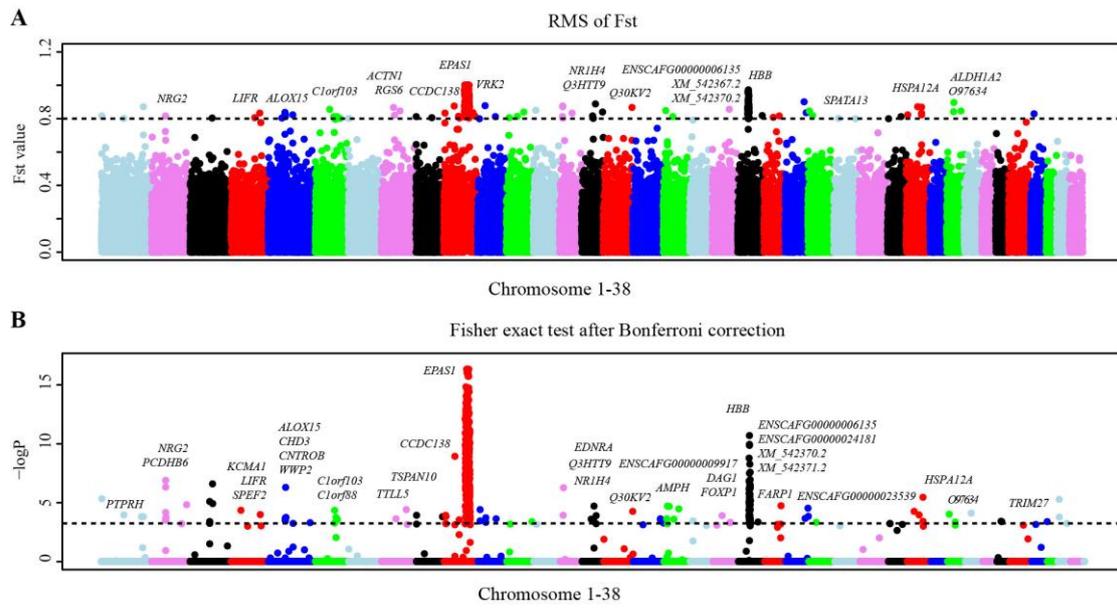


Figure 3

