Polymorphisms in the Egl nine homolog 3 (EGLN3) and Peroxisome proliferator activated receptor-alpha (PPARα) genes and their correlation with hypoxia adaptation in Tibetan chickens

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Abstract

Peroxisome proliferator activated receptor-alpha (PPARα) and Egl nine homolog 3 (EGLN3) play critical roles in facilitating the adaptation to a hypoxic environment. However, the relationship between EGLN3 and PPARα variants and hypoxic adaptation remains poorly understood in Tibetan chickens. To better understand the effects of genetic variation, we sequenced exons of PPARα and EGLN3 in 138 Lowland chickens (LC) from 7 breeds that were located in Emei, Miyi, Shimian, Wanyuan, Pengxian, and Muchuan in the Sichuan province, and Wenchang in the Hainan province (altitudes for these locations are below 1800 meters). Total 166 Tibetan chickens (TC) from 7 subpopulations that were located in Shigatse, Lhoka, Lhasa, Garze, Aba, Diqing and Yushu in the Tibetan area were also sequenced (altitudes greater than 2700 meters). One single-nucleotide polymorphism (rs316017491, C>T) was identified in EGLN3 and was shared by TC and LC with no significant difference for allele frequencies between them (P>0.05). Six single-nucleotide polymorphisms (SNP1, A29410G; SNP2, rs13886097; SNP3, T29467C; SNP4, rs735915170; SNP5, rs736599044; and SNP6, rs740077421) including one non-synonymous mutation (SNP2, T>C) were identified in PPARα. This is the first report of SNP1 and SNP3. There was a difference between TC and LC for allele frequencies (P<0.01), except for SNP1, SNP4, and SNP5) The fix index statistic test indicated that there was population differentiation between TC and LC for allele frequencies (P<0.05). Phylogenetic analysis showed that the genetic distance among chickens, finch and great tit were close for both EGLN3 and PPARα. Bioinformatics analysis of PPARα showed that SNP2 leads to an amino acid substitution of Ile for Met, which results in the protein being more likely to be hydrolyzed. Thus, genetic variation in PPARα may play a role in the ability of TC to adapt to...
a high altitude environment; however we were unable to identify a relationship between polymorphisms in EGLN3 and environmental adaptability.

Introduction

Tibetan chickens, an aboriginal chicken breed distributed in the highland at over 3,000 m, have adapted to the harsh living conditions, characterized by cold weather, low partial pressure of oxygen and strong ultraviolet radiation [1]. Compared with the breeds that inhabit the Lowland, Tibetan chickens have more erythrocytes with enhanced oxygen affinity, richer blood vessel density and less mean corpuscular volume. All of these changes were produced by strong selection pressures during the history of domestication [2, 3], which may directly affect the genetic structure of this population.

Peroxisome proliferator-activated receptors (PPARs), as members of the nuclear hormone receptor superfamily, play a key role in energy metabolism [4]. The ability to consume oxygen and to produce adenosine triphosphate (ATP) during energy metabolism greatly influences the ability of animals to adapt to hypoxia [5]. There are three isotypes named PPARα, PPARβ, and PPARγ. PPARα is the main regulator of lipid metabolism [6]. Carbohydrate and lipid metabolism are two important components of energy metabolic pathways. Animals utilize one as an optimal-fuel strategy to cope with cold hypoxic environments [7]. Previous studies indicated that the genes undergoing positive selection in the ground tit on the Tibetan plateau were mostly involved in fatty-acid metabolic pathways [8]. Most animals use fatty acids as energetic substrate, as mitochondrial β-oxidation contributes to energy production via oxidative phosphorylation, thereby generating ATP [5, 9]. The activated-PPARα modulates this pathway by up-regulating the gene expression of some key factors such as fatty acid transporter protein (FATP), carnitine palmitoyl transferase I (CPT I), and acetyl-CoA synthetase (ACS) [10].

In addition to energy metabolic factors, the hypoxia-inducible factor-1α (HIF-1α) is vital to oxygen regulation. Egl nine homolog 3 (EGLN3), also called proline hydroxylase domain 3 (PHD3), controls the expression of the HIF-1α gene [11, 12]. When oxygen is present, PHD3 hydroxylates specific proline residues on HIF-1α, initiated by von Hippel-Lindau protein (pVHL), leading to ubiquitination and destruction of the HIF-1α protein. In a hypoxic environment, the activity of EGLN3 decreased, leading to accumulation of HIF-1α and formation of erythrocytes, which improved oxygen transportation [13].

We hypothesized that sequence variation in PPARα and EGLN3 genes may contribute to the adaptation to hypoxic conditions in Tibetan chickens. Thus, we identified SNPs in the coding sequences of each gene in Tibetan chicken (TC) and Lowland chicken (LC)s and examined their association.

Materials and methods

Sampling and DNA extraction

In total, 304 blood samples were collected from 7 highland locations in Qinghai, Tibet, and Yunnan, and the Sichuan province, including Shigatse, Lhoka, Lhasa, Garze, Aba, Diqing, and Yushu, and 7 lowland native chicken breeds in Emei, Miyi, Shimian, Wanyuan, Pengxian, and Muchuan in the Sichuan province and Wenchang in the Hainan province (Fig 1). Blood was
collected from the brachial vein and genomic DNA was extracted via the phenol-chloroform method [14]. The altitude, longitude, latitude, and population size of each location are shown in Table 1.

Sampling occurred on local farms with owner permission. All procedures for sample collection were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University under permit number DKY- S20163651.
DNA amplification and sequencing

Primer pairs flanking the coding region of exons were designed by Primer Premier 6.0 [15]. The details for these primers are summarized in Tables 2 and 3. PCR was performed in 25 μL reactions that contained 50 ng DNA template, 1× buffer (including 1500 μmol L⁻¹ Mg²⁺Cl², 200 μmol L⁻¹ dNTPs, and 1.5 U of Taq DNA polymerase) and 1 μmol L⁻¹ of each primer. Cycling parameters were as follows: initial denaturation at 96˚C for 4 min, followed by 35 cycles of 95˚C for 30 s, then annealing (temperatures provided in Tables 2 and 3) for 1 min, and 72˚C for 90 s, and a final extension at 72˚C for 10 min. PCR products were sequenced in both directions by the Beijing Genomics Institute (BGI).

Table 1. Altitude, longitude, and latitude of the sampling locations for 7 Tibetan subpopulations and 7 Lowland chicken breeds.

| Populations | Sampling locations | Sample sizes | Altitude (m) | Longitude (E) | Latitude (N) |
|-------------|--------------------|--------------|--------------|---------------|--------------|
| Tibetan chicken (TC) |                     |              |              |               |              |
| Shigatse (RKZ)  | Shigatse, Tibet     | 11           | 3900         | 89.60         | 28.92        |
| Lhoka (SN)      | Lhoka, Tibet        | 24           | 3700         | 90.03         | 28.27        |
| Lhasa (LS)      | Lhasa, Tibet        | 28           | 3650         | 91.01         | 29.26        |
| Garze (GZ)      | Garze, Sichuan      | 7            | 3390         | 99.22         | 28.34        |
| Aba (AB)        | Aba, Sichuan        | 25           | 3300         | 102.33        | 31.27        |
| Diqing (DQ)     | Diqing, Yunnan      | 19           | 3280         | 99.53         | 28.08        |
| Yushu (YS)      | Yushu, Qinghai      | 52           | 2700         | 96.6          | 33.2         |
| Lowland chicken (LC) |                |              |              |               |              |
| Emei (EM)       | Emei, Sichuan       | 9            | 1800         | 103.41        | 29.49        |
| Miyi (MY)       | Panzhihua, Sichuan  | 21           | 1400         | 101.45        | 26.45        |
| Shimian (SM)    | Yaan, Sichuan       | 27           | 1120         | 102.13        | 29.40        |
| Jiuyuan (JY)    | Wanyuan, Sichuan    | 15           | 900          | 108.21        | 31.84        |
| Pengxian (PX)   | Yaan, Sichuan       | 30           | 600          | 102.98        | 29.98        |
| Muchuan (MC)    | Muchuan, Sichuan    | 16           | 540          | 103.90        | 29.02        |
| Wenchang (WC)   | Wenchang, Hainan    | 20           | 10           | 110.87        | 19.72        |

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Table 2. Primer information for detecting SNPs in PPARα coding regions.

| Names | Target regions | Primer sequences (5’-3’*) | Product length (bp) | Annealing temperature (˚C) |
|-------|----------------|---------------------------|---------------------|---------------------------|
| P1    | Exon1          | F: ACCTGTCAGGAGATTCACATT  | 687                 | 50.5                      |
|       |                | R: AAGGAGGCTTACAGACTCAT   |                     |                           |
| P2    | Exon2          | F: GCTATGATTATCCACTACGTGAC| 702                 | 58.4                      |
|       |                | R: ATGGCTCTTGGATGAA       |                     |                           |
| P3    | Exon3          | F: CTCAAGGCTTCCAGTTTCTT  | 727                 | 57.8                      |
|       |                | R: GCAAGCACAATACAGAT     |                     |                           |
| P4    | Exon4          | F: TCATCATGTCAGTCTGAGT   | 605                 | 53.6                      |
|       |                | R: CCTACTATAACTTAGGGCTCTT|                     |                           |
| P5    | Exon5          | F: ACACAGGGAGTTCAGAGTG  | 727                 | 54.6                      |
|       |                | R: CACCAACTTCTTTTACTTTCC |                     |                           |
| P6    | Exon6          | F: TTACTGAAGACGGTATTG   | 655                 | 56.6                      |
|       |                | R: AGCCTCCAGTACTACTAGC  |                     |                           |

1 The forward sequence of the primer.
2 The reverse sequence of the primer.

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Table 3. Primer information for detecting SNPs in EGLN3 coding regions.

| Names | Target regions | Primers sequence (5'-3') | Production length (bp) | Annealing temperature (˚C) |
|-------|----------------|--------------------------|------------------------|---------------------------|
| E1    | Exon1          | F1: CAAGATGCGGTGGAAGTG   | 687                    | 50.5                      |
|       |                | R1: CTGGTATTGAGGAGAAGAT  |                        |                           |
| E2    | Exon2          | F: AGGCTGTGACTAGTCATA   | 702                    | 58.4                      |
|       |                | R: AGGAGAGCTCTAGACAGC   |                        |                           |
| E3    | Exon3          | F: CCAGTGGTGTCTATAC   | 727                    | 57.8                      |
|       |                | R: ATCTGATGTTGTTAGGAG   |                        |                           |
| E4    | Exon4          | F: CCTCATGACCACCTGTTC | 605                    | 53.6                      |
|       |                | R: ACACTCTACATTACACTAACG|                        |                           |
| E5    | Exon5          | F: GGAGCAGAAGGGAGAACTAT | 727                    | 54.6                      |
|       |                | R: CCAGCACTTTACTTCAGAT  |                        |                           |

1 The forward sequence of the primer.
2 The reverse sequence of the primer.

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Sequence data analysis

Sequence variations, including nucleotide composition and locations were identified by MEGA 5.10 [16]. The sequences were edited and aligned by DNAsStar [17]. PPARα and EGLN3 genome sequences of Chinese red jungle fowl that were obtained from NCBI GenBank (NC_006088.4 and NC_006092.4) were used as the reference sequences. Allele frequencies of EGLN3 and PPARα genes in TC and LC groups were analyzed by Pearson’s Chi-square tests. These parameters were calculated using SPSS software Version 22 and \( P < 0.05 \) was considered significant. We used Arlequin 3.5 to calculate \( F_{st} \) and analyzed population genetic differentiation [18]. Phylogenetic analysis of nucleotide sequences was carried out by MEGA, J modeltest, BEAST2 and Figtree. J modeltest was used to estimate the best model for establishing the Phylogenetic tree and we used BEAST2 to construct the Phylogenetic tree. Figtree and MEGA were used to embellish the tree. The nucleotide sequences of EGLN3 and PPARα in 8 representative vertebrates were used to construct the Phylogenetic tree and were retrieved from Ensembl [19].

Protein secondary and tertiary structure prediction

Protein structure was predicted using SWISS-MODEL (https://www.swissmodel.expasy.org/). DNAsStar was used for analyzing hydrophilicity. The complete genome of Cochin-Chinese Red Jungle Fowl was used as the reference sequence (ENSGALG00000041470).

Results

Sequence variations in EGLN3 and PPARα

One SNP (rs316017491, C > T) was identified in EGLN3. Allele frequencies of EGLN3 in TC and LC groups are shown in Table 4. The distribution of this SNP in each population is shown in Fig 2A and Table A in S1 File. The SNP is a synonymous substitution (Table 5). Pearson’s chi-square test results showed that there was no significant difference between TC and LC for allele distribution (\( P > 0.05 \)).

Six SNPs were identified in PPARα. Their allele frequencies in groups TC and LC are shown in Table 4. This is the first report of SNP1 and SNP3. Distributions of six SNPs in each subgroup are shown in Fig 2B–2G and Tables B-G in S1 File. One non-synonymous mutation (rs13886097, T > C) and five synonymous mutations were found in PPARα (Table 5). All
SNPs were observed in both TC and LC. That the minor allele frequency of all loci was greater than 1% suggests that mutation sites are ubiquitous. There were significant differences in allele frequencies between TC and LC for SNP2, SNP3, and SNP6 ($P < 0.01$), whereas there were no significant difference in allele frequencies between TC and LC for SNP1, SNP4, and SNP5 ($P > 0.05$).

Hardy-Weinberg equilibrium (HWE) test results showed that except for SNP1, SNP2, and SNP3 of $PPAR\alpha$, the other SNPs were consistent with HWE in TC groups ($P > 0.05$), whereas there was no SNP consistent with HWE ($P < 0.01$) in LC (Table 6). The observed heterozygosity of all SNPs was from 0.079 to 0.475 in TC and from 0 to 0.63 in LC.

Population genetic differentiation
Fix index statistic test ($F_{ST}$) values for each SNP locus of $EGLN3$ and $PPAR\alpha$ are displayed in Table 7. There was population differentiation between groups TC and LC for SNP2, SNP3, and SNP6 of $PPAR\alpha$ ($P < 0.05$), while for other SNPs there were enough heterozygotes in the metapopulation ($P > 0.05$). Further analysis for SNP2, SNP3, and SNP6 indicated that the variation mainly occurred in the interior of the population ($P < 0.05$) and their values were 77.75%, 81.27%, and 96.18%, respectively (Table 8).

Phylogenetic tree
Nucleotide sequences of $EGLN3$ from mouse, cow, horse, macaque, dog, chicken, great tit, and finch were used in phylogenetic analyses. Results showed that the phylogenetic tree was generally divided into two branches. One branch contains the chicken, great tit, and finch and

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Table 4. Allele frequencies of mutation loci in $EGLN3$ and $PPAR\alpha$ genes.

| Genes | SNP | Allele distribution | P-Value | MAF |
|-------|-----|---------------------|---------|-----|
|       |     | Allele | TC $^1$ | LC $^2$ |       |
| $EGLN3$ | SNP rs316017491 | C | 183(0.614) | 143(0.572) | 0.361 | 0.40511 |
| $PPAR\alpha$ | SN1 (A29410$^4$G) | A | 243(0.929) | 221(0.944) | 0.596 | 0.06262 |
|       | SNP rs13886097 | T | 54(0.215) | 24(0.103) | 0.001$^{**}$ | 0.16049 |
|       | SNP3 (T29467C) | C | 198(0.785) | 210(0.897) | 0.000$^{**}$ | 0.09053 |
|       | SNP4 rs735915170 | T | 267(0.862) | 190(0.896) | 0.293 | 0.12452 |
|       | SNP5 rs736599044 | A | 264(0.852) | 190(0.890) | 0.176 | 0.13027 |
|       | SNP6 rs740077421 | C | 267(0.861) | 199(0.939) | 0.008$^{**}$ | 0.10727 |

$^1$TC Tibetan chickens. $^2$LC Lowland chickens. $^3$The figures in brackets represent allele frequencies. $^4$The number represents the SNP position in the DNA sequence. $^5$Pearson’s Chi-square test. $^6$MAF represents the minor allele frequency. $^{**}$P-value less than 0.01.

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### Fig 2. Allele frequencies of the SNPs scanned in genes **EGLN3** and **PPARα** for the populations at different altitude locations.

(A) Pattern of allele frequencies at the SNP in **EGLN3**. "C" and "T" represent the frequencies of the ancestral and mutant alleles of the candidate SNP in each chicken population, respectively.

(B) Pattern of allele frequencies at the SNP1 in **PPARα**. "A" and "G" represent the frequencies of the ancestral and mutant alleles of the candidate SNP in each chicken population, respectively.

(C) Pattern of allele frequencies at the SNP2 in **PPARα**. "T" and "C" represent the frequencies of the ancestral and mutant alleles of the candidate SNP in each chicken population, respectively.

(D) Pattern of allele frequencies at the SNP3 in **PPARα**. "T" and "C" represent the frequencies of the ancestral and mutant alleles of the candidate SNP in each chicken population, respectively.

(E) Pattern of allele frequencies at the SNP4 in **PPARα**. "T" and "A" represent the frequencies of the ancestral and mutant alleles of the candidate SNP in each chicken population, respectively.

(F) Pattern of allele frequencies at the SNP5 in **PPARα**. "A" and "G" represent the frequencies of the ancestral and mutant alleles of the candidate SNP in each chicken population, respectively.

(G) Pattern of allele frequencies at the SNP6 in **PPARα**. "C" and "T" represent the frequencies of the ancestral and mutant alleles of the candidate SNP in each chicken population, respectively.

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### Table 5. Mutation information for **EGLN3** and **PPARα**.

| SNP locus | Genomic location | Nucleotide variant | Amino acid variant |
|-----------|------------------|--------------------|--------------------|
| **EGLN3** |                  | Wild Mutant        | Wild Mutant        |
| SNP rs316017491 | Chr5:35744822  | C T                | Phe Phe            |
| **PPARα** |                  |                    |                    |
| SNP1\(^1\) (A29410\(^2\)G) |    | A G                | Arg Arg            |
| SNP2 rs13886097 | Chr1:71358406  | T C                | Ile Met            |
| SNP3 (T29467C) |                  | T C                | Ile Ile            |
| SNP4 rs73591510 | Chr1:71360891  | T A                | Ser Ser            |
| SNP5 rs73659904 | Chr1:71692243  | A G                | Val Val            |
| SNP6 rs74007741 |                  | C T                | Thr Thr            |

\(^1\) SNP1 and SNP3 are unreported;
\(^2\) The number represents the SNP location in the nucleotide sequence.

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Table 6. Hardy-Weinberg equilibrium (HWE) tests of SNPs in *EGLN3* and *PPARα* for Tibetan chickens and Lowland chickens.

| Genes | SNP                  | Tibetan chickens |              |              | Lowland chickens |              |              |
|-------|----------------------|------------------|--------------|--------------|------------------|--------------|--------------|
|       |                      | $\chi^2$         | Pearson's $P$| $H_o$        | $H_e$            | $\chi^2$     | Pearson's $P$| $H_o$ | $H_e$ |
| *EGLN3* | SNP rs316017491      | 0.004            | 0.950        | 0.47518      | 0.47401          | 10.514       | 0.001*       | 0.63  | 0.48963 |
| *PPARα* | SNP1 (A29410G)       | 20.769           | 0.000*       | 0.07937      | 0.13192          | 42.488       | 0.000*       | 0.04274 | 0.10573 |
|        | SNP2 rs13886097      | 29.306           | 0.001*       | 0.1746       | 0.33755          | NA           | NA           | 0     | 0.18478 |
|        | SNP3 (T29467C)       | 12.223           | 0.000*       | 0.190476     | 0.27689          | NA           | NA           | 0     | 0.01784 |
|        | SNP4 rs735915170     | 0.002            | 0.964        | 0.23871      | 0.23791          | 36.559       | 1.481E-09*   | 0.075472 | 0.18637 |
|        | SNP5 rs736599044     | 0.018            | 0.893        | 0.258065     | 0.2522           | 36.559       | 1.481E-09*   | 0.075472 | 0.19701 |
|        | SNP6 rs740077421     | 1.914            | 0.167        | 0.212903     | 0.23936          | NA           | NA           | 0.122642 | 0.11456 |

* represents $P$-value of less than 0.05.

1NA means not able to be calculated. (The value of $\chi^2$ is not calculated because the frequency of a certain genotype was 0 in the Lowland chickens)

2$H_o$ represent the observed heterozygosity.

3$H_e$ represent the expected heterozygosity.

Table 7. $F_{st}$ values for the SNPs in *EGLN3* and *PPARα* for Tibetan chickens and Lowland chickens.

| Genes | SNP locus | $F_{st}$ value | $P$-value |
|-------|-----------|----------------|-----------|
| *EGLN3* | SNP rs316017491 | 0.001 | 0.286 |
| *PPARα* | SNP1 (A29410G) | -0.002 | 0.603 |
|        | SNP2 rs13886097 | 0.041 | 0.000** |
|        | SNP3 (T29467C) | 0.138 | 0.000** |
|        | SNP4 rs735915170 | -0.001 | 0.407 |
|        | SNP5 rs736599044 | 0.005 | 0.160 |
|        | SNP6 rs740077421 | 0.025 | 0.006** |

** represents $P$-value less than 0.01.

Table 8. Variance analysis of SNP2 in *PPARα* of TC and LC.

| SNP   | Source of variation | Sum of squares | Variance components | Percentage variation | Fixation indices | $P$ Value |
|-------|---------------------|----------------|---------------------|----------------------|------------------|-----------|
| SNP2  | Among groups        | 1.514          | -0.000              | -0.001               | -0.000           | 0.327     |
|       | Among populations within groups | 13.365 | 0.031 | 22.251 | 0.222 | 0.000 |
|       | Within populations  | 50.602         | 0.107               | 77.749               | 0.222            | 0.000     |
|       | Total               | 65.481         | 0.138               |                      |                  |           |
| SNP3  | Among groups        | 3.034          | 0.011               | 12.620               | 0.138            | 0.003     |
|       | Among populations within groups | 2.994 | 0.005 | 6.106 | 0.070 | 0.003 |
|       | Within populations  | 50.602         | 0.072               | 81.274               | 0.187            | 0.000     |
|       | Total               | 56.621         | 0.089               |                      |                  |           |
| SNP6  | Among groups        | 0.709          | 0.002               | 2.245                | 0.022            | 0.072     |
|       | Among populations within groups | 1.764 | 0.002 | 1.571 | 0.016 | 0.069 |
|       | Within populations  | 47.587         | 0.093               | 96.182               | 0.038            | 0.018     |
|       | Total               | 50.061         | 0.097               |                      |                  |           |

1Tibetan chickens and Lowland chickens.

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another includes the macaque, dog, horse, cow, and mouse (Fig 3A). We found that the genetic
distances of EGLN3 among chicken, great tit, and finch were close.

The same analysis of PPARα was performed on mouse, cow, horse, macaque, dog, chicken,
great tit, and finch. Similarly, the Phylogenetic tree was generally divided into two branches.
Mouse, cow, horse, dog, and macaque formed a branch and the other species constituted
another independent branch, with high homology among chicken, great tit, and finch (Fig 3B).

**Bioinformatics analysis of PPARα**

The SNP2 of PPARα resulted in an amino acid change (Ile > Met). The amino acid substitution
occurred in the ligand-binding domain (LBD). Further study of this mutation site indicated
that amino acid residues had changed (Fig 4) and that the mutated protein had higher
hydrophilicity (Fig 5).

**Discussion**

Chicken (*Gallus gallus*) is not only an important domestic bird for egg and meat production,
but also a valuable model for evolutionary and developmental biology studies [20]. Tibetan
chickens have inhabited the Tibetan plateau for thousands of years, and during that time have
developed adaptability to hypoxia [21, 22]. Mutations in DNA and changes in functionality of
proteins are responsible for these physiological adaptations to the hypoxic environment.

Herein, we analyzed the polymorphisms in EGLN3 and PPARα genes in LC and TC popula-
tions. One and six SNPs were detected in EGLN3 and PPARα, respectively. The MAF values
for all SNPs were greater than 0.05, which is of great significance. For the EGLN3 SNP, there
was no significant difference between TC and LC in allele frequencies, whereas for SNP2, SNP3, and SNP6 in PPARα, there were significant differences between TC and LC in their respective allele frequencies. The mutant allele frequencies of SNP2 and SNP3 in LC were higher than those in Tibetan chickens and Hardy-Weinberg equilibrium (HWE) test results showed that all SNPs in LC were not consistent with HWE, indicating that the genetic structure of Lowland chickens may be affected by environmental or artificial factors[23]. The fixed index is a theoretical measure of whether the actual frequency of genotypes in a population departs from the genetic balance[24]. The result of fix index statistic tests showed there was a significant difference in the Fst value for SNP2, SNP3, and SNP6 between TC and LC, demonstrating that the three sites were specific in different populations and may be candidates for high altitude hypoxia adaptability. Arlequin was used to analyze the source of variation and the result showed that variation was mainly derived from individuals. These results suggest that geographic isolation among these groups diminished gradually, and likely did not play a major role in the genetic differentiation among populations [23].

Phylogenetic analyses showed that genetic relationships among chicken, great tit, and finch are close for EGLN3 and PPARα, which is consistent with the results of zoological classification [25]. This homology represents the proximity of species relationship, reflecting the importance of the structural stability of the EGLN3 and PPARα gene among species.

Bioinformatics analyses indicated that except for SNP2 in PPARα, the other SNPs were synonymous mutations. Although synonymous mutations do not cause structural variation in the protein, it can change the amount of expression and modulate the translation efficiency of the downstream target protein [26].

![Fig 4. Three dimensional modeling of the amino acid sequence for PPARα. One non-synonymous mutation (Ile > Met) was identified.](https://doi.org/10.1371/journal.pone.0194156.g004)
In the present study, we identified one non-synonymous mutation at SNP2 (Ile > Met). The variation occurred in the ligand-binding domain (LBD), which contributes to the dimerization interface of the receptor and in addition, binds co-activator and co-repressor proteins [27].

The PPARα protein is highly hydrophobic [28], but the mutation detected in the present study increased its hydrophilicity and made it more likely to be hydrolyzed. As activated-PPARα modulates lipid metabolism by up-regulating the expression of key genes such as fatty acid transporter protein (FATP), carnitine palmitoyl transferase I (CPT I), and acetyl-CoA synthetase (ACS), we inferred that this genetic variation may alter the efficiency of lipid catabolism.

Fig 5. Protein hydrophobicity analyses for the PPARα protein. (A) Hydrophobic analysis before mutation; (B) Hydrophobic analysis after mutation; Positive values represent hydrophobic and negative values represent hydrophilic.

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In conclusion, genetic analysis of PPARα and EGLN3 genes in Tibetan and Lowland chickens suggests that the non-synonymous SNP2 of PPARα may play a role in the ability of Tibetan chickens to adapt to a high altitude environment.

Supporting information

S1 File. Allele and genotype frequencies of the SNPs in the PPARα and EGLN3 genes.

Table A. Allele and genotype frequencies of the SNP in the EGLN3 gene. Table B. Allele and genotype frequencies of the SNP1 in the PPARα gene. Table C. Allele and genotype frequencies of the SNP2 in the PPARα gene. Table D. Allele and genotype frequencies of the SNP3 in PPARα gene. Table E. Allele and genotype frequencies of the SNP4 in PPARα gene. Table F. Allele and genotype frequencies of the SNP5 in the PPARα gene. Table G. Allele and genotype frequencies of the SNP6 in the PPARα gene.

(DOCX)

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References

1. Lorenzo FR, Huff C, Myllymäki M, Olenchok B, Swierczek S, Tashi T, et al. A genetic mechanism for Tibetan high-altitude adaptation. Nat Genet. 2014; 46(9):951–956. https://doi.org/10.1038/ng.3067 PMID: 25129147

2. Zhang H, Wu CX, Chamba Y, Ling Y. Blood characteristics for high altitude adaptation in Tibetan chickens. Poult Sci. 2007; 86 (7):1384–1389. https://doi.org/10.1093/ps/86.7.1384 PMID: 17575186

3. Hochachka PW, Rupert JL, Monge C. Adaptation and conservation of physiological systems in the evolution of human hypoxia tolerance. Comp Biochem Physiol A: Physiol. 1999; 124(1):1–17.

4. Matsuda S, Kobayashi M, Kitagishi Y. Expression and function of ppars in placenta. Ppar Research. 2013; 2013(3):275–283.

5. Sun J, Zhong H, Chen SY, Yao YG, Liu YP. Association between MT-CO3 haplotypes and high-altitude adaptation in Tibetan chicken. Gene. 2013; 529(1):131–137. https://doi.org/10.1016/j.gene.2013.06.075 PMID: 23890731

6. Garcia TS, Honório TS, Káthia M. Two-dimensional quantitative structure-activity relationship studies on bioactive ligands of peroxisome proliferator-activated receptor δ. J Braz Chem Soc. 2011; 22(22):65–72.

7. Cheviron ZA, Bachman GC, Connaty AD, Mcclelland GB, Storz JF. Regulatory changes contribute to the adaptive enhancement of thermogenic capacity in high-altitude deer mice. Proc Natl Acad Sci USA. 2012; 109(22):8635–8640. https://doi.org/10.1073/pnas.1120523109 PMID: 22586089
8. Qu Y, Tian S, Han N, Zhao H, Gao B, Fu J, et al. Genetic responses to seasonal variation in altitudinal stress: whole-genome resequencing of great tit in eastern Himalayas. Sci Rep. 2015; 5(1):91–97.
9. Grabel'ni ch OI, Pivovarova NY, Pobezhimova TP, Kolesnichenko AV, Voinikov VK. The role of free fatty acids in mitochondrial energetic metabolism in winter wheat seedlings. Russ J Plant Physiol. 2009; 56(3):332–342.
10. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev. 1999; 20(5):649–688. https://doi.org/10.1210/edrv.20.5.0380 PMID: 10529899
11. Epstein AC, Gleadle JM, Mcneill LA, Hewitson KS, O’Rourke J, Mole DR, et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 2001; 107(1):43–54. PMID: 11595184
12. Tian YM, Mole DR. Characterization of different isoforms of the HIF prolyl hydroxylase PHD1 generated by alternative initiation. Biochem J. 2006; 397(1):179–186. https://doi.org/10.1042/BJ20051996 PMID: 16509823
13. Xie L, Xiao K, Whalen EJ, Forrester MT, Freeman RS, Fong G, et al. Oxygen-regulated β2-adrenergic receptor hydroxylation by EGLN3 and ubiquitylation by pVHL. Sci Signal. 2009; 2(78):2315–2320.
14. Mü llenbach R, Lagoda PJ, Welter C. An efficient salt-chloroform extraction of DNA from blood and tissues. Tig. 1989; 5(12):391. PMID: 2623762
15. Singh VK, Mangalam AK, Dwivedi S, Naik S. Primer premier: program for design of degenerate primers from a protein sequence. Bio Techniques. 1998; 24(2):318–319.
16. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGAS: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011; 28(10):2731–2739. https://doi.org/10.1093/molbev/msr121 PMID: 21546353
17. Burland TG. DNASTAR's Lasergene sequence analysis software. Methods Mol Biol. 2000; 132(1):71.
18. Excoffier L, Lischer HE. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour. 2010; 10(3):564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x PMID: 21565059
19. Yates A, Akanni W, Amode MR, Barrett D, Billis K, Carvalho-Silva D, et al. Ensembl 2016. Nucleic Acids Res. 2016; 44(1):710–716.
20. Mei L, Zhao CJ. Study on Tibetan Chicken embryonic adaptability to chronic hypoxia by revealing differential gene expression in heart tissue. Sci China: Life Sci. 2009; 52(3):284–295.
21. Zhang H, Chang-Xin WU, Yangzom C, Xue-Ying MA, Jun-Ying LI, Tang XH, et al. Hatchability of miniature laying chicken and its hybrids at high altitude. Sci Agric Sin. 2006; 39(10):1507–1510.
22. Bao HG. A comparison of mitochondrial respiratory function of tibet chicken and silky chicken embryonic brain. Poult Sci. 2007; 86(10):2210–2215. https://doi.org/10.1093/ps/86.10.2210 PMID: 17878451
23. Long A, Ying Q, Gu T, Zhu Q, Liu Y, Wang Y, et al. Genetic Variation of Nine Chicken Breeds Collected from Different Altitudes Revealed by Microsatellites. J Poultry Sci. 2016; 54(1):18–25.
24. Hart CW. The ocular fixation index. Ann Otol Rhinol Laryngol. 1973; 82(6):848–851. https://doi.org/10.1177/000348947308200618 PMID: 4357419
25. Liu Z, Qing-Shen LI, Cai Y, Wang-JF, Huang-QJ, Chai WQ, et al. Study on genetic structure and genetic diversity of three populations of trout (oncorhynchus mykiss). J Hydroecol. 2010; 03(15):48–53.
26. Futuyma DJ. Evolutionary biology. Q Rev Biol. 1998; 24(4):354.
27. Kimchisarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV. A "Silent" Polymorphism in the MDR1 Gene Changes Substrate Specificity. Sci. 2007; 315(5811):525–528.
28. Beato M. The nuclear receptor superfamily: the second decade. Cell. 1995; 83(6):835–839. PMID: 8521507