p53* gene cloning and response to hypoxia in the plateau zokor, *Myospalax baileyi

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Abstract

The plateau zokor (*Myospalax baileyi*) is a specialized subterranean rodent that lives on the Qinghai-Tibet Plateau. The species has evolved a series of strategies to adapt to its hypoxic environment and hypercapnia. *p53* is a tumour suppressor gene that plays a crucial role in the cellular response to hypoxia by inducing cell cycle arrest, cell apoptosis, DNA damage repair and angiogenesis. To investigate the sequence characteristics of *p53* and the response to hypoxia in plateau zokor, we cloned the *p53* coding DNA sequence, analysed it, and measured the expression level of *p53* at different altitudes in plateau zokor and rats. Our results show that the coding DNA sequence is 1179 bp, consisting of 392 amino acid residues. Compared to human *p53*, the subterranean rodents have two mutation sites in common with the human hotspots in the DNA-binding domain. Compared to subterranean rodents, plateau zokor have a mutation at residue 309. In addition, subterranean rodents have two convergent sites at residues 78 and 84. The expression levels of *p53* in plateau zokor tissues increase significantly from 2260 m to 3300 m, but there was no significant difference in rats at those altitudes. Our results suggest that subterranean rodents have two mutation sites in common with the human hotspots in the DNA-binding domain, the mutation of Gly309Asp is a unique mutation site of plateau zokor *p53*, and there are two convergent sites enhancing subterranean rodent adaptation to hypoxic conditions. In addition, *p53* is sensitive to the oxygen concentration in plateau zokor, and hypoxia upregulates the levels of *p53*. Generally, plateau zokor use this strategy to adapt to a hypoxic environment.

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Keywords

Asiatic burrowing rodent; hypoxic; p53; rodent; TP53; tumor protein; tumour suppressor

Introduction

p53, a tumour suppressor gene, plays a major role in the cellular response to DNA damage and hypoxia (Resnick & Inga, 2003; Ashur-Fabian et al., 2004; Resnick et al., 2005; Menendez et al., 2006). It is also one of the most frequently mutated proteins in cancers (Hernandez et al., 2003; Klein, 2004). Human *p53* has 393 residues, and it contains a transcriptional activation domain (TAD), a sequence-specific DNA-binding domain (DBD), a tetramerization domain (TD), a C terminus (CT), and a proline-rich domain (PRD) (Bargonetti et al., 1993; Cho et al., 1994; Thut et al., 1995; Walker & Levine, 1996). The DNA-binding domain is the core domain of *p53*, and a majority of mutations occur in this region (Pavletich et al., 1993; Zhao et al., 2001). The *p53* gene has two types, wild and mutant. The wild-type *p53*-encoded protein suppresses tumourigenesis by regulating cell cycle arrest, cell apoptosis (Vousden & Lu, 2002; Klein, 2004), DNA damage repair and angiogenesis (Graeber et al., 1996; Kinzler & Vogelstein, 1996). This protein has been called the “guardian of the genome” and a “gatekeeper for growth and division” (El-Deiry, 1998). However, the mutant *p53*-encoded protein loses its anti-tumour function and can also promote tumourigenesis, including in several human cancers (Vousden & Lu, 2002; Menendez et al., 2006). The biological effects of *p53* can be explained by its ability to activate the expression of a number of target genes. These target genes can mediate cell cycle arrest, as with *p21*, *14-3-3- δ* , and *Rep-rimo*, induce cell apoptosis, as with *Apaf-1*, *Fas*, and *Bax*, or inhibit angiogenesis and metastasis, as with *PAI*, *TSP1*, and *KAI* (El-Deiry, 1998).

Subterranean rodents are small mammals that spend their entire lives underground in sealed burrows. Severe hypoxia and hypercapnia is common in burrows (Fan & Shi, 1982; Nevo, 1999, 2011; Nevo et al., 2001; Shams et al., 2005). In evolution, subterranean rodents have over the long term developed a series of phenotypic, physiological and genomic strategies to adapt to the harsh environment. Firstly, the eyes of subterranean rodents are degenerated, but they display photosensitivity in their photoperiodic response. These rodents have also developed skeletal muscle that is conducive to digging burrows (Pevet et al., 1984; Nevo, 1999; Fang et al., 2014). Secondly, in these animals the surface area of the pulmonary alveoli and the alveolar capillary volume are enlarged. This change benefits the lungs by increasing the capacity for oxygen uptake (Widmer et al., 1997; Wang et al., 2008a). Thirdly, the increased erythrocyte count and haemoglobin and myoglobin content allows for efficient oxygen transport (Arieli et al., 1986; Wei et al., 2001a, b, 2006). Fourthly, they have reduced the distance for oxygen diffusion to the mitochondria, which ensures efficient oxygen delivery by the structural adaptation of tissues (Arieli & Ar, 1981; Edoute et al., 1988). A series of genes have also been shown to be adapted at the molecular level to the underground habitat. This

modification includes a higher expression level for genes like hypoxia-inducible factor 1 α (*HIF-1 α*), vascular endothelial growth factor (*VEGF*), and erythropoietin (*EPO*) (Shams et al., 2004, 2005; Ke & Costa, 2006; Zheng et al., 2011; Wang et al., 2012), and the substitution of amino acids in haemoglobin, haptoglobin and myoglobin (Gurnett et al., 1984; Kleinschmidt et al., 1984, 1985; Ben-Shlomo & Maeda, 1989). In addition, subterranean rodents display exceptional longevity. *Heterocephalus glaber* and *Spalax judaei* have maximum lifespans exceeding 30 years (Buffenstein & Jarvis, 2002; Buffenstein, 2008; Kim et al., 2011; Tian et al., 2013) and 21 years (Edrey et al., 2012), respectively. In addition to longevity, reporter assays have shown a striking resistance to cancer. Multi-year observations of large samples did not detect a single incidence of cancer (Buffenstein & Jarvis, 2002; Buffenstein, 2008; Delaney et al., 2013).

Previous *p53* studies revealed that an arginine (R) was substituted by lysine (K) in residue 172 (Arg174 in human) in *Spalax*. This substitution is similar to certain mutations found in human tumours; therefore, it implies that *Spalax* undergoes similar adaptations to hypoxia to those undergone by *p53* in human cancers. The mutation reduces the transcription of apoptosis genes, such as *Apaf-1*, *Puma*, *Pten* and *Noxa*, and enhances the cell cycle arrest and *p53* stabilization/homeostasis genes *Mdm2*, *p21* and *CycG* (Ashur-Fabian et al., 2004; Avivi et al., 2007). Genome and transcriptome analysis of the *p53* signal pathway has shown that eight *p53* target genes are differentially regulated by hypoxia in *Spalax* and rat (Fang et al., 2014).

The plateau zokor, *Myospalax baileyi*, is a specialized subterranean rodent living on the Qinghai-Tibet Plateau. This species inhabits sealed burrows at an altitude of 2800 to 4200 m (Wang et al., 1979; Fan & Shi, 1982). Like other subterranean rodents, the plateau zokor has strategies to adapt to hypoxic and hypercapnic underground environments. In *M. baileyi*, the *p53* expression level was significantly decreased in the liver when mimicking oxygen levels at an altitude of 7000 m (8.0% O₂) for 8 h compared to an altitude of 3352 m (Wang et al., 2013; Zhao et al., 2013). Comparison of *p53* sequence data and functional studies found that a mutation in codon 104 from serine (S) to asparagine (N) activated the apoptosis-related genes *IGFBP3*, *Apaf-1* and *Bax*, but had no significant influence on cell cycle arrest genes, including *p21* and *Mdm2* (Zhao et al., 2013).

To date, the sequence characteristics of *p53* and the response to hypoxia in the plateau zokor are not understood well. In this study, we clone the coding DNA sequence (CDS) of *p53*, analyse and characterize *p53* in other rodents, and measure *p53*'s expression differences between high and low altitudes in plateau zokor tissues using quantitative real-time PCR.

Materials and methods

Animals and sample collection

Plateau zokors were live-trapped in the Zongjiagou region in Huangyuan country, Qinghai Province, China. They were divided into two groups: (1) high altitude (3300 m group), which were zokors collected from the Zongjiagou region in Huangyuan country at an altitude of 3300 m with an oxygen content of 193.4 g/m³; (2) low altitude (2260 m), which were zokors collected from Zongjiagou in the Huangyuan region and raised in Xining City, Qinghai Province, China at an altitude of 2260 m for eight days with an oxygen content of 226.7 g/m³. Sprague-Dawley (SD) rats were bought from Lanzhou, Gansu Province, China. Rats were divided into two groups: (1) high altitude (3300 m Group), raised in Zongjiagou region in Huangyuan country for eight days; (2) low altitude (2260 m), raised in Xining City at an altitude of 2260 m for eight days. The sample size was 8 for each of the above groups. All animals were anaesthetized with sodium pentobarbital (5%) and sacrificed using cervical dislocation immediately before dissection. Liver, lung, stomach, intestine and skeletal muscles were rapidly removed and quickly frozen in liquid nitrogen. All procedures involved in the handling and care of animals were in accordance with the China Practice for the Care and Use of Laboratory Animals and approved by the China Zoological Society (permit number: GB 14923-2010).

Cloning of the p53 CDS sequence of plateau zokor

The plateau zokor *p53* CDS was cloned from mRNA extracted from the liver. Primers were designed according to the *Homo sapiens* (DQ263704), *Rattus norvegicus* (AH002222), *Mus musculus* (AB020317), *Cricetulus griseus* (U50395), *Eospalax cansus* (JX998172), *H. glaber* (KM486789) and *Microtus oeconomus* (JX998171) *p53* cDNA sequences. Total RNA was isolated using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA). The RNA concentration and purity were assessed using UV spectrophotometry ($1.8 < A_{260}/A_{280} < 2.0$). RNA integrity was checked using electrophoresis. A reverse transcription reaction was performed starting from 3.8 µg of total RNA and using the First Strand cDNA Synthesis kit (TIANGEN, China). The PCR primers were designed as follows: forward, 5'-ATGGAGGAACCCAGTCAG-3' and reverse, 5'-CAGGCGGAGGTGTGGAGTCG-3', with an amplicon length of 519 bp; forward, 5'-GCACGCAGTTCTGGAGGT-3' and reverse, 5'-AATTTCCTCGAAGTCGGTGGT-3', with an amplicon length of 682 bp. A PCR reaction system of 25 µl was used which contained 1 µl template, 2.5 µl 10× Taq buffer (Mg²⁺-free), 2 µl MgCl₂ (25 mM), 2 µl dNTP mixture (2.5 mM/each), 1 µl forward and 1 µl reverse primers (10 µM), 0.25 µl Taq polymerase (5 U/µl) (Takara Bio Inc., Otsu, Shiga, Japan) and 15.25 µl doubly distilled H₂O. PCR included denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 52°C (51°C) for 30 s and 72°C for 45 s, and finishing with chain extension at 72°C for 5 min. The amplified product was separated on a 1.5% agarose gel. The target product was recycled and purified

with the TIANGen Midi Purification Kit (Tiangen Biotech (Beijing) Co., Beijing, China), cloned into the pMD 19-T vector (Takara Bio) and transformed into DH5 α competent cells. Positive clones were sequenced. The GenBank accession number is KX823344.

Multiple alignment and phylogenetic tree construction

The *p53* sequences were obtained from GenBank (table S1). A phylogenetic tree of *p53* was constructed with ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2>) and MEGA 7.0 software (Kumar et al., 2016) using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap re-sampling was applied to assess support for individual nodes using 1000 replicates. An amino acid sequence alignment of *p53* proteins from *H. sapiens*, *M. baileyi*, *E. cansus*, *S. judaei*, *H. glaber* and *R. norvegicus* was generated using Multalin software (<http://multalin.toulouse.inra.fr/multalin/>) (Corpet, 1988).

HIF-1 α sequences of *H. sapiens* (NM_001530.3), *M. baileyi* (DQ229099.1), and *R. norvegicus* (NM_024359.1) were obtained from GenBank. An amino acid sequence alignment of the *HIF-1 α* oxygen-dependent degradation (ODD) domain from *H. sapiens*, *M. baileyi* and *R. norvegicus* was generated using Multalin software (<http://multalin.toulouse.inra.fr/multalin/>) (Corpet, 1988).

Selection pressure analysis

Sequence alignments were obtained using ClustalX1.81 (Jeanmougin et al., 1998), and format conversion was performed with MEGA 7.0 software (Kumar et al., 2016). The maximum-likelihood method was used to estimate the positive sites using branch-site models in codeml from the PAML package based on the mammalian species tree. The significance of the likelihood ratio test (LRT) statistic was determined using a χ^2 distribution, and the positively selected sites were identified using Bayes Empirical Bayes (BEB) analysis (Zhang et al., 2005; Yang, 2007).

Convergent evolution analysis

Ten mammalian species were used to infer the convergent sites. We constructed ancestral *p53* amino acid sequences using the Ancestors programme in the MEGA 7.0 software (Tamura et al., 2011). Ancestral inferences appeared reliable because mean posterior probabilities for the entire protein exceeded 99% for all nodes. We attempted to identify convergent changes by comparing ancestral and extant *p53* protein sequences. Next, we calculated the probability that the observed convergent sites exceeded the expectation due to random chance using the Jones-Taylor-Thornton (JTT) and Poisson models (Zhang & Kumar, 1997).

RNA extraction and quantification of p53 mRNA using qRT-PCR

Total RNA was isolated from the liver, lung, stomach, intestine and skeletal muscle using TRIzol reagent (Invitrogen Corp.). The RNA concentration and purity were

assessed using UV spectrophotometry ($1.8 < A_{260}/A_{280} < 2.0$). RNA integrity was checked using electrophoresis. A reverse transcription reaction was performed starting with 3.8 μg of total RNA and the First Strand cDNA Synthesis kit (Tiangen).

Quantitative real-time RT-PCR was performed using the SYBRPremix Ex Taq™ II (Takara Bio) protocol on a Bio-Rad Connect real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) to quantify the expression level of *p53*. qRT-PCR was performed at 95°C for 3 min followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. β -actin was used as an internal control. The PCR primers for *p53* and β -actin were designed as follows: *zokor-p53*: forward, 5'-GCCCAAGAAGAAGCCACTAC-3' and reverse, 5'-CCTGCTCTCTCCTGACTCCT-3', with an amplicon length of 142 bp; *rat-p53*: forward, 5'-CGACTATACCACTATCCACTACA-3' and reverse, 5'-GTCTTCCA GCGTGATGATG-3', with an amplicon length of 97 bp; β -actin: forward, 5'-TCA CCAACTGGGACGATATG-3' and reverse, 5'-GTTGGCCTTAGGGTTCAGAG-3', with an amplicon length of 119 bp. The relative expression level of *p53* mRNA was computed based on the internal control gene using the $2^{-\Delta\Delta\text{CT}}$ method (Livak & Schmittgen, 2001).

Statistical analysis

Statistical analyses were performed using SAS 8.2 software. The expression levels of *p53* between high and low altitudes were compared using Student's *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Comparison and phylogenetic tree of *Myospalax baileyi* *p53* sequences

The *p53* CDS was cloned and sequenced from *M. baileyi*. The complete coding sequence was 1179 bp, and the deduced protein consisted of 392 amino acid residues. Alignment by ClustalW2 found that the *M. baileyi* *p53* protein is identical to 98.98%, 95.41%, 87.02%, 82.99% with *E. cansus*, *S. judaei*, *H. glaber* and *R. norvegicus* *p53* proteins, respectively. The phylogenetic tree demonstrates that plateau *zokor* had the highest homology with *E. cansus*, followed by *S. judaei* (fig. 1).

Multiple sequence alignment analysis using Multalin software showed that compared with human *p53*, four amino acid sequences of subterranean rodents (*M. baileyi*, *E. cansus*, *S. judaei*, *H. glaber*) had seven mutation sites in common within the DNA-binding domain: Met131Leu (M131L, 133 in human – numbering by actual position; numbering of the amino acids including gaps was used for the figure), Gln163Lys (Q163K, 165 in human), Val201Ala (V201A, 203 in human), Gln246Arg (Q246R, 248 in human), Leu265Arg (L265R, 267 in human), His271Arg (H273R, 273 in human), and Leu287Phe (L287F, 289 in human) (fig. 2).

Figure 1. Phylogenetic tree based on the amino acid sequence of *p53* from *Myospalax baileyi* together with 36 other species. The *p53* sequences of the other species are from GenBank. Neighbour-joining method with 1000 bootstrap replicates was used. Bootstrap values for the internal nodes are given.

Compared with *E. cansus p53*, *M. baileyi* has two mutation sites within the DNA-binding domain: Ser104Asn, and Ala121Thr; compared with *S. judaei p53*, *M. baileyi* has three mutation sites within the DNA-binding domain: Ser104Asn, Pro127Cys, and Lys172Arg. By comparison with *H. glaber p53*, there are fifteen mutation sites within the DNA-binding domain of *M. baileyi*: His98Gln, His108Arg, Gln113His, Val127Cys, Val143Leu, Glu146Asp, Pro148Thr, Arg207Lys, Thr208His, Asp219Glu, Leu220Pro, His288Arg,

Figure 2. Amino acid sequence alignment of p53 protein with *Homo sapiens*, *Myospalax baileyi*, *Eospalax cansus*, *Spalax judaei*, *Heterocephalus glaber* and *Rattus norvegicus*. Amino acids with more than 90% identity are shown in red, 50%-90% identity in blue and the others in black. Arrows indicate that *Myospalax baileyi* has a mutation at residue 309 in the black box.

Gly292Glu, Pro297Leu, and Thr298Pro; and compared with *R. norvegicus* p53, the DNA-binding domain of *M. baileyi* has fifteen mutation sites: His108Arg, Gln113His, Ile126Pro, Ser127Cys, Thr146Asp, Trp174Cys, Gly183Ser, Pro199Leu, Tyr200Arg, Arg207Lys, Gln208His, Tyr227Cys, Lys233Asn, Asp266Asn, and Glu291Gly.

Multiple sequence alignment analysis showed that the mutation of codon 309 from glycine (G) to aspartic acid (D) in *M. baileyi* is unique to the plateau zokor compared to *E. cansus*, *S. judaei* and *H. glaber* p53.

Table 1.

Likelihood values, parameter estimates, and sites under positive selection for *p53* in plateau zokor.

Model	Parameter estimates	$\ln L$	$2\Delta \ln L$ (<i>P</i> -value)	Positive selection site
Null A	$p = 0.785, p1 = 0.215,$ $(p2 + p3 = 0), \omega0 = 0,$ $\omega1 = 1.0, \omega2 = 1.0$	−8750.89	0 ($P = 1.000$)	Not available
Model A	$p = 0.785, p1 = 0.215,$ $(p2 + p3 = 0), \omega0 = 0,$ $\omega1 = 1.0, \omega2 = 1.0$	−8750.89		Not found

Positive selection sites analysis

To detect the positively selected sites of *p53* in plateau zokor, we treated the plateau zokor branch as the foreground branch. Using the LRT based on the branch-site model for gene *p53*, we observed that there are no positive selection sites in plateau zokor *p53* (table 1).

Convergent evolution analysis

To detect the sites that are convergent in subterranean rodents in response to hypoxic environments, we attempted to identify convergent changes by comparing ancestral and extant *p53* protein sequences. We observed that two sites experienced convergent evolution in subterranean rodents. The 78th position of *p53* was proline (P) on the ancestral branch. However, it was replaced by serine (S) on the subterranean rodent branch of *M. baileyi* and *S. judaei*. This change was caused by a C to T transition at the first position of the codon at site 78. In addition, the 84th position was alanine (A) on the ancestral branch, and it was replaced by proline (P) in the subterranean rodent branch (fig. 3). According to a statistical test, the 78th and 84th positions were likely convergent sites, rather than chance substitution ($P < 0.01$).

Expression of plateau zokor p53 under hypoxia

As shown in fig. 4, the relative expression level of *p53* mRNA in plateau zokor tissues was significantly higher in the high-altitude group (3300 m) than the low-altitude group (2260 m) (liver: $t = 7.50, P < 0.0001$; lung: $t = 17.41, P < 0.0001$; stomach: $t = 10.01, P < 0.0001$; intestine: $t = 12.95, P < 0.0001$; skeletal muscle: $t = 7.98, P < 0.0001$). However, there was no significant difference between the high- and low-altitude groups in rat (liver: $t = 1.67, P = 0.1131$; lung: $t = 1.59, P = 0.1283$; stomach: $t = 0.75, P = 0.4615$; intestine: $t = 1.17, P = 0.2577$; skeletal muscle: $t = 0.53, P = 0.5993$).

Figure 3. Evolution of convergent sites in *p53* sequences. Amino acids and codons of sites 78 and 84 are shown. Amino acids in *Myospalax baileyi* and *Spalax judaei* are highlighted in red, and in the other species in blue.

Multiple alignment of HIF-1 α sequences

The complete coding sequence (CDS) of *H. sapiens* *HIF-1 α* was 2481 bp, encoding 826 amino acids, that of *M. baileyi* was 2460 bp, encoding 819 amino acids, and of *R. norvegicus* it was 2472 bp, encoding 823 amino acids. The ODD domain consisted of 203, 201 and 202 amino acids in *H. sapiens*, *M. baileyi* and *R. norvegicus*, respectively.

Alignment by ClustalW2 found that in human, the motif at amino acids 397 to 436 is up to 90.00% identical with plateau zokor and rat, and the other motif in human (positions 507 to 579) is 97.26% and 95.89% identical with those in plateau zokor and rat, respectively. Compared with human, plateau zokor and rat had four common mutation sites within the motif 397 to 436: Asn416Asp, Asp421Glu, Glu427Asp, Leu436Phe, and they had two mutation sites in common within the motif 507 to 579: Tyr522Asp, Glu530Val. In addition, rat had a mutation of codon 552 from Thr (T) to Ala (A).

The sequence alignment showed that the amino acid sequences of the plateau zokor *HIF-1 α* ODD domain were 92.61% identical to those in rat. Compared with plateau zokor, rat had 12 mutation sites within the ODD domain: Asp440Asn, Asp441Glu, Thr444Asn, Asn465Ser, Pro480Ser, Asn481Ser, Ala482Pro, Glu486Gly, Thr552Ala, Ser586Asn, Asn589Ser, Ala599Val. The motif 397 to 436 of plateau zokor is 100% identical with that of rat, and the other motif (507 to 579) of plateau zokor is 98.63% identical with that of rat. Compared with plateau zokor, rat had one mutation sites within the motif 507 to 579: Thr552Ala (fig. 5).

Discussion

The plateau zokor is a specialized rat on the Qinghai-Tibet Plateau. This species was reported to have adaptations in a series of genes involved in hypoxia response, including *p53* (Fan & Shi, 1982; Zheng et al., 2011; Wang et al., 2012; Zhao et al.,

Figure 4. Quantification of *p53* mRNA levels in tissues of plateau zokor and rat at different altitudes. A-E. Electrophoresis results of real-time PCR of *p53* and β -actin in plateau zokor and rat tissues. F-J. Quantification of *p53* mRNA levels in plateau zokor and rat at different altitudes. Panels indicate liver (A, F), lung (B, G), stomach (C, H), intestine (D, I) and skeletal muscle (E, J). Abbreviations and symbols: M, marker; L, low-altitude group (2260 m); H, high-altitude group (3300 m); **, $P < 0.01$; *, $P < 0.05$; ns, not significant ($P > 0.05$). The sample size was 8 for each group.

Figure 5. Amino acid sequence alignment of *HIF-1α* ODD domain in *Homo sapiens*, *Myospalax baileyi* and *Rattus norvegicus*. Amino acids with more than 90% identity are highlighted in red, 50-90% identity in blue and the others in black. The arrow indicates residue 552.

2013). According to our phylogenetic gene tree, the plateau zokor is placed with rodentia and shares many homologies with *E. cansus*. These two species are grouped on a small branch with *S. judaei* as sister group. In an analysis of reporter assays, *E. cansus* and *S. judaei* were grouped on a small branch subtended by plateau zokor (Zhao et al., 2013). These species all grouped into a large branch with *R. norvegicus*. *Heterocephalus glaber* belongs to the family of subterranean rodents; however, this species was far removed from other subterranean rodents. Studies of the phylogenetic relationship based on the genome and transcriptome analysis found that the plateau zokor diverged from the rat approximately 52 million years ago (Shao et al., 2015). A molecular phylogenetic tree based on the *p53* and cytochrome b protein sequences reported previously showed that plateau zokor and *E. cansus* were grouped on a branch with *S. judaei* as sister group (Zhao et al., 2013). *Eospalax cansus* is a typical subterranean rodent, living on the Loess Plateau in China (Li, 1989). Severe hypoxia and hypercapnia are common characteristics of burrows (Fan & Shi, 1982;

Nevo, 1999). During long-term evolution, *M. baileyi* and *E. cansus* developed a series of strategies to evolve adaptively and convergently in the hypoxic environment. They have a higher oxygen utilization rate in their tissues to adapt to hypoxic conditions, with a high oxygen pressure and saturation in arterial blood, and a low oxygen pressure and saturation in venous blood; the number of red blood cells, and the hemoglobin and myoglobin concentrations are also significantly higher. In addition, they have substitutions of amino acids in hemoglobin and myoglobin to increase the affinity to oxygen, and have enhanced their capacity to adapt to hypoxic environments (Wei et al., 2006a, b; Yang et al., 2006; Wang et al., 2008b). Thus, our results are consistent with previously published reports and imply that the molecular evolution of the *p53* gene in plateau zokor is similar to that in *E. cansus*, and this also indicates that subterranean rodents have evolved adaptively and convergently to hypoxia.

Acting as a tumour suppressor gene, *p53* has mutations that are also observed in approximately 50% of human cancers (Hernandez et al., 2003; Klein, 2004). Several mutations were reported at the mutation ‘hotspots’ at residues R175, G245, R248, R249, R273, and R282. The mutation occurs in more than 90% of human cancers (Bullock & Fersht, 2001; Freed-Pastor & Prives, 2012). Compared to the amino acid sequence of *p53* protein in *E. cansus*, *S. judaei* and *H. glaber*, we found that in plateau zokor codon 309 is mutated from glycine (G) to aspartate (D); this mutation is unique for plateau zokor. We predict that the Gly309Asp mutation may play a significant role in the adaptation of the plateau zokor to its extreme environment, but this warrants further investigation. We also observed that the subterranean rodents (*M. baileyi*, *E. cansus*, *S. judaei*, *H. glaber*) had seven mutation sites in common compared to human *p53*. There were two mutation sites in common with the human hotspots: Gln246Arg (248 in human) and His271Arg (273 in human). These two mutations are reported to be the most frequently altered residues in *p53* protein. In *p53*, these mutations cause a loss of wild-type *p53* tumour suppressor activity and promote tumourigenesis, resulting in cell cycle G1 arrest in cells (Cho et al., 1994; Hollstein et al., 1994; Vousden & Lu, 2002; Menendez et al., 2006). Using site-directed mutagenesis to study the function of *p53*, the Arg174Lys amino acid substitution in *Spalax* was found to reduce transcription of the apoptosis genes and enhance cell cycle arrest and *p53* stabilization/homeostasis genes (Ashur-Fabian et al., 2004; Avivi et al., 2007). The Ser104Asp amino acid substitution in plateau zokor activated the apoptosis genes and had no significant influence on cell cycle arrest genes, including *p21* and *Mdm2* (Zhao et al., 2013). The mutations in different subterranean rodents were species-specific and could be attributed to their specific microenvironment. Therefore, we propose that the variations do not promote tumourigenesis in subterranean rodents, but enhance their adaptability to the hypoxic environment.

Subterranean rodents live in dark, hypoxic and hypercapnic underground environments (Fan & Shi, 1982; Nevo, 1999, 2011; Nevo et al., 2001; Shams et al., 2005), and *p53* plays an important role in hypoxic environments. Genome and

transcriptome data have shown that *p53* is not positively selected in subterranean rodents (Kim et al., 2011; Fang et al., 2014), and previous studies have shown that plateau zokor *p53* does not have positively selected sites (Zhao et al., 2013). In this study, the LRT based on branch-site model A for gene *p53* indicated there are no positive selection sites in plateau zokor. In addition, we observed that *p53* amino acid sequences have two convergent sites in *M. baileyi* and *S. judaei*. We propose that the two substitution sites might enhance subterranean rodent adaptation to hypoxic environments.

Studies have demonstrated that the expression level of *p53* is related to the degree of hypoxia. Under normal conditions, wild-type *p53* is expressed at low levels in most cells and has a short half-life. In contrast, its level increases and stabilizes in response to hypoxia (Giaccia & Kastan, 1998; Koumenis et al., 2001). However, later study showed that hypoxic induction of *p53* requires the concomitant induction of *HIF-1 α* , demonstrating a direct interaction between *p53* and *HIF-1 α* (Hansson et al., 2002; Fels & Koumenis, 2005; Sánchez-Puig et al., 2005). The oxygen-dependent degradation (ODD) domain lies within amino acids 401 to 603 in human *HIF-1 α* (Huang et al., 1998). In the ODD domain, the two proline residues (P402 and P564), play a pivotal role in regulating *HIF-1* activity and expression by the ubiquitin-proteasome pathway, and act as switches for the oxygen-dependent regulation of *HIF-1 α* (Huang et al., 1998; Masson et al., 2001). Studies have identified two *p53*-binding sequence motifs in human *HIF-1 α* ; one motif (397 to 436) is adjacent to the ODD domain, the other motif (507 to 579) is coincident with the ODD domain of *HIF-1 α* (Hansson et al., 2002). Under normoxic conditions, the expression levels of *p53* and *HIF-1 α* are low. When the oxygen concentration decreases, *HIF-1 α* stabilizes, and the *p53* level remains low or lower than before. Under severe hypoxia or anoxic conditions, *p53* is accumulated and stabilizes and binds to the ODD domain of *HIF-1 α* (Fels & Koumenis, 2005; Sánchez-Puig et al., 2005). When comparing oxygen levels at a mimicked altitude of 7000 m (8.0% O₂) for 8 h with an altitude of 3352 m, the *p53* expression level was significantly decreased in plateau zokor liver and significantly increased in rat. However, there was no significant difference between 10.8% O₂ and 16.0% O₂ (Wang et al., 2013; Zhao et al., 2013). In this study, we found that the relative expression levels of *p53* in the tissues of liver, lung, stomach, intestine and skeletal muscle of the plateau zokor are increased significantly from 2260 m to 3300 m, but there was no significant difference under the same conditions in rat. Thus, the expression level of *p53* showed different patterns among plateau zokor and rat in various tissues. This could be due to different regulatory mechanisms in the two species that are adaptive for the hypoxic environment. Doing a multiple sequence alignment of the two *p53*-binding sequence motifs in *HIF-1 α* , our results show that plateau zokor and rat are highly homologous to human, with the two proline residues (P402 and P564) not mutated in plateau zokor or rat. Compared with plateau zokor, rat had one mutation site within the motif 507 to 579: a polar amino acid (Thr, T) was substituted by a non-polar amino acid (Ala, A) in residue 552 in rat. The results imply that the

substitution in the motif 507 to 579 decreases the affinity of *HIF-1 α* to *p53*-binding sites in rat, and the *p53*-binding sites in *HIF-1 α* of plateau zokor are stronger than that of rat.

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Table S1.

Species examined in this study.

Species name	Accession Number
<i>Homo sapiens</i>	DQ263704
<i>Rattus norvegicus</i>	AH002222
<i>Mus musculus</i>	AB020317
<i>Eospalax cansus</i>	JX998172
<i>Spalax judaei</i>	AJ783406
<i>Heterocephalus glaber</i>	KM486789
<i>Cricetulus griseus</i>	U50395
<i>Microtus oeconomus</i>	JX998171
<i>Cavia porcellus</i>	NM_001172740
<i>Meriones unguiculatus</i>	AB033632
<i>Mesocricetus auratus</i>	NM_001281661
<i>Rhesus monkey</i>	L20442
<i>Macaca fascicularis</i>	U48957
<i>Macaca mulatta</i>	U48956
<i>Tupaia chinensis</i>	NM_001287369
<i>Canis lupus familiaris</i>	AB020761
<i>Felis catus</i>	D26608
<i>Delphinapterus leucas</i>	AF475081
<i>Sus scrofa</i>	NM_213824
<i>Ovis aries</i>	FJ855223
<i>Bubalus bubalis</i>	JF792632
<i>Bos taurus</i>	X81704
<i>Bos grunniens</i>	KF682140
<i>Cynops orientalis</i>	HM627863
<i>Xenopus laevis</i>	X77546
<i>Oryzias latipes</i>	U57306
<i>Platichthys flesus</i>	Y08919
<i>Oncorhynchus mykiss</i>	NM_001124692
<i>Pelteobagrus fulvidraco</i>	HQ419002
<i>Ictalurus punctatus</i>	NM_001200076
<i>Strongylocentrotus purpuratus</i>	JF704085
<i>Brachionus koreanus</i>	KJ526612
<i>Spodoptera frugiperda</i>	HM773026
<i>Bombyx mori</i>	HM773025
<i>Marsupenaeus japonicus</i>	AB559569