Gene 523 (2013) 187–191

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Short communication

Expression profiling of abundant genes in pulmonary and cardiac muscle tissues of Tibetan Antelope (*Pantholops hodgsonii*)

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ARTICLE INFO

Article history:
Accepted 7 March 2013
Available online 20 April 2013

Keywords:
Chiru
EST
Positive selection

ABSTRACT

The Tibetan Antelope (TA), which has lived at high altitude for millions of years, was selected as the model species of high hypoxia-tolerant adaptation. Here we constructed two cDNA libraries from lung and cardiac muscle tissues, obtained EST sequences from the libraries, and acquired extensive expression data related energy metabolism genes. Comparative analyses of synonymous (Ks) and nonsynonymous (Ka) substitution rates of nucleus-encoded mitochondrial unigenes among different species revealed that many antelope genes have undergone rapid evolution. Surfactant-associated protein A (SP-A) and surfactant-associated protein B (SP-B) genes in the AT lineage experienced accelerated evolution compared to goat and sheep, and these two genes are highly expressed in the lung tissue. This study suggests that many specific genes of lung and cardiac muscle tissues showed unique expression profiles and may undergo fast adaptive evolution in TA. These data provide useful information for studying on molecular adaptation to high-altitude in humans as well as other mammals.

1. Introduction

Tibetan antelopes (TA, commonly called chiru), are aboriginal Pantholops, Bovidae, artiodactyla animals of the Qinghai–Tibet Plateau. TA has superior sports ability and greater resistance to hypoxia compared with other aboriginal animals in high altitude (e.g., these antelope can run 70–100 km continuously at the speed around 60 km per hour) (Schaller & Kang, 2008; Chang et al., 2012). During a long period of evolution, TA obtained genetic features of high-altitude hypoxia adaptation at the physiological, morphological and genetic levels (Liu et al., in press; Yang et al., 2007; Zhang et al., 2011). In the present study, we obtained gene expression profiles from lung and cardiac muscles of TA, classified energy-related gene expression patterns, and analyzed the evolution of tissue-specific genes.

2. Materials and methods

One adult male TA was captured from the Kekexili Natural Reservation (altitude 4600 m) in Qinghai Province, People's Republic of China, and was scarified. Organs were immediately removed and stored in liquid nitrogen until used. All the instruments were treated under 180 °C for 8 h, and the reagents were dissolved with DEPC treated water. 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 were approved by State Forestry Administration of China National Bureau of Wild Animal Protection (No. 2009-46).

2.1. cDNA libraries

cDNA libraries of lung and cardiac muscle tissues were constructed by using mRNAs purified with Oligotex mRNA Isolation Kits (Qiagen) from total RNAs. Sequences were obtained with ABI3730 sequencer and dye-primer chemistry.

2.2. Phylogenetic analyses

Complete cytochrome b sequences from nine mammals were used for phylogenetic analyses: By using Model test 3.7 (Posada & Crandall, 1998), we obtained an optimal model of sequence substitution by comparing likelihood scores, used the model for parameter estimates, and constructed a neighbor-joining tree with PAUP 4.0b10 (Swofford, 2002).

Abbreviations: cDNA, complementary deoxyribonucleic acid; COXI, mitochondrion-encoded cytochrome coxidase I; COXII, mitochondrion-encoded cytochrome coxidase II; DEPC, diethylprocarbonate; ESTs, expressed sequence tags; Ka, nonsynonymous substitution rate; Ks, synonymous substitution rate; NEM, nuclear-encoded mitochondrial; SP-A, surfactant-associated protein A; SP-B, surfactant-associated protein B; TA, Tibetan antelope; TPM, transcripts per million.

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http://dx.doi.org/10.1016/j.gene.2013.03.011

2.3. EST assembly and annotation

We assembled high-quality ESTs using the Phred–Phrap–Consed package (http://www.phrap.org/) after data-filtering to remove cloning vector and mitochondrion-encoded sequences as well as other repetitive sequences based on Repeat Masker. Removing short ESTs less than 100 bp in length, we grouped ESTs with >90% identity over >40 bp in length into the same cluster using CAP3. The relative expression level was described and normalized as transcripts per million or TPM.

2.4. Ka and Ks calculation

In a BLASTX (E values <1e-5) search against HMPDb database, we collected 98 nuclear genome-encoded mitochondrial unigenes in our libraries as well as 293 and 341 cDNAs in cow and mouse (length >100 bp), respectively. The synonymous (Ks) and nonsynonymous (Ka) substitution rates for these nuclear-encoded mitochondrial (NEM) unigenes were calculated with LPB93 method (Li, 1993) for three pairs of species: chiru–human, cow–human, and mouse–human.

2.5. Evolutionary analysis of surfactant-associated protein A (SP-A), and surfactant-associated protein B (SP-B) genes

To acquire the mRNA sequences of SP-A and SP-B from goat, we designed primers according to the sheep sequences (accession nos. AF076633, AF107544 and AF076634). The primers used are: SPA-F (forward, 5′-ATGCTGCTGTGCTCTTTGAC-3′; genome position from 46 to 66), SPA-R (reverse 5′-TGCCTTCACTTCTGATAGCC-3′; 894–914), SPB-F1 (forward, 5′-GCTGCCCATGCTCTGTG-3′; 36–53), SPB-R1 (reverse, 5′-ATCAC AGCCTGGATTCGTT-3′; 619–629), SPB-F2 (forward, 5′-TGTGATTCCCA AGGGTGTACT-3′; 624–645), SPB-R2 (reverse 5′-TGGCTGTGCGTTCTCA TC-3′; 1123–1141), SPC-F (forward, 5′-CAGCAAGATGGATGTGG-3′; 6–22) and SPC-R (reverse, 5′-AGGATGCTTTAATCTTTGGT-3′; 767–787). The SP-A and SP-B sequences of other mammalian species were retrieved from the public data (http://www.ncbi.nlm.nih.gov).

3. Results

3.1. ESTs and expression profiling

In total, 7509 ESTs from cDNA libraries of lung and cardiac muscle tissues (Table 1) were acquired, and 6265 high-quality sequence reads were assembled into 765 (21.2%) clusters and 2847 (78.8%) singlets. Among the unigene clusters, 253 (33.1%) were expressed in both libraries, 448 (58.5%) and 64 (8.4%) were unique to the lung and cardiac muscles, respectively. Among 2847 singlets, 582 (20.4%) and 2265 (79.6%) were expressed in cardiac muscle and the lung, respectively (Table 2). Most of the shared ESTs between libraries are housekeeping genes, such as ribosomal and cytoskeleton genes. To further reveal whether TA gene expression profiles are associated with the unique living environment and capability of adapting, we compared EST-based expression patterns of medium- and high-abundance genes with those of human, mouse, and other available animals hosted by both UCSC annotation database and NCBI DDD databases. Based on the alignment, we managed to discover three different types of differentially-expressed genes: tissue-specific genes, species-specific genes, and TA-associated differentially-expressed genes. According to the relative transcripts per million (TPM), we found that all medium- and high-abundance genes exhibited differential-expression patterns between the two tissues from antelope, human, and mouse; several genes such as telethonin, troponin C, thymosin beta 4, and pulmonary surfactant-associated protein family were observed differentially expressed among different species.

Table 1
EST datasets and their distributions among cDNA libraries.

	Cardiac muscle	Lung	Added
Total sequenced reads	1956	5553	7509
Low quality reads (Q20, <100 bp)	230	608	838
Mitochondrial sequences	360	46	406
Total high-quality sequences[a]	1366	4899	6265

[a] High-quality sequences were trimmed of vector, low-quality sequence, mitochondrial sequences and filtered for minimum length 100 bp.

Table 2
ESTs assembly results.

	CAP3a
Total sequences analyzed[b]	6265
Number of assembled sequences[a]	3612
Number of contigs	765
Number of singlets	2847
Total contig sequences	765
Only expressed in lung library	448
Only expressed in cardiac muscle library	64
Expressed in two libraries	253
Total singlet sequences	2847
Expressed in lung library	2265
Expressed in cardiac muscle library	582

[a] Default settings were used except minimum overlap was 40 bp and 90% identity (default is 30 bp, 75% identity).
[b] 6265 high-quality sequences.

3.2. Phylogeny and evolutionary analyses

We constructed a phylogenetic tree based on cytochrome b genes with PAUP*410b (Fig. 1), and found the antelope is closer to sheep and goat. To study the evolutionary forces that influence nucleus-encoded mitochondrial (NEM) proteins, we calculated Ka/Ks of NEM unigenes for three paired species: antelope vs. human, cow vs. human, and

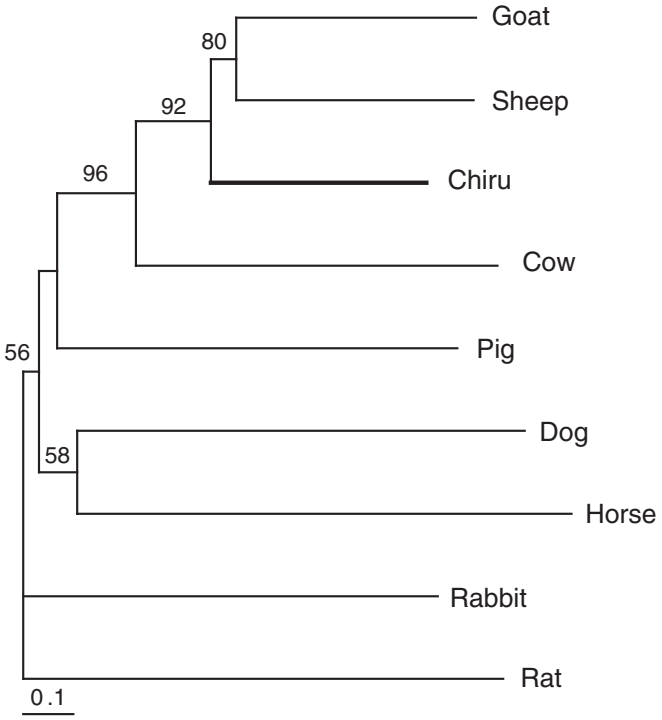

Fig. 1. Neighbor-joining tree based on cytochrome b genes. The tree was constructed by using PAUP*(4b10), with parameters estimated by MODELTEST3.7. Numbers on the nodes are the bootstrap values (>50%) based on 1000 replicates. Rat sequence was used as out group. The antelope sequence was highlighted in bold. The species included were chiru (*Pantholops hodgsonii*), sheep (*Ovis aries*), goat (*Capra hircus*), cow (*Bos taurus*), pig (*Sus scrofa*), horse (*Equus caballus*), dog (*Canis lupus*), rabbit (*Oryctolagus cuniculus*) and rat (*Rattus norvegicus*).

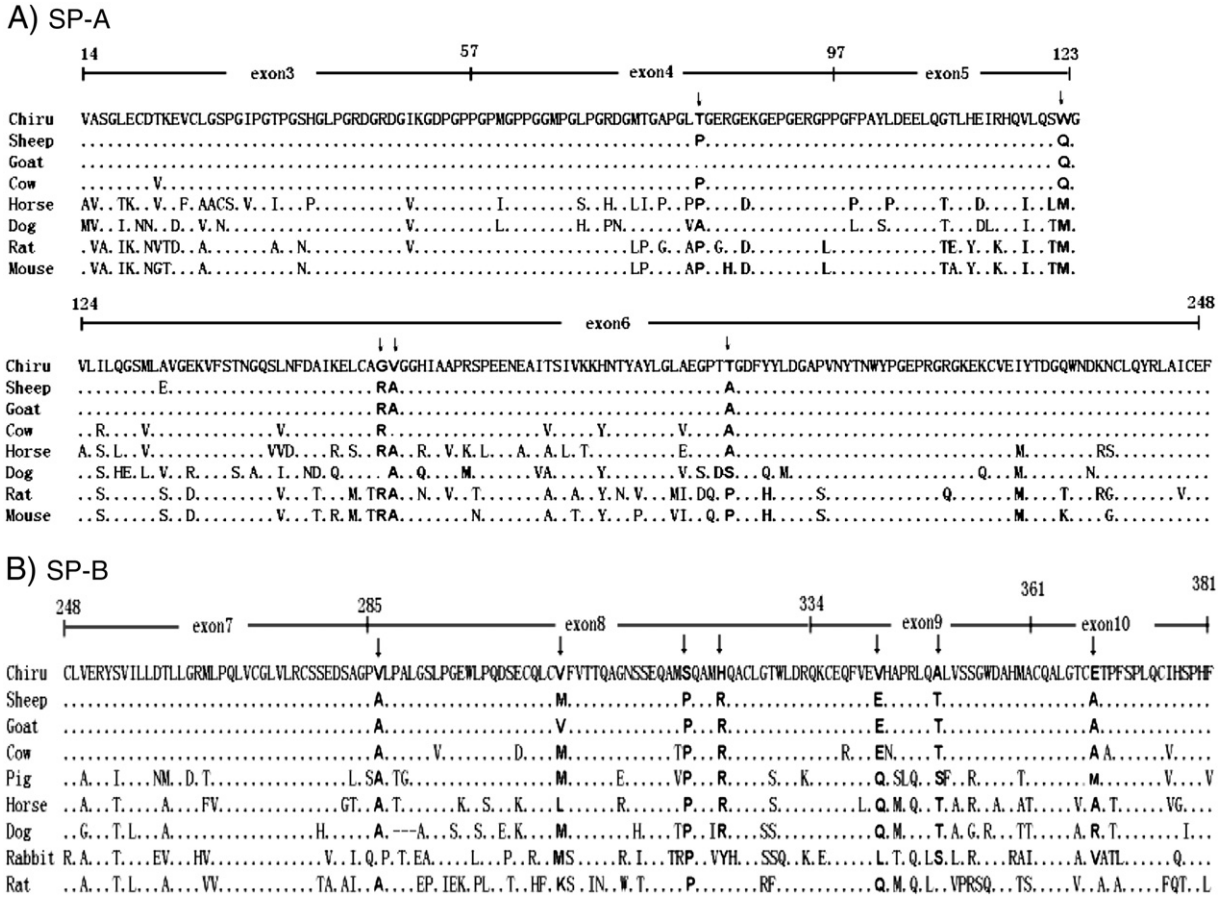

Fig. 2. Protein alignments of surfactant-associated protein A (A, SP-A) and surfactant-associated protein B (B, SP-B) genes sequenced in antelope and other mammalian species. The numbers indicate relevant amino acid residues. The arrows indicate amino acid variations in antelope.

mouse vs. human. The median Ka/Ks ratios were 0.21 for antelope vs. human, 0.16 for cow vs. human, and 0.14 for mouse vs. human. The result suggested that the NEM unigenes in antelope underwent a relative faster pace of evolution than those genes in cow and mouse.

3.3. Surfactant-associated protein A and B genes

After calculating the Ka/Ks ratios between TA and sheep, we analyzed SP-A and SP-B as candidate genes that might have been under positive selection. By comparing with protein sequences of sheep, goat, cow and a few other mammals, we analyzed amino acid mutations of SP-A and SP-B genes. In the case of SP-A, five novel amino acid mutations were observed in chiru at the following position: 82 (Pro to Thr), 122 (Gln to Trp), 157 (Arg to Gly), 158 (Ala to Val) and 195 (Ala to Thr) (Fig. 2A). Seven amino acid substitutions happened in SP-B protein, including 286 (Ala to Val), 308 (Met to Val), 323 (Pro to Ser), 327 (Arg to His), 346 (Glu to Val), 353 (Thr to Ala) and 372 (Ala to Glu) (Fig. 2B). To see if the accelerated evolution of SP-A and SP-B occurs only in the TA lineage, we constructed their phylogenetic trees to reveal detailed mutation patterns in different evolutionary lineages (Fig. 3). When calculated following Pamilo–Bianchi–Li's method, Ka/Ks ratios in the chiru lineage were 1.38 (SP-A) and 1.05 (SP-B), respectively. Similar results were observed when calculated ω (dN/dS) values using the maximum likelihood method (Yang, 1998). The likelihood ratio test indicated that the chiru lineage has a significant ω-value than non-chiru lineages ($2\triangle LnL = 16.40$, $P < 0.005$ for SP-A and $2\triangle LnL = 25.30$, $P < 0.005$ for SP-B).

4. Discussion

Organisms living in the Qinghai–Tibetan Plateau have evolved capabilities to survival under limited oxygen supplemental conditions over thousands of years. It has shown that the TA can survive in the hypoxia and has already developed unique adaption mechanisms. Examples of those genetic features are (i) high degree of arterial oxygen saturation, (ii) rich type II cells lung tissue, (iii) high tolerance of hypoxia stimulation for cardiovascular system, (iv) specific change of genetic locus, fragment length, repeat sequence relating to oxygen transfer genes of mitochondria, and (v) N132S and S134G locus mutations of hemoglobin α-chain (Chang et al., 2010; Liu et al., 2011; Yang et al., 2007). In the present study, we found that a large number of genes related to energy metabolism were highly expressed in the pulmonary and cardiac muscle tissues of Tibetan antelope. Comparative analyses of Ks and Ka substitution rates of nucleus-encoded mitochondrial unigenes indicate that the NEM unigenes in antelope underwent a rapid evolution compared to those genes in other species. We also found that the SP-A and SP-B genes underwent strong positive selection within the AT lineage, and several novel amino acid mutations were also observed in pulmonary surfactant protein A and B genes in TA, which include five amino acids mutated in SP-A gene and seven amino acids mutated in SP-B gene (Fig. 2).

The surfactant-associated proteins are the phospholipid-rich lipid-protein that synthesized and secreted by type II pneumocytes, and stored in the lamellar bodies of the alveoli (Brasch et al., 2004; Roszell et al., 2012; Saxena et al., 2005). The function of the these proteins is to reduce surface tension and maintained alveolar integrity during respiration by decreasing the surface tension at the air–fluid

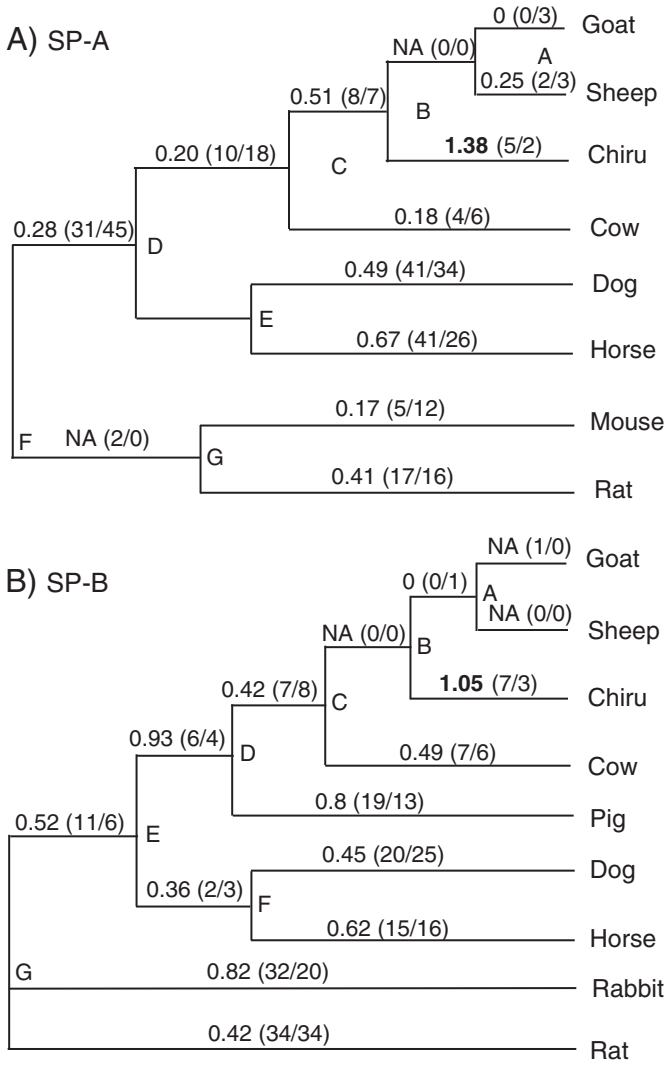

Fig. 3. Ka/Ks ratios of different evolutionary lineages among selected mammalian taxa. The Pamilo–Bianchi–Li method was used in estimating Ka/Ks ratios. The numbers of nonsynonymous and synonymous substitutions are shown in the parentheses.

interface on the alveolar epithelium (Griese, 1999), and to also contribute to the innate immune system (Haagsman, 2002). Our previous studies on the ultrastructural of lung tissues indicated that a large number of lamellar bodies were observed in alveoli of lungs in TA, whereas the lamellar bodies of Tibetan sheep (TS) were much less than TA (Ge, 2007). An abundant of lamellar bodies in the lungs suggests that the type II pneumocyte is produce and secrete large amounts of pulmonary surfactant that is highly responsible for lowering the surface tension in the lung to maintain a higher amount of pulmonary ventilation during high speed running at extremely hypoxic environments in TA. A strong positive selection of pulmonary surfactant protein genes and enrichment of alveolar lamellar bodies in the TA clearly demonstrated that adaptation to high-altitude hypoxia in the TA is not only physiological, but also genetically related.

Mitochondrion plays an important role in energy metabolism, and the function in mitochondria is of great interest for studying high-altitude adaptation (Donnelly and Carroll, 2005; Luo et al., 2008). The pulmonary and cardiac muscle cDNA libraries of AT contained a large amount of energy metabolism related genes. To study the evolutionary forces that influence nuclear encoded mitochondrial proteins, we calculated Ka/Ks of nuclear-encoded mitochondrial unigenes for three pairs of species: chiru vs. human, cow vs. human, and mouse vs. human, and found that the nuclear encoded mitochondrial unigenes in TA underwent rapid evolution compared to those genes in cow and mouse. These results are quite consistent with our pervious study (Xu et al., 2005) that many energy metabolism genes, such as COXI and COXII (mitochondrion-encoded cytochrome coxidase I and II) are probably under positive selection in antelope and yak, which are also native to the Tibetan Plateau.

4.1. Limitation of the study

This study suffered from several obvious limitations: First, due to the lack of sufficient database in closely related species, identification of more fast evolving genes in the TA lineages was very difficult. Second, the unigenes used in this study only came from one individual TA. Whether the amino acid substitutions were fixed in TA populations or not, it could not be identification at present. Our findings should therefore be considered preliminary and a relatively larger prospective study would be needed to further confirm these findings.

5. Conclusion

The pulmonary and cardiac muscle cDNA libraries of TA contained a large amount of energy metabolism related genes, and those genes were expressed in lung and cardiac muscle tissues. Some nuclear-encoded mitochondrial proteins have undergone rapid evolution. The SP-A and SP-B genes were highly expressed in the lung tissues and underwent accelerated evolution in the TA lineage, suggesting the pulmonary surfactant proteins that produced by type II pneumocytes might play important roles in the high-altitude adaptation. The annotated EST datasets also provided a large number of potential unigenes for further biological studies on high altitude adaptation.

Conflict of interest

We declare that there is no any conflict of interest with the present study.

Acknowledgments

This project has been supported by the National Basic Research Program of China (No. 2012CB518200), the Program of International S&T Cooperation of China (No. 2011DFA32720), and the National Natural Science Foundation of China (No. 31160232 & 31160219). We would like to thank Dr. T Simonson, School of Medicine, University of California, San Diego, for english correction, and also acknowledge a number of colleagues for valuable assistance and insightful comments from Qinghai University, Beijing Genomics Institute.

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