MOLECULAR CLONING AND CHARACTERISTICS OF THE *PSPH*, *SNRPA1* AND *TPM1* GENES IN BLACK-BONED SHEEP (*OVIS ARIES*)

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ABSTRACT

The first animal with black traits to be studied was the Black-boned chicken. In China, we identified a flock of sheep with black traits. In the present study, the complete coding sequences of three genes, PSPH, SNRPA1 and TPM1, of Black-boned sheep (Ovis aries) were amplified using the reverse transcription polymerase chain reaction, according to the conserved sequence information for cattle and other mammals and known highly homologous sheep ESTs. The results showed that the Black-boned sheep PSPH gene encodes a protein of 225 amino acids, which has high homology with the PSPH proteins of seven species: cattle (99 %), humans (94 %), rat (93 %), mouse (93 %), African clawed frog (77 %), Atlantic salmon (70 %) and zebrafish (68 %). The Black-boned sheep SNRPA1 gene encodes a protein of 255 amino acids, which has high identity with the SNRPA1 proteins of four species: cattle (99 %), humans (99 %), mouse (97 %) and chicken (91 %). The Black-boned sheep TPM1 gene encodes a protein of 284 amino acids, which has high homology with the TPM1 proteins of four species: cattle (82 %), rabbit (82 %), mouse (82 %) and humans (81 %). Using phylogenetic analysis, it was shown that the Black-boned sheep PSPH, SNRPA1 and TPM1 proteins are closely related to their cattle counterparts. The tissue expression analyses revealed that the Black-boned sheep PSPH, SNRPA1 and TPM1 genes were expressed in a range of tissues, including leg muscles, the kidneys, the skin, the longissimus dorsi muscle, the spleen, the heart and the liver. These data serve as a foundation for further insight into these three genes in this rare sheep breed.

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Introduction

The mechanisms underlying the phenomenon of hyperpigmentation could possibly be sought in genes which are over-expressed in melanoma. Studies of mRNA expression in melanoma show over 100 upregulated genes, including *PSPH1*, *SNRPA1*, and *TPM1* (13).

Phosphoserine phosphatase (PSPH) is a member of a subfamily of the phosphotransferases. This enzyme is responsible for the third (last) step in L-serine biosynthesis. It catalyzes the Mg^{2+}-dependent hydrolysis of L-phosphoserine and is also involved in the conversion between L-serine and L-phosphoserine (2, 19). Additionally, PSPH has a role in neural stem cell proliferation and may regulate the signaling between neural stem cells and other cells within the stem cell niche (19). Lack of this protein is thought to be linked to Williams syndrome (15, 20).

Small nuclear ribonucleoprotein polypeptide A-prime (SNRPA1) is one of the specific protein constituents of the U2 snRNP (small nuclear ribonucleoprotein) particle (23). It is associated with sn-RNP U2 and helps the A-prime protein to bind the stem loop IV of U2 snRNA (22). Kittler et al. (16) identified 37 genes required for cell division, one of which was *SNRPA1*. These genes contain several splicing factors whose knockdown generates mitotic spindle defects (16). In addition, a putative nuclear-export terminator was identified to accelerate cell proliferation and mitotic progression after knockdown (11, 16).

Tropomyosin-1 alpha chain or alpha-tropomyosin (TPM1) belongs to the tropomyosin family of highly conserved, widely distributed actin-binding proteins which play a role in the contractile system of striated and smooth muscles and the cytoskeleton of non-muscle cells. TPM1 is associated with the troponin complex, in the calcium-dependent regulation of vertebrate striated muscle contraction. And smooth muscle contraction is regulated by interaction with caldesmon. In non-muscle cells, TPM1 is involved in stabilizing the cytoskeleton actin filaments (1, 10, 14, 21).

The first animal with black traits to be studied was the Black-boned chicken (silky fowl). Intriguingly, we have reported the discovery of Black-boned sheep (*Ovis aries*) in a flock of sheep distributed in the Nanping County of Yunnan Province, China (6, 7, 8). These sheep have dark coloured (black) tissues, compared with the reddish coloration typical for ordinary sheep (*Ovis aries*), and the coloration was shown to be due to the presence of excessive melanin (5, 7), as in the silky fowl. Cross-breeding studies have shown the trait for dark coloration in sheep to be inherited (18, 29). Moreover, in 2009, the Black-boned sheep were certificated as a novel livestock genetic resource by the Ministry of Agriculture, China.

The functions of the PSPH, SNRPA1 and TPM1 proteins, which are encoded by the *PSPH*, *SNRPA1* and *TPM1* genes, respectively, and the association of these three genes with growth, health, cell morphology, melanogenesis regulation and other important functions, suggest that they could be potentially related to melanocyte function and melanin synthesis in Black-boned sheep. This justifies their cloning from Black-boned sheep, which are a natural and rare animal model for hyperpigmentation research.

In the present experiment, we cloned the whole coding sequences of Black-boned sheep *PSPH*, *SNRPA1* and *TPM1* genes according to the conserved sequence information for cattle and other mammals and highly homologous sheep ESTs. We also conducted sequence analysis of established nucleotide sequences, some necessary function analysis, and finally examined the expression of these genes in a range of Black-boned sheep tissues. These data could serve as a foundation for further research into the function of these three genes in the rare Black-boned sheep breed.

Materials and Methods

Sample collection, RNA extraction and first-strand cDNA synthesis

Samples of leg muscle, kidney, skin, *longissimus dorsi* muscle, spleen, heart and liver were collected from six adult Black-boned sheep (*Ovis aries*). Total RNA extraction and first-strand cDNA synthesis were conducted as described in our previous studies (17, 25, 28).

Isolation of the *PSPH*, *SNRPA1* and *TPM1* genes

To design a primer pair for amplification of the complete coding sequence of *PSPH*, the sequences published in GenBank for *PSPH* from humans (GenBank accession number: AK315235), cattle (GenBank accession number: NM_001046355), mice (GenBank accession number: NM_133900), rat (GenBank accession number: NM_001009679), and their highly homologous sheep ESTs: EE800625, EV439875 and EE770071. The Primer Premium 5.0 software was used. Similarly, another two pairs of primers for sheep *SNRPA1* and *TPM1* genes isolation were designed based on the conserved coding sequences from cattle and their highly homologous sheep EST sequences.

Reverse transcription PCR (RT-PCR) was performed to isolate Black-boned sheep *PSPH*, *SNRPA1* and *TPM1* genes, using the cDNAs from different tissues described above. The 25 μL reaction system was: 2.0 μL of cDNA, 2.0 μL of 10 mmol/L mixed dNTPs, 2.5 μL of 10× Taq DNA polymerase buffer, 0.8 μL of 10 pmol forward primer, 0.8 μL of 10 pmol/L reverse primer, 0.4 μL Taq DNA polymerase (5 U/μL), and 16.5 μL of sterile water. These primer sequences and their annealing temperature for RT-PCR are shown in **Table 1**. Then, the PCR products for Black-boned sheep *PSPH*, *SNRPA1* and *TPM1* genes were cloned into a PMD18-T vector and sequenced bidirectionally.

TABLE 1

Primers and annealing temperature used for isolation for the sheep *PSPH*, *SNRPA1* and *TPM1* genes

Gene	Primer sequences	Ta / °C
PSPH	Forward 5'- ATG GTC TCC CAT TCA GAG -3' Reverse 5'- TTA TTC TTC CAG TGC TCCC-3'	54
SNRPA1	Forward 5'- ATG GTG AAG CTG ACG GCG-3' Reverse 5'- TCA GGA CCC ATT GGC AAC -3'	56
TPM1	Forward 5'- ATG GAC GCC ATC AAG AAG -3' Reverse 5'- TTA TAT GGA AGT CAT GTC GTT-3'	55

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR for tissue expression profile analysis was performed as previously described (4, 9, 24). To eliminate the effect of cDNA concentration, we repeated the RT-PCR five times, using 1 μL, 2 μL, 3 μL, 4 μL, and 5 μL of cDNA as templates. We selected the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the internal control. The control primers used were: 5'-AAGTTCAACGGCACAGTCA-3' (GAPDH 5' primer) and 5'-TCATAAGTCCCTCCACGAT-3' (GAPDH 3' primer). To ensure that no false positive PCR fragments were generated from pseudogenes in the contaminating genomic DNA, GAPDH primers were derived from different exons in the same gene. PCR primer combinations were tested using ovine genomic DNA as a negative control and an approximately 364 bp PCR fragment was amplified when cDNA was contaminated by genomic DNA. Semi-quantitative RT-PCR was performed with the same primers as those used for the isolation RT-PCR of the Black-boned sheep *PSPH*, *SNRPA1* and *TPM1* genes (see above). The PCR reactions were optimized for a number of cycles to ensure product intensity within the linear phase of amplification.

Bioinformatic analysis

The cDNA sequence prediction was conducted using the GenScan software (http://genes.mit.edu/GENSCAN.html). The protein prediction and alignment and phylogenetic analysis were performed using the Conserved Domain Architecture Retrieval Tool of BLAST at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST) and the ClustalW software (http://www.ebi.ac.uk/clustalw). Secondary structures of deduced amino acid sequences were predicted by SOPMA (http://npsa-pbil.ibcp.fr/). The theoretical isoelectric point (pI) and molecular weight (Mw) of the three putative proteins of the sheep genes were computed using the Compute pI/Mw Tool (http://www.expasy.org/tools/pi_tool.html).

Results and Discussion

RT-PCR result for Black-boned sheep *PSPH*, *SNRPA1* and *TPM1* genes

The amplification products obtained through RT-PCR with different tissue cDNAs, for Black-boned sheep *PSPH*, *SNRPA1*

and *TPM1* genes, were 678 bp, 768 bp and 855 bp, respectively (**Fig. 1**).

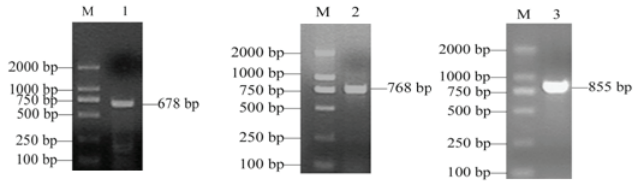

Fig. 1. RT-PCR result for Black-boned sheep *PSPH* (left), *SNRPA1* (middle) and *TPM1* (right) genes. M: DL2000 DNA Marker.

Sequence analysis

The cDNA nucleotide sequence BLAST analysis showed that these three genes were not homologous to any of the known sheep genes and their sequences were then deposited into the GenBank database (Accession Nos.: EU583474, EU583475 and EU583476). The sequence predictions were carried out using the GenScan software and the results showed that the 678 bp, 768 bp and 855 bp cDNA sequences represent three single genes which encode 225, 255 and 284 amino acids, respectively. The theoretical pI of sheep PSPH, SNRPA1 and TPM1 was 5.13, 8.92 and 4.69; and the theoretical Mw of these three putative proteins was 24839.34, 28297.48 and 32694.58.

Further BLAST analysis revealed that Black-boned sheep PSPH has high homology with the PSPH proteins of seven species: cattle (99 %), humans (94 %), rat (93 %), mouse (93 %), African clawed frog (77 %), Atlantic salmon (70 %), and zebrafish (68 %). Black-boned sheep SNRPA1 was found to have high homology with the SNRPA1 proteins of four species: cattle (99 %), humans (99 %), mouse (97 %), and chicken (91 %). Black-boned sheep TPM1 was shown to have high homology with the TPM1 proteins of four species: cattle (82 %), rabbit (82 %), mouse (82 %), and humans (81 %).

The prediction of the secondary structures by SOPMA (3) indicated that the deduced PSPH contains 83 α-helices, 30 extended β strands, and 112 random coils; SNRPA1 consists of 128 α-helices, 27 extended β strands, and 100 random coils; and TPM1 has 272 α-helices, two extended β strands, and 10 random coils (**Fig. 2**).

Based on the results of the alignment of PSPH, SNRPA1 and TPM1, phylogenetic trees were constructed using the ClustalW software (**Fig. 3**). The phylogenetic tree analysis showed that sheep PSPH, SNRPA1 and TPM1 have close genetic relationships with cattle PSPH, SNRPA1 and TPM1.

Gene expression profiles

Gene expression profile analysis was carried out and the results showed that the *PSPH* gene was over-expressed in the spleen of Black-boned sheep. There was almost no expression in the heart, the liver, the kidneys, leg muscles, *longissimus dorsi* muscle, and the skin. The expression of the *SNRPA1* gene in Black-boned sheep was intermediate in the heart, the spleen, leg muscles, and the *longissimus dorsi* muscle; and weak in the kidney and the liver. There was almost no expression in the skin. The third studied gene, *TPM1*, was over-expressed in the heart, leg muscles, the *longissimus dorsi* muscle, and the skin

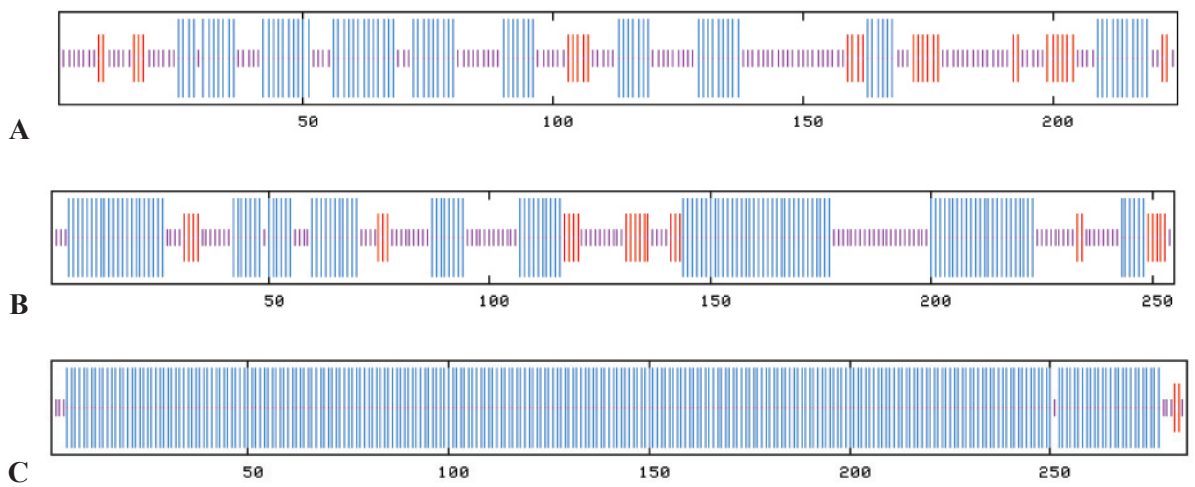

Fig. 2. Predicted secondary structure of black-boned sheep PSPH (**A**), SNRPA1 (**B**) and TPM1 (**C**) proteins by SOPMA. Helices, strands and coils are indicated, respectively, with the longest, the medium and the shortest vertical lines.

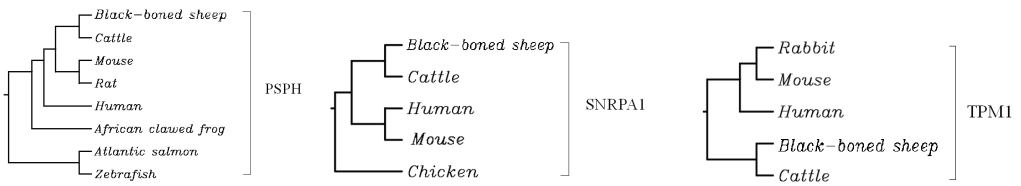

Fig. 3. Phylogenetic trees for different PSPH, SNRPA1 and TPM1 proteins.

of Black-boned sheep, and hardly expressed at all in the liver, the spleen, and the kidneys (**Fig. 4**).

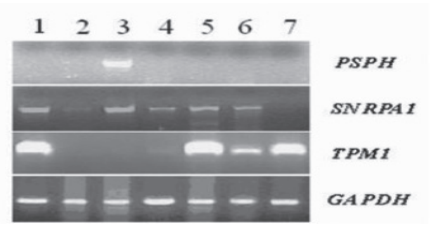

Fig. 4. Tissue expression distribution of the *PSPH*, *SNRPA1* and *TPM1* genes in Black-boned sheep. Lane 1: heart; Lane 2: liver; Lane 3: spleen; Lane 4: kidney; Lane 5: *longissimus dorsi* muscle; Lane 6: leg muscle; Lane 7: skin. The *GAPDH* expression is the internal control.

Comparative genomics is a useful tool for retrieving novel genes

In the present study, we firstly obtained the full coding regions of the *PSPH*, *SNRPA1* and *TPM1* genes from Black-boned sheep, based on comparative genomics. The *PSPH*, *SNRPA1* and *TPM1* genes have been reported to have significant roles in melanoma (13). Thus, our work can provide molecular basis for association analysis of the DNA polymorphism of the three genes with the black traits of Black-boned sheep.

Comparative genomics analyzes the relationship between genome structure and function among different biological species or strains. The function of human genes has been determined by comparing their counterparts in whole reference sequences of the human and mouse genome and some results have shown that virtually all (99 %) of the protein-coding genes in humans align with homologs in mouse, and over 80 % are clear 1:1 orthologs (12, 26, 27). This high conservation in protein-coding regions implies that the same sequences could be expected in different mammals such as cattle, humans, rat and mouse. This provides us with a useful tool to retrieve the functional regions of different genes for sheep, according to the conserved sequence information for the mouse, humans, cattle or other animals and to predict what those functions are.

In this experiment, the complete coding sequences of the sheep *PSPH*, *SNRPA1* and *TPM1* gene were isolated based on the genomic sequence information of the mouse, cattle or other animals and some referenced sheep ESTs. Sequence identification further validated that the method of comparative genomics is a useful tool for cloning unknown genes, especially the conserved coding region of genes in the sheep genome. Our results showed that Black-boned sheep PSPH, SNRPA1 and TPM1 are highly homologous with PSPH, SNRPA1 and TPM1 from cattle or other animals and sheep PSPH, SNRPA1 and TPM1 also have the same domains as their corresponding highly homologous proteins from cattle or other animals. This implies that black-boned sheep PSPH, SNRPA1 and TPM1 most likely have similar functions as PSPH, SNRPA1 and TPM1 of cattle or other animals. We also found that sheep PSPH, SNRPA1 and TPM1 do not show complete identity to their counterparts in cattle or other animals. This implies that there are most probably some differences in the functions of these proteins from those of their homologues in cattle or other animals.

The phylogenetic tree analysis revealed that the sheep PSPH, SNRPA1 and TPM1 proteins have a close genetic relationship with those of other species. Although different genes may have different evolutionary models even though they are in one individual or in one species, we found that these sheep proteins have a close relationship with those of other animals. This supports our choice of methods for cloning the target protein-encoding regions of Black-boned sheep based on the information available for the conserved encoding regions from other animals.

The results from the tissue expression profile analysis showed that the studied genes are differentially expressed in different tissues of Black-boned sheep. This differential expression indicates that the biological activities associated with these genes are diversely presented at the same time in different tissues.

Conclusions

To the best of our knowledge, this is the first report of the cloning of the ovine *PSPH*, *SNRPA1* and *TPM1* genes and the conducting of the necessary functional analysis and tissue expression profiles. The obtained information provides the primary foundation for further insight into these three sheep genes.

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