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Molecular cloning, mRNA expression and tissue distribution analysis of *Slc7a11* gene in alpaca (*Lama paco*) skins associated with different coat colors

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ABSTRACT

Slc7a11 encoding solute carrier family 7 member 11 (amionic amino acid transporter light chain, xCT), has been identified to be a critical genetic regulator of pheomelanin synthesis in hair and melanocytes. To better understand the molecular characterization of *Slc7a11* and the expression patterns in skin of white versus brown alpaca (*lama paco*), we cloned the full length coding sequence (CDS) of alpaca *Slc7a11* gene and analyzed the expression patterns using Real Time PCR, Western blotting and immunohistochemistry. The full length CDS of 1512 bp encodes a 503 amino acid polypeptide. Sequence analysis showed that alpaca xCT contains 12 transmembrane regions consistent with the highly conserved amino acid permease (AA_permease_2) domain similar to other vertebrates. Sequence alignment and phylogenetic analysis revealed that alpaca xCT had the highest identity and shared the same branch with *Camelus ferus*. Real Time PCR and Western blotting suggested that xCT was expressed at significantly high levels in brown alpaca skin, and transcripts and protein possessed the same expression pattern in white and brown alpaca skins. Additionally, immunohistochemical analysis further demonstrated that xCT staining was robustly increased in the matrix and root sheath of brown alpaca skin compared with that of white. These results suggest that *Slc7a11* functions in alpaca coat color regulation and offer essential information for further exploration on the role of *Slc7a11* in melanogenesis.

1. Introduction

In adult animals, both hair and skin colors are dependent on the types of pigments produced by melanocytes located in the bottom layer of the skin's epidermis (Barsh and Cotsarelis, 2007). Melanocytes found in the skin of mammals and birds generate two distinct types of melanin, eumelanin, which produce black to brown coloration, and pheomelanin, which appears yellow to reddish brown in color (Ito and Wakamatsu, 2008; Ito et al., 2000). The quality and ratio of eumelanin to pheomelanin alter the pigmentation and result in the colors of skin, fleece, feather, and iris of eye (Ito and Wakamatsu, 2011). Current researches indicate that a large number of genes regulate hair and skin colors in human and other vertebrate species (Lamason, 2005; Park et al., 2014; Passeron et al., 2007), however the molecular and cellular mechanisms regulating coat colors in fiber-producing species have not been completely elucidated.

Alpacas have a great variety of natural coat colors, but genetics and biological mechanisms of coat color regulation in this species remain unclear. Researchers have previously examined polymorphisms of melanocortin-1 receptor protein (*Mc1r*) (Feeley and Munyard, 2009; Powell et al., 2008) and agouti signaling protein (*Asip*) (Bathrachalam et al., 2012; Chandramohan et al., 2013) in alpacas with different coat colors and speculated that the polymorphisms present in these genes might alter coat color. Earlier, we explored the expression level of *Mc1r* and *Asip* as well as other genes related to coat color in white and brown alpacas. We demonstrated that the expression level of many genes including *Mc1r*, tyrosinase related protein 1(*Tyrp1*), tyrosinase (*Tyr*), tyrosinase related protein 2 (*Tyrp2*), inducible nitric oxide synthase (*Nos2*), paired box proteins 3 (*Pax3*) and *Wnt3A*, was elevated in the skin of brown alpacas compared to level found in white animals, however, further genes that regulate coat color remain to be evaluated.

Abbreviations: *Slc7a11*, solute carrier family 7 member 11; cDNA, DNA complementary to RNA; bp, base pair(s); AA_permease_2, amino acid permease; *Asip*, agouti signaling protein; *Mc1r*, melanocortin-1 receptor protein; *Tyrp1*, tyrosinase related protein 1; *Tyr*, tyrosinase; *Tyrp2*, tysrosinase related protein 2; *Nos2*, inducible nitric oxide synthase; *Pax3*, paired box proteins 3; NCBI, National Center for Biotechnology Information; ORF, open reading frame; DNase, deoxyribonuclease; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BCA, bicinchoninic acid; aa, amino acid(s); KDa, kilodalton(s); A, adenosine; C, cytidine; G, guanosine; T, thymidine; ANOVA, one-way analysis of variance; CT, comparative threshold cycle; pI, isoelectric point.

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Although multiple genes have been examined for regulation of coat color, little is known about the potential role of the gene *Slc7a11*. The gene *Slc7a11*, encoded xCT, was the light chain of system x_c^- with the activity of cystine/glutamate transporter (Shih and Murphy, 2001) and displayed higher conservation among many mammals, including sheep, bovine, human, mouse and chimpanzee. Functionally, *Slc7a11* has been shown to have important roles in cellular proliferation (Liu et al., 2007), response to oxidative stress (Bridges et al., 2001), and as an important target for the treatment of Alzheimer's disease (Qin et al., 2006). In terms of coat color regulation, *Slc7a11* has also been identified to play a crucial role in pheomelanin synthesis by increasing intracellular cystine level (Chintala et al., 2005). Recent work by He et al. demonstrated that testicular injection of *Slc7a11* into sheep resulted in progeny with brown/yellow patches in their coats (He et al., 2012). Although accumulating evidences have confirmed that *Slc7a11* gene had function in melanin production in mice and sheep, the expression pattern and localization of this gene have not been fully elucidated in alpacas. In this study, we have isolated and characterized the full length CDS of *Slc7a11*, analyzed its temporal and spatial expression types in the skin of alpacas with different coat colors and observed the localization of its expressed protein within the hair follicle using immunohistochemistry. The results of these studies contribute to understand the function of *Slc7a11* in coat color regulation and melanogenesis.

2. Materials and methods

2.1. Sample collection and total RNA extraction

The husbandry of alpacas and collection of their skin samples for use in described experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals (http://www.cioms.ch/index.php/texts-of-guidelines) and approved by Henan Normal University Council on Animal Care Guidelines. Three white and three brown healthy adult alpacas (*Lama paco*) were selected from the alpaca farm in Shanxi Agriculture University (Shanxi, China) for sample collection. Three pieces of skins (2 cm × 3 cm) were collected surgically from the hindquarters of each alpaca and immediately stored in liquid nitrogen. Total RNA from the samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA integrity was evaluated using gel electrophoresis and the RNA concentration was measured using a Nanodrop spectrophotometer an OD at 260 nm.

2.2. Cloning of Slc7a11 cDNA

In order to clone the alpaca *Slc7a11*, 4 μg of total RNA from the skin was used to synthesize the first cDNA using PrimeScript II RTase reverse transcriptase (TakaRa, Japan) following the manufacturers' instructions. Using comparison among the published *Slc7a11* cDNA sequences, two pairs of degenerate primers were designed for cloning alpaca *Slc7a11* (Table 1). The 25 μL PCR reaction included 12.5 μL 2 × Es Taq MasterMix (CWBIO, China), 1.0 μL of both forward and reverse primers, 2 μL diluted (10 times) cDNA and 8.5 μL water. The following cycling parameters were used for PCR: 95 °C held for 2 min, followed by 35 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min. The PCR products were analyzed by 1% agarose gel electrophoresis in the presence of ethidium bromide. The PCR products were gel-purified and cloned into the pUC-T vector (CWBIO, China), transformed into DH5α competent cells (CWBIO, China), extracted and sequenced.

Table 1
Primer sequences used in this study.

Primer name	Sequence (5′ to 3′)	Tm (°C)	Size (bp)
RT-PCR			
SLC7A11-1F	ATGGTCAGAAAGCCTGTGGTGTC	67.7	1031
SLC7A11-1R	CATAGAATAACCTGGAGACAGC	60.3	
SLC7A11-2F	GTTTGCTGTCTCCAGGTTATTCT	62.8	526
SLC7A11-2R	CAAGGATTGAATGCCAAGATTTC	60.8	
Real time PCR			
SLC7A11-R-F	GCTGGGCTGATTTATCTTCG	61.2	220
SLC7A11-R-R	TAAACCACTTAGGTTTCTTG	55.1	
β-actin-F	CTAAGGAGAAGGGCCAGTCC	63.9	177
β-actin-R	CTCAAGTTGGGGGACAAAAA	60.7	

F, sense primers; R, antisense primers.

2.3. Sequence analysis

The nucleotide and the deduced amino acid sequences were analyzed against the National Center for Biotechnology Information (NCBI) database using the BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The CDS of the alpaca *Slc7a11* gene was predicted using the Open Reading Frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Alignment of amino acid sequences was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2) and the phylogenetic tree was generated by the neighbor-joining method with 1000 bootstrap replicates in MEGA 6.06. The transmembrane regions of xCT and conserved domains were predicted by TMHMM and Pfam programs from ExPASy proteomics tools (http://www.expasy.org/resources).

2.4. Real time quantitative PCR

Total RNA was isolated from six alpaca skin samples (3 white, 3 brown). 1 μg DNase-treated RNA was converted to cDNA using PrimeScript RT reagent kit mix (TakaRa, Japan). The cDNA was then used for Real Time PCR quantification of mRNAs using the gene specific forward and reverse primers (Slc7a11-R-F, Slc7a11-R-R, 10 μM). *β-actin* was served as an endogenous control (primers listed in Table 1). Quantitative Real-Time PCR was performed in triplicate on the Stratagene Mx3005P system. The 25 μL PCR reaction included 12.5 μL SYBR Premix Ex TaqTM II (TaKaRa, Dalian, China), 1.0 μL of each specific forward and universal primers, 0.5 μL of ROX reference dye, 1.0 μL of diluted (8 times) cDNA and 9 μL of water. The following cycling parameters were used for real time PCR: 95 °C held for 30 s followed by 40 cycles of 95 °C for 5 s, 56 °C for 20 s and 72 °C for 15 s. Melting curve analyses were performed following amplifications. Quantification of *Slc7a11* transcript abundance was performed using the comparative threshold cycle (CT) method established by Livak et al. (Livak and Schmittgen, 2001) (Suppl. Table 1).

2.5. Western blotting analysis

Total protein was extracted from six alpaca skin samples using a protein extraction kit (Beyotime, China) according to manufacturer instructions. Protein concentrations were measured by the BCA method using bovine serum albumin (BSA) as the standard. Extracts were heat denatured at 99 °C for 5 min. Equal amounts (25 μg/lane) of protein from each sample were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene fluoride membranes. Membranes were then incubated with primary antibodies at the following dilutions: anti-xCT (H-121, 1:200, sc-98552, Santa Cruz, USA), anti-β-actin (1:1000, CWBIO, China). Primary antibodies were diluted in fresh 5% skim milk blocking buffer and incubated overnight at 4 °C. After washing in TBS with 0.1% Tween-20, the membranes were incubated with horseradish peroxidase conjugated secondary antibody (1:10,000, CWBIO, China) for 2 h at room temperature. Following washes, the membranes were detected using eECL Western Blot kit (CWBIO, China) and exposed to film. The intensity of signals for each protein was quantified using Image-Pro Plus Software, version 6.0 (Media Cybernetics, USA) and normalized to values obtained for β-actin (Suppl. Table 2). All experiments were performed in triplicates.