Advances in Fibre Production Science in South American Camelids and other Fibre Animals

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Preface

Animal fibres from South American camelids and other fibre or wool bearing species provide important products for use by the human population. The contemporary context includes the competition with petrocarbon-based artificial fibres and concern about excessive persistence of these in the natural environment. Animal fibres present highly valuable characteristics for sustainable production and processing as they are both natural and renewable. On the other hand, their use is recognised to depend on availability of appropriate quality and quantity, the production of which is underpinned by a range of sciences and processes which support development to meet market requirements.

Such support includes the efforts of the Animal Fibre Working Group (AFWG) of the European Federation of Animal Sciences (EAAP) which was instituted in 2007 and tasked with creating a network for investigation and dissemination of information in Europe and internationally. One task has been the organisation of scientific meetings, and continuing the tradition of previous European Symposia on South American camelids. These include the recent 5th Meeting in Sevilla (Spain: 2010) and 6th Meeting at EAAP, Nantes (France: 2013). References to these and other meetings, workshops and publications may be found on the AFWG website: http://www.eaap.org/presentation/scientific-structure/commissions-working-groups/animal-fiber-working-group/.

The present publication derives from the 7th European Symposium on South American Camelids and 3rd European Meeting on Fibre Animals (http://www.sympcam.org/). This meeting was held in the conference facility of the Domus Pacis Hotel, Assisi, Italy, on 12 to 14 June 2017. It was organised by Prof Dr Carlo Renieri and his colleagues Dr Attilio De Cosmo, Dr Francesco Fantuz, Dr Antonietta La Terza, Prof Alessandro Valbonesi (University of Camerino), Dr Marco Antonini (ENEA), and Maurizio Gubbiotti (University Marconi, Roma) with support from the scientific board comprising AFWG colleagues. We wish to thank Dario Pediconi, Cristina Nocelli, Irene Pazzaglia, Stefano Pallotti (University of Camerino) who helped us during the symposium. We also thank all participants who readily agreed to chair sessions or to participate in the Round Table.

We are very grateful to Loro Piana (http://www.sympcam.org/loropiana/) for generous funding support which enabled the attendance of international speakers and provided scholarships for three young scientists from Latin American countries.

Individual papers and abstracts, where full papers were not available, were printed from the manuscripts supplied by the authors. The assistance of Marvin Heuduck (Göttingen University) and the editors of Göttingen University Press in the editorial process is acknowledged.

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The editors

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Abstracts

Characterization and Expression Analysis of SLC7A11 in Llamas

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Abstract. The llama (*Lama glama*) is a South American camelid which is gaining worldwide recognition for its fiber. Coat color is one of its most cherished and commercially important characteristics; however, there is very little information about the molecular mechanisms that control pigmentation in llamas. SLC7A11 (solute carrier family 7 number 11) encodes the light chain of cystine/glutamate exchanger, xCT, which has a major role in pheomelanin synthesis Reports on these issues indicate for instance, that in mouse a mutation in SLC7A11 gene produce a diluted coat color due to a reduction in pheomelanin production (Chintala et al. (2005).Furthermore, differences in the skin expression levels of SLC7A11 have been observed in brown and white alpacas (Tian et al., 2015). The aim of this study is to describe SLC7A11 coding region and to analyze its variation and expression in llamas of different coat color. For this purpose, skin biopsies from 13 animals were collected, the RNA extracted and total cDNA obtained. cDNA was used for PCR amplification and sequencing of the full coding region. In addition, expression levels were analyzed by Real Time PCR. Coding region of SLC7A11 consisted of 1,512 bp that encoded a 503-amino acid protein. Protein sequence analysis showed 12 transmembrane helix regions with cytoplasmic N-terminal and C-terminal residues. Analysis with BLASTP showed 91-99 % identity to other mammals as well as a highly conserved amino acid permease domain. Seven SNPs were observed in the llama DNA sequences, 6 synonymous and 1 non synonymous. Finally, preliminary results indicated that expression levels of SLC7A11 in undiluted phenotypes differ significantly from those in diluted and white, but no differentiation was found between diluted and white phenotypes.

Resumen. La llama (*Lama glama*) es un camélido sudamericano que está empezando a ganar reconocimiento mundial por su fibra. El color de la fibra es una de las características de mayor valor comercial, sin embargo, hay muy pocos estudios sobre los mecanismos moleculares que controlan la pigmentación en llamas. El gen SLC7A11 (transportador soluble de la familia 7, número 11) codifica la cadena liviana del intercambiador de cistina/glutamato, xCT, y cumple un rol importante en la síntesis de feomelanina. Chintala et

al. (2005) encontraron que una mutación este gen es responsable del fenotipo subtle gray (sut) en ratón. Los mutantes sut muestran un color de capa diluido que se debe a una reducción de la síntesis de feomelanina. Además, alpacas marrones y blancas presentan diferencias en los niveles de expresión de SLC7A11 (Tian et al., 2015). El propósito de este trabajo es describir la región codificante de SLC7A11 y analizar su variación y expresión en llamas con distinto fenotipo de color Para ello se tomaron biopsias de piel, se extrajo el ARN y se obtuvo el cADN total. Este último fue empleado para reacciones de PCR y posterior secuenciación de la región codificante completa. Los niveles de expresión se analizaron mediante PCR en tiempo real. La región codificante de SLC7A11 está compuesta por 1512 pb y codifica una proteína de 503 aminoácidos. El análisis de la secuencia de la proteína muestra 12 regiones de tipo hélice transmembrana con los residuos C-terminal y N-terminal de orientación citoplasmática. Mediante el análisis con BLASTP se observó entre 91-99 % de identidad con otros mamíferos y la presencia de un dominio aminoácido permeasa altamente conservado.Se identificaron siete SNPs en las secuencias de ADN de llamas, 6 sinónimos y 1 no sinónimo. Finalmente, de acuerdo a resultados preliminares de expresión, los niveles de SLC7A11 en fenotipos no diluidos fueron significativamente diferentes de los hallados en fenotipos diluidos y blancos.

Keywords: lamas, coat color, SLC7A11

Introduction

The fiber of llama (*Lama glama*) is increasingly appreciated by the textile industry. Its cost depends primarily on the diameter and the color of the fiber. However, there is little knowledge about the molecular basis of coat color determination, which makes it very difficult for breeders to obtain the expected color phenotypes or reduce those of less commercial value.

In mammals, the basic coat colors are defined by the relationship between two pigments: eumelanin (from black to brown) and pheomelanin (from red to yellow). Eumelanin/pheomelanin ratio is regulated mainly by the ligand-receptor system of the agouti signaling protein (ASIP) and the melanocortin 1-receptor (MC1R). The binding of alpha-melanocyte stimulating hormone (α-MSH) to MC1R leads to eumelanin synthesis while binding of ASIP inhibits signal transduction, causing the melanocytes to produce pheomelanin (Lu et al., 1994). However, the final color phenotype will also depend on the expression and interaction of many other genes involved in processes such as the development and differentiation of melanocytes, melanosome formation and pigment synthesis, pigment transport and transference to tissues and survival of melanocyte stem cells. Mutations affecting genes involved in any of these processes can disrupt the normal pigmentation pathway producing diluted and white phenotypes.

The different coat color phenotypes in llamas were described by Frank (2001) and Frank et al. (2006). Based on these descriptions and performing classical crossbreeding analysis, those authors studied the segregation of color phenotypes and postulated that pigmented phenotypes are segregated by the Agouti locus.

Nevertheless, the molecular basis of coat color determination in these species has not yet been established. Recently, we have sequenced the coding region of MC1R and ASIP in llamas and studied the association between polymorphisms in these genes and coat color variation (Daverio et al., 2016). An interesting finding in that work was the detection of association between MC1R*2 haplotype and white coat. However, white llamas were also homozygous for haplotype MC1R*3, which was found in colored phenotypes as well. This suggests that other genes might be involved in the production of white phenotypes. In most species, mutations in MITF and KIT genes are responsible of white phenotype (Haase et al., 2007; Pielberg et al., 2002), but we have studied the complete coding region of these genes in llamas and found no differences between white and colored phenotypes (Anello et al., 2015, 2016).

Extreme dilution of pheomelanin has been proposed as a possible mechanism for white or cream phenotypes in other species. Sponenberg and Rothschild, (2001) described in dogs an Intense (I) locus that is thought to dilute only pheomelanin. However, I locus has not been characterized yet. Several genes have been involved in color dilution in different mammal species; one of them is SLC7A11 (solute carrier family 7 number 11). This gene encodes the light chain of cystine/glutamate exchanger, xCT, which has a major role in pheomelanin synthesis. Cystine is a precursor for pheomelanin synthesis but it is not necessary for the synthesis of eumelanin. Therefore, mutations in SLC7A11 are supposed to produce only pheomelanin dilution, without affecting eumelanin. There are only a few reports about this gene and even less that analyses its relationship with pigmentation. One of them is the one carried out by Chintala et al. (2005). They found that a mutation in SLC7A11 gene was responsible for the subtle gray (sut) phenotype in mouse; sut mutants show a diluted coat, due to a reduction in pheomelanin production. In another work, Tian et al. (2015) studied brown and white alpacas and found that there were differences in the expression levels of SLC7A11 between these two phenotypes.

Based on the above information, the aim of the present study is to describe SLC7A11 coding region and to analyze its variation and expression in llamas of different coat color phenotype.

Materials and Methods

Skin biopsies from llamas with white (n=6) and pheomelanic (n=7) phenotypes were collected by disposable biopsy punch (3 mm diameter), following the Argentinean Ethical Guidelines for Biomedical investigation in Animals from Laboratory, Farm or obtained from Nature (Resolution D N° 1047/05 from CONICET, Argentina). Biopsies were conserved in RNAlater (SIGMA, Germany) until their further extraction. Total RNA was extracted by homogenization in TRIzol® and addition of chloroform to separate phases. The aqueous phase was used for the alcoholic precipitation of RNA. Then, it was washed and resuspended

in RNase free water. Reverse transcription to obtain cDNA was performed in a 20 µl reaction volume, using 1 µg/µl RNA , RevertAid Reverse Transcriptase (Thermo Fisher Scientific) and random primers (Biodynamics), following the manufacturer's instructions.

PCR primers were designed over conserved regions from others mammal sequences available in GenBank, to fully cover SLC7a11 coding region and flanking 5´and 3´UTRs Amplification reactions were carried out in 25 µl PCR mix containing 1X PCR Buffer (Invitrogen, Carlsbad, CA, USA), 2 mM MgCl2, 0.2 mM dNTPs, 60 µg BSA, 1 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.5 µM of each primer and 50 ng of cDNA. The cycling profile consisted of an initial denaturation step at 94 °C for 3 min, 30 cycles of 40 sec at 94 °C, 50 sec at 55–57 °C, 40 sec at 72 °C and a 5-min final extension at 72 °C. PCR products were checked on a 1 % agarose gel stained with GelRedTM, purified by PEG precipitation and sequenced. Sequences obtained were aligned and analyzed using Geneious (v.6.1.8, Biomatters). Additionally, comparison with the alpaca sequence available at GenBank (KM095134) was carried out. Protein sequence analysis was performed with TMHMM server (http://www.cbs.dtu.dk/services/TMHMM) for the prediction of transmembrane regions, Pfam (http://pfam.xfam.org) for the identification of conserved domains and BLASTP (blast.ncbi.nlm.nih.gov) for protein homology.

For the expression analysis the samples were divided into three color groups: undiluted pheomelanic (n=3), diluted pheomelanic (n=3) and white (n=6). Quantitative Real Time PCR was carried out using specific primers designed over the llama SLC7A11 coding region; 18S gene was used as endogenous control. Amplification reaction consisted of 20 µl, including 4 µl of HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solís Biodyne), 0.5 mM of each primer and 1 ng cDNA. The cycling parameters were: 15 min at 95 °C, 40 cycles of 15 sec at 95 °C, 20 sec at 60 °C, 20 secs at 72 °C, and a final gradient from 95 °C to 72 °C. Every sample was analyzed in triplicate on a RotorGene Q (Qiagen) equipment. Quantification of SLC7A11 transcript abundance was performed using the comparative threshold cycle (CT) method established by Livak and Schmittgen (2001). Finally, ANOVA analysis of variance was used to assess if differences in expression were significant.

Results

Complete coding region of the llama SLC7A11 gene consists of 1,512 bp divided into 12 exons. The protein encoded has 503 amino acids and, when aligned with other mammal species, it presented high identity with its homologues: from 99 % with Vicugna pacos and Camelus ferus to 91 % with Mus musculus. The protein displays one highly conserved functional domain Amino Acid Permease_2 located at amino acids 72-461, and 12 transmembrane helix regions with citoplasmatic N-terminal and C-terminal residues.

We detected 3 positions in the llama SLC7a11 gene that differ from the ones present in the alpaca sequence: c.15 T>C, c.18 G >T and c.445 C> T. The first two are located in exon 1 and represent synonymous substitutions, while the third one is located in exon 3 and it produces a change from His to Tyr .

Seven SNPs were found along the llama sequences, of which 6 are synonymous changes and one is a non-synonymous substitution. Table 1 shows the extension of each exon in the coding sequence (CDS) and the location of the polymorphisms.

Table 1: *SLC7A11* exons and polymorphisms in llama

Exon	Extension in CDS	Polymorphisms	Change in protein
Exon 1	1 - 277	-	-
Exon 2	278 - 403	c.298 T>C c.381 C>T	no no
Exon 3	404 - 520	c.418 G>T	p.140 A>S
Exon 4	521 - 646	c.522 T>C	no
Exon 5	647 - 746	-	-
Exon 6	747 - 791	-	-
Exon 7	792 - 915	c.837 T>C	no
Exon 8	916 - 1019	c.984 T>C	no
Exon 9	1020 - 1116	-	-
Exon 10	1117 - 1266	-	-
Exon 11	1267 - 1444	c.1380 G>T	no
Exon 12	1445 - 1512	-	-

We also studied the relative expression of *SLC7A11* in the skin of undiluted, diluted and white phenotypes (Figure 1). SLC7A11 expression levels in the non-diluted group differ significantly from those in diluted and white ($p<0.05$) animals. Although expression level was consistently lower in white llamas than in the diluted ones, the difference was not significant.

Discussion

There are very few studies addressing SLC7A11 gene variation and its role in mammal's pigmentation. Here, we described the coding region of this gene in llamas and found results that support previous information (Tian et al., 2015). SLC7A11 codes for a highly conserved protein with an AA_permease_2 domain, which presents 12 transmembrane helices and it has the function of transport of amino acids. Variation observed in the llama SLC7A11 gene was mainly due to synonymous substitutions and the unique non-synonymous SNP found does not seem to be associated to color dilution.

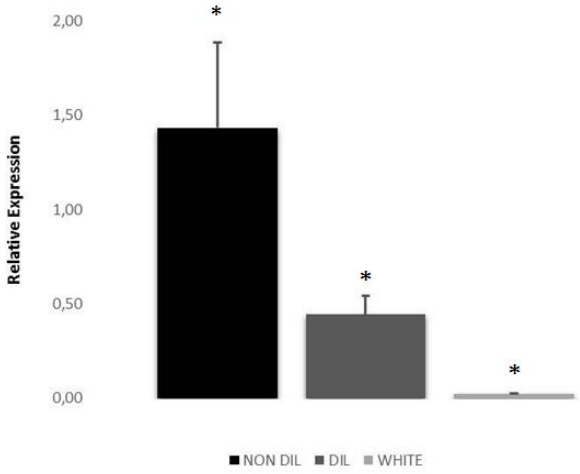

Figure 1: Relative expression of SLC7A11 in llamas.

SLC7A11 expression levels in undiluted llamas differ significantly from those in diluted and white. This is consistent with the protein function: it allows cystine to enter the cell and cysteine is necessary for the pheomelanin synthesis. Therefore, less expression of SLC7a11 should be translated into a reduction in pheomelanin synthesis. However, though it is evident that level of the diluted group is in between the others two, mRNA expression levels between diluted and white were not significantly different. This could be due to a small difference that would need a larger sample to be detectable. Moreover, as pheomelanic coats can vary from dark red to very light brown, the results obtained may depend on the manner in which the animals from the diluted group were chosen. In other words, at the lower end of the dilution range, expression values close to those of the group of white animals would be expected, while at the other end values should be more similar to those of animals with undiluted coats. This discussion raised the question if white coat could be an extreme dilution of pheomelanin, There is not enough information to answer this question yet, but considering the results from this work and previous findings from other genes (Daverio et al., 2016, Anello et al. 2015, Anello et al., 2016) it is likely that more than one genetic mechanism is responsible for white color in llamas.

Further research in needed to understand how SLC7a11 expression is controlled. We cannot rule out regulatory mutations affecting SLC7A11, but it is also possible that another gene, located upstream in the pigmentation pathway is down-regulating the expression of SLC7A11in white and diluted llamas.

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Publication of this book is supported by the Animal Fibre Working Group of the European Federation of Animal Science (EAAP). 'Advances in Fibre Production Science in South American Camelids and other Fibre Animals' addresses issues of importance to scientists and animal breeders, textile processors and manufacturers, specialised governmental policy makers and students studying veterinary, animal and applied biological sciences.

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