Small Ruminant Research 143 (2016) 29–34

Contents lists available at ScienceDirect

Small Ruminant Research

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

Short communication

Novel mutations in *Vicugna pacos* (alpaca) *Tyrp1* are not correlated with brown fibre colour phenotypes

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ARTICLE INFO

Article history:
Received 12 April 2016
Received in revised form 12 July 2016
Accepted 17 August 2016
Available online 17 August 2016

Keywords:
Tyrosinase related protein 1
Tyrp1
Alpaca
Brown
Fibre colour
Melanin

ABSTRACT

The tyrosinase related protein 1 (*Tyrp1*) gene is a logical candidate to house the causative mutation for the brown fibre colour phenotype that exists in alpacas. Polymorphisms in *Tyrp1* have been associated with brown fibre colour phenotypes in several species including mice, cows, cats and sheep. To investigate the role of *Tyrp1* on coat colour variation in alpacas, the complete coding region of *Tyrp1* was sequenced in a cohort of black and brown alpacas. Alpaca *Tyrp1* is 1614 nucleotides long and encodes a 537 amino acid protein. Eleven novel polymorphisms were identified within the coding region, and eight within introns. Analysis of the only non-synonymous polymorphism showed no association with fibre colour (T262R; c.785C > G, p = 0.90). There was no association observed between any of the polymorphisms and fibre colour. An homology model of Tyrp1 suggests that the T262R mutation causes minimum disruption of structure. These results provide evidence for the absence of a eumelanic brown phenotype caused by *Tyrp1* variants in the Australian alpaca population.

1. Introduction

The genetic variants responsible for variation in alpaca pigmentation remain largely unknown, despite recent advances (e.g. Munyard, 2011) and a growing demand from the industry for information that will help them to reliably breed the colour of fibre desired. Mutations within alpaca coat colour genes have been reported by a few research groups (Feeley and Munyard, 2009; Feeley et al., 2011; Powell et al., 2008; Sponenberg, 2001), but as yet there is still insufficient knowledge to fully inform breeding programs. Coat colour is of particular importance in the alpaca industry because of the financial impact that colour has on fibre value. There is currently no consensus on the nomenclature of colour in alpacas (either in the industry or in the scientific community), but it is generally accepted that >20 colours/patterns exist in the species. These colours range from white to black, with multiple shades of fawn and brown, plus all of these "base colours" with patterns such as roan, "grey" (not a colour, but a specific pattern of dilution), appaloosa and white spotting. White fibre is the most valuable, however the increasing demand for environmentally friendly fibre has seen an increase in the value of other colours.

Coat colour in mammals is determined largely by the amount, type and distribution of specialised pigment granules known as melanins (Furumura et al., 1996; Hoekstra, 2006). Mammals are capable of producing two chemically distinct melanin types known as eumelanin and pheomelanin (Furumura et al., 1996; Hoekstra, 2006). These pigments, while sharing a common precursor, tyrosine, differ in their biochemical properties (Capozzi et al., 2006; Thiruvenkadan et al., 2008). Eumelanin ranges from black to brown while pheomelanin is a sulphur rich compound that is responsible for red to yellow colour (del Marmol and Beermann, 1996; Thiruvenkadan et al., 2008).

Melanin synthesis occurs in specialised pigment cells called melanocytes, and is under the catalytic control of a number of enzymes including Tyrp1. Tyrp1 is responsible for the oxidation of a eumelanin monomer, formed during pigment synthesis, which then allows for the formation of a more stable, black eumelanin. Melanin production can still occur without the activity of Tyrp1, however the product is a less stable intermediate polymer which is brown rather than black (Kobayashi et al., 1998; Olivares et al., 2001). Loss of function mutations in *Tyrp1* are therefore associated with brown phenotypes (Berryere et al., 2003; del Marmol and Beermann, 1996; Gratten et al., 2007; Kobayashi et al., 1998; Lyons et al., 2004). *Tyrp1* brown phenotypes have been reported in cats

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http://dx.doi.org/10.1016/j.smallrumres.2016.08.012
0921-4488/

(Lyons et al., 2004; Schmidt-Küntzel et al., 2005), sheep (Gratten et al., 2007), dogs (Schmutz et al., 2002), cattle (Berryere et al., 2003) and mice; however they do not exist in horses. The role of *Tyrp1* in alpaca pigmentation is currently unknown.

One of the most common non-white colours in alpacas is described as "brown" by alpaca breeders. Our previous work investigating the melanin content of brown alpaca fibre indicated that eumelanic brown was not the cause of the majority of "brown" alpaca phenotypes, however some "brown" phenotypes could not be ruled-out as being caused by Tyrp1 (Cransberg et al., 2013). The objective of this study was to investigate sequence variation in the alpaca *Tyrp1* gene in black and brown alpacas to ascertain if there were polymorphisms that could potentially cause the eumelanic brown phenotype.

2. Materials and methods

2.1. Animals and DNA extraction

Blood samples were collected from a total of 37 alpacas bred in the states of Western Australia and New South Wales, in Australia. Genomic DNA was extracted from 200 μL of EDTA anticoagulated blood using the AxyPrep Blood Genomic DNA Miniprep Kit (Axygen, Union City, CA., USA) according to the manufacturer's instructions. Analysis was carried out on an initial sample group of 4 black and 19 brown animals. Fibre colour phenotype was determined according to a colour chart provided by the Australian Alpaca Association for colour registration. *Tyrp1* polymorphisms in other species lead to a characteristic phenotype often termed warm brown or chocolate brown and are usually recessive (Lyons et al., 2004). Following this premise, the brown sample group was selected to contain animals that were most likely to have a eumelanic background, either through visual inspection to have a colour that appeared to meet the "chocolate" description, or because they resulted from two non-brown parents (i.e. recessive inheritance). Consequently, the "brown" animals were all uniformly coloured over the whole body (i.e. not reddish/brown body with black extremities), and had skin that was not black. The black animals were likewise uniformly coloured (i.e. did not have tan undersides, nor any additional pattern such as roan or grey or white spotting) and had black skin. Following analysis of initial results, a further 10 black and 4 brown animals with the same phenotype restrictions/descriptions were genotyped for the exon 4 mutations.

2.2. Amplification and sequencing of alpaca Tyrp1

PCR primers were designed to amplify the seven coding exons of the alpaca *Tyrp1* gene (Table 1). These primers were designed based on the VicPac1.0 genome assembly (http://www.ensembl.org/index.html) and were designed to hybridise approximately 100 bp outside of the predicted splice sites for these exons, thereby amplifying the complete coding region of alpaca *Tyrp1*. At the time this work began, complete sequence information for exon 4 was not available, so primers were designed to amplify a larger, 2.5 kb section, of the gene which included the region predicted to encode for exon 4. The resulting sequence was then used to design internal primers to sequence from both the 5′ and 3′ directions to capture the entire exon 4 sequence, and approximately 2 kb of the surrounding introns (Table 1). All PCRs were carried out in an Eppendorf Mastercycler (Eppendorf, North Ryde, NSW, Australia), in 10 μL reactions containing 67 mmol/L Tris.HCl (pH 8.8), 16.6 mmol/L $[NH_4]_2SO_4$, 0.45% v/v Triton X-100, 0.2 mg/mL gelatin, 0.2 mmol/L dNTP (Fisher Biotech, Wembley, WA, Australia), 0.2 mmol/L each of forward and reverse primer, 1U *Taq* DNA polymerase (Fisher Biotec), 1.5 mmol/L $MgCl_2$ and 20 ng genomic DNA.

Table 1
Primer pairs used for amplification and sequencing of TYRP1 exons from genomic DNA.

Primer	Sequence	Product size	Temp (°C)
Ex2F	5′ CGGCTACATGGATTGACTTCC 3′	850 bp	60
Ex2R	5′ TTCACTTTGAGGTGGGTTGG 3′		
Ex3F	5′ TGAAATTGCTTGGTCAGTGC 3′	520 bp	61
Ex3R	5′ GCTCATCTCTACCCACGCTC 3′		
Ex4F	5′ GAAGTGTTTCCCAGCAAGG 3′	2503 bp	65
Ex4R	5′ TTTGACCTGGGAGTTCTC 3′		
Ex4F-2	5′ CCTCTGTAGTCTGTAGTCAT 3′	Seq only[a]	60
Ex4 R2	5′ AAGAGTTTGGGATTGGCAGA 3′	Seq only	60
Ex4R 3	5′ TCCAGTGATCTGAGTGCCAC 3′	Seq only	60
Ex4F-3	5′ AACGTTTATTCTGCGTATGTTTTT 3′	Seq only	60
Ex4F-4	5′ GCTTCACACCAAAACCCACT 3′	Seq only	60
Ex4IF	5′ AAGCCAAGCAAAGGGAGAAT 3′	560 bp	65
Ex4IR	5′ GCTTCACACCAAAACCCAC 3′		
Ex5F	5′ CCACATTACCTCAGGCAAGC 3′	2500 bp	62
Ex5R	5′ ATGACCAGTGATGGGAGA 3′		
Ex5IF	5′ AATCACAGAAGTTGGACATGG 3′	Seq	60
Ex5IR	5′ ATCAATCTGGCATTCAAAGGT 3′	Seq	60
Ex6F	5′ TGTTGAGCCTGCAAAAA 3′	304 bp	55
Ex6R	5′ TGTTTCCCAATATCATCACTGT 3′		
Ex7F	5′ TTTTGGGTCACCTTCAGAACA 3′	287 bp	57
Ex7R	5′ GGGTAACACATTTGCTTTTGG 3′		
Ex8F	5′ TTTGCTCTCATTTCCTTTTTCA 3′	306 bp	57
Ex8R	5′ AGCTTTTAATTCCAACCTGTGC 3′		

[a] Seq only: primers used as internal sequencing primers.

Thermal cycles were: initial denaturation at 95 °C for 3 min, followed by 30 cycles, each consisting of 94 °C for 30 s, annealing for 30 s (see Table 1 for temperature) and 72 °C for 45 s; with a final extension at 72 °C for 10 min. Amplified DNA was analysed by electrophoresis in 1.5% (w/v) agarose gels in TAE buffer, stained with ethidium bromide and visualised by UV transillumination. PCR products were purified using the AxyPrep PCR Cleanup Kit (Axygen, Union City, CA, USA). Amplification of *Tyrp1* coding exons from each animal was carried out in five independent 10 μL reactions, which were pooled before purification and sequencing. Sequencing reactions were carried out using *Tyrp1* primers for each exon (Table 1) with Big Dye Terminator v3.1 Technology (Applied Biosystems, Scoresby, Vic., Australia) and analysed on a 3730 DNA analyser (Applied Biosystems). Exons 4 and 5 were also sequenced using an additional set of internal primers to achieve full-length bi-directional coverage (Table 1).

2.3. Sequence assembly and analysis

Splice sites were determined using SpliceView, coupled with the known bovine and human *Tyrp1* exons (Fig. 1) (http://zeus2.itb.cnr.it/~webgene/wwwspliceview.html). Complete *Tyrp1* coding sequences for each animal were compiled using Geneious software (version 7.1, Biomatters, Auckland, New Zealand). The DNA and protein sequences from all animals were aligned using the Geneious Alignment algorithm, with options set to: Global alignment with free gaps, 65% similarity cost matrix, gap open penalty of 12 and gap extension penalty of 3. Single nucleotide polymorphisms (SNPs) were identified manually from this alignment. These data were also compared with genes and proteins from other species by GenBank NCBI BLASTn and BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Associations between polymorphisms and fibre colour were determined using Fishers exact test and Hardy-Weinberg equilibrium was tested using the Chi-square test with 1° of freedom.

2.4. Determination of gene structure and protein domains

The position of protein domains in alpaca Tyrp1 were determined using ScanProsite (http://prosite.expasy.org/scanprosite/)