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Characterisation of the Melatonin Receptor 1A (*MTNR1A*) gene in the Rasa Aragonesa sheep breed: Association with reproductive seasonality

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ABSTRACT

The ovine *Melatonin Receptor 1A (MTNR1A)* gene was structurally characterised and association between its variants and the reproductive seasonality was examined in a daughter design comprising three families of Rasa Aragonesa sheep breed. Sequencing of six Rasa Aragonesa ewes with extreme values for seasonality trait revealed 28 polymorphisms: 11 SNPs in the coding region (all in Exon 2), and 17 SNPs in the promoter region MTNR1A. All the substitutions in the coding region were found most likely lacking any phenotypic effect, because they are conservative mutations or were not part of the transmembrane domain. The silent mutations, which had shown association with reproductive seasonality in other breeds, were also found and genotyped in Rasa Aragonesa. The T allele of SNP606/*RsaI* of MNTR1A gene was associated with a greater percentage of oestrous cyclic ewes in the Rasa Aragonesa breed, indicating that this SNP may be in linkage disequilibrium with a mutation responsible for this trait close to *MTNR1A*, or in regulatory sequences of the gene. In this sense, several SNPs affecting a binding element for some transcription factors have been identified in the promoter region. The SNPs at 422 and 527 positions could constitute a binding element for some transcription factors (TFs), located in an EF2 and SRY consensus sites in the promoter region, respectively. Haplotype h_5 showed significant differences with the h_2 haplotype (66% compared to 49.2%) on oestrous cyclicity, thus these results are consistent with genotypic associations for each SNP. Haplotype with T, A and T alleles for SNPs 422, 677 (promoter region) and 612 (Exon 2) showed an increase of the percentage of oestrous cyclic ewes. Although some of these mutations have been associated with seasonal reproduction, further studies with a more appropriate animal design as well as functional studies of TF binding activity are needed.

1. Introduction

Many sheep breeds from Mediterranean area have seasonal patterns of oestrous behaviour and ovulation. Maximal reproductive activities occur from August to March. This reproductive seasonality induces great variation in lamb production and, therefore, in the market price of lamb meat. Hormonal treatments are widely used in some countries to induce out-of season reproduction, but the increasing demand for free-hormone products leads to a search for alternative methods. In the case of Rasa Aragonesa as in other Mediterranean breeds a percentage of ewes in good management and feeding conditions have spontaneous ovulations in spring, and can be naturally mated throughout the year (Folch et al., 1990; Folch and Alabart, 1999). This spring ovulatory activity is under

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genetic control (Hanocq et al., 1999) but selection for improved fertility in accelerated lambing systems is particularly challenging because of the complexity of the system (Notter, 2002). In this context, the use of genetic markers of photoperiod sensitivity is a promising approach to decrease seasonality of reproduction in sheep.

The melatonin receptor subtype 1A (MNTR1A) gene has been repeatedly proposed as a candidate gene and seems to play a key role in the control of photoperiod-induced seasonality mediated by the circadian concentrations of melatonin (Dubocovich, 1988; Weaver et al., 1996). Different studies in different sheep breeds have found two silent mutations in positions 606 and 612, associated with the seasonal reproductive trait (Pelletier et al., 2000; Notter et al., 2003; Chu et al., 2003; Faigl et al., 2008; Mura et al., 2010; Mateescu et al., 2009; Teyssier et al., 2010; Carcangiu et al., 2009, 2011). However, Hernandez et al. (2005) did not find any relationship between MNTR1A polymorphisms and reproductive seasonality in Ile-de France ewes, indicating that the effect of these polymorphisms could depend on the breed and/or environmental conditions.

In the present research, the association between MNTR1A polymorphisms and reproductive seasonality were investigated in a daughter design comprising three families of Rasa Aragonesa breed. For this purpose, detection and characterisation of polymorphisms were performed in the whole coding region and promoter of the MTNR1A gene in Rasa Aragonesa ewes. Secondly, an association study between some of the polymorphisms and reproductive seasonality was performed.

2. Materials and methods

All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367-91) for the use and care of animals in research.

2.1. Animals and experimental design

This experiment was conducted from January to August in the facilities of Centro de Investigación y Tecnología Agroalimentaria (CITA), a research Centre located in Zaragoza (Spain). Rasa Aragonesa is an autochthonous Mediterranean breed of sheep from the northeast of Spain, with about 500,000 animals recorded, mainly reared in extensive or semi-extensive farming systems and oriented to meat production. A total of 80 single reared ewes from a daughter design comprising three sire families of the Rasa Aragonesa sheep breed (26, 25 and 29 animals per family represented by rams A, B and C, respectively) were used. Sires heterozygous at position 606 and 612 were chosen. The ewes have born from January to April and came from 21 different farms. Ewes from each ram were selected to be as unrelated as possible on the basis of pedigree information. The age of all ewes was 3 years old, with similar body conditions (ranging from 2.5 to 3.0 of 5.0) and were maintained under the same management and fed ad libitum while grazing pasture. Weight and body condition score were recorded weekly from the beginning of the experiment and measured according to Russel et al. (1969) by two trained technicians. No hormonal treatments were applied to ewes during the study.

From January to August 2009, four vasectomised rams fitted with harnesses and marking crayons were mixed with the ewes and daily oestrous detection was performed (Radford et al., 1960). Thus, after natural mating, oestrus was recorded as a colour mark on the rump of the ewes, easy to identify visually. As ovulatory cycles occur approximately every 17 days, the colour of the marker crayon was changed every 2 weeks to avoid confusing marks between consecutive oestrus.

2.2. Structural characterisation of the MNTR1A gene

Genomic DNA was extracted from ovine lymphocytes according to the salting-out procedure described by Miller et al. (1988). Primers designed from sheep sequences NM_001009725 and AF078545 were used to amplify total coding and 5′ UTR regions and partial 3′ UTR and promoter genomic regions of the MNTR1A gene (Table 1). Genomic DNA (50 ng) of six Rasa aragonesa ewes with extreme values for the reproductive seasonality trait and the three rams was amplified in a final PCR volume of 25 μl containing 5 pmol of each primer, 200 nM dNTPs, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, 0.1% Triton X-100 and 0.75 U Taq polymerase (Taq, Biotools). Standard amplification cycles were used. The PCR products were sequenced using an ABI Prism 3700 (Applied Biosystems) and standard protocols. Direct sequencing of the PCR products of a small sample of ewes were used to search polymorphism in the experimental population (two ewes for each family) with extreme values for seasonality trait was performed as above, and standard protocols were used to search for polymorphisms. Homology searches were performed using the BLAST algorithm (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments were performed using CLUSTALW software (http://www.ebi.ac.uk/clustalw/). Studies of putative regulatory elements within the promoter were performed using TF Search (http://www.cbrc.jp/research/db/TFSEARCH.html) and Signal Scan (http://www-bimas.cit.nih.gov/ molbio/signal/) software.

MNTR1A genotyping was performed in the 80 ewes and the three sires for the SNPs at positions 606 and 612 (according to GenBank reference sequence U14109), and the SNPs in the promoter region. A fragment of Exon 2 was amplified containing the two SNPs at positions 606 and 612 (Table 1). PCR product was digested separately for SNPs 606 and 612 with *RsaI* and *MnlI* restriction enzymes, respectively.

Because of the large number of SNPs found in the promoter of *MTNR1A*, a PCR comprising all promoter polymorphisms was designed and amplification products were sequenced with forward primer (Table 1).

2.3. Statistical analyses

Oestrous cyclic and acyclic ewes in a given month were coded with “1” and “0”, respectively. An ewe was considered oestrous cyclic when at least one mark was detected