Meta Gene 2 (2014) 450–458

Contents lists available at ScienceDirect

Meta Gene

Sequence analysis and identification of new variations in the coding sequence of melatonin receptor gene (*MTNR1A*) of Indian Chokla sheep breed

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ARTICLE INFO

Article history:
Received 12 February 2014
Received in revised form 2 May 2014
Accepted 12 May 2014
Available online 28 June 2014

Keywords:
Genotyping
Polymorphism
Seasonality
Anestrus

ABSTRACT

Melatonin receptor 1A gene is the prime receptor mediating the effect of melatonin at the neuroendocrine level for control of seasonal reproduction in sheep. The aims of this study were to examine the polymorphism pattern of coding sequence of *MTNR1A* gene in Chokla sheep, a breed of Indian arid tract and to identify new variations in relation to its aseasonal status. Genomic DNAs of 101 Chokla sheep were collected and an 824 bp coding sequence of Exon II was amplified. RFLP was performed with enzyme RsaI and MnlI to assess the presence of polymorphism at position C606T and G612A, respectively. Genotyping revealed significantly higher frequency of *M* and *R* alleles than *m* and *r* alleles. *RR* and *MM* were found to be dominantly present in the group of studied population. Cloning and sequencing of Exon II followed by mutation/polymorphism analysis revealed ten mutations of which three were non-synonymous mutations (G706A, C893A, G931C). G706A leads to substitution of valine by isoleucine Val125I (U14109) in the fifth transmembrane domain. C893A leads to substitution of alanine by aspartic acid in the third extracellular loop. G931C mutation brings about substitution of amino acid alanine by proline in the seventh transmembrane helix, can affect the conformational stability of the molecule. Polyphen-2 analysis revealed that the polymorphism at position 931 is potentially damaging while the mutations at positions 706 and 893 were benign.

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http://dx.doi.org/10.1016/j.mgene.2014.05.005

It is concluded that G931C mutation of MTNR 1A gene, may explain, in part, the importance of melatonin structure integrity in influencing seasonality in sheep.

Introduction

Animals live under the influence of seasonal changes in climatic variables often linked to changes in latitude and altitude of a place with respect to the total insolation received. The daily photoperiod and the annual cycles in environmental temperatures are the striking variables in temperate regions while the annual cycles in rainfall, with the consequent cycles of food availability are found in the tropical region (Vivien-Roels and Pévet, 1983). Animals do regulate their reproductive activity by virtue of differential melatonin secretion in response to photoperiodism. Melatonin is produced by the pineal gland in proportion to the period of darkness (Malpaux et al., 2001). Melatonin regulates the circadian rhythmic cycles and reproductive changes in seasonal reproductive animals (Barrett et al., 1997; Ortavant et al., 1985; Reppert et al., 1994). Melatonin exerts its reproductive and circadian effects by virtue of its binding to high affinity melatonin receptors, which are G-protein coupled receptors prominently located in the pars tuberalis (PT) region and pre-mamillary hypothalamus (PMH). Pars tuberalis region, where the highest density of melatonin receptor (*MTNR1A*) has been documented, was found to be associated with prolactin secretion (Dardente, 2007; Dupré et al., 2008), whereas the PMH region observed to be closely associated with the control of GnRH secretion (Chabot et al., 1998; Malpaux et al., 1998). Melatonin receptor (*MTNR1A*) is a high affinity receptor which was first cloned in mammals by Reppert et al. (1994). There are other melatonin receptor subtypes also (*MTNR1B & 1C*) but only *MTNR1A* seems to be involved in the regulation of seasonal reproductive activity (Dubocovich et al., 2003; Weaver et al., 1996). Messer et al. (1997) reported two polymorphic RFLP sites (606 & 612) within the coding sequence of Ovine *MTNR1A* gene due to digestion by RsaI and MnlI, respectively. Studies of the association between the polymorphism of this gene and reproductive activity were carried out in sheep (Pelletier et al., 2000), goat (Migaud et al., 2002) and buffalo (Carcangiu et al., 2011a, 2011b). Some breeds of sheep like Merinos d'Arles (Pelletier et al., 2000); small tailed Han sheep (Chu et al., 2003), Awasi (Faigl et al., 2008), and Sarda (Carcangiu et al., 2009) showed a reproductive association with the polymorphic sites identified by Messer et al. (1997) while the Ile-de France ewes (Hernandez et al., 2005) did not show any association with them. Interestingly Martínez-Royo et al. (2012) had gone for characterization of *MTNR1A* gene in Rasa Aragonesa breed of sheep and have found 11 SNPs in the coding region and 17 SNPs in the promoter region. They associated the 'T' allele of SNP606/RsaI with the greater percentage of cyclic ewes in the Rosa Aragonesa breed. In the light of above studies we can conclude that the phenotypic elicitation of polymorphic genotypes varied with the breed and environmental conditions prevailing at a given region. In the present study, the aims were to verify the presence of polymorphic MnlI and RsaI sites in the coding sequence of melatonin receptor gene and to study the genetic variability of the *MTNR1A* gene in Chokla sheep breed.

Material and methods

Experimental animals and management

The study was undertaken in a homogenous flock of 101 ewes of Chokla breed maintained at the experimental farm of Central Sheep and Wool Research Institute (C.S.W.R.I.), Avikanagar, Rajasthan, India 26.1633° N, 75.7869° E. All the ewes were 2 to 4 years old. The experimental ewes were apparently healthy and without any reproductive disorders. Ewes were kept under natural photoperiod since birth and were fed on natural pasture integrated with concentrated feed. The latitude of the place corresponds to sub-tropical environmental conditions; therefore the Chokla breed is known to ovulate throughout the year with intermittent acyclity rarely observed.

Genomic DNA isolation and primer sequences

2 ml blood was collected from the jugular vein of each ewe in a microcentrifuge tube containing 200 μl of Acid Citrate Dextrose solution (ACD) as an anti-coagulant. Genomic DNA was extracted from 200 μl of blood using a commercial kit (AxyPrep™ Blood Genomic DNA miniprep Kit, Axygen Biosciences). Yield and purity of gDNA were estimated with a UV spectrophotometer. Samples were stored in −20 °C until use. Published primers specific for *MTNR1A* gene were used (Messer et al., 1997), which corresponds to positions 285–304 (Sense primer 5′-TGT GTT TGT GGT GAG CCT GG-3′) and 1108–1089 (Anti-sense primer 5′-ATG GAG AGG AGG GTT TGC GTT TA-3′) of the sequence by Reppert et al. (1994) in Exon II of the ovine *MTNR1A* gene (GeneBank U14109). Primers were commercially synthesized by Xcelris Labs Ltd. Ahmedabad, India.

PCR amplification of exon-II of MTNR1A gene

Reaction of PCR was carried out in 25 μl volume containing 200 ng gDNA, 1XPCR Buffer (50 mmol KCl, 10 mmol Tris HCl, (pH 8.0), 0.1% (w/v), Triton X-100), 2.0 mmol $MgCl_2$, 0.2 mmol of each dNTP, 10 pmol of each primer and 2 U of TaqDNA polymerase (Fermentas, Canada). PCR conditions were as follows: initial denaturation at 94 °C for 5 min followed by 35 repeated cycles of denaturation at 94 °C for 1 min, annealing 62 °C for 1 min, extension 72 °C for 1 min and final extension at 72 °C for 10 min on a peltier thermal cycler (M.J. Research). PCR products were resolved by electrophoresis on 2% agarose gel (Axygen Biosciences) in parallel with 100 bp DNA marker (qARTA. Bio) ladder.

PCR-RFLP and genotyping

All the PCR products were digested separately using 5 U of MnlI (Fermentas, Canada) enzyme and 5 U of RsaI (Fermentas) enzyme. The digestion reaction was carried out in 30 μl volume, containing PCR product 10 μl, 2μl of 1× Buffer G (Fermentas) (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH-7.9) for MnlI enzyme, whereas 2μl of Tango™ Buffer (10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH-7.0) for RsaI. The reaction mixture was incubated at 37 ºC for overnight, followed by a deactivation process at 65 °C for 20 min. Resulting fragments were separated by electrophoresis on 4% agarose gel (Axygen Biosciences), in parallel with a 50 bp DNA marker GeneRuler™ (Fermentas). Genotyping was performed in all the 101 samples in case of MnlI while in case of RsaI, it was performed for 99 samples. Two samples due to improper digestion could not be assessed.

Sequencing & SNP characterization

For sequencing, a total of 50 samples of amplified products was purified using PCR purification kit (Qiagen) and sequenced using Applied Biosystem 3730 DNA analyzer. The obtained sequences were aligned using the Megalign algorithm of LASERGENE software. The BLAST analysis was performed for all the sequences to obtain sequence similarity and percentage identity with other sequences. All the 50 chromatograms were analyzed for the identification of SNPs using LASERGENE software (DNASTAR) taking U14109 sequence as reference.

Polyphen-2 analysis

Polyphen-2 is a tool which predicts possible impact of an amino acid substitution on the structure and function of a protein using straightforward physical and comparative considerations (Adzhubei et al., 2010). All the non-synonymous mutations were analyzed for their effect on the structure of the protein, i.e. relative stability of the protein structure with respect to the change in amino-acid substitution. Polyphen-2 analyzed the impact of mutations identified measuring them on a scale of 0–1 determining whether they are benign, moderately deleterious or potentially damaging.

Results

PCR amplification of the gene

Genomic DNA of all the samples was analyzed by UV spectrophotometric qualitative analysis in terms of their 260/280 ratio and all of them were found to be in satisfactory range. PCR amplification of all samples was carried out using sense and anti-sense primer sequences and it resulted in an 824 bp single band corresponding to Exon-II of the *MTNR1A* melatonin receptor gene (Fig. 1).

RFLP analysis of the exon-II gene using MnlI & RsaI

Restriction fragment length polymorphism was performed by carrying out digestion with *MnlI* & *RsaI* to identify the presence of polymorphism at position 612 and 606 respectively with respect to reference sequence U14109. *MnlI* digestion resulted in cleavage sites at position 221, 254, 324, 560, 582, 610, and 693. The MnlI/G612A was found to be polymorphic due to the transition of 'G' nucleotide to 'A' nucleotide. *MnlI* cleavage site was disrupted due to G to A transition, resulting in a single 303 bp band denoted as '*m*' allele while the presence of guanine (G) position at 612 resulted in complete digestion to produce two bands of 236 and 67 bp identifies as '*M*' allele. These two alleles resulted in three genotypes '*MM*', '*Mm*' and '*mm*' in Chokla sheep breed (Fig. 2).

Enzyme digestion using *RsaI* evidenced four-cleavage site which resulted in resolution of five bands at positions 411, 267, 67, 56 and 23 bp respectively. The RsaI/C606T was polymorphic due to substitution of cytosine (C) by thymine (T) nucleotide. Digestion with *RsaI* resulted in polymorphic fragments of 267 and 23 bp when restriction site is present (allele *R*) or a single (290 bp) fragment when the restriction site was absent. Thus, three genotypes '*RR*', '*Rr*' and '*rr*' were observed in the Chokla sheep breed (Fig. 3).

Allele and genotype frequency of MTNR1A gene

Genotype distribution was *78MM*, *22Mm* and *1mm*, *78RR*, *17Rr*, and *4rr* for *MnlI* and *RsaI* digestions, respectively. Genotypic frequencies of the population were found to be in Hardy–Weinberg equilibrium as

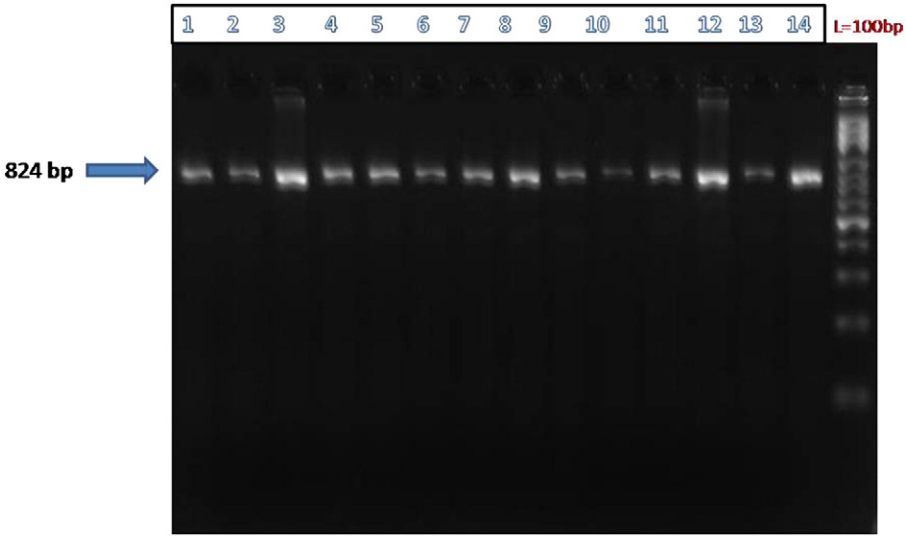

Fig. 1. PCR amplification of exon II OF MTNR1A gene.

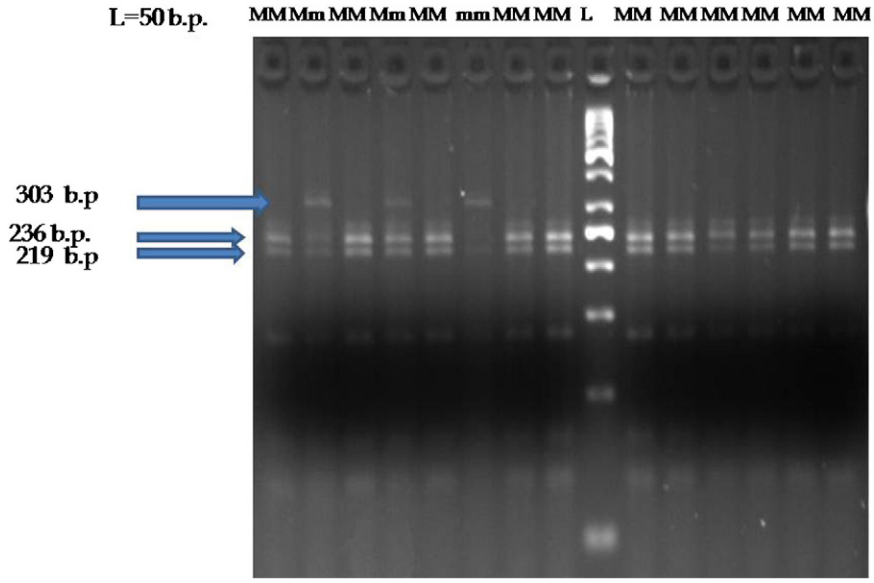

Fig. 2. RFLP Genotyping of MTNR1A gene by MnlI.

tested by web based tool of online encyclopedia for genetic epidemiology studies (Rodriguez et al., 2009). *RR* and *MM* genotypes were the dominant genotypes in tested group of animals. Allele and genotype frequencies are depicted in the (Tables 1a and 1b).

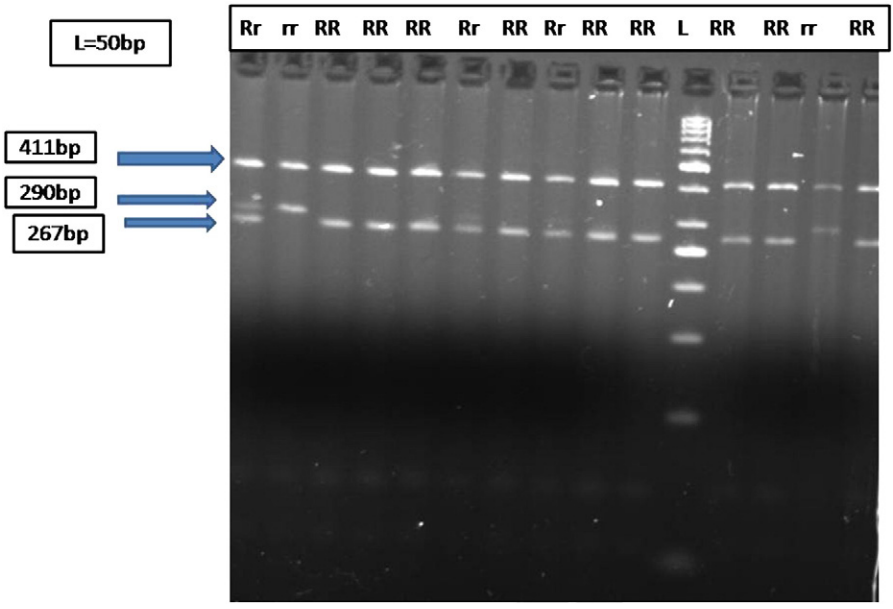

Fig. 3. RFLP Genotyping of MTNR1A gene by RsaI.

Table 1a
Genotypes identified and their frequencies in Chokla breed.

Genotype	No. of animals	Genotype frequency
RR	78	0.757
Rr	17	0.226
rr	4	0.017
MM	78	0.774
Mm	22	0.211
mm	1	0.014

Sequencing and identification of new variations

Samples (n = 50) of amplified product of exon-II were gel purified and sequenced. One representative sequence submitted to the NCBI database with GeneBank accession no. KC757711.1. Sequences obtained showed close similarity with goat (98%), cattle (97%) and buffalo (97%). Sequencing revealed several mutations/polymorphisms, which are presented in (Table 2). Most of the polymorphisms/mutations did not lead to amino acid changes except at 706, 893 and 931. Polyphen-2 analysis revealed that the polymorphism at position 931 is potentially damaging with a score of 0.912 (sensitivity: 0.81; specificity: 0.94) (Fig. 4) while the mutations at positions 706 and 893 were benign with a score of 0 and 0.1 respectively.

Discussion

Melatonin exerts its role as the regulator of reproductive activity by virtue of variation in its nocturnal secretion from the pineal gland, as an effect of photoperiodic changes in the environment. The reception of these signals by neuroendocrine cells is mediated through melatonin receptors of *MTNR1A* and *MTNR1B* subtypes, but it is *MTNR1A* which is chiefly associated with regulation of seasonal reproductive activity. Therefore, *MTNR1A* Exon II, which forms a major portion of coding sequence, was chosen to be characterized in a breed of sheep which is a native of sub-tropical arid tract of the Indian Thar Desert. Exon II portion of the coding sequence was amplified and characterized. Our results demonstrated the presence of polymorphic locus at positions of 606 and 612, which were detected by restriction fragment length polymorphism (RFLP) using enzyme *RsaI* and *MnlI,* respectively. A study of Pelletier et al. (2000) demonstrated an association between mm genotype and seasonal anestrus ewes of Merino, d'Arles breed in spring, whereas a study of Notter et al. (2003) showed that ewes carrying at least one '*M*' allele showed greater spring fertility. Thereafter, Mateescu et al. (2009) and Carcangiu et al. (2009) showed a correlation of *MM* and *RR* genotype to greater spring fertility and thus as an allele (both *R* and *M*), they have been hypothesized to be associated with out of season breeding ability of sheep. In our studies on Chokla sheep allelic frequency showed a significantly higher frequency of '*M*' (0.88) and '*R*' (0.87) in comparison to '*m*' (0.12) and '*r*' (0.13) alleles. Chu et al. (2006) did a descriptive study on allelic frequencies of non-seasonal (small tail Han sheep and Hu sheep breeds) and seasonal (Suffolk, Dorset and German mutton merino sheep). He reported significantly higher allelic frequencies of '*M*' and '*R*' allele in non-seasonal sheep breeds than that of seasonal sheep breeds. Carcangiu et al. (2009) reported '*M*' and '*R*' allelic frequency to be 0.78 and 0.66, respectively in Sarda sheep breed. Chokla animals are known to breed throughout the

Table 1b
Allele frequencies in Chokla breed.

Alleles	Allele frequencies
R	0.87
r	0.13
M	0.88
m	0.12

Table 2
SNPs identified in the Chokla sheep breed.

S. no	Positions of mutations[a]	Name of mutations	Amino acid change and position	Minor allele frequency (%)
1.	453	U14109:c.453G > T	None	20
2.	606	U14109:c.606C > T	None	20
3.	612	U14109:c.612G > A	None	20
4.	675	U14109:c.675G > A	None	2
5.	706	U14109:c.706G > A	Valine to isoleucine	20
6.	783	U14109:c.783G > A	None	10
7.	801	U14109:c.801G > A	None	10
8.	891	U14109:c.891C > T	None	30
9.	893	U14109:c.893C > A	Alanine to aspartic acid	10
10.	931	U14109:c.931G > C	Alanine to proline	30

Positions of mutations, nucleotide base changes and amino acid substitutions in Exon II of *MTNR1A* gene in Chokla.
[a] Sequence U14109 has been taken as reference.

year although majority of them are breeding during July–August immediately after onset of monsoon when plenty of natural grazing is available (Acharya, 1982). It may be due to the fact that some nutritional factors may play a role in enhancing ovulation and fertility during this period, although animals are breeding throughout the year in natural photoperiodic conditions of the region. Thus, they are considered to be aseasonal animals and the genotype frequency we obtained for *'R'* and *'M'* alleles may point towards their reproductive non-seasonal status, needing further investigation, considering the significantly higher frequencies for '*R*' and '*M*' alleles. Frequencies of *'M'* and *'R'* alleles were significantly higher than those of the French breeds (Pelletier et al., 2000) and Cross-bred Dorset (Mateescu et al., 2009). The second objective was to identify the sequence variations in *MTNR1A* gene of Chokla breed. Sequencing of the Chokla sheep Exon II region led to identification of ten mutations. Eight of which are identical (G453T, C606T, G612A, G706A, G783A, G801A, G891A and G893A) to that reported by Carcangiu et al. (2009). Mutations C606T and G612A have already been detected and genotyped using RFLP. Two other mutations (G675A and G931C) are first time reported in this study. We did not observe mutations C426T and G555A in Chokla sheep breed as observed by the Pelletier et al. (2000) in Merino d'Arles ewes. We observed that the mutations G706A, C893A and G931C led to an amino acid change at position 220, 282 and 295, respectively. The mechanistic/causal relation between *MTNR1A* gene polymorphism and seasonality of reproduction is yet to be established. Martínez-Royo et al. (2012) had reported polymorphism in the promoter region of the gene and found several SNPs affecting the binding element for some transcription factors in the promoter region. G706A, a mutation leads to substitution of valine by isoleucine in the fifth transmembrane helix of the receptor, has been shown to be in close contact with Histidine 211, the mutation of which modified the affinity of receptor to ^{125}I melatonin (Conway et al., 1997). Recently, Trecherel et al. (2010) reported that this substitution does alter the c-AMP signal transduction pathway. The mutation at position G893A led to substitution of amino acid alanine by aspartic acid in the third extracellular loop. Another important mutation G931C, which is being reported first time, seems to be one of the major conformational destabilizing mutations (CDM). Polyphen-2 analysis revealed that this mutation was potentially damaging with a score of 0.912. It led to substitution of alanine by proline in the seventh helical transmembrane domain. Helical domains, embedded in hydrophobic lipid environment, are made up of a stretch of 20–30 hydrophobic amino acids. Loop regions between these helical domains

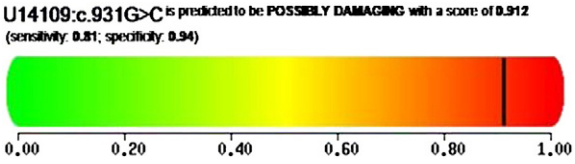

Fig. 4. Polyphen 2 Analysis of G931C mutation.

are usually hydrophilic. Substitution of alanine by proline may be destabilizing to the stable conformation of the *MTNR1A* gene for the following reasons: First, in proline the nitrogen ring is part of a rigid ring and rotation about N – Cα bond is not possible, acting as a kink in the helical structure. Second, proline has low hydrophobicity index (Kyte and Doolittle, 1982 index) of (−1.6) in comparison to alanine (+1.8); this may impede the interaction with lipid moieties inside the membrane by hydrophobic interaction. Third, proline residue in linked state does not have free substituent hydrogen to participate in hydrogen bonding of helical structure. The functional consequences of alanine to proline substitution on *MTNR1A* gene in relation to seasonal reproductive behavior in Chokla ewes deserve future investigations.

Conclusion

Chokla sheep *MTNR1A* receptor gene existed in four allelic isoforms with higher incidence of *MM* and *RR* genotypes. SNP characterization revealed certain important mutations, some of which were helix destabilizing and affecting conformational stability of the receptor. The next step will be to associate the structural mutations with the activity of the receptor and to study the regulatory sequences and interaction of the *MTNR1A* gene with other possible interactive genes.

Acknowledgments

The research is funded by the Department of Biotechnology, Government of India (Grant/Project No. BT/PR14462/AAQ/01/442/2010).

The authors are thankful to the Department of Animal Genetics, Central Sheep and Wool Research Institute for allowing the blood sample collection from animals. Special acknowledgement to Director, CSWRI Avikanagar for giving required permissions and support to facilitate the conduction of experiment. Dr Yogesh Gadekar, Scientist, is acknowledged for helping in collection of biological samples. The support of S.C. Tanwar, S.S. Rajput and Ramraj as skilled supporting staff is acknowledged.

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