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Polymorphisms of the melatonin receptor 1A gene that affects the reproductive seasonality and litter size in Small Tail Han sheep

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Abstract

Previous researches have shown that *MTNR1A* plays an essential role in sheep reproduction. However, most researches focused more on the reproductive seasonality of sheep, and few scientists had studied the association of polymorphisms of the *MTNR1A* gene with ovine litter size and reproductive seasonality. Therefore, we chose *MTNR1A* gene to detect its novel sequence polymorphisms and population genetics and analyse their association with seasonal reproduction and litter size in ewes. The mRNA expression level in hypothalamus, pituitary and ovary was also detected. In this study, five polymorphisms (g.15118664G > T, g.15118683C > T, g.15118756C > T, g.15118774C > T and g.15118951G > A) were identified in exon 2. Most importantly, the g.15118683C > T and g.15118951G > A were significant difference between year-round oestrous sheep and seasonal oestrous sheep ($p < .01$), and g.15118756C > T had a great effect on litter size of Small Tail Han sheep ($p < .05$). In addition, the mRNA expression level of *MTNR1A* in the hypothalamus of polytocous Small Tail Han sheep was significantly higher than that in monotocous Small Tail Han sheep ($p < .01$) and the expression of *MTNR1A* in the hypothalamus of year-round oestrous sheep was significantly higher than that in seasonal oestrous sheep ($p < .01$). Polymorphisms in exon 2 may regulate the reproductive seasonality and litter size of ewes by influencing gene expression to regulate the reproductive seasonality and litter size of ewes. Our studies provided useful guidance in marker-assisted selection of the litter size in Small Tail Han sheep.

KEYWORDS
litter size, reproductive seasonality, sheep, SNPs

1 | INTRODUCTION

Small ruminants that live at temperate latitudes utilize the photoperiod as a temporal signal to initiate changes in their reproductive status (Bittman, Karsch, & Hopkins, 1983). Changes in day length are perceived and translated into a physiological signal by the pineal gland through the night-time secretion of melatonin (Reiter, 1980). In mammalian reproduction, melatonin has a particular effect on ovaries by stimulating receptor sites within the hypothalamus, pituitary and gonadal axis (Carla Cristina et al., 2013; Malpaux, Daveau, Maurice-Mandon, Duarte, & Chemineau, 1998). Melatonin action is mediated through specific receptors. In mammals, there are two

high-affinity melatonin receptor subtypes, MTNR1A and MTNR1B, but only the MTNR1A seems to be involved in the regulation of reproductive activity (Carcangiu et al., 2011; Mura et al., 2014). With the deepening of researches, several studies have found the relationships between MTNR1A and reproduction activity in different animal species (Alsiddig et al., 2016; Carcangiu, Vacca, et al., 2009; Zetouni et al., 2014). In chicken, melatonin receptor subtypes were identified in ovaries, suggesting that melatonin directly affects ovarian function through activation of multiple receptors (Sundaresan et al., 2009). In geese, the expression levels of *MTNR1A* initially increased and later decreased during the follicular development cycle, indicating that melatonin receptors participated in activating small white follicles and small yellow follicles to develop into subsequent greater hierarchical follicles (He et al., 2014). In sheep, most studies were centred on the positions of 606 and 612 in the *MTNR1A* gene exon 2, whose mutation could result in association with seasonal reproduction (Chu, Cheng, Liu, Fang, & Ye, 2006; Giantsis, Laliotis, Stoupa, & Avdi, 2016; Luridiana et al., 2015; Pelletier et al., 2000). Polymorphisms within the *MTNR1A* gene in the Sarda sheep breed, which exhibits an anoestrous period in late winter/spring, led to advances in reproductive resumption (Carcangiu, Vacca, et al., 2009). In Dorset ewe lambs, the polymorphisms at this gene were shown to influence the beginning of puberty (Mateescu, Lunsford, & Thonney, 2009). In the Aragonesa breed, however, only the polymorphism in position of 612 was associated with a greater percentage of oestrous cyclic ewes between January and August (Martínez-Royo, Lahoz, Alabart, Folch, & Calvo, 2012). In the Ile de France ewes, the polymorphism in position of 606 was not associated with a difference in the onset, cessation or length of the breeding season among the animals of the two homozygous genotypes (Hernandez et al., 2005). Thus, numerous studies have investigated the relationship between *MTNR1A* and reproduction traits in different species and made it as a potential candidate gene for QTLs. However, these results in sheep also indicated that the relationship between the *MTNR1A* gene polymorphism and the reproduction can be varied with the breed.

As we all know, the prolificacy of sheep is an important economic trait. Generally, the litter size in the first parity is relatively lower, and the previous study indicated that the average litter size of four Swiss sheep breeds ranged from 1.36 to 1.57 in the first parity, from 1.52 to 1.75 in the second parity and from 1.56 to 1.86 in the third parity (Hagger, 2002). In the Hu sheep, the litter size in the first parity averages 1.72 and second parity litter size is 2.17 (Guan et al., 2011). Small Tail Han sheep, a famous Chinese indigenous breed for its prolificacy, average litter size is 2.67 (Guo, 2018). However, previous researches on the mechanism of melatonin regulation of animal reproduction have mainly focused on the reproductive seasonality in hypothalamus and pituitary (Gunwant et al., 2018), although there were some studies that have shown the expressional profile of *MTNR1A* in the ovine ovaries in different sheep breeds (Jiang et al., 2017; Martine, Agnès, & Beno, 2005), a few studies have shown that the polymorphism of the *MTNR1A* gene is significantly related to the litter size of ewes (Chu, Cheng, Liu, Fang, & Ye, 2008; Wang, 2013).

Therefore, this experiment aimed to detect the SNPs in *MTNR1A* and, subsequently, analyse polymorphisms in 6 different Chinese native sheep breeds, and explored their association with ovine reproduction traits (litter size) in addition to the reproductive seasonality. The expression levels of *MTNR1A* gene in 3 major tissues controlling reproduction at different fecundity sheep were also conducted. Our study aimed to identify a genetic marker conceivably valuable for marker-assisted selection.

2 | MATERIALS AND METHODS

2.1 | Animals preparation, sample collection and DNA extraction

All the experimental procedures mentioned in the present study were approved by the Science Research Department (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS) (Beijing, China). Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS (No. IASCAAS-AE-03, 12 December 2016).

As detailed in Table 1, 737ewes from six sheep breeds were selected for genotyping. Jugular vein blood samples were collected for DNA extraction using the phenol–chloroform method and then dissolved in ddH_2O.

2.2 | Primer design and genotyping

The primers for genotyping were designed using MassARRAY Assay Design v3.1 from Beijing Compass Biotechnology Co., Ltd. According to

TABLE 1 Information of six sheep breeds selected for genotyping

Breed	Number	Type	District
Small Tail Han sheep	380	Polyembryony and year-round oestrus	Yuncheng, Shandong Province, China
Hu sheep	101	Polyembryony and year-round oestrus	Xuzhou, Jiangsu Province, China
Cele black sheep	52	Polyembryony and year-round oestrus	Cele, Hetian, Xinjiang Uygur Autonomous Region, China
Prairie Tibetan sheep	161	Single birth and seasonal oestrus	Dangxiong, Tibet Autonomous Region, China
Sunite sheep	21	Single birth and seasonal oestrus	Wulate Zhongqi, Bayannaoer, Inner Mongolia Autonomous Region, China
Tan sheep	22	Single birth and seasonal oestrus	Yanchi, Ningxia Hui Autonomous Region, China

the sheep *MTNR1A* sequence (GenBank accession no. NC_019483.2). Genotyping results were validated by PCR and sequencing. The qPCR primers were designed using primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and sheep *MTNR1A* mRNA (GenBank accession no. NM_001009725.1) and ACTB mRNA sequences (GenBank accession no. NM_001009784). All primers were synthesized by Beijing Tianyi Huiyuan Biotechnology Co. Ltd. Primers' information is listed in Table 2. Five loci in the *MTNR1A* gene (g.15118664G > T, g.15118683C > T, g.15118756C > T, g.15118774C > T and g.15118951G > A) were selected for genotyping in 737 samples from Small Tail Han, Hu, Cele black, Prairie Tibetan, Sunite and Tan sheep. Genotyping was performed using a MassARRAY® SNP analysis (http://www.sequenom.com) (Johansen, Andersen, Børsting, & Morling, 2013). The polymerase chain reaction system and temperature were described in detail in a previous study (Zhou et al., 2018). Only those samples with a >95% success rate and only those SNPs with a genotype success rate of >95% were included in the analysis.

2.3 | RNA extraction, cDNA synthesis and qPCR

According to the litter size and the oestrous characters (Zhou et al., 2018), three ewes under each group were selected for expression study. RNA was extracted from three tissues (hypothalamus, pituitary and ovary) which are especially vital for mammal reproduction using the RNAprep Pure Tissue Kit (Tiangen). Quantity and quality of total RNA were determined using a NanoDrop 2000 and 1.2% agarose gel electrophoresis. The cDNAs were synthesized by PrimeScript™ RT Reagent Kit (TaKaRa) according to the kit instructions. The qPCR was performed on a Roche LightCycler® 480II (Roche) and carried out in 20 μl containing 10 μl SYBR Premix Ex Taq II (TaKaRa), 0.8 μl each primer, 2 μl cDNA and 6.4 μl ddH_2O. The qPCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s. ACTB was used as a reference gene.

2.4 | Statistical analysis

Genotype and allele frequency, polymorphism information content (PIC), heterozygosity (HE) and effective number of alleles (NE) were calculated. Then, the distributions of genotypes for each SNP in the studied populations were tested for deviation from Hardy–Weinberg equilibrium by the Hardy–Weinberg law. Statistical analyses were performed using SAS (V. 9.2) (SAS Institute Inc.). Differences among three groups of samples were tested by the least significant difference test. p values <.05 were considered to be significant. The adjusted linear model was: $y_{ijn} = \mu + P_i + G_j + I_{PG} + e_{ijn}$, where y_{ijn} is the phenotypic value of litter size; μ is the population mean; P_i is the fixed effect of the ith parity (i = 1, 2, 3); G_j is the fixed effect of the jth genotype (j = 1, 2, 3); I_{PG} is the interaction effect of parity and genotype; and e_{ijn} is the random residual. The gene expression data were normalized to the reference gene *ACTB*, and relative

TABLE 2 Primers information

Primer name	Sequences (5′–3′)	Product size	Usage
MTNR1A-1-F	ACGTTGGATGGTAACTAGCCACAAACAGCC	99bp	PCR for g.15118664G > T
MTNR1A-1-R	ACGTTGGATGCTTCATTGGTCTCGTTGTGG		
MTNR1A-1-E	CATCCTGGGTGCCATGCTG		Extension reaction
MTNR1A-2-F	ACGTTGGATGTTTGCTGGGCTCCTCTGAAC	116bp	PCR for g.15118683C > T
MTNR1A-2-R	ACGTTGGATGAACTAGCCACAAACAGCCAC		
MTNR1A-2-E	CTGAACTTCATTGGTCTC		Extension reaction
MTNR1A-3-F	ACGTTGGATGCAGCAAATGGCAAAGAGGAC	99bp	PCR for g.15118756C > T
MTNR1A-3-R	ACGTTGGATGACAAACCGAAACTGAAGCCC		
MTNR1A-3-E	CCACAAACATGGTGACAAAATT		Extension reaction
MTNR1A-4-F	ACGTTGGATGGTGAAACCGGACAACAAACC	112bp	PCR for g.15118774C > T
MTNR1A-4-R	ACGTTGGATGCAGCAAATGGCAAAGAGGAC		
MTNR1A-4-E	TTGCAACCGGACAACAAACCGAAACT		Extension reaction
MTNR1A-5-F	ACGTTGGATGTTCCTGATCTGGACGCTGAC	117bp	PCR for g.15118954G > A
MTNR1A-5-R	ACGTTGGATGGCGTGAAGGTACAGGAATAG		
MTNR1A-5-E	AGGCGGGGACCCTGCAGTA		Extension reaction
MTNR1A-6-F	GCATTGAGGCAGCTGTTGAA	534bp	PCR for SNPs identification
MTNR1A-6-R	CGTTTTCAGCATCACGGGAA		
MTNR1A-7-F	CCTCAGATACGGCAAGCTG	127bp	qPCR
MTNR1A-7-R	GATCCTCGGGTCATACTGCA		
ACTB-F	GCTGTATTCCCCTCCATCGT	97bp	qPCR
ACTB-R	GGATACCTCTCTTGCTCTGG		

expression level was calculated by the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008).

3 | RESULTS

3.1 | Polymorphisms of the coding region of the *MTNR1A* gene in Small Tail Han sheep

In this study, 5 SNPs in the exon 2 of the *MTNR1A* gene were identified by sequencing amplicon using primer 6 (MTNR1A-6-F and MTNR1A-6-R) in Table 2. Sequencing peak of different genotypes of the 5 SNPs (g.15118664G > T, g.15118683C > T, g.15118756C > T, g.15118774C > T and g.15118951G > A) is shown in Figure 1. The basic information and allele frequencies in Small Tail Han sheep are shown in Figure 2. Two SNPs (g.15118664G > T and g.15118683C > T) were identified as those involved in amino acid changes. The chi-square test demonstrated that all SNPs were under Hardy–Weinberg equilibrium ($p > .05$).

3.2 | Population genetic analysis of polymorphism in the *MTNR1A* gene

Besides Small Tail Han sheep (Figure 2), population genetic characteristics of 5 SNPs in the other 5 sheep breeds were also analysed, and the results are listed in Table 3. It revealed that g.15118664G > T and g.15118951G > A loci were moderately polymorphic (0.25 < PIC <.5), while others were at a low rate of polymorphisms in Small Tail Han sheep (Figure 2e). The g.15118756C > T and g.15118774C > T in Sunite sheep as well as g.15118951G > A locus in Hu sheep, Prairie Tibetan sheep, Cele black sheep and Tan sheep were moderately polymorphic (0.25 < PIC <.5). The chi-square test indicated that all SNPs were under Hardy–Weinberg equilibrium ($p > .05$) except g.15118683C > T in Tan sheep. In addition, we classified six breeds into two categories, year-round oestrus and seasonal oestrus, based on the oestrous characteristics, and the results of comparison of the population genetic analysis are shown in Table 4. The results indicated that the g.15118683C > T and g.15118951G > A were significantly different between year-round oestrous sheep and seasonal oestrous sheep ($p < 0.01$).

3.3 | Association of polymorphisms of 5 loci with litter size in Small Tail Han sheep

The results of association analysis between 5 loci with litter size in Small Tail Han sheep are shown in Table 5. At g.15118756C > T, sheep with TT genotype had a large litter size than those with CC and CT genotype ($p < .05$). At other loci, no significant differences in litter size between different genotypes were found. However, it can be seen that the mutant homozygotes were higher than the other two genotypes in g.15118664G > T, g.15118683C > T and g.15118774C > T, although it does not reach a significant level.

3.4 | Expression of *MTNR1A* in sheep with different oestrous characters and fecundity

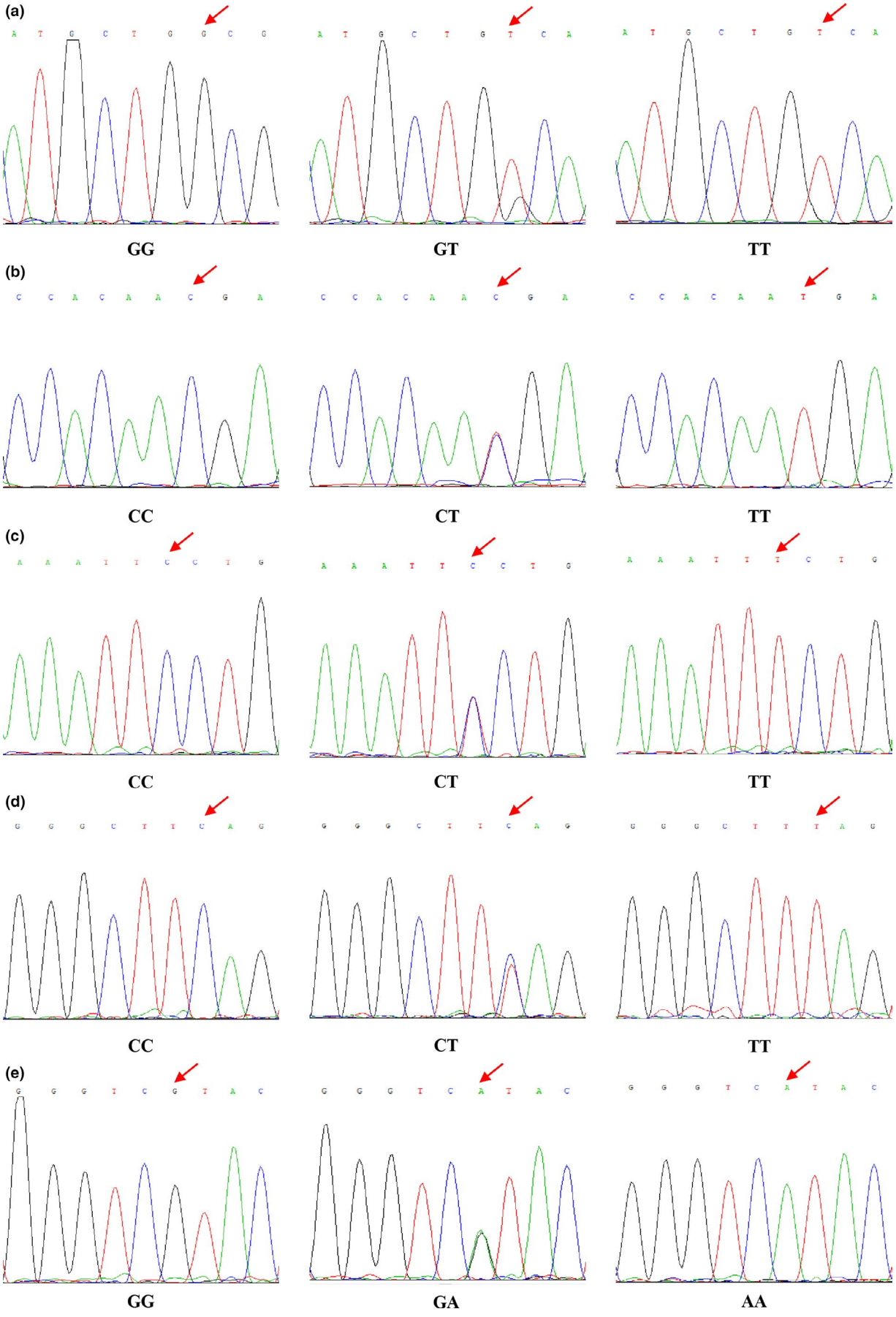
The expression of *MTNR1A* gene in hypothalamus, pituitary and ovary tissues in polytocous Small Tail Han sheep and monotocous Small Tail Han sheep was detected. The result is shown in Figure 3. *MTNR1A* gene was expressed in all three tissues with the highest level in the hypothalamus, followed by pituitary and ovary. The expression of MTNR1A in the hypothalamus of polytocous Small Tail Han sheep was significantly higher than that in monotocous Small Tail Han sheep ($p < .01$); it also reached a significant level in ovary ($p < .05$). However, there was no significant difference in pituitary ($p > .05$). The expression of MTNR1A gene in hypothalamus, pituitary and ovary tissues in seasonal oestrous Sunite sheep and year-round oestrous Small Tail Han sheep is also shown in Figure 3. MTNR1A gene was expressed in all three tissues with the highest level in the hypothalamus, followed by pituitary and ovary. The expression of MTNR1A in the hypothalamus of Small Tail Han sheep was significantly higher than that in Sunite sheep ($p < .01$); it also reached a significant level in the pituitary ($p < .05$). However, there was no significant difference in the ovary in two breeds ($p > .05$).

4 | DISCUSSION

4.1 | Polymorphism of *MTNR1A* gene

Polymorphisms of *MTNR1A* gene have a great influence on the reproduction of goat (Carcangiu, Vacca, et al., 2009; Chu et al., 2007), cattle (Elraey et al., 2011), pig (Ramírez et al., 2009), goose (Alsiddig et al., 2016), buffalo (Gunwant et al., 2018; Jiang et al., 2017), chicken (Sundaresan et al., 2009) and sheep (Pelletier et al., 2000, Chu, Ji, & Chen, 2003, Chu et al., 2006, Chu et al., 2008, Mateescu et al., 2009, Wang, 2013, Lei, Di, Liu, & Chu, 2015, Giantsis et al., 2016, Calvo et al., 2018). The earliest researchers found two polymorphic loci in the 824bp of exon 2 of the *MTNR1A* gene in many sheep breeds using restriction endonucleases *MnlI* and *RsaI* (Messer et al., 1997; Notter, Cockett, & Hadfield, 2003). In our previous studies, these two restriction enzymes were used to digest 824bp of exon 2 in year-round oestrous and seasonal oestrous sheep, and the two sites were all found (Chu et al., 2006, 2003; Ji, Chu, Chen, Zhou, & Zhu, 2003). Subsequently, A. Martínez-Royo found 11 SNPs in the coding region by sequencing, in which 9 SNPs (g.15119131G > A, g.15118951G > A, g.15118882G > T, g.15118851C > T, g.15118774C > T, g.15118666G > A, g.15118664G > T, g.15118464G > A and g.15118428T > C) were detected in Hu sheep;

FIGURE 1 The 5 mutations were detected in exon 2 of Small Tail Han sheep. (a-e) sequences of different genotypes of g.15118664G > T, g.15118683C > T, g.15118756C > T, g.15118774C > T and g.15118951G > A in Small Tail Han sheep; the red arrow indicates the location of the mutation site


(a)
GG
GT
TT
(b)
CC
CT
TT
(c)
CC
CT
TT
(d)
CC
CT
TT
(e)
GG
GA
AA

interestingly, these sites are all on exon 2 (Martínez-Royo et al., 2012; Wang, Deng, et al., 2015) . In the present study, we firstly carried out polymorphism detection in exon 2, the most widely studied exon of *MTNR1A*, and found five SNPs in sheep, each locus contained three genotypes (Figure 1), and both g.15118664G > T and g.15118683C > T loci were involved in amino acid changes (Figure 2b). Interestingly, three of the five loci had been detected in Altay sheep and Hu sheep (Wang, Shi, et al., 2015), and the other two loci were also found in the Rasa Aragonesa sheep (Calvo et al., 2018).

However, the researchers did not mention whether these loci play biological functions in ovine reproduction. In order to better understand the functions of the loci, we conducted population genetic analysis of five loci in six sheep breeds according to the typing results. Except for g.15118664G > T and g.15118951G > A loci, all others were at a low rate of polymorphisms in Small Tail Han sheep. In other five breeds, only the g.15118756C > T and g.15118774C > T in Sunite sheep as well as g.15118951G > A locus in Hu sheep, Prairie Tibetan sheep, Cele black sheep and Tan sheep were moderately polymorphic (.25 < PIC <.5). These results indicated that different genotypes of five loci were widely found in various sheep breeds, which provides valuable information to further study their functions.

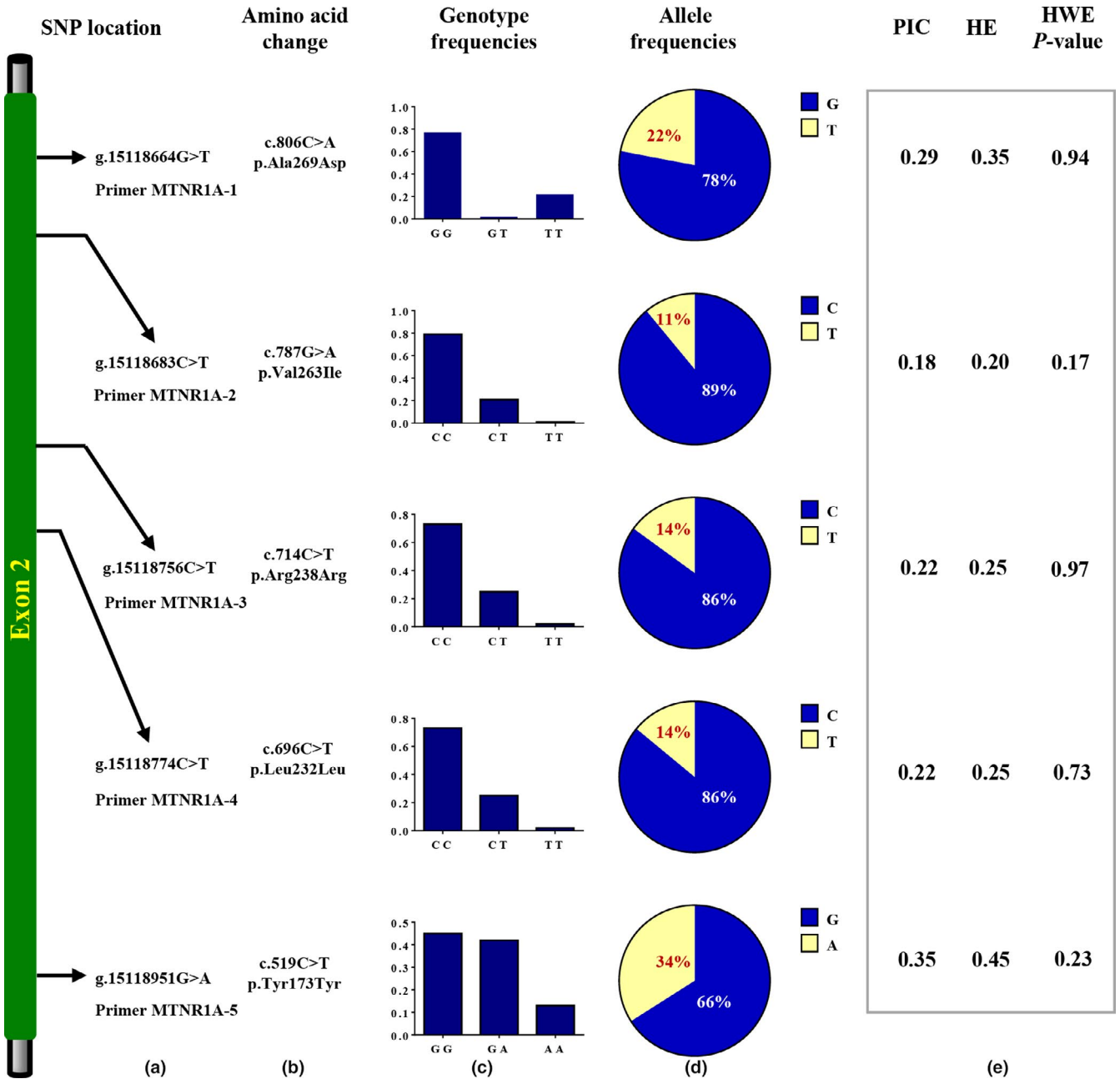


FIGURE 2 Polymorphisms of the coding region (exon 2) of the MTNR1A gene in Small Tail Han sheep. (a) The detected SNPs and specific primers. A total of 5 SNPs are found in the exon 2 of MTNR1A gene; (b) the change of amino acids; (c–d) the genotype and allele frequencies of the MTNR1A gene; (e) the test of polymorphism information content (PIC), heterozygosity (HE) and Hardy–Weinberg equilibrium (HWE)

TABLE 3 Population genetic analysis of 5 loci of *MTNR1A* gene in five sheep breeds

Locus	Breed	Genotype frequency			Allele frequency		PIC	HE	NE	Chi-square test (*p*-value)
g.15118664G > T		GG	GT	TT	G	T	–	–	–	–
	Hu sheep	0.81	0.18	0.01	0.90	0.10	0.16	0.18	1.22	.99
	Prairie Tibetan sheep	0.77	0.22	0.01	0.88	0.12	0.19	0.22	1.28	.73
	Cele black sheep	0.92	0.08	0.00	0.96	0.04	0.07	0.07	1.08	.77
	Sunite sheep	0.76	0.24	0.00	0.88	0.12	0.19	0.21	1.27	.54
	Tan sheep	0.82	0.18	0.00	0.91	0.09	0.15	0.17	1.20	.64
g.15118683C > T		CC	CT	TT	C	T	–	–	–	–
	Hu sheep	0.85	0.15	0.00	0.93	0.07	0.13	0.14	1.16	.42
	Prairie Tibetan sheep	0.96	0.04	0.00	0.98	0.02	0.04	0.04	1.04	.81
	Cele black sheep	0.92	0.08	0.00	0.96	0.04	0.07	0.07	1.08	.77
	Sunite sheep	0.95	0.05	0.00	0.98	0.02	0.05	0.05	1.05	.91
	Tan sheep	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0
g.15118756C > T		CC	CT	TT	C	T	–	–	–	–
	Hu sheep	0.80	0.19	0.01	0.90	0.10	0.17	0.19	1.23	.92
	Prairie Tibetan sheep	0.77	0.22	0.01	0.88	0.12	0.19	0.22	1.28	.73
	Cele black sheep	0.92	0.08	0.00	0.96	0.04	0.07	0.07	1.08	.77
	Sunite sheep	0.62	0.33	0.05	0.79	0.21	0.28	0.34	1.51	.96
	Tan sheep	0.82	0.18	0.00	0.91	0.09	0.15	0.17	1.20	.64
g.15118774C > T		CC	CT	TT	C	T	–	–	–	–
	Hu sheep	0.80	0.19	0.01	0.90	0.10	0.17	0.19	1.23	.92
	Prairie Tibetan sheep	0.77	0.22	0.01	0.88	0.12	0.19	0.22	1.28	.73
	Cele black sheep	0.92	0.08	0.00	0.96	0.04	0.07	0.07	1.08	.77
	Sunite sheep	0.62	0.33	0.05	0.79	0.21	0.28	0.34	1.51	.96
	Tan sheep	0.82	0.18	0.00	0.91	0.09	0.15	0.17	1.20	.64
g.15118951G > A		GG	GA	AA	G	A	–	–	–	–
	Hu sheep	0.44	0.38	0.18	0.63	0.37	0.36	0.47	1.88	.08
	Prairie Tibetan sheep	0.30	0.54	0.16	0.57	0.43	0.37	0.49	1.96	.18
	Cele black sheep	0.75	0.21	0.04	0.86	0.14	0.22	0.25	1.33	.30
	Sunite sheep	0.48	0.38	0.14	0.67	0.33	0.35	0.44	1.80	.51
	Tan sheep	0.59	0.36	0.05	0.77	0.23	0.29	0.35	1.54	.87

Note:: PIC, HE and NE represent polymorphism information content, heterozygosity and effective number of alleles, respectively; $p > .05$ indicates the locus was under Hardy–Weinberg equilibrium.

4.2 | Reproductive seasonality and *MTNR1A* gene

MTNR1A, a receptor of melatonin, plays important roles in regulating the physiological rhythm and reproductive seasonality of mammals (Lei et al., 2015). Many researches indicated that polymorphisms of *MTNR1A* gene had effects on the reproductive seasonality in sheep (Calvo et al., 2018; Carcangiu, Mura, et al., 2009; Chu et al., 2006; Giantsis et al., 2016; Mateescu et al., 2009; Pelletier et al., 2000), goat (Carcangiu, Vacca, et al., 2009; Chu et al., 2007) and buffalo (Gunwant et al., 2018; Luridiana et al., 2012). *MTNR1A* is one potential candidate gene involved in the control of ovine reproductive seasonality. The previous studies found homozygous genotype for the polymorphic *MnlI* site at position 605 of exon 2 was associated with year-round oestrus in ewes (Pelletier et al., 2000); if not, this site was associated with seasonal anovulatory activity in ewes (Chu et al., 2006; Notter et al., 2003). Besides, another position of 612 (exon 2) showed an increase in the percentage of oestrous cyclic ewes (Martínez-Royo et al., 2012) and influenced spring reproductive resumption in the Sarda sheep breed (Mura et al., 2014). In our study, we divided the six sheep breeds into two groups (year-round oestrous sheep and seasonal oestrous sheep) according to the oestrous characteristics and found that genotype frequency and allele frequency were significantly different between year-round oestrous sheep and seasonal oestrous sheep ($p < .01$) in the g.15118683C > T and g.15118951G > A loci for the first time. The results preliminarily showed that they may be related to the oestrus or reproductive seasonality and play important roles in year-round oestrous sheep. However, the similar

TABLE 4 Frequencies of 5 loci in *MTNR1A* gene in sheep with different oestrous characters

Locus	Characteristics of oestrus	Genotype frequency			Allele frequency		Chi-square test (*P*-value)
		GG	GT	TT	G	T	
g.15118664G > T	Year-round oestrous sheep	0.83	0.09	0.08	0.88	0.12	0.71
	Seasonal oestrus	0.78	0.21	0.01	0.89	0.11	
		CC	CT	TT	C	T	
g.15118683C > T	Year-round oestrous sheep	0.85	0.15	0.00	0.93	0.07	7.22E−07
	Seasonal oestrus	0.97	0.03	0.00	0.99	0.01	
		CC	CT	TT	C	T	
g.15118756C > T	Year-round oestrous sheep	0.82	0.17	0.01	0.90	0.10	0.93
	Seasonal oestrus	0.73	0.25	0.02	0.86	0.14	
		CC	CT	TT	C	T	
g.15118774C > T	Year-round oestrous sheep	0.82	0.17	0.01	0.90	0.10	0.97
	Seasonal oestrus	0.73	0.25	0.02	0.86	0.14	
		GG	GA	AA	G	A	
g.15118951G > A	Year-round oestrous sheep	0.55	0.34	0.11	0.72	0.28	6.60E−03
	Seasonal oestrus	0.46	0.43	0.11	0.67	0.33	

TABLE 5 LSM ± SE of litter size in Small Tail Han sheep with different genotypes

Locus	Genotype	Litter size of the first parity	Litter size of the second parity	Litter size of the third parity
g.15118664G > T	GG	2.17 ± 0.05 (267)	2.34 ± 0.06 (255)	2.83 ± 0.09 (99)
	GT	2.04 ± 0.10 (77)	2.28 ± 0.11 (72)	2.80 ± 0.17 (30)
	TT	2.67 ± 0.37 (6)	2.95 ± 0.37 (6)	3.00 ± 0.52 (3)
g.15118683C > T	CC	2.15 ± 0.06 (272)	2.31 ± 0.06 (260)	2.76 ± 0.09 (97)
	CT	2.10 ± 0.10 (78)	2.33 ± 0.11 (72)	2.97 ± 0.16 (34)
	TT	2.67 ± 0.52 (13)	2.77 ± 0.53 (6)	3.00 ± 0.64 (3)
g.15118756C > T	CC	2.18 ± 0.06b (253)	2.26 ± 0.06b (244)	2.67 ± 0.11b (96)
	CT	1.99 ± 0.09b (82)	2.23 ± 0.11b (75)	2.64 ± 0.19b (31)
	TT	2.86 ± 0.32a (7)	3.08 ± 0.34a (5)	3.30 ± 0.52a (3)
g.15118774C > T	CC	2.19 ± 0.06 (260)	2.36 ± 0.06 (249)	2.84 ± 0.09 (99)
	CT	2.02 ± 0.10 (84)	2.22 ± 0.10 (78)	2.74 ± 0.16 (31)
	TT	2.67 ± 0.37 (6)	2.69 ± 0.37 (5)	3.00 ± 0.52 (3)
g.15118951G > A	GG	2.14 ± 0.07 (152)	2.36 ± 0.08 (145)	2.72 ± 0.12 (54)
	GA	2.18 ± 0.08 (141)	2.28 ± 0.08 (133)	2.95 ± 0.13 (57)
	AA	2.05 ± 0.14 (41)	2.28 ± 0.15 (39)	2.69 ± 0.23 (16)

Note: LSM, least squares mean; SE, standard error; numbers in the parentheses next to litter size represent the amount of sheep of each genotype; different small letters in the same group mean significant difference ($p < .05$).

effects in other 3 loci were not observed, which was completely consistent with previous studies, possibly because these mutations were not in the transmembrane region (Martínez-Royo et al., 2012; Wang, Deng, et al., 2015). Whether those 3 mutations affect ovine seasonal oestrus and other reproductive traits by altering the structure of MTNR1A requires further researches.

4.3 | Litter size and polymorphism of *MTNR1A* gene

The previous studies also found that the polymorphisms of *MTNR1A* gene may affect the litter size in Small Tail Han sheep (Chu et al., 2008) and pig (Wang et al., 2006). Although these studies were not very clear, it also provides a new research direction. In the recent years, studies have found that *MTNR1A* gene could alter the number of lambing in ewes indirectly through influencing pregnancy (Luridiana et al., 2014), or involved in the production of eggs in birds (Alsiddig et al., 2016; Feng et al., 2018). Present association analysis revealed that mutation at g.15118756C > T had a great effect on litter size (the first, second and third parity) in Small Tail Han sheep ($p < .05$) (Table 5), which indicated that polymorphism of ovine *MTNR1A* gene may play a fundamental role in litter size of sheep. More interestingly, it could be seen the mutant homozygotes

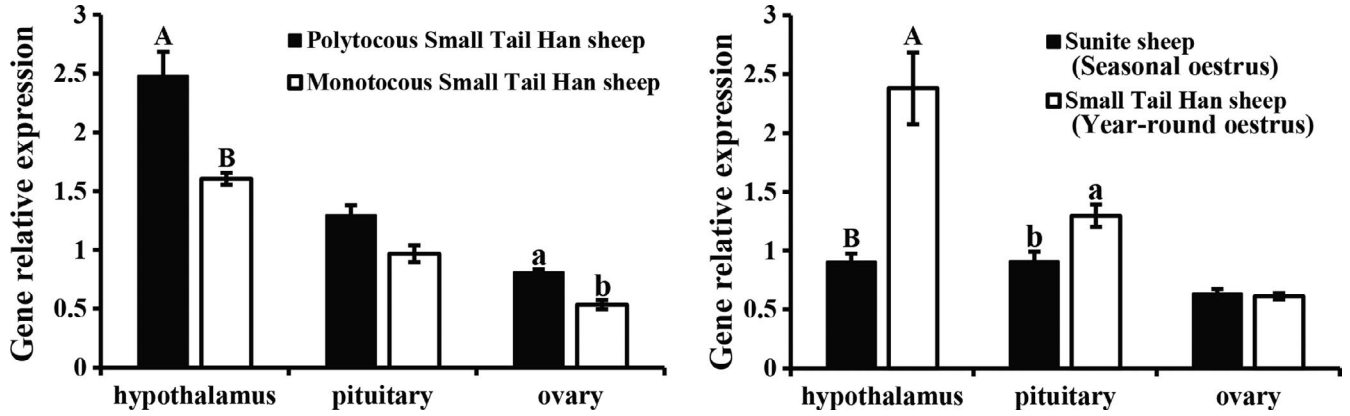


FIGURE 3 Expression of MTNR1A gene in HPG axis. Note: Different capital letters in the same group mean highly significant difference ($p < .01$); different small letters in the same group mean significant difference ($p < .05$)

were higher than the other two genotypes in g.15118664G > T, g.15118683C > T and g.15118774C > T in the first parity, although it does not reach a significant level, as well as in the second or third parity in above 3 loci (Table 5). The reason for this may be the limited sample size, and we will use larger samples to further verify the functions of those loci. Collectively, the results of the current study suggested that the polymorphisms of ovine *MTNR1A* gene play an important role in litter size of ewes; SNPs in this gene could be used as markers in marker-assisted selection for ovine reproductive traits.

4.4 | Expression of *MTNR1A* in sheep with different fecundity and different oestrous characters

Melatonin exerts its role as the regulator of reproductive activity by virtue of variation in its nocturnal secretion from the pineal gland. The reception of these signals by neuroendocrine cells is mediated through melatonin receptors of MTNR1A and MTNR1B, but it is MTNR1A which is chiefly associated with regulation of ovine seasonal reproductive activity (Saxena, Jha, Meena, & Naqvi, 2014), and most studies found MTNR1A plays a vital role in hypothalamus, pituitary and ovary tissues (Carla Cristina et al., 2013; Lei et al., 2015) and MTNR1A was expressed in hypothalamic–pituitary–ovarian axis tissues with entire oestrous cycles in Ganjia Tibetan sheep (Yang et al., 2019). Based on the results of the association analysis of oestrus and litter size, we selected two comparison groups to analyse the expression of *MTNR1A* in hypothalamus, pituitary and ovary tissues; the expression tendency among the three tissues was completely consistent with previous studies (Guo, Liu, Zhang, & Wang, 2010; Jiang et al., 2017; Yang et al., 2019). Interestingly, the expression of *MTNR1A* in hypothalamus of polytocous Small Tail Han sheep was significantly higher than that in monotocous Small Tail Han sheep ($p < .01$), and this significant level was also achieved in the year-round oestrous Small Tail Han sheep and seasonal oestrous Sunite sheep ($p < .01$) (Figure 3). These results indicated a higher expression of *MTNR1A* in the hypothalamus, and *MTNR1A* may have a positive effect on litter size and year-round oestrus. Therefore, polymorphisms of ovine *MTNR1A* gene exon 2 may have effects on its expression in hypothalamus, which may further influence the reproductive seasonality and litter size of ewes.

5 | CONCLUSIONS

Both g.15118683C > T and g.15118951G > A of *MTNR1A* may affect the oestrus or reproductive seasonality in sheep breeds. The homozygous mutation (TT) in g.15118756C > T locus can significantly increase litter size in Small Tail Han sheep. The differential expression of *MTNR1A* in ovine hypothalamus with different fecundity or oestrous characters suggested that polymorphisms in exon 2 may influence gene expression to regulate the reproductive seasonality and litter size of ewes. Therefore, our studies could be useful in marker-assisted selection of the litter size in Small Tail Han sheep.

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CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Mingxing Chu and Qiuyue Liu designed the research; Xiaoyun He and Zhuangbiao Zhang analysed or interpreted the data; Xiaoyun He drafted the paper.

DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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