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Association of methionine synthase gene polymorphisms with wool production and quality traits in Chinese Merino population[1,2]

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ABSTRACT: Methionine synthase (MTR) plays a crucial role in maintaining homeostasis of intracellular methionine, folate, and homocysteine, and its activity correlates with DNA methylation in many mammalian tissues. Our previous genomewide association study identified that 1 SNP located in the *MTR* gene was associated with several wool production and quality traits in Chinese Merino. To confirm the potential involvement of the *MTR* gene in sheep wool production and quality traits, we performed sheep *MTR* tissue expression profiling, SNP detection, and association analysis with sheep wool production and quality traits. The semiquantitative reverse transcription PCR analysis showed that the *MTR* gene was differentially expressed in skin from Merino and Kazak sheep. The sequencing analysis identified a total of 13 SNP in the *MTR* gene from Chinese Merino sheep. Comparison of the allele frequencies revealed that these 13 identified SNP were significantly different among the 6 tested Chinese Merino strains ($P < 0.001$). Linkage disequilibrium analysis showed that SNP 3 to 11 were strongly linked in a single haplotype block in the tested population. Association analysis showed that SNP 2 to 11 were significantly associated with the average wool fiber diameter and the fineness SD and that SNP 4 to 11 were significantly associated with the CV of fiber diameter trait ($P < 0.05$). Single nucleotide polymorphism 2 and SNP 5 to 12 were weakly associated with wool crimp. Similarly, the haplotypes derived from these 13 identified SNP were also significantly associated with the average wool fiber diameter, fineness SD, and the CV of fiber diameter ($P < 0.05$). Our results suggest that *MTR* is a candidate gene for sheep wool production and quality traits, and the identified SNP might be used in sheep breeding.

Key words: methionine synthase, sheep, single nucleotide polymorphism, wool quality trait

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INTRODUCTION

The Merino is an economically influential breed of sheep prized for its wool, and sheep wool production is very important in many countries. Identification of genetic markers and important genes responsible for wool production and quality traits is essential for genetic improvement of wool sheep (Dominik, 2005; Purvis and Franklin, 2005; Lamy et al., 2009).

The methionine synthase (*MTR*) gene encodes the enzyme 5-methyltetrahydrofolate- homocysteine methyltransferase that catalyzes the final step in methionine biosynthesis from homocysteine (Papatheodorou and Weiss, 2007; Zhang et al., 2012). Methionine is the direct precursor of S-adenosylmethionine (**SAM**), the methyl donor involved in transmethylation reactions and related epigenetic mechanisms (Blaise et al., 2007; Anderson et al., 2012).

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Methionine synthase regulates the homeostasis of intracellular methionine, folate, and homocysteine (Greenberg et al., 2011; Gueant et al., 2013) and plays a vital role in animal development. $MTR^{-/-}$ mice died during embryo formation (Swanson et al., 2001), and *MTR* mutations in human led to methylcobalamin deficiency complementation group G and the patients manifested polymorphic symptoms (Leclerc et al., 1998; Chen et al., 2001; Gueant-Rodriguez et al., 2003; Li et al., 2006; Huang et al., 2008; Ding et al., 2013; Yang et al., 2013).

There are no previous reports describing genetic variation of the *MTR* gene in sheep. In a previous study, we performed a genomewide association study to identify the genetic markers and important genes responsible for wool production and quality traits in Chinese Merino (Wang et al., 2014). The result showed that 1 SNP located in the *MTR* region (s55389.1) was significantly associated with the average wool fiber diameter, CV of fiber diameter, and wool crimp. The objective of this study was to confirm the association of *MTR* with wool production and quality traits in Chinese Merino. Here, we investigated the *MTR* tissue expression pattern and identified novel *MTR* SNP and performed the association analysis of these SNP with wool production and quality traits. Our results showed that *MTR* is an important candidate gene for the wool production and quality traits.

MATERIALS AND METHODS

Animal Resource and Phenotyping

Animals used in the current study belong to the Chinese Merino (Xinjiang Junken type), which consists of 6 different Merino sheep strains: superfine wool (**SF**), prolific wool (**PW**), prolific meat (**PM**), A, B, and U. The A strain was selected for large body size and high wool yield, the B strain was selected for long wool fiber, and the U strain was selected for long wool fiber, high wool yield, and lower fiber diameter (Shi et al., 2010). A total of 743 ewes—181 from SF, 138 from PW, 134 from PM, 151 from A, 103 from B, and 36 from U—were genotyped and phenotyped in this study. In addition, 18 ewes (3 from each of SF, PW, PM, A, B, and Kazak sheep) were used for *MTR* gene expression analysis. Of these 18 sheep, the 3 sheep from the SF strain were slaughtered at 240 d of age, and the body side skin, skeletal muscle, small intestine, ovary, heart, lung, liver, spleen, pituitary gland, kidney, rumen, and pineal gland samples were collected, and the other 15 sheep were slaughtered for collecting only body side skin samples. All collected tissue samples were snap-frozen in liquid nitrogen and stored at –80°C until analyzed. All animals were fed ad libitum with a grazing diet and maintained under the same conditions of environment, feeding, and management. Procedures involving animals and their care were conducted in conformity with National Institutes of Health guidelines (NIH Publication No. 85-23, revised 1996) and were approved by the Laboratory Animal Management Committee of the Northeast Agricultural University (Harbin, China) and Xinjiang Academy of Agriculture and Reclamation Science (Shihezi, China).

The ear notch samples and wool samples were collected at shearing. The wool production and quality traits (the average wool fiber diameter, fineness SD, CV of fiber diameter, wool fiber length, sticky sweating, wool crimp, fleece weight, and clean wool yield) were measured following the guidelines of the China Fiber Inspection Bureau and International Wool Textile Organization (Cottle, 2010). Birth date, birth weight, birth rank (single, twin, or triplet), gender, and subsequent growth data including the birth weight, BW before shearing, BW after shearing, and the birth weight of F_1 hybrids were also collected for the phenotyped individuals.

Deoxyribonucleic Acid and RNA Extraction and cDNA Synthesis

Sheep genomic DNA was extracted from the ear notch samples using a standard phenol-chloroform procedure (Sambrook and Russell, 2002) and stored for genotyping. Total RNA from the frozen tissues was isolated with Trizol reagent (Invitrogen, Rockville, MD) according to the manufacturer's instructions, and RNA quality was assessed by denaturing formaldehyde agarose gel electrophoresis. First-strand cDNA synthesis was performed using Promega Improm-II reverse transcription System (Promega, Madison, WI) following the manufacturer's instructions. Each reaction was transcribed from 1 μg total RNA.

Semiquantitative Reverse-Transcription PCR

Semiquantitative Reverse Transcription PCR (**RT-PCR**)was used to detect *MTR* tissue expression. The following primers were used: *MTR*, forward primer: 5′-GGGGCAAATACCCGAACCGA-3′ and reverse primer: 5′-ACCTGAGTGCAGAGGGGCGAT-3′ and *GAPDH*, forward primer: 5′-CTGACCTGCCGCC-TGGAGAAA-3′ and reverse primer: 5′-GTAGAAGAG-TGAGTGTCGCTGTT-3′. The PCR cycling parameters for both the *MTR* and the *GAPDH* gene were initial denaturation at 94°C for 5 min followed by 28 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 15 s and final extension at 72°C for 7 min. The conditions were chosen so that none of the cDNA analyzed reached a plateau at the end of the amplification protocol. The resulting PCR products were resolved by electrophoresis on 2% agarose gels with a final concentration of

0.5 μg/mL ethidium bromide and photographed under UV light. Bands were quantified densitometrically (integrated optical density [**IOD**]) using Quantity One 4.6.2 software (Bio-Rad, Hercules, CA). Methionine synthase gene expression was expressed as IOD normalized against the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

Detection of Polymorphisms of the Sheep MTR *Gene*

In an effort to identify SNP in a cost-effective manner, SNP discovery was achieved by sequencing PCR products of the pooled DNA samples from 60 Chinese Merino individuals. Polymerase chain reaction primers (forward primer: 5′-CTAAATGACTCCCACAGAT-3′ and reverse primer: 5′-TTGAGCGAACTATTTCTACT-3′) were designed for the partial amplification of a 1,981-bp fragment of the sheep *MTR* gene (introns 26 and 27 and exon 26), based on the genome sequence from the assembly of chromosome 25 reported in GenBank (accession number XM_004021372) and the *Ovis aries* genome sequencing (http://genome.ucsc.edu). The PCR products were purified and sequenced directly using Sanger sequencing by Invitrogen (Shanghai, China). The sequences were aligned using the Align X function of Vector NTI (Informax, Rockville, MD). Single nucleotide polymorphisms were identified by double peaks at a single base in the chromatograms.

Genotyping of the Sheep MTR *Gene*

A multiplexed SNP single base extension (**SBE**) assay was designed by using Sequenom Assay Design 3.1 software (Sequenom, San Diego, CA) according to the publisher's instructions. Genotyping was performed by using a 384-well plate format on the Sequenom MassARRAY platform (Bioyong Technologies Inc., Beijing, China) during the year 2012. Primers used for the assay are listed in the Supplemental File S1 (see the online version of the article at http://journalofanimalscience.org). The raw data files generated by Mass Array Sequenom were analyzed for the intensity peaks of calibrant to ascertain the quality of the data as previous described (Thomas et al., 2007; Gabriel et al., 2009). An overall call rate of >95% was maintained. For every 96 samples (a quadrant of the Sequenom chip), 4 samples were duplicated and the call rates were checked for concordance. The calls in the negative control (no DNA) were also monitored in all the runs. The reproducibility was 100% in present study.

Statistical Analysis

Data are expressed as mean (SD) for each parameters measured in each group. Differences between groups were analyzed by Student's *t* test and power analysis was performed using Statistical Analysis System (SAS Inst. Inc., Cary, NC). Significance was evaluated based on an experiment-wise type I error rate of 0.05, unless otherwise adjusted by Bonferroni correction.

Genotype and allele frequencies were estimated by gene counting. The allele frequencies in subjects for each SNP were tested for departure from Hardy–Weinberg equilibrium using the χ^2 test.

Haplotype analyses and graphical representation of the linkage disequilibrium (**LD**) structure as measured by coefficient D′ and r^2 using expectation–maximization algorithm were performed with the Haploview software (version 4.2; Barrett et al., 2005). Haplotypes for each individual were obtained in SAS/GENETICS using the PROC HAPLOTYPE procedure. In large samples, D′ = 1 indicates complete LD, that is, no evidence for recombination between the SNP pairs and such SNP are good surrogates for each other; D′ = 0 indicates no LD. "Strong" LD was defined as having a pairwise D′ > 0.85. Haplotype-block structure was examined using the criteria described by Gabriel et al. (2002), which use the 90% confidence bounds of D′ to define sites of historical recombination between SNP. Patterns of LD were visualized using Haploview.

According to the characteristics of the Chinese Merino population, models used to analyze the data were assumed to be $Y = \mu + G + L + A + G \times L + G \times A + L \times A + e$, in which Y is the observed value for each individual; μ is the population mean; genotype or haplotype (G), line (L), and age (A) were the fixed effects; $G \times L$, $G \times A$, and $L \times A$ were the interaction effects of G by L, G by A, and L by A, respectively; and e was the random error. Data were subjected to the GLM procedures of John's Macintosh Program 4.0 (JMP, SAS Inst. Inc., Cary, NC), which was used to examine the correlation between genotypes and haplotypes and continuous traits and to evaluate the least squares means. In addition, for discrete traits, for example, sticky sweating, it was calculated using the PROC CATMOD program in SAS/GENETICS. The P-values < 0.05 were considered statistically significant, and P-values < 0.01 were highly significant, unless otherwise specified.

RESULTS

Tissue Expression Profiling of the Sheep MTR *Gene*

To test the potential involvement of MTR in sheep wool production and quality traits in Chinese Merino, we first profiled *MTR* gene expression in various tissues of the SF strain of Chinese Merino by using semiquantitative reverse transcription PCR. The results showed that the *MTR* gene was ubiquitously expressed in all tissues

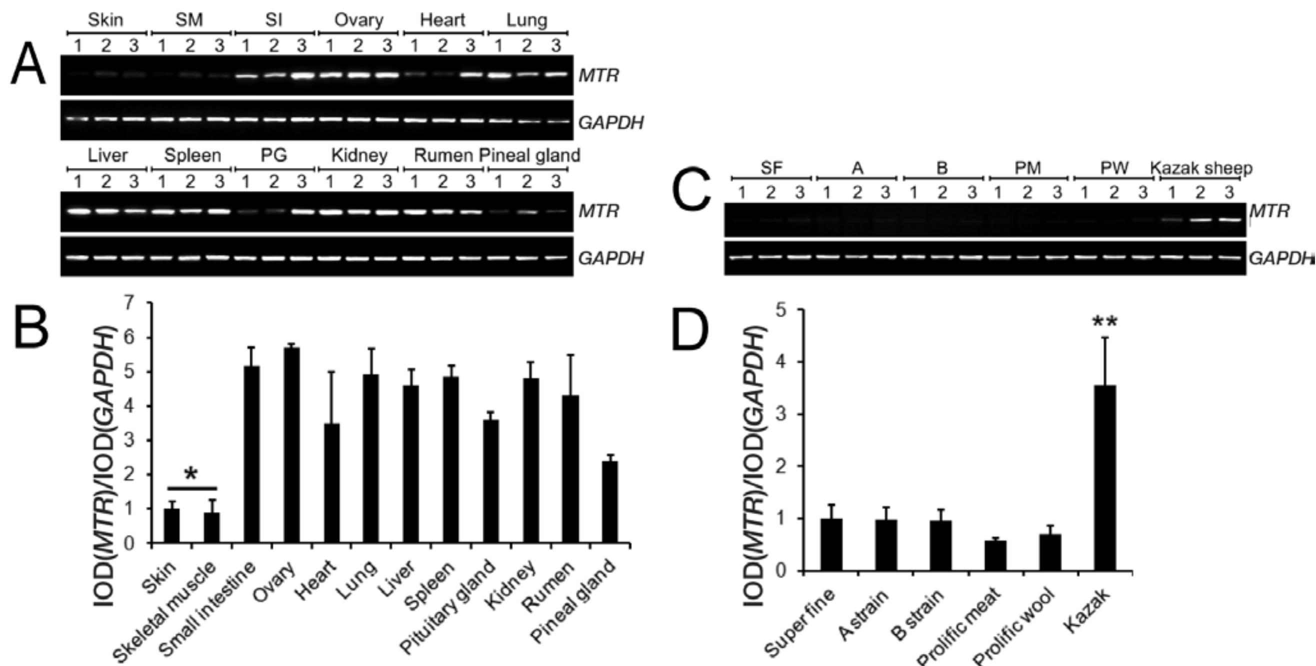

Figure 1. Semiquantitative reverse transcription PCR analysis of *MTR* gene expression. The housekeeping gene *GAPDH* was used as an internal control to normalize *MTR* gene expression in semiquantitative *RT-PCR* analysis. (A) Tissue expression profiling of the *MTR* gene in 12 different sheep tissues and organs of the superfine wool (SF) strain. SM = skeletal muscle; SI = small intestine; PG = pituitary gland. (B). Histogram of relative expression of the *MTR* gene in the SF strain of Chinese Merino (n = 3) as described in Fig. 1A. Each bar, along with the SD, represents relative mean normalized fluorescence data from 3 different animals. Significant difference was observed in the *MTR* expression levels between skin and the other tissues (except skeletal muscle). IOD = integrated optical density. (C) Skin *MTR* gene expression in the 5 Chinese Merino strains and Kazak sheep (n = 3). Very significant differences were observed in *MTR* gene expression in body side skin between wool (Chinese Merino) and hair (Kazak) sheep. PM = prolific meat; PW = prolific wool. (D) Histogram of relative expression of the skin *MTR* gene in the 5 strains of Chinese Merino and Kazak sheep (n = 3) as described in Fig. 1C; $^*P < 0.05$; $^{**}P < 0.01$.

tested. Comparatively, *MTR* was highly expressed in small intestine, ovary, lung, liver, spleen, kidney, and rumen but lowly expressed in skin and skeletal muscle ($P < 0.05$; Fig. 1A and 1B). Then, we compared *MTR* gene expression levels of the body side skin from the 6 tested strains at 240 d of age, when the hair follicles are at an anagen-like stage (Galbraith, 2010). The results showed that the *MTR* gene was lowly expressed in the body side skin, and no difference was observed in all 5 tested Chinese Merino strains. Merino and Kazak sheep have strikingly different wool production and quality traits. Interestingly, we observed a 4.25-fold difference in the skin *MTR* gene expression between Chinese Merino (5 strains) and Kazak sheep ($P < 0.01$; Fig. 1C and 1D).

Identification of Novel SNP in the MTR *Gene*

By PCR and sequencing, we searched for SNP in introns 26 and 27 and exon 26 of the sheep *MTR* gene from the pooled genomic DNA samples. We identified 13 novel SNP in the analyzed region. These 13 SNP were designated SNP 1 to 13, and the detailed SNP information is summarized in Table 1. Of these 13 novel SNP, 12 SNP were located in introns and only 1 SNP (SNP 4) was located in an exon.

Frequency of Alleles and Genotypes

We then genotyped these 13 identified novel SNP in 743 individuals of the 6 Chinese Merino strains (SF, PW, PM, A, B, and U) using the SBE assay and performed allele and genotype frequency analysis. The results showed that the minor allele frequency of these 13 novel SNP ranged from 10.6 to 44.8%. Table 1 shows the allele frequencies and Hardy–Weinberg test *P*-values in all tested individuals. All the 13 identified SNP, with the exception of SNP 12 ($P = 0.3419$), were not in Hardy–Weinberg equilibrium based on the combined data. The frequencies of the alleles and genotypes of the 13 identified SNP are shown in Supplemental Table S1 (see the online version of the article at http://journalofanimalscience.org). The χ^2 test results showed that the allele frequencies for these 13 SNP were significantly different among the 6 studied strains ($P < 0.001$; Supplemental Table S1). The allele frequency distribution of these SNP varied significantly between the SF strain and the other 5 strains ($P < 0.001$; data not shown). Interestingly, the genotype GG of SNP 1 was not identified in the SF strain and genotype GG of SNP 12 was not identified in the SF, PW, B, and U strains. All these 13 identified SNP exhibited heterozygous genotypes.

Table 1. Summary of the 13 identified novel SNP in the sheep *MTR* gene

SNP ID[1]	Chromosome position,[2] bp	Region	db SNP ss# ID[3]	Alleles	MAF[4]	HW[5] *P*-value
SNP 1	9,561,932	Intron 26	ss900693204	A/G	0.150	5.8×10^{-7}
SNP 2	9,562,057	Intron 26	ss900693206	G/A	0.448	5.6×10^{-19}
SNP 3	9,562,256	Intron 26	ss900693212	G/A	0.271	1.9×10^{-7}
SNP 4	9,562,558	Exon 27	ss900693213	C/T	0.374	1.0×10^{-4}
SNP 5	9,562,667	Intron 27	ss900693217	C/T	0.413	0.0016
SNP 6	9,562,883	Intron 27	ss900693219	T/G	0.409	0.0010
SNP 7	9,563,005	Intron 27	ss900693221	A/G	0.410	0.0013
SNP 8	9,563,072	Intron 27	ss900693223	C/T	0.415	1.3×10^{-5}
SNP 9	9,563,122	Intron 27	ss900693225	T/C	0.413	0.0017
SNP 10	9,563,258	Intron 27	ss900693227	A/G	0.406	0.0023
SNP 11	9,563,279	Intron 27	ss900693228	A/G	0.410	1.0×10^{-4}
SNP 12	9,563,514	Intron 27	ss900693230	A/G	0.107	0.3419
SNP 13	9,563,552	Intron 27	ss900693232	G/A	0.106	3.5×10^{-25}

[1]ID = identifier.

[2]Nucleotides are numbered according to the *Ovis aries* genome (build UCSC, Aug. 2012 [ISGC Oar_v3.1/oviAri3], http://genome.ucsc.edu/cgi-bin/hgBlat) and located in chromosome 25 at 9,561,771 to 9,563,755 bp, bp = base pair.

[3]db = database; ss# = submitted SNP #.

[4]MAF = minor allele frequency. A/B implies that B is the minor allele.

[5]HW = Hardy–Weinberg test.

Linkage Disequilibrium Analysis

Linkage disequilibrium analysis was performed on all these 13 identified SNP in the 6 studied Chinese Merino strains, and LD was estimated based on the pairwise LD coefficient, the D′ value. When |D′| is higher than 0.85, 2 loci are considered in strong LD (Guryev et al., 2006; Slatkin, 2008). The estimated D′ values for each strain are depicted using a color scheme in Supplemental Fig. S1 (see the online version of the article at http://journalofanimalscience.org). Dark shading indicates strong LD. As shown in this figure, in the SF, PW, U, and B strains, SNP 2 to 11 were identified as a large LD block. In the A strain, SNP 1 to 11 lay within a haplotype block. In the PM strain, SNP 3 to 12 were identified as a large LD block. Single nucleotide polymorphisms 12 and 13 were not in strong LD with the other SNP in the PW, U, and B strains. Linkage disequilibrium analysis identified SNP 1 to 3, SNP 7, SNP 12, and SNP 13 as haplotype tag SNPs for the sheep *MTR* gene, and SNP 7 was a good surrogate for SNP 4 to 6 and SNP 8 to 11.

Association of SNP in the MTR *Gene with Wool Production and Quality Traits*

Our previous study showed that *MTR* was associated with several sheep wool production and quality traits in Chinese Merino (Wang et al., 2014). To further validate this result, we performed association analysis with the 13 identified SNP and wool production and quality traits. The association analysis showed that, in agreement with our previous finding (Wang et al., 2014), SNP 2 to 11 were highly significantly associated with the average wool fiber diameter and the fineness SD in the tested population ($P < 0.01$) except for SNP 3, which was only significantly associated with the average wool fiber diameter ($P = 0.0472$). Single nucleotide polymorphisms 4 to 11 were significantly associated with the CV of fiber diameter trait ($P < 0.05$). Single nucleotide polymorphism 2 and SNP 5 to 12 were significantly or almost significantly associated with wool crimp trait, and SNP 8 and 9 were associated with the clean wool yield trait ($P = 0.0419$ and $P = 0.0412$; Table 2). In addition, very significant associations ($P < 0.01$) were observed between SNP 13 and the fleece weight and the BW after shearing.

The effect (least squares means) of the *MTR* genotypes on wool production and quality traits is shown in Table 2. We designated the allele A (SNP 1), G (SNP 2), G (SNP 3), C (SNP 4), C (SNP 5), T (SNP 6), A (SNP 7), C (SNP 8), T (SNP 9), A (SNP 10), A (SNP 11), A (SNP 12), and G (SNP 13) as wild-type "A" and the mutated allele as mutant type "B." Of these 13 identified SNP, the wild-type allele A of SNP 4 to 11 was the preponderant allele in SF, whereas the mutant allele B was the preponderant allele in the other 5 strains. For the SNP significantly associated with the tested wool production and quality traits, there were significant trait differences between individuals carrying genotypes AA and BB, with the exception of SNP 12 on the wool crimp. Generally, sheep with the AA genotype of these SNP tended to have much lower average wool fiber diameter, fineness SD, CV of the average wool fiber diameter, clean wool yield, fleece weight, and BW after shearing but higher wool crimp levels compared with sheep with genotype BB (Table 2). The effect of genotype AB was intermediate between those of genotypes AA and BB.

Table 2. Effects (least squares means [SE]) of *MTR* genotypes on production and wool quality traits. The complete trait data are only included for the traits associated with the identified SNP.

			Genotype (no.)		
SNP ID[1]	Traits	*P*-value	GG (279)	GA (239)	AA (204)
SNP 2	Average wool fiber diameter, µm	<0.0001‡	20.46 (0.13)^{a}	20.60 (0.13)^{a}	21.47 (0.17)^{b}
	Fineness SD	<0.0001‡	4.06 (0.05)^{a}	4.11 (0.04)^{a}	4.38 (0.06)^{b}
	Wool crimp, crimps/2.5 cm	0.0176†	12.37 (0.15)^{a}	12.27 (0.16)^{a}	11.70 (0.20)^{b}
			GG (403)	GA (221)	AA (80)
SNP 3	Average wool fiber diameter, µm	0.0472†	20.45 (0.11)^{a}	20.76 (0.14)^{b}	21.07 (0.28)^{b}
	Fineness SD	0.0079‡	4.07 (0.04)^{a}	4.23 (0.05)^{b}	4.29 (0.10)^{b}
			CC (119)	CT (271)	TT (290)
SNP 4	Average wool fiber diameter, µm	0.0016‡	20.26 (0.23)^{a}	20.57 (0.12)^{a}	21.08 (0.13)^{b}
	Fineness SD	<0.0001‡	3.92 (0.08)^{a}	4.09 (0.04)^{a}	4.32 (0.05)^{b}
	CV of fiber diameter	0.0187†	19.38 (0.34)^{a}	19.89 (0.18)^{a}	20.43 (0.20)^{b}
	Clean wool yield, %	0.0745			
			CC (146)	CT (313)	TT (274)
SNP 5	Average wool fiber diameter, µm	<0.0001‡	20.28 (0.21)^{a}	20.53 (0.11)^{a}	21.19 (0.14)^{b}
	Fineness SD	<0.0001‡	3.92 (0.07)^{a}	4.08 (0.04)^{b}	4.35 (0.05)^{c}
	CV of fiber diameter	0.0096‡	19.37 (0.32)^{a}	19.91 (0.17)^{a}	20.47 (0.21)^{b}
	Wool crimp, crimps/2.5 cm	0.0522			
	Clean wool yield, %	0.0553			
			TT (140)	TG (298)	GG (269)
SNP 6	Average wool fiber diameter, µm	0.0003‡	20.41 (0.22)^{a}	20.56 (0.12)^{a}	21.20 (0.14)^{b}
	Fineness SD	<0.0001‡	3.95 (0.08)^{a}	4.09 (0.04)^{a}	4.35 (0.05)^{b}
	CV of fiber diameter	0.0178†	19.40 (0.34)^{a}	19.88 (0.18)^{a}	20.44 (0.21)^{b}
	Wool crimp, crimps/2.5 cm	0.0661			
			AA (151)	AG (288)	GG (272)
SNP 7	Average wool fiber diameter, µm	0.0002‡	20.27 (0.21)^{a}	20.56 (0.12)^{a}	21.18 (0.14)^{b}
	Fineness SD	<0.0001‡	3.90 (0.07)^{a}	4.07 (0.04)^{b}	4.36 (0.05)^{c}
	CV of fiber diameter	0.0030‡	19.29 (0.32)^{a}	19.83 (0.18)^{a}	20.51 (0.21)^{b}
	Wool crimp, crimps/2.5 cm	0.0351†	12.56 (0.24)^{a}	12.27 (0.13)^{ab}	11.88 (0.16)^{b}
	Clean wool yield, %	0.0761			
			CC (144)	CT (312)	TT (276)
SNP 8	Average wool fiber diameter, µm	0.0001‡	20.28 (0.21)^{a}	20.55 (0.11)^{a}	21.19 (0.14)^{b}
	Fineness SD	<0.0001‡	3.91 (0.07)^{a}	4.08 (0.04)^{b}	4.36 (0.05)^{c}
	CV of fiber diameter	0.0054‡	19.32 (0.32)^{a}	19.89 (0.17)^{a}	20.49 (0.21)^{b}
	Wool crimp, crimps/2.5 cm	0.0522			
	Clean wool yield, %	0.0419†	NE[2]	NE	NE
			TT (145)	TC (311)	CC (272)
SNP 9	Average wool fiber diameter, µm	<0.0001‡	20.29 (0.21)^{a}	20.53 (0.11)^{a}	21.19 (0.14)^{b}
	Fineness SD	<0.0001‡	3.91 (0.07)^{a}	4.09 (0.04)^{b}	4.36 (0.05)^{c}
	CV of fiber diameter	0.0056‡	19.30 (0.32)^{a}	19.93 (0.17)^{a}	20.50 (0.21)^{b}
	Wool crimp, crimps/2.5 cm	0.0438†	12.50 (0.24)^{a}	12.28 (0.13)^{a}	11.88 (0.16)^{b}
	Clean wool yield, %	0.0412†	NE	NE	NE
			AA (137)	AG (302)	GG (271)
SNP 10	Average wool fiber diameter, µm	0.0003‡	20.40 (0.22)^{a}	20.54 (0.12)^{a}	21.18 (0.14)^{b}
	Fineness SD	<0.0001‡	3.95 (0.08)^{a}	4.08 (0.04)^{a}	4.35 (0.05)^{b}
	CV of fiber diameter	0.0147†	19.41 (0.34)^{a}	19.87 (0.18)^{a}	20.46 (0.21)^{b}
	Wool crimp, crimps/2.5 cm	0.0530			
	Clean wool yield, %	0.0858			
			AA (143)	AG (291)	GG (270)
SNP 11	Average wool fiber diameter, µm	0.0001‡	20.41 (0.21)^{a}	20.50 (0.12)^{a}	21.20 (0.14)^{b}
	Fineness SD	<0.0001‡	3.94 (0.07)^{a}	4.09 (0.04)^{a}	4.35 (0.05)^{b}
	CV of fiber diameter	0.0129†	19.34 (0.33)^{a}	19.94 (0.18)^{a}	20.45 (0.21)^{b}
	Wool crimp, crimps/2.5 cm	0.0670			
	Clean wool yield, %	0.0677			
			AA (587)	AG (135)	GG (11)
SNP 12	Wool crimp, crimps/2.5 cm	0.0259†	12.65 (0.10)^{a}	11.87 (0.20)^{b}	NE
			GG (476)	GA (47)	AA (36)
SNP 13	BW after shearing (kg)	0.0010‡	38.64 (0.36)^{a}	41.74 (1.78)^{b}	NE
	Fleece weight (kg)	0.0056‡	4.38 (0.06)^{a}	4.51 (0.29)^{ab}	NE

$^{a–c}$Means within a row with no common superscript are different ($P < 0.05$).

[1]ID = identifier.

[2]NE = not estimable.

†$P < 0.05$; ‡$P < 0.01$.

Table 3. The influence of *MTR* haplotypes on several wool quality traits (least squares means [SE]). Only traits associated with the identified haplotypes are presented in this table

Haplotype[1] (no.)	Frequency, %	Average wool fiber diameter, µm	Fineness SD	CV of fiber diameter
H1: AGG**CCTACTAA**[2]AG (555)	36.5	20.40 (0.09)^{a}	4.01 (0.03)^{ab}	19.71 (0.13)^{a}
H2: AAA**TTGGTCGG**[3]AG (237)	14.9	20.94 (0.23)^{b}	4.16 (0.08)^{abc}	19.87 (0.33)^{ab}
H3: AAG**TTGGTCGG**[3]AG (189)	13.5	20.87 (0.15)^{b}	4.21 (0.05)^{c}	20.20 (0.23)^{abc}
H4: GAA**TTGGTCGG**[3]AG (176)	12.0	20.76 (0.15)^{b}	4.22 (0.05)^{c}	20.33 (0.22)^{bcd}
H5: AGG**TTGGTCGG**[3]AA (103)	6.9	20.76 (0.20)^{ab}	4.29 (0.07)^{c}	20.80 (0.29)^{c}
H6: AGG**TTGGTCGG**[3]GG (81)	4.9	20.46 (0.23)^{ab}	3.99 (0.08)^{b}	19.56 (0.34)^{ad}
H7: AGG**CCTACTAA**[2]AA (33)	2.9	20.70 (0.44)^{ab}	4.37 (0.15)^{c}	21.04 (0.66)^{bc}
P-value[4]		0.0467	<0.0001	0.0036

$^{a-d}$Means within a column with no common superscript are different ($P < 0.05$).

[1]Seven major haplotypes (>1%) accounted for 91.6% of the alleles are listed.

[2]Indicates the allele combination (CCTACTAA) of SNP 3 to SNP 11, which is bold.

[3]Indicates the allele combination (TTGGTCGG) of SNP 3 to SNP 11, which is underlined.

[4]Represents the *P*-value of association between these haplotypes and wool quality traits.

Haplotype Association Analysis

To further confirm our findings, we performed haplotype analysis and association analysis. Haplotype analysis revealed 7 most common *MTR* haplotypes in the tested population, accounting for 91.6% of all haplotypes (Table 3). Among these haplotypes, haplotype H1 (36.5%) had the highest proportion. The haplotype association analysis results are summarized in Table 3. Haplotypes were significantly associated with some of the wool production and quality traits analyzed, including the average wool fiber diameter, fineness SD, and the CV of fiber diameter ($P < 0.05$). Sheep with haplotype H1 had a significantly lower average wool fiber diameter than the sheep with haplotypes H2, H3, and H4. In contrast to the sheep with haplotype H1, sheep with haplotypes H4, H5, and H7 had a tendency for the increased variation level of the average fiber diameter (fineness SD and CV of fiber diameter). In addition, the presence of haplotypes H1, H2, and H6 favored fineness SD levels with a very high significance ($P < 0.0001$). Collectively, to some extent, sheep with haplotype H1 produced better quality wool than did sheep with the other haplotypes.

DISCUSSION

In the present study, we profiled sheep *MTR* gene tissue expression and performed *MTR* SNP detection and association analysis with wool production and quality traits in Chinese Merino. A total of 13 *MTR* SNP were identified in Chinese Merino sheep. Single SNP and haplotype association analysis showed that the majority of the identified SNP were associated with wool production and quality traits, which is consistent with our previous finding (Wang et al., 2014).

Methionine synthase catalyzes the remethylation of homocysteine to methionine. Methionine is an essential amino acid and precursor of SAM, which is a universal methyl-group donor involved in methylation reactions. It has been shown that methionine plays a crucial role in wool growth (Sahoo, 2011) and its specific effects on wool growth are most probably related to the formation of SAM (Black and Reis, 1979; Reis, 1982). Wool is produced by hair follicles, which consist of several layers of epithelial cells. The wool growth involves epithelial cell proliferation and differentiation. A recent study has shown that MTR has a marked expression in the proliferation site of various epithelia such as the skin basal layer of the Malpighian epithelia and, conversely, its activity is low in the epithelium areas such as the skin layer of differentiated keratinocytes, where proliferation is low (Gueant et al., 2013). Chinese Merino and Kazak sheep have striking different wool production and quality traits. In the present study, we observed that the skin *MTR* gene was differentially expressed between Chinese Merino and Kazak sheep (Fig. 1C and 1D). Taken together, all these data lead us to hypothesize that MTR may play a role in hair follicle development and hair formation and that differential MTR gene expression might be related to the differences in sheep wool production and quality traits.

The SNP association analysis showed that most of these 13 identified SNP were significantly associated with wool production and quality traits. The wild-type allele A of SNP 4 to 11 was the preponderant allele in SF. The sheep with the AA genotype of SNP 4 to 11 had much lower average wool fiber diameter, fineness SD, and the CV of fiber diameter compared with sheep with genotype BB in the tested population ($P < 0.05$; Table 2), and the sheep with the AA genotype of SNP 2 and SNP 5 to 12 had high wool crimp levels compared with sheep with the BB genotype ($P < 0.05$). Given that SF has the finest and softest wool among these Chinese merino strains (Shi et al., 2010), we presume that the allele A of SNP 4 to 11 is related to lower average wool fiber diameters and higher crimp of wool and that the allele A of these SNP could be used as the beneficial allele for

genetic improvement of wool production and quality traits in Chinese Merino population. It has been known that there is a negative correlation between average wool fiber diameter and wool crimp in sheep (Naylor, 1995). Therefore, selection for allele A/genotype AA of SNP 4 to 11, which were associated with lower average wool fiber diameter, might increase wool crimp.

In the current study, we identified 13 novel SNP in the sheep *MTR* gene, and most of these identified SNP were significantly associated with wool production and quality traits. Of these 13 SNP, only SNP 4 was located in an exon, causing a silent mutation, and the other 12 SNP were located in introns. It is unclear how these SNP affect wool production and quality traits. The significant associations for these SNP may be due to their strong LD with true unknown functional variants or causal SNP within or outside of the *MTR* gene. However, we cannot exclude the possibility that that some of these SNP are functional and affect *MTR* gene expression, causing changes in wool production and quality traits. It has been reported that some silent mutations affect mRNA processing or protein translation and that some intron SNP affect mRNA processing, stability, and protein translation (Duan et al., 2003; Le Hir et al., 2003; Parmley et al., 2006). It is worthwhile to test functionality of these SNP in the future.

In summary, *MTR* SNP and their haplotypes are associated with several sheep wool production and quality traits. The identified SNP and haplotypes may be useful for marker-assisted selection for high quality wool in Chinese Merino sheep.

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