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Polymorphism of the *Keratin 31* Gene in Different Gansu Alpine Fine-Wool Sheep Strains by High Resolution Melting Curve Analysis

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Abstract: Keratin Intermediate Filament (KIF) is a key member of the keratin family of proteins. In this study, High-Resolution Melting (HRM) analysis and DNA sequencing were used to detect polymorphisms of the Keratin intermediate-filaments 31 (*KRT 31*) gene in three Gansu Alpine fine wool sheep strains (fine, super fine and dual purpose strain) (n = 448) and to determine impacts of genotype for KRT 31 on expression of wool traits. Results showed that there were a total of two Single Nucleotide Polymorphisms (SNPs) identified as KRT 31 SNP c.166A>G and c.191T>C. The mutation site c.166 (SNP c.166A>G) results in a missense mutation from Asn to Ser. The mutation at site c.191 (SNP c.191T>C) does not alter the amino acid sequence. The χ^2-test showed that the genotype distributions in these three strains of the SNP c.166A>G and SNP c.191T>C were not all in agreement with Hardy Weinberg equilibrium. Furthermore, analysis of the impact of *KRT 31* gene polymorphism on wool traits in three Gansu Alpine fine wool sheep strains indicated that the mean fiber diameter for KRT31c.166A>G genotype AA and AG was smaller than genotype GG ($p<0.05$) suggesting this mutation could potentially be exploited in gene marker-assisted selection programmes within the wool industry to select for animals with decreased fiber diameter.

Key words: Gansu Alpine fine wool sheep, KRT 31, high resolution melting, wool traits, analysis

INTRODUCTION

The main structural components of hair/wool fibre are Keratins (KRT) and Keratin Associated Proteins (KAPs) the former assembling into bundles to form Keratin Intermediate Filaments (KIFs) while the later form the matrix between the KIFs via extensive disulfide bond cross linking (Powell and Rogers, 1997). Over 54 *keratin* genes have been identified in mammals including two large families and one small family (Pruett *et al*., 2004; Chamcheu *et al*., 2011). The two large families are designated I and II based on sequence similarity and the acid or neutral characteristics of the amino acids. In sheep, the type I and II of *keratin* genes are located at chromosomal locations 11q25~q29 and 3q14~q22, respectively (Powell *et al*., 1983). Type I genes are about 4~5 kb with 6 introns and type II genes are about 7~9 kb with 8 introns (Powell and Rogers, 1997; Yu *et al*., 2010). Genetic differences at KRT regulation sites may play key roles in determining phenotypic variation in wool quality and productivity (Arora *et al*., 2008; Yu *et al*., 2009; Yu *et al*., 2010; Li *et al*., 2009; Ma *et al*., 2006; Mckenzie *et al*., 2012). Polymorphism of KRT have been reported in different sheep and goat breeds (Itenge-Mweza *et al*., 2007; Li *et al*., 2009; Mckenzie *et al*., 2012). Yu *et al*. (2009) firstly found that Keratin 31 (KRT 31) located on chromosome 11 was asymmetrically expressed in sheep secondary follicles and was associated with deviation in follicle and wool fiber morphology as well as wool fineness and bending. However, but to date very little evidence of genetic variation in the ovine *KRT 31* gene have been described in any species. Therefore, the present study was planned to initiate the research on gene based marker selection for enhancing sheep production by exploring KRT 31 polymorphism in Gansu Alpine fine wool sheep using High Resolution Melting (HRM) analysis (Erali *et al*., 2008; Yue *et al*., 2011).

MATERIALS AND METHODS

Experimental animal and phenotypic data: Gansu Alpine fine wool sheep was developed in the Huang Cheng District of Gansu Province, China by crossing Mongolian or Tibetan with Xinjiang fine wool and then with some fine wool breeds from Union of Soviet Socialist Republics

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(USSR) such as Caucasian and Salsk. This breed has been approved by the Gansu Provincial Government in 1980 (Ma *et al*., 2006). Since, then for improving the wool quality this breed were crossed with Australian Merino, New Zealand Merino and German Mutton Merino. Nowadays, the modern Gansu Alpine fine wool sheep can be classified into three strains according to the wool which is fine (19.0 μ < fibre diameter <21.0 μ) superfine (fibre diameter <19.0 μ) and dual purpose strains (21.0 μ <fibre diameter). Blood or samples were collected from fine (n = 159) superfine (n = 113) and dual purpose strains ewes (n = 176), respectively.

Wool traits analysis: Mid side fleece samples were taken from Gansu Alpine fine wool sheep. Mean Fiber Diameter (FD, μm) and Coefficient of Variation of Fiber Diameter (CVFD, %) were analyzed following GB/T 21030-2007 procedures, the clean fleece yield was analyzed following GB 6978 procedures while Staple Length (SL, mm) and Staple Strength (SS, N/ktex) were measured following GB/T 4711-1984 procedures at the Textile Animal Fiber Laboratory of CAAS (Chinese Academy of Agricultural Sciences, Lanzhou, China) by the Quality Monitoring and Test Center for Animal Fur and Products at the Ministry of Agriculture (Lanzhou Institute of Animal and Veterinary Pharmaceutics Sciences). Wool crimp was appraised on site and rated as follows: 1 for maximum crimp, 2 for moderate crimp and 3 for minimal crimp.

High resolution melting analysis of *KRT 31* gene: Blood samples were taken from Gansu Province of China using FTA® paper (Whatman Inc.) from 448 Gansu Alpine fine wool sheep (ewe) along with phenotypic data on seven fleece traits form 2010-2012 year. For the blood samples on FTA® paper, DNA was extracted from duplicate 2.0 mm diameter disk. The disks were transferred into a 0.2 mL PCR tube. An aliquot of 80 μL of 50 mM NaOH solution was added to the tube containing the blood disks and it was incubated for 10 min at 98°C. The tube was inverted occasionally during incubation. The solution was then discarded and the disk was washed in 100 μL TE^{-1} buffers twice. After the removal of the TE^{-1}, the disk was air dried (Zhou *et al*., 2006).

Oligos were synthesized at Takra Biotechnology (DaLian) Co., Ltd. Primers were designed using Primer 3.0 (Rozen and Skaletsky, 2000). Primers and probes were chosen to detect KRT 31 mutations according to the sequence of KRT 31 (GenBank No.: BC119951, EU216425). The primer pairs and the genotyping methods utilized are described in Table 1. The unlabeled probes had either a 3'-phosphate or the 3'-amino modifier (Takra Biotechnology (DaLian) Co., Ltd.) incorporated to prevent extension during PCR. Probes were designed to analyze the mutations.

PCR was performed in the Veriti™ 96 well Thermal Cycler (ABI) and optimized. All DNA from sheep was amplified in a 10 μL final volume containing 1×TIANGEN BIOTECH Taq PCR Master Mix, 0.1 μmol L^{-1} forward primer, 0.1 μmol L^{-1} reverse primer and 1×LCGreen^{Plus+} for mutation scanning. All sheep of DNA were amplified in a 15 μL final volume containing 1×TIANGEN BIOTECH Taq PCR Master Mix, 0.1 μmol L^{-1} forward primer, 0.5 μmol L^{-1} reverse primer, 0.5 μmol L^{-} probe and 1×LCGreen^{Plus+} for mutation genotyping. The PCR program consists of an initial preheating at 95°C for 5 min to activate the Taq DNA polymerase followed by 45 amplification cycles. Each cycle is comprised of an annealing step at 62.5°C for 15 sec an elongation step at 72°C for 15 sec and denaturation at 94°C for 45 sec. The PCR products were separated in 2% agarose gel for 1 h and then photographed under UV.

The Lightscanner (Idaho Technology) is an instrument that measures high resolution DNA melting curves from samples in a 96 well PCR Plates. This is achieved by monitoring the fluorescence change of the fluorescent DNA intercalating dye, LCGreen^{Plus+} as the sample is melted. Turnaround time per sample is approximately 1-2 min depending on how broad the temperature range is required to be. The Lightscanner was heated at 0.1°C sec^{-1}. *KRT 31* gene was simultaneously analyzed between 40 and 98°C with a turnaround time of approximately 7 min/96 samples. The Light Emitting Diode (LED) power was auto adjusted to 90% fluorescence.

Sequencing of *KRT 31* gene PCR products: The samples were directly sequenced from the Lightscanner PCR

Table 1: Oligo nucleotides used for detection and sequencing

Methods	Primers	Primers sequences (5'-3')	Annealing temperature (°C)	3' modification
Mutation	MF	AACTTGGACTCTGTGTTCAGCC	66.5	None
Scanning	MR	CCTCGATGGTCCGGAAGTAG		None
	UPF166	AACTTGGACTCTGTGTTCAGCC		None
	UPR166	GGTCTCCTTCTCGTTGCCGTTG	65	C6 amino
	UPT166	GCTGCCCACATTGGCGGGGATT		None
Unlabeled probes	UPF191	GGTCTCCTTCTCCTTGCCGTTG		None
	UPR191	AATTCCAGGCCCTGCGTG	65	None
	UPT191	TGCCGTTGAAGGAGCCCTCACAGAACCAGTT		C6 amino

Unmodified oligo nucleotides were used as primers for PCR amplification and sequencing and oligo nucleotides with 3' modifications were used as probes

amplification. The PCR products were mixed with Nucleic Acid Purification kit (TIANGEN BIOTECH) to remove the remaining primers and bidirectionally sequenced with forward and reverse primers using ABI PRISM terminator cycle sequencing kit Version 1.1 (Takra Biotechnology (DaLian) Co., Ltd.) on the ABI PRISM 3730 genetic analyzer (Takra Biotechnology (DaLian) Co., Ltd.).

Statistical methods and analysis: The allele frequency and genotype frequencies at KRT 31 locus were analyzed with SPSS Software (Version17.0). The linear model used in analyses of the impact of KRT 31 genotype on wool traits in Gansu Alpine fine wool sheep (n = 448) included effects of strain, farm, age of sheep and KRT 31 genotype with random residual used as error.

$$Y_{ijk} = \mu + Strain_i + Age_j + Farm_K + Genotype_L + e_{ijkl}$$

Where:

Y_{ijk} = Value of phenotype in individual
μ = Mean of population
$Strain_i$ = Effective value of the ith strain (F, SF and DP strain)
Age_j = Fix effect of the jth age (3, 4, 5, 6, 7 and 8)
$Farm_K$ = Fix effect of the kth farm (1, 2, 3 and 4)
$Genotype_k$ = Fixed effects of the kth genotype
e_{ijkl} = Random residual effect of each observation

RESULTS AND DISCUSSION

Results of KRT 31 SNP genotyping analysis by using the high resolution melting method: Wool traits such as fineness, length, strength and crimp are important economic traits influencing wool quality. With the development of modern molecular biology technology, studies of sheep wool traits are shifting from quantitative genetics to molecular genetics. Genetic mechanisms of wool fiber traits and the accurate mapping of QTLs on sheep chromosomes have been important research areas. McLaren *et al.* (1997) located KAP6.1, KAP7 and KAP8 on sheep chromosome 1 and KAP1.1, KAP1.2, KAP1.3, KAP3.2 on chromosome 2 through linkage analysis (McLaren *et al.*, 1997). Henry *et al.* (1998) analyzed several traits including yearling weight, mean fiber diameter and wool production through backcrossing of super fine Merino and Romney Marsh sheep breeds. They scanned the genome with 216 microsatellite markers and analyzed one locus (not named) related to wool fiber diameter (Henry *et al.*, 1998). Ponz *et al.* (2001) found QTLs that control wool traits by segment mapping approach. They analyzed 40 microsatellite markers in the French synthetic sheep strain INR401 and found that a QTL on chromosome 6 controlled wool fiber diameter while a QTL on chromosome 7 controlled the variation coefficient of fiber diameter (Ponz *et al.*, 2001). The earlier studies chose KAP as a candidate gene for wool traits but SNP sites that control wool fiber fineness are still unknown. Yu *et al.* (2009) firstly found asymmetrical expression of KRT 31 in sheep secondary follicles which may be relate to wool fineness, crimp and bulb deviation of follicles and wool fiber morphology (Yu *et al.*, 2009). Researchers chose *KRT 31* gene as a candidate gene for fine wool traits and analyzed it with the HRM SNP fast assay. The purpose of this study was to identify SNP sites related to fine wool traits and provide a theoretical basis and technical method for early marker assisted selection for fine wool economic traits. Genomic DNA from 448 Gansu Alpine fine wool sheep was analyzed in the *KRT 31* gene by using HRM analysis for a rapid screening of mutations. Optimized PCRs produced a single product (388 bp) as determined by electrophoresis in 2% agarose gel electrophoresis. The fragment of KRT 31 was scanned by HRM analysis to indentify polymorphic variants. The melting curves obtained clearly indicated the presence of SNP in the fragment of the *KRT 31* gene (Fig. 1). The results confirmed that two polymorphic sites in the 388 bp fragment of *KRT 31* gene by sequencing. A mutation site (c.166A>G) results in a change from Asn to Ser (Fig. 2a). The mutation at site 191 (c.191T>C) does not alter the amino acid sequence (Fig. 2b). HRM unlabeled probe scanning showed that the PCR products could be genotyped into three genotypes for each site: AA, AG, GG (c.166A>G; Fig. 3a) and CC, CT, TT (c.191T>C; Fig. 3b), respectively.

Polymorphism in the *KRT 31* gene in Gansu Alpine fine wool sheep and its relationship with wool traits: Allele and genotype frequencies of KRT 31 SNP c.166A>G and c.191T>C were statistically analyzed in three strains of Gansu Alpine sheep. The A allele at SNP c.166A>G was the predominant allele in fine and super fine wool types with allele frequencies of 0.62 and 0.65, respectively. The AA homozygote was the predominant genotype in both the fine strain and super fine strain with a genotype frequency of 0.45 and 0.45, respectively the A/G heterozygote was the predominant genotype in dual purpose types with a genotype frequency of 0.47 (Table 2). The T allele at SNP c.191T>C was the predominant allele in the fine, super fine and dual purpose strains with allele frequencies of 0.60, 0.52 and 0.55, respectively. The TT homozygote was the predominant genotype in fine and dual purpose strains with genotype frequencies of 0.43 and 0.39, respectively. The T/C heterozygote was the predominant genotype in the super

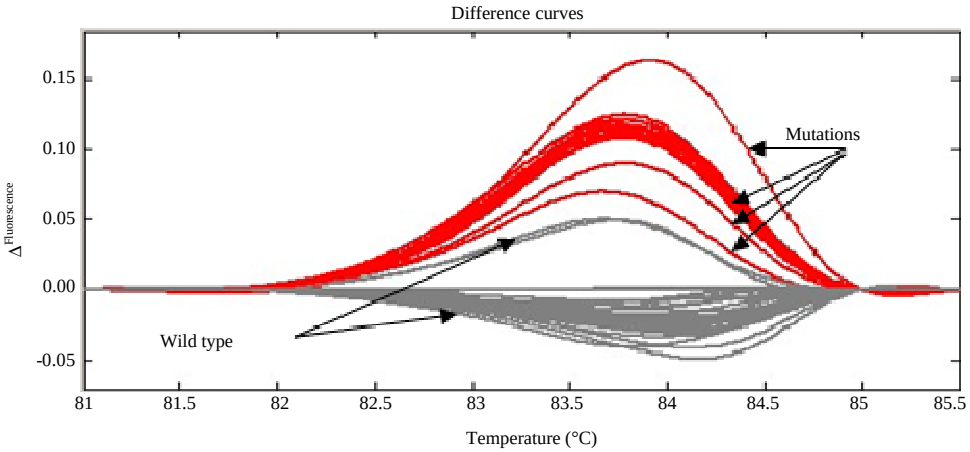

Fig. 1: High-resolution melt analysis for mutation screening in Gansu Alpine fine-wool sheep *KRT 31* gene. Melting curve normalization was carried out with Light Scanner Software on the Light Scanner (Idaho Inc., UT). Figure shows a difference plot of fluorescence against temperature. Wild-type amplicons are shown in grey, putative mutants in red

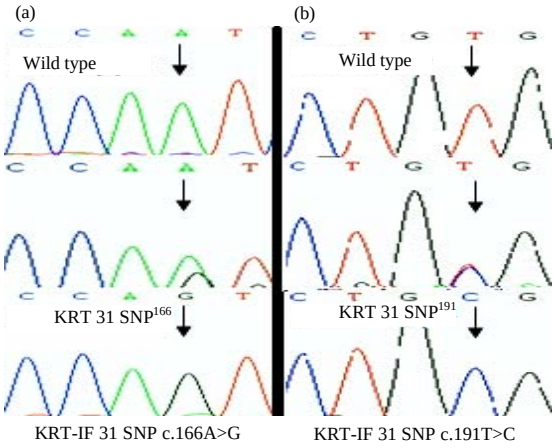

Fig. 2: A partial sequencing maps from different genotypes in Gansu Alpine fine-wool sheep KRT 31 SNP c.166A>G and c.191T>C. a) sequencing maps from different genotypes in Gansu Alpine fine-wool sheep KRT 31 SNP c.166A>G. The wild type is AA, mutation genotype is GG; b) sequencing maps from different genotypes in Gansu Alpine fine-wool sheep KRT 31 SNP c.191T>C. The wild type is TT, mutation genotype is CC

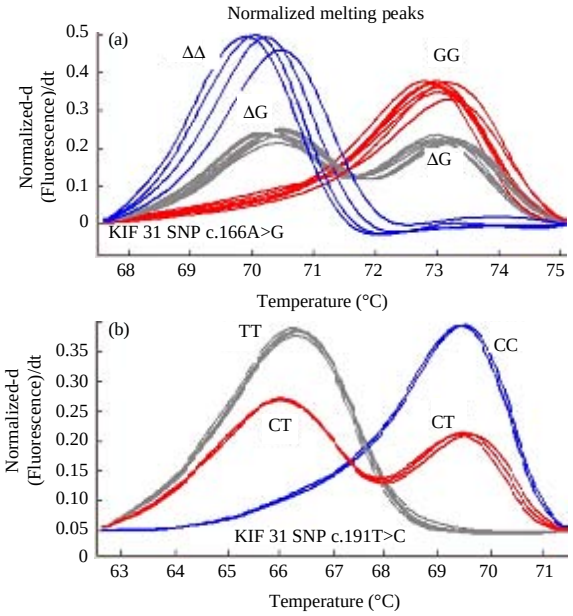

Fig. 3: Genotyping results for KIF 31 SNP c.166A>G and c.191T>C in HRM assays. a) the blue curve genotype is A/A at Single Nucleotide Polymorphism (SNP) position KRT 31 SNP c.166A>G. The light grey curve genotype is A/G at SNP position KRT 31 SNP c.166A>G. The red curve genotype is G/G at SNP position KRT 31 SNP c.166A>G; b) the blue curve genotype is C/C at Single Nucleotide Polymorphism (SNP) position KRT 31 SNP c.191T>C. The light grey curve genotype is T/T at SNP position KRT 31 SNP c.191T>C. The red curve genotype is C/T at SNP position KRT 31 SNP c.191T>C

fine strain with a genotype frequency of 0.48 (Table 2). The SNP c.166A>G in fine strain was in Hardy Weinberg equilibrium by χ^2 statistics ($p>0.05$) but in the other two strains it was Hardy Weinberg disequilibrium ($p<0.05$) (Table 2). The SNP c.191T>C in fine and dual purpose strains was in Hardy Weinberg equilibrium ($p>0.05$)

Table 2: Distribution of KIF 31 SNP c.166A>G and c.191T>C genotype and allele frequency in three lines of Gansu Alpine fine-wool sheep

		SNP c.166A>G						SNP c.191T>C					
		AF		GF				AF		GF			
Lines	n	A	G	AA	AG	GG	p-values	T	C	TT	TC	CC	p-values
FL	159	0.62	0.38	0.45 (72)	0.33 (52)	0.22 (35)	0.100	0.60	0.40	0.43 (68)	0.23 (55)	0.35 (36)	0.080
SFL	113	0.65	0.35	0.45 (51)	0.40 (44)	0.16 (18)	0.020	0.52	0.48	0.28 (32)	0.48 (54)	0.24 (27)	0.001
DPL	176	0.50	0.50	0.26 (46)	0.47 (83)	0.27 (47)	0.003	0.55	0.45	0.39 (68)	0.29 (57)	0.32 (51)	0.120

F: Fine Line; SFL: Super Fine Line; DPL: Dual-Purpose Line; AF: Allele Frequency; GF: Genotype Frequency; p-value: p-value of χ^2-test for Hardy-Weinberg equilibrium

Table 3: Genetic polymorphism on KIF 31 SNP c.166A>G and c.191T>C in three lines of Gansu Alpine fine-wool sheep

	SNP c.166A>G			SNP c.191T>C		
Lines	Ho	He	PIC	Ho	He	PIC
FL	0.53	0.47	0.36	0.52	0.48	0.36
SFL	0.54	0.46	0.35	0.51	0.49	0.37
DPL	0.50	0.50	0.38	0.50	0.50	0.37

Ho: gene Homozygosity; He: gene Heterozygosity; PIC: Polymorphism Information Content; PIC>0.5 indicates high polymorphism, 0.25<PIC<0.5 indicates middle polymorphism, PIC<0.25 indicates low polymorphism

Table 4: Association analysis of wool traits and KIF 31 SNP c.166A>G and c.191T>C

	SNP c.166A>G			SNP c.191T>C		
Traits	AA	AG	GG	TT	TC	CC
FD (μm)	20.28±0.21^a	20.33±0.22^a	21.01±0.24^b	20.39±0.13	20.20±0.15	20.45±0.16
CV-FD (%)	22.48±0.50	22.88±0.53	22.69±0.57	21.80±0.51	21.85±0.43	21.86±0.56
CR (%)	48.64±1.02	47.57±1.06	48.83±1.15	47.88±1.04	48.28±0.89	48.14±1.14
SS (N/ktex)	6.98±0.40	7.04±0.42	6.49±0.46	6.93±0.45	6.56±0.38	6.33±0.49
SL (mm)	46.31±0.53	46.15±0.55	45.57±0.60	45.51±0.55	45.73±0.47	45.35±0.60
WC	2.25±0.15	2.25±0.16	2.11±0.17	2.28±0.16	2.24±0.14	2.23±0.17

FD: Fiber Diameter; CV-FD: Coefficient of Variation of Fiber Diameter; CR: Cleaning Rate; SS: Staple Strength; SL: Staple Length; WC: Wool Crimp; values with different superscripts within the same line differ significantly at p<0.05 (a, b)

but in the super fine strain it was disequilibrium (p<0.05) (Table 3 and 4). This study is the first study to report extensive sequence variation in the ovine *K33* gene. Researchers revealed a missense mutation in whole cording region of sheep *KRT 31* gene (SNP c.166A>G) which is a novel SNP. Although, the polarity of amino acid was unchanged after mutation, the spatial structure was predicted to be changed. The mutation at site c.191 (SNP c.191T>C) does not alter the amino acid sequence. Furthermore, gene frequency and genotyping frequency of SNP c.166A>G and c.191T>C showed that the A allele on SNP c.166A>G was the predominant allele in fine and super fine types, the AA genotype was the predominant genotype in fine and super fine types and the AG genotype was the predominant genotype in the dual purpose type (Table 4). The mean fiber diameter for genotype AA and AG was smaller than genotype GG (p<0.05). This may because the A allele was favored during selection for the main trait of fine fiber diameter in Gansu Alpine fine wool sheep suggesting this site have the potential to impact on wool quality.

Furthermore, analysis of the impact of *KRT 31* gene polymorphism on wool traits in three Gansu Alpine fine wool sheep strains indicated that it was not significantly different among three genotype SNP c.166A>G and SNP c.191T>C for CV-FD, CR, SS, SL, WC, respectively (p>0.05). However, the mean fiber diameter for KRT 31 c.166A>G genotype AA and AG was smaller than genotype GG (p<0.05) suggesting this mutation could potentially be exploited in gene marker-assisted selection programmes within the wool industry to select for fine wool sheep with decreased fiber diameter. This study was however limited to t three strains of Gansu Alpine fine wool sheep and further investigation is required.

CONCLUSION

The present study revealed two SNPs identified as KRT 31 SNP c.166A>G and c.191T>C. The mutation of KRT 31 c.166A>G appears to be associated with c fiber diameter (p<0.05) and may be useful in decreased fiber diameter of fine wool sheep through marker assisted selection.

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