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Association of the KAP 8.1 Gene Polymorphisms with Fibre Traits in Inner Mongolian Cashmere Goats*

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ABSTRACT : The objective of this study was to investigate polymorphisms of keratin-associated protein 8.1 (KAP8.1) gene and its effect on fibre traits of Chinese Inner Mongolian Cashmere goats. The fibre traits data investigated were cashmere fibre diameter, combed cashmere weight, cashmere fibre length and guard hair length. Five hundred and forty animals were used to detect polymorphisms in the complete coding sequence of the hircine KAP8.1 gene by means of PCR-SSCP. The results identified six genotypes, AA, BB, CC, AB, AC and BC, coded for by three different alleles A, B and C. Two SNPs in the coding region were confirmed by sequencing, which were T113G and G116C respectively. The relationships between the genotypes and cashmere fibre diameter, combed cashmere weight, cashmere fibre length and guard hair length were analyzed. There were significant differences between the associations of the different genotypes with cashmere weight ($p<0.01$), cashmere length ($p<0.05$) and hair length ($p<0.01$). Cashmere fibre diameter was the only trait that was not associated with the genotypes. The animals of genotype AB and BB had the higher cashmere weight compared with the genotype AA. By further analysis, it appeared that the KAP8.1 genotype effects on fibre traits may be due to a mutation at the 113 locus. These results suggested that polymorphisms in the hircine KAP8.1 gene might be a potential molecular marker for cashmere weight in Cashmere goats. (**Key Words :** Keratin Associated Protein 8.1, Polymorphisms, Cashmere Weight, PCR-SSCP, Inner Mongolian Cashmere Goats)

INTRODUCTION

The Inner Mongolian Cashmere goat is one of the most excellent breed of Cashmere goat in China and is an important source of income for the Inner Mongolia region with the largest portion derived from cashmere (Bai et al., 2006). The value of cashmere is determined largely by clean cashmere weight and fibre diameter with an increasing market premium for 15 μm diameter and finer cashmere. The main objective of the Inner Mongolian Cashmere goat breeder therefore is to breed Cashmere goats for increased cashmere fleece weight with a fine fibre diameter. Cashmere fibre grown in goats (*Capra hircus*), is the finest and softest animal fibre, with an average diameter of 15 μm, and is used exclusively in luxurious textile products (McCarthy, 1998). World market demands still exceed the supply, so prices are consistently stable and higher than wool and mohair. China is the largest world producer of cashmere. About 30% of the total production comes from the Inner Mongolian population of Cashmere goats that vary in production traits among individuals and flocks (Zhou et al., 2003).

The cashmere fibre is a complex structure composed primarily of proteins from the keratin family. These proteins are responsible for the major structural and mechanical properties of the cashmere fibre. The keratin association proteins (KAPs) are composed of a large number of multigene families, which are expressed in various types of epithelia (Shimomura et al., 2002). KAPs have been classified on the basis of their amino acid composition as high sulfur (16-30 mol % cysteine), ultra-high sulfur (>30 mol % cysteine), and high glycine/tyrosine proteins (Powell and Rogers, 1997). The high glycine-tyrosine (HGT) proteins in fibre are significantly varied both within and between species ranging from 1% to 12% in sheep wool, 18% in mouse hair to more than 30% in echidna quills

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(Frenkel et al., 1974; Gillespie, 1990). The wide range in the content of these proteins in wool and hair raises intriguing questions concerning the regulation and function of these proteins in the matrix structure of the fibre (Liu et al., 2007; Zhao et al., 2009). The keratin-associated protein 8.1 (KAP8.1) is one member of the HGT proteins that belong to one of the three classes of KAP proteins (Kuczek and Rogers, 1987; Fratini et al., 1992; Aoki et al., 1997). The hircine KAP8.1 gene has a coding sequence of 189 bp, which lacks introns and mainly is expressed in the wool follicle cortex. KAP8 gene has been mapped to ovine chromosome 1 (Wood et al., 1992). Genes encoding HGT proteins have been located on chromosome 21q22.1 (Rogers et al., 2002) in human. Many studies have reported polymorphism in members of the KAP gene families (Rogers et al., 1994; Rogers et al., 1994; McLaren et al., 1997; Beh et al., 2001; Itenge-Mweza et al., 2007; Yu et al., 2008; Zhao et al., 2009). Furthermore, there have been some reports associating variation in the KAP loci with variation in fibre diameter (Parsons et al., 1994; Beh et al., 2001; Liu et al., 2007), staple strength (Rogers et al., 1994) and wool colour and brightness (McKenzie et al., 2001). However, few candidate genes for cashmere production traits have been reported in Cashmere goats. The objective of this study was to identify polymorphisms in the KAP8.1 gene and to evaluate the association of these polymorphisms with cashmere traits in Inner Mongolian Cashmere goats.

MATERIALS AND METHODS

Animals

The animals used in this study consisted of 540 Inner Mongolia Cashmere female goats from 24 selected sires. All goats were ear tagged at birth with both coloured and aluminium tags and their pedigree was recorded. The goats were raised in the breeding Cashmere goat farm in the southwestern of the Inner Mongolia region of China. All animals were kept extensively in a desert pasture all year round with the similar rearing and feeding conditions.

Genomic DNA preparation

Blood samples were collected by venepuncture into 10-ml EDTA vacutubes. The blood samples were stored frozen at -20°C. DNA was isolated from 5 ml of thawed blood using the method of Sambrook et al. (1989). The DAN samples were diluted to 100 ng/μl in 10 mM Tris-HCl pH 8 ready for used in PCR and stored at -20°C.

Phenotypic data collection

The fibre data including combed cashmere weight, cashmere fibre length and guard hair length, were collected from farm records. Moulted cashmere was harvested once a year by combing in late April, and weighed using an electronic scale. Cashmere and guard hair length were measured prior to combing at the same side of the shoulder without stretching the fibre. Patch samples of 10 cm^2 moulted cashmere on the side of the shoulder were obtained before combing. Hairs in the samples of moulted cashmere were separated and the cashmere samples were washed in ether solution to remove contaminants such as soil and grease. The cashmere fibre diameter was measured using an Auda 2000 optical wool fiber gauging instrument (Shenaoda Technology Company, Shijiazhuang China) taking the mean from 1,000 fibres per animal. Measurements were performed by the Wool Analysis Laboratory of Liaoning Cashmere Breeding Center.

PCR amplification and SSCP analysis

PCR was performed to amplify sequences fragments of KAP8.1. Three pairs of PCR primers (Table 1) were designed using the software Oligo 6.0, according to the mRNA sequence of KAP8.1 (GenBank accession no. AY510122). The three overlapping PCR fragments covered the complete coding region and the 3' un-translated region of the hircine KAP8.1 gene. The 25-μl PCR volume included 50 ng DNA template, 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 0.5 U TaqDNA polymerase (Dingguo Biotechnology Company, Beijing, China). The PCR protocol was 94°C for 5 min followed by 35 cycles of 94°C for 30 s, annealing (Table 1) for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min.

The PCR products were then directly genotyped by SSCP. One microlitre of PCR product from each individual was mixed with 5 μl denaturing buffer (98% formamide,

Table 1. PCR primers used for characterization of the hircine KAP8.1 gene

Primers	Sequence of primers	Starting nucleotide position (GenBank accession no. AY510122)	Fragment length (bp)	Annealing temperature (°C)
1	5'- ATACTGAGGAAATTCATTCCCTGC -3' 5'- GCCCCAGAGCCGTTGTAG -3'	1	199	60
2	5'- CTATGGCTACAACGGCTCTG -3' 5'- CCCTTGAGACTCTGGTGCC -3'	176	187	63
3	5'- CACATCGGCACCAGAGT -3' 5'- TAGCGGTTGAGACAAGTTTATT -3'	339	207	60

0.05% xylene, cyanole FF and 0.05% bromophenol blue) and denatured at 98°C for 5 min, followed by a rapid chill on ice for 10 min. The denatured PCR products were electrophoresed for 16 h at 8 V/cm on 12% acrylamide gels. DNA bands on the gel were visualized by the silver staining technique.

Cloning and sequencing of PCR products to confirm mutation

The PCR products of individuals that showed different conformation patterns were purified using the DNA Fragment Quick Purification/Recover Kit (Dinggou Biotechnology Company, Beijing, China), and cloned using pGEM-T Easy Vector Kit (Promega, Madison, WI, USA), then sequenced using an 3730 sequencer (Applied Biosystems 3730xl DNA Analyzer).

Statistical analysis

All data were subjected to general linear models analysis using the SAS software package (version 8.2). The mixed linear model was as follows:

$$Y_{ijkl} = \mu+G_i+Y_j+A_k+S_l+e_{ijkl}$$

Where Y_{ijkl} is an observation of the dependent variable (cashmere fibre diameter, combed cashmere weight, cashmere fibre and guard hair length); μ is the population mean for the variable; G_i is the fixed effect of i^{th} genotype (i = 1 (AA), 2 (AB), 3 (AC), 4 (BB), 5 (BC) or 1 (AA), 2 (AB), 3(BB)); Y_j is the fixed effect of j^{th} year (j = 1, 2, …, 8); A_k is the fixed effect of k^{th} age (k = 1, 2, …, 8); S_l is the random effect of l^{th} sire and e_{ijkl} is the random residual term. Treatments comparisons were made using least square means by Tukey options. The allele and genotype frequencies were calculated and Hardy-Weinberg equilibrium was tested by comparing expected and observed genotype frequencies using a Chi-square test.

RESULTS

The analysis of polymorphism

Three fragments, amplified by PCR using the primers described in Table 1, showed the expected lengths. The polymorphisms of the PCR products in all individuals were analyzed by PCR-SSCP. Only the products of primer 1 exhibited polymorphisms. In the primer 1 fragment, six conformation patterns were identified, denominated AA, AB, AC, BB, BC and CC genotypes by analyzing the SSCP results (Figure 1).

Nucleotide sequence

The nucleotide sequence analyses of the six genotypes revealed that three different alleles, A (G-G), B (T-G) and C (G-C), which contained two SNPs (Figure 2) were identified in the KAP8.1 mRNA sequence (GenBank accession no. AY510122). The mutations were described as T113G and C116G, which were synonymous mutations in KAP8.1 protein, namely Proline (P) and Leucine (L) respectively.

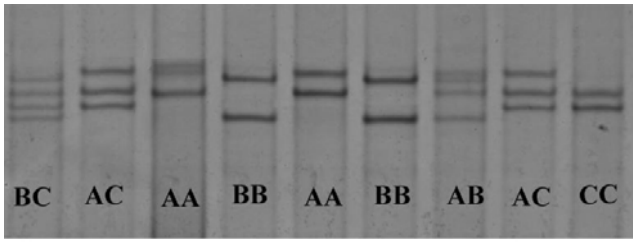

Figure 1. Genotypes of SSCP in the hircine KAP8.1. Individuals with one slow band and one fast band were designed as genotype AA, BB and CC, and two bands were designed as genotype AB, AC and BC, respectively, besides one mutual band of genotype AC.

Association between genotypes and fibre traits

The frequencies of genotypes and alleles were showed in Table 2. The frequencies of allele A, B and C were 0.62, 0.33 and 0.05 respectively in the population (n = 540). Allele A and genotype AB were predominant in the breed. The x^2 test showed that the genotype frequencies were in agreement with Hardy-Weinberg equilibrium in the population ($p>0.05$).

Statistical analyses showed that the different genotypes were significantly associated with cashmere weight ($p<0.01$), cashmere length ($p<0.05$) and hair length ($p<0.001$), but not with cashmere fibre diameter ($p>0.05$) (Table 3). The genotype CC was not included in statistical analysis due to small sample size (only 2 animals). Goats with genotype BB and AB exhibited significantly higher cashmere weight when compared with genotype AA ($p<0.01$). The mean difference was about 20 grams in cashmere weight between genotype AA and genotype BB or AB. Genotypes with allele B had significantly increased cashmere weight. The Cashmere goats with genotype BB had longer cashmere length than genotype AA ($p<0.05$). The animals with genotype AB exhibited the shorter hair length when compared with genotype AA and BB ($p<0.01$).

The analysis of gene effects on the cashmere traits in Table 3, may have been affected by allele B. Therefore according to sequence results, we attempted to combine the alleles and analyze the association between genotypes and cashmere traits. At the 113 locus of genotype AA, AC and CC were homozygous GG, so genotypes AA, AC and CC were combined into to genotype AA; genotype AB and BC were heterozygous GT and were combined into genotype AB and BC to genotype AB; genotype BB was homozygous TT and was combined into genotype BB. Using the same

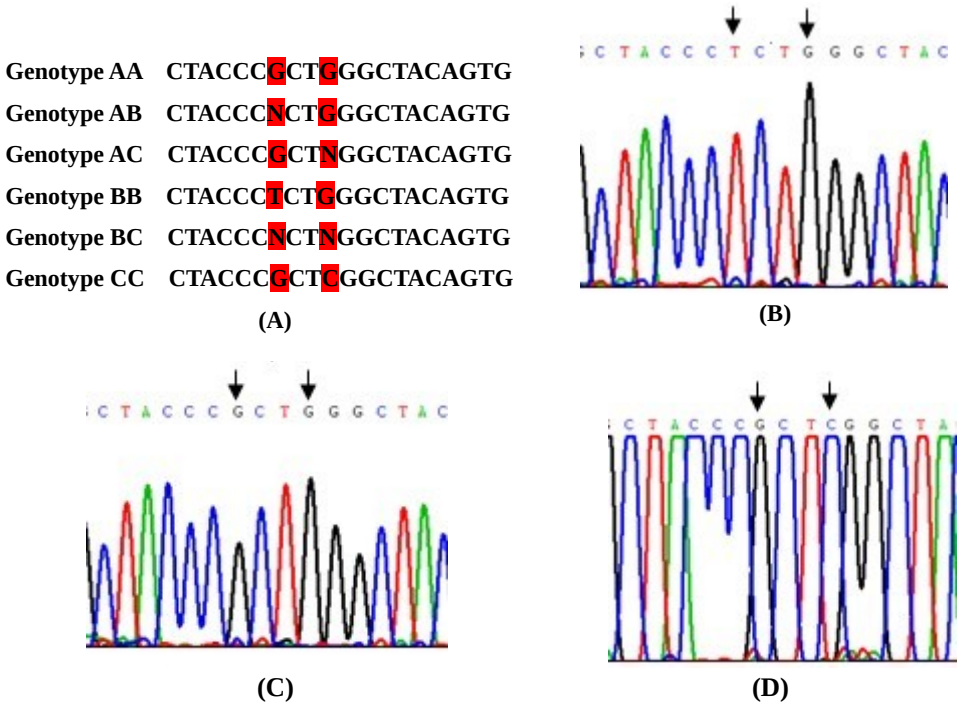

Figure 2. Nucleotide sequence comparison of the PCR products amplified by primer 1. (A) BLAST results of the nt sequence of the six genotypes in this study with nt sequence in GenBank; (B) nt sequence of genotype BB (arrows' positions are consistent with nt sequence in GenBank); (C) and (D) nt sequence of genotype AA and CC (the arrow pointed to the mutation site). AA genotype contains two 113G116G single strands (allele A); BB genotype contains two 113T116G single strands (allele B); CC genotype contains two 113G116C single strands (allele C); AB genotype contains one 113G116G single strand and one 113T116C single strand; AC genotype contains one 113G116G single strand and one 113G116C single strand; BC genotype contains one 113T116G single strand and one 113G116C single strand.

Table 2. Distribution of genotype and allele frequencies of hircine KAP8.1 gene

Category	Genotypes						Alleles		
	AA	AB	AC	BB	BC	CC	A (G-G)	B (T-G)	C (G-C)
No. of animals	204	219	35	61	19	2			
Frequency	0.3778	0.4056	0.0648	0.1130	0.0352	0.004	0.6153	0.3346	0.0502

Table 3. Associations between genotypes of KAP8.1 gene and cashmere fibre traits (LSM±SE)

Genotype	Fibre diameter (μm)	Cashmere weight (g)	Cashmere length (cm)	Hair length (cm)
AA	15.04±0.15	601.31±20.52^{a}	5.80±0.09^{a}	17.03±0.54^{a}
AB	15.05±0.14	620.02±20.53^{b}	5.82±0.09^{ab}	16.35±0.54^{b}
BB	15.07±0.18	620.58±21.46^{b}	5.92±0.10^{b}	17.38±0.58^{a}
AC	15.16±0.21	606.71±22.75^{ab}	5.88±0.11^{ab}	17.20±0.61^{a}
BC	15.16±0.24	623.24±24.15^{ab}	5.81±0.12^{ab}	16.90±0.65^{ab}

Means within a column with no common superscript lowercases differed significantly ($p<0.05$).

method at the 116 locus, genotypes AA and AB and BB were combined into genotype GG, and genotypes AC and BC were combined into genotype GC.

The statistical results are given in Table 4 and suggest that the SNP at the 113 locus was significantly associated with cashmere weight ($p<0.01$), cashmere length ($p<0.05$) and hair length ($p<0.001$) except for cashmere fibre diameter ($p>0.05$) (not listed in Table 4). However the SNP of G116C did not affect cashmere traits and was a neutral mutation. Cashmere traits least square means for KAP8.1 T113G genotypes are same as in Table 3. The T allele at T113G was associated with an increase in cashmere weight and cashmere fibre length. The animals with genotype BB had an increase of 18 g in cashmere weight ($p<0.01$) and 0.11 cm in cashmere length ($p<0.05$) compared with the genotype AA animals.

DISCUSSION

The KAP genes that code for the structural proteins of the wool fibre may be candidate genes with major effects on wool production variations. Wood et al. (1992) reported a CA repeat variation at the KAP8 locus in sheep. It was also indicated that the allelic variation at the KAP8 locus and fibre diameter were significantly associated (Parsons et al., 1994; Liu et al., 2007). The follicular expression of KAP varies considerably in different species (Gillespie, 1972). Therefore, HGT-KAPs genes may be candidates for cashmere QTLs.

In the current study, six genotypes and three alleles were identified by SSCP (Figure 1), sequencing showed that two mutations of the KAP8.1 gene, which supports previous research (Zhao et al., 2009). The results revealed that the genotypes AA and AB were predominant in Inner Mongolian Cashmere goats, the frequency being 38% and 40% respectively. Whereas genotype CC was the least common with only two animals detected. The allele frequencies were A>B>C. The frequency of each genotype detected in our research was similar to the results of Zhao et al. (2009) in Inner Mongolia White Cashmere goats. The distribution of allele and genotype frequencies in our sample was in accord with the Hardy-Weinberg equilibrium (x^2 = 0.17, $p>0.05$) indicating that the locus is under panmixia random mating. The genotypic frequencies and haplotypic frequencies of the KAP8.1 gene were found to be significantly different between the Chinese Inner Mongolia White Cashmere goat and the Shaanbei White Cashmere goat (Zhao et al., 2009), and that polymorphism was not present in this gene in the Liaoning Cashmere goat, Licheng goat, and Liaoning-Kelan crossed goat (Zhao et al., 2007), which leads us to believe that the KAP genes varies considerably in different breeds.

The current results are the first to reveal that the polymorphisms of KAP8.1 are associated with cashmere weight, cashmere length and hair length (Table 3). The goats with genotypes AB and BB had over 2.7% higher cashmere weight than genotype AA. Since the small number of observations on the genotypes AC, BC and CC might not yield convincing statistical inferences, according to the sequencing results, we attempted combine the alleles and further analyzed the association between genotypes and cashmere traits at the 113 and 116 loci respectively. The statistical results showed that the SNP at 113 locus was significantly associated with cashmere weight, cashmere length and hair length. Whereas the SNP at 116 locus may be a neutral mutation which does not affect the cashmere traits. The results of Table 4 suggested that the genotype BB was significantly associated with cashmere weight and cashmere length, which is consistent with the data in Table 3. Therefore, the genotype BB may be the most advantageous genotype for cashmere weight. Interestingly, alleles at the 113 locus were not associated with variation in cashmere fibre diameter. The strong association of 113 alleles with cashmere weight but not cashmere fibre diameter suggests that these traits are controlled by different genes and may be selected for separately (Adelson et al., 2002). The higher cashmere weight may be related to longer cashmere length. This is important and indicates Cashmere goats may be selected to produce higher cashmere weights of fine cashmere fibre at the same time.

The SNPs in the KAP8.1 gene in this study were all synonymous mutations and are therefore traditionally viewed as being phenotypically silent since they do not alter the amino acid sequence of the subsequent protein. However a number of recent studies have demonstrated that synonymous mutations may affect gene function by altering mRNA secondary structure, stability, splicing (Chamary and Hurst, 2005; Salomons et al., 2007) and protein expression (Shah et al., 2008). Two silent SNPs at KAP8.1 gene could result in two synonymous mutations in KAP8.1 protein,

Table 4. Association between genotypes of KAP8.1 gene at site T113G and cashmere fibre traits (LSM±SE)

Genotype	Cashmere weight (g)	Cashmere length (cm)	Hair length (cm)
AA	601.94±20.45^a	5.81±0.09^b	17.05±0.54^a
AB	620.09±20.48^b	5.82±0.09^b	16.40±0.54^b
BB	620.38±21.43^b	5.92±0.10^a	17.37±0.58^a

Means within a column with no common superscript lowercases differed significantly ($p<0.05$).

which maybe lead to protein with the same amino acid sequence but different structural and functional properties (Komar, 2007) and could affect cashmere traits. These mutations at the 113 locus of the nucleotide sequence of a gene significantly associated with the cashmere production indicate that it could be an important functional site for KAP8.1 and further studies are needed to investigate it. Investigations are required on the gene function at the protein level and its interactions with other candidate genes.

In conclusion, three alleles and six genotypes of the KAP8.1 gene were identified in Inner Mongolia Cashmere goats. There were two synonymous mutations at 113 locus and 116 locus of the KAP8.1 gene. The study showed that the KAP8.1 gene was strongly associated with cashmere weight, cashmere length and hair length. The SNP T113G was found to be effected cashmere trait however SNP G116C might be a neutral mutation. The animals with genotype AB and BB had higher cashmere weight and fine fibre diameter, indicating that the polymorphisms in the hircine KAP8.1 gene may be a potential molecular marker for cashmere weight in Cashmere goats.

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