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Missense Mutation of KAP16.6 Gene on Three Goat Breeds in China and Their Associations with Cashmere Production Traits

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Abstract: In the present study, the keratins and Keratin-Associated Proteins (KAPs) are one of the largest gene families in mammalian genomes encode, which is a heterogeneous group of proteins that make up about 90% of the cashmere fiber. Also, it regarded as a candidate gene of cashmere production traits. In this study, we aimed to detect polymorphisms of KAP16.6 gene and to investigated their associations with cashmere production traits (fiber diameter, cashmere yield, down cashmere thickness, body weight after combed cashmere) of three local goat breeds in China. In Xinjiang goat, statistical evaluation revealed significant differences ($p<0.05$) between the fiber diameter and cashmere yield trait of GG genotype. In Nanjiang cashmere goat, it is no significant differences ($p>0.05$) between cashmere production traits. The missense mutation of KAP16.6 gene in 816 cashmere goat samples was firstly detected in three of Xinjiang local goat breeds. Also, parts of these samples were sequenced. The results showed that frequencies of the KAP16.6-G allele in Xinjiang goat (n = 220), Nanjiang cashmere goat (n = 310) and BoGeDa cashmere goat breeds (n = 286) were 0.705, 0.603 and 0.600, respectively. The χ^2 test showed that the genotype distributions in these three cashmere goat breeds were in agreement with Hardy-Weinberg equilibrium. According to the classification of PIC, BoGeDa cashmere goat was more polymorphic at this locus. Then a missense mutation was described at KAP16.6 locus in Xinjiang local goat breeds for the first time. The results possibly revealed that the size polymorphism existed in the three Xinjiang local goat breeds.

Key words: Goat, keratin association protein16.6, cashmere traits, polymorphism, missense mutation, Single Nucleotide Polymorphism (SNP)

INTRODUCTION

This research is based on the keratins and Keratin-Associated Proteins (KAPs) in population genetics as most of the hair wool traits have polygenetic nature. Mammalian skin consists of three major compartments: epithelium, dermis and hypodermis. It is important that epidrmeis is a derivative of the surface ectoderm, as a protective barrier and specific appendages including hair, nails and different eccrine glands (Leon *et al*., 2004). The cuticle, the cortex and the medulla is a main structure of all hairs and wool (Bond *et al*., 1996; Langbein *et al*., 1999, 2001). Cashmere fiber is composed of the cuticle and the cortex. The internal cortical cells are long polyhedral spindle-shaped structures (Jones, 2001), which mechanically are the most important component of any α-keratin fiber (Feughelman, 2002). The flattened overlapping, cuticle cells surround the cortex and forms the external layer of the fiber (Marshall *et al*., 1991). The structure of the cashmere fiber largely involves the expression of hair keratins and their keratins-associated proteins (Langbein *et al*., 1999, 2001). In particular, the keratins and Keratin-Associated Proteins (KAPs) are a large heterogeneous group of proteins that make up about 90% of the wool fiber (Powell and Rogers, 1994). And human hair is very resistant to external stimuli and high

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stability due to keratins (Barba *et al*., 2009). So, the keratins and Keratin-Associated Proteins (KAPs) play an essential role in hairs and wool.

The KAP genes are small in size <1 kb, generally contain a single exon. Also, KAPs are encoded by a large number of multigene families. Furthermore, Keratin proteins are the main structural componentsof wool and can be divided into 2 groups, the Intermediate Filament Proteins (IFPs) and the matrix proteins (Powell *et al*., 1992). Also, the KAPs have been divided into three categories, the high sulfur KAPs (<30 mol% cysteine content), the ultra-high sulfur KAPs (>30 mol% cysteine content) and the high tyrosine/glycine KAPs (Powell and Rogers, 1996). The hair keratins represent the type I (acidic) and type II (basic) two multigene families. They form the 8-10 nm Intermediate Filaments (KIF) of trichocytes by co-polymerization of type I and II members, which are differentially expressed during hair fiber development (Langbein *et al*., 1999, 2001). Previous studies have introduced these KAPs are using the abbreviations KAP1.n through KAP23.n for these members known at that time with n referring to a number identifying individual members, also subdivided into 23 distinct families (Powell and Rogers, 1993) and >100 KAP genes have been isolated from human and other mammalian species. Genetic markers for the keratin and keratin-associated protein genes have been associated with variation in fiber diameter and staple strength (McLaren *et al*., 1997).

Keratin-Associated Proteins 16.6 gene (KAP16.6) is one of the high sulfur KAPs, which were essentially localized to the hair cortex and which also showed matrix, cuticular expression and might play an important role in the hair forming compartment (Michael *et al*., 2001, 2002), so KAP16.6 gene is very important for the hair structure. Apparent molecular weights of the high sulfur proteins were 26.5-43.0 kd, but it is probably higher than the real values 75-150% (Marshall, 1983). The high sulfur proteins predominantly found in the cuticle with some also found in the cortex (Alan, 2005).

Xinjiang is one of the biggest cashmere-produced country in China. The Xinjiang goat is a native indigenous breed. The breed of Nanjiang cashmere goat is hybrid offspring of Liaoning cashmere goat, which is famous for its high cashmere yield, strong adaptability, stable heritage and better improved effect throughout the Xinjiang indigenous goat in the world and BoGeDa cashmere goat is hybrid offspring of Liaoning cashmere goat breed and Xinjiang indigenous goat and was named by Ministry of livestock of the People's Republic of Xinjiang (Xinjiang Autonomous Region) in 1997 of China.

To date, no polymorphisms of KAP16.6 gene have been reported on Xinjiang local cashmere goat in China. Therefore, it was a preliminary and interesting research to analyze the genetic variations of KAP16.6 gene in 816 goat individuals of Xinjiang in China. Herein, we are the first to identify the novel genetic variation of cashmere goat KAP16.6 gene by PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) and DNA sequencing methods, which will possibly contribute to conducting association analysis and evaluating them as genetic markers in cashmere production and other performance for animal breeding and genetics.

MATERIALS AND METHODS

Animal source: The samples from these three cashmere goat breeds (Xinjiang goat, n = 220; Nanjiang cashmere goat, n = 310; BoGeDa cashmere goat, n = 286) in this study. The Xinjiang goat were from the breeding centre of KuErLe of XinJiang in China, the Nanjiang cashmere goat were from AkeSu Goat Research Center of XinJiang and BoGeDa cashmere goat were from Urumuqi of XinJiang in China. Many records of cashmere traits and body weight were collected for statistical analysis.

DNA preparation and primer design: Genomic DNA of 816 cashmere goat were isolated from 2% heparin-treated blood samples and stored at -80°C, following standard procedures (Sambrook and Resseu, 2001). According to the sequence of KAP16.6 (GenBank accession number AY510118), one pair of PCR primers was designed with Primer5.0, as follow:

Forward: 5'-TGCCATTACAGCAACCAC -3'
Reverse: 5'-GGTAGCAGATGTTGGGTT -3'

It was used to amplify 254 bp PCR products for cashmere goat KAP16.6 gene of the whole CDS region.

PCR amplification: One pair of PCR primers were designed using Primer 5.0 software to amplify the whole CDS region of Capra KAP16.6 gene (high sulfur KAPs) (GenBank accession number AY510118), the size of the PCR products was 254 bp. The 25μL volume contained: 50ng genomic DNA, 0.5 μM of each primer, 1× Buffer (including1.5mM $MgCl_2$), 200 μM dNTPs and 0.625 units of Taq DNA polymerase (MBI). The PCR was performed using the following program: 94°C for 5min followed by 33 cycles of 94°C for 40 sec, annealing for 35 sec and 72°C for 35 sec and a final extension at 72°C for 10 min.

The polymorphisms of HindIII-PCR-RFLP in the KAP16.6 gene and DNA sequencing: Aliquots of 10 μL PCR products of KAP16.6 were incubated with 6 units restriction enzyme HindIII (Promega) for 6 h at 37°C, then electrophoresed on 2% agarose gels with 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA), containing 200 ng mL^{-1} ethidium bromide. A 7 μL aliquot of PCR products was added to 1.5 μL of loading dye

(0.025% bromophenol blue, 0.025% xylene cyanol, 40% (w v^{-1}) sucrose) and the gels were run at a constant voltage (100 v) for 0.4-0.6 h.

The PCR fragments from different patterns in the three breeds were amplified by the pair of primers, then sequenced in both directions by ABI PRIZM 254 bp DNA sequencer (PerkinElmer) and analyze the sequences with BioXIM software (version 2.6).

Statistical methods and analysis: Based on the genotypes of KAP16.6 in Xinjiang local breed locus, genotypic frequencies, allelic frequencies and Hardy-Weinberg equilibriums were directly calculated. Differences in genotypic frequencies at KAP16.6 locus among Xinjiang indigenous goat and cashmere goat populations in China were analyzed using a χ^2-test, which were performed by SPSS software (version 16.0). Population genetic indexes, such as He (gene heterozygosity), Ho (gene homozygosity), Ne (effective allele numbers) and PIC (Polymorphism Information Content) were calculated according to Nei and Roychoudhury (1974) and Nei and Li (1979), respectively.

$$H_o = \sum_{i=1}^{n} P_i^2;\ H_e = 1 - \sum_{i=1}^{n} P_i^2;\ Ne = 1 \Big/ \sum_{i=1}^{n} P_i^2$$

$$PIC = 1 - \sum_{i=1}^{m} P_i^2 - \sum_{i=1}^{m-1} \sum_{j=i+1}^{m} 2P_i^2 P_j^2$$

Furthermore, statistical analysis was performed on records of cashmere traits in Xinjiang Goat (XJG, n = 220) and Nanjiang cashmere goat (NJG, n = 310). The software SPSS (version 16.0) (Norusis, 2008) was used to analyze the relationship between the genotypes and cashmere traits in goat. The reduced linear model with fixed effects was established and included effects of ewe, ram within ewe, age and genotype, as well as interaction between ram and genotype was involved. Reduced linear model:

$$Y_{ijklm} = u + S_i + D_{ij} + A_k + G_l + (SG)il + E_{ijklm}$$

Where,

Y_{ijklm} = The trait measured on each of the ijklmth animal
u = The overall population mean
S_i = The fixed effect associated with the ith ram
D_{ij} = The fixed effect associated with jth ewe with ram i
A_k = Fixed effect due to the kth age
G_l = The fixed effect associated with lth genotype (KAP16.6/GG and GA genotype)
(SG)il = Interaction between the ith ewe and the lth genotype
E_{ijklm} = The random error

An effect associated with farm, sex were not matched in the linear model, as the preliminary statistical analyses indicated that these effect did not have a significant influence on variability of traits in analyzed populations.

RESULTS AND DISCUSSION

There have no polymorphisms of KAP16.6 gene of Xinjiang local cashmere goat were reported. In this study, the CDS region of KAP16.6 gene demonstrated polymorphic patterns (namely, genotype GG and GA) in three populations by PCR-RFLP method and DNA sequencing, the result revealed a mutation G>A (Fig. 1 and 2). In detail, the G>A mutation was located in (g.216 G>A) nucleotide position of GenBank Accession no. AY510118 at KAP16.6 locus. Then, the mutation was confirmed by restriction digestion (HindIII-AAG/CTT), which resulted in a missense mutation in KAP16.6 protein, namely, AGC (Serine) > AAC (Asparagine).

In this study, different polymorhic PCR products were sequenced of cashmere KAP16.6 gene. Then, the sequencing result was compared with capara KAP16.6 gene DNA sequence (GenBank Accession no. AY510118). The result showed that the GG genotype sequence was the same as the KAP16.6 gene DNA sequence in Genbank, while in GA genotype one mutation was located in the whole CDS region of KAP16.6 gene in Xinjiang local goat breeds, which leads to a deletion restriction-reaction polymerase locus. The genotypes (GG and GA) were firstly detected at KAP16.6 CDS region (Fig. 1). Genotypic and allele frequencies of KAP16.6 gene in three breeds were showed in Table 1 and 2. The genotypic frequencies at KAP16.6 locus were significantly different

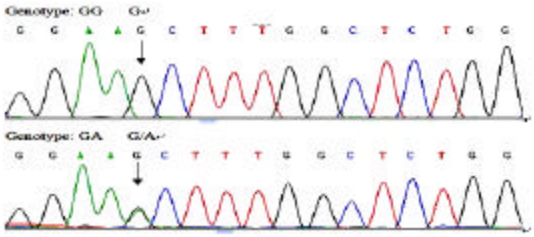

Fig. 1: A partial Sequencing maps from different genotypes in cashmere goat KAP16.6 gene

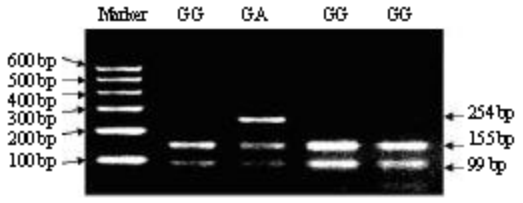

Fig. 2: PCR-RFLP patterns of the KAP16.6 gene in goats (Genotype GG, Genotype GA)

Table 1: Genotype distribution and allelic frequencies at the KAP16.6 gene locus

	Observed genotypes			Allelic frequencies		
Breeds	GG	GA	N	G	A	X^2 (HWE)
NJG	64	246				
	0.206	0.794	310	0.603	0.397	134.119
BGD	57	229				
	0.9476	0.0419	286	0.600	0.400	127.482
XJG	90	130				
	0.199	0.801	220	0.705	0.295	38.689

Shade: Genotype frequencies at the KAP16.6 gene locus, X^2, HWE: Hardy-Weinberg Equilibrium X^2 value; NJG: NanJiang cashmere Goat, BGD: BoGeDa cashmere Goat; XJG: XinJiang cashmere Goat

Table 2: Genetic index in three Xinjiang local goat breed in China

Breeds	Types	Ho	He	Ne	PIC
NJG	Cashmere	0.521	0.479	1.918	p=0.364
BGD	Cashmere	0.520	0.480	1.924	p=0.365
XJG	Cashmere	0.584	0.416	1.713	p=0.330

Ho: Gene Homozygosities; He: Gene Heterozygosities; Ne: Effective Allele Number, PIC: Polymorphic Information Content

between three goat breeds and the frequencies of allele KAP16.6-G was 0.603 in Nanjiang cashmere goat, 0.600 in BoGeDa cashmere goat and it was 0.705 in Xinjiang goat, respectively. G haplotype and GA genotype were predominant in three goat breeds. The X^2 test showed that the genotype distributions of KAP16.6 gene were in agreement with Hardy-Weinberg equilibrium in three breeds (Table 1). Based on a X^2 test, genotypic frequencies of the various polymorphism at KAP16.6 gene were found to be significantly different in the three breeds ($X^2 = 35.632$, d.f. = 2, $p<0.001$).

Gene homozygosity, gene heterozygosity, effective allele numbers and PIC of KAP16.6 gene in the three breeds were showed in Table 2. According to the classification of PIC (high polymorphism if PIC value>0.5, median polymorphism if 0.25<PIC value<0.5 and low polymorphism if PIC value<0.25) (Botstein *et al*., 1980), KAP16.6 gene in three of Xinjiang local goat population were at median polymorphic level. Gene heterozygosity, effective allele numbers and PIC of KAP16.6 gene in BoGeDa cashmere goat population (p = 0.365) were higher than that of Xinjiang goat population (p = 0.330) and Nanjiang cashmere goat population (p = 0.364).

In this study, we associated with body weight after combed, fiber diameter, down cashmere thickness, cashmere yield data and revealed that the polymorphism of KAP16.6 gene firstly. Further analysis suggested that the animals with Nanjiang cashmere goat no significant differences were observed between the GG and GA genotypes of the cashmere production traits (p>0.05) (Table 3). In Xinjiang goat, GG genotype of the fiber diameter and cashmere yield trait was significant differences (p<0.05) that the age from 2-4 years old cashmere goat. And the fiber diameter of the GG genotype is finenest than the GA genotype. The down cashmere

Table 3: Association of genotypes at the KAP16.6 gene with growth traits in NanJiang cashmere goat

	Genotypes at KAP16.6 gene		
Cashmere traits	GG (n = 64)	GA (n = 246)	p-value
BWC (kg)	21.156±0.335	21.451±0.171	>0.05
CFD (μm)	15.742±0.107	15.727±0.055	>0.05
DCT (mm)	4.794±0.088	4.658±0.045	>0.05
CY (g)	487.266±12.873	461.037±6.566	>0.05

BWC: Body Weight after Combed; CFD: Cashmere Fiber Diameter, DCT: Down Cashmere Thickness; CY: Cashmere Yield, *Values with different superscripts within the same line differ significantly at p<0.05 (a, b, c). SE: Standard Error of means

Table 4: Association of genotypes at the KAP16.6 gene with growth traits in XinJiang goat

		Genotypes at the KAP16.6 gene	
Cashmere traits	Ages	GG (n = 90)	GA (n = 130)
CFD (μm)	1	15.644±0.217b	15.532±0.230b
	2	15.471±0.180b	16.220±0.472c
	3	15.016±0.468a	16.243±0.272c
	4	16.823±0.478d	16.398±0.163c
DCT (cm)	1	3.156±0.199	3.319±0.211
	2	3.258±0.166	3.603±0.433
	3	3.818±0.430	3.707±0.250
	4	4.308±0.439a	3.500±0.150b
BWC (kg)	1	29.556±1.271	27.562±1.348
	2	30.846±1.057	27.825±2.762
	3	31.161±2.739a	28.394±1.596b
	4	31.738±2.798a	29.437±0.953b
CY (g)	1	148.333±8.455b	126.562±8.967a
	2	147.692±7.035b	126.825±18.378a
	3	152.306±18.222b	146.753±10.619b
	4	173.885±18.612c	156.562±6.341b

BWC: Body Weight after Combed; CFD: Cashmere Fiber Diameter; DCT: Down Cashmere Thickness; CY: Cashmere Yield, *Values with different superscripts within the same line differ significantly at p<0.05 (a, b, c, d). SE: Standard Error of means

thickness trait between the GG and GA genotype is significant differences (p<0.05) with the 4 years old cashmere goats. The bodyweight after combed cashmere production trait between the GG and GA genotype is significant differences (p<0.05) among the age of three to four years old cashmere goat (Table 4).

The KAP genes, which encode major structural proteins in the hair, wool and nail, are obvious candidate genes for molecular genetic maker. This research it is firstly attempted to detect polymorphism at KAP16.6 gene in three Xinjiang local cashmere breeds. As is known, the fiber diameter, cashmere yeild and down cashmere thickness were important cashmere production traits in cashmere goat. Furthermore, there were some studies reported on the polymorphisms in the human high sulfur and ultra-high sulfur hair keratin-associated proteins, which play an important role in determining various wool traits and KAP genes have been associated with variation in fiber diameter (McLaren *et al*., 1997; Parsons *et al*., 1994). Some studies have reported that genetic variation

at KAP loci might play an important role in determining various wool traits and KAP genes have been associated with variation in fiber diameter (Parsons *et al*., 1994; McLaren *et al*., 1997), which would possibly facilitate the further research on the fiber traits of the cashmere goat. Also, genetic markers for the keratin and keratin-associated protein genes can be associating with variation in fiber diameter and staple strength.

We reported on the detection of missense mutations in the KAP16.6 gene that Ser to Asn and results showed that the polymorphisms of KAP16.6 gene may not a casual. Also, the SNP here may not a casual mutation. The mechanism causing this elevated mutation rate is not known, which indicated that mutations in structural proteins such as keratins are usually dominant. That may be linked to cashmere goat in the coding or regulatory regions of the gene which is not a causal mutation for the cashmere goat growth traits. According to the classification of PIC polymorphic level that the KAP16.6 gene in three of Xinjiang local goat population, which may not be caused by nonrandom mating and among the three Xinjiang local goat breed may had been not selected some of the excellent individuals, which in agreement with the Hardy-Weinberg equilibrium.

Furthermore, the mutations of KAP16.6 gene were associations with cashmere production traits, which was important factor to fiber diameter, cashmere thickness, bodyweight after combed cashmere production, cashmere yield traits. Statistical results show that individuals with genotype GG and GA is no significant in Nanjiang ($p>0.05$). Statistical results show that individuals with genotype GG had greater cashmere yield and fiber diameter than those with genotype GA ($p<0.05$) in Xinjiang goat apart from Nanjiang cashmere goat breed. The result which the variance test was applied to assess statistical were given in Table 3 and 4. This shows that the SNPs locus of KAP16.6 gene probable have business relations to increase the cashmere yield and decrease the fiber diameter in some animal. The research results remind us of associating to human Hair disorders and shedding and also has certain reference value on the diagnosis of Hair disorders and shedding. However, further research would be required to verify this.

KAP16.6 gene seems to be promising as it plays an important role in cashmere production traits. Despite all the available information concerning KAP16.6 protein in human and mouse, there is no information known in cashmere goat or other livestock. Considering the economic importance of the cashmere production traits to the livestock industry, it appears clearly essential to further research on KAP16.6 protien in the livestock.

CONCLUSION

This study revealed a missense mutation in whole CDS region of capara KAP16.6 protein gene, which is a novel SNP. This mutation may changed the spatial structure of kertain protein. And associated with fiber diameter and down cashmere thickness trait that the SNP is significantly. Considering the A haplotype was recessive in three goat breeds, we should reject the A allele in the breeding schemes of goat. So, the KAP16.6 gene is a potential genetic marker and can be used for the marker-assisted selection in cashmere goat breeding work. This study will be practical for the improvement of Xinjiang local goat breeds and the breeding of genuine cashmere in China.

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