



Developmental expression patterns and association analysis of sheep *KAP8.1* and *KAP1.3* genes in Chinese Merino sheep

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ABSTRACT

The keratin-associated protein (*KAP*) is one of the main structural proteins of wool fibre, which is responsible for the major structural and mechanical properties of the wool fibre. Here, the quantitative real-time PCR was used to investigate the developmental changes of *KAP8.1* and *KAP1.3* mRNA expression levels in skin tissue of Chinese Merino lambs. In addition, the genetic variations of sheep *KAP8.1* and *KAP1.3* genes and their associations with wool traits were also tested by least square means in 197 adult Chinese Merino sheep. The results showed that the developmental expression patterns of *KAP8.1* and *KAP1.3* genes were similar, presenting the highest levels at day 0 followed by decline, the sheep *KAP1.3* was highly expressed in the skin, while the *KAP8.1* was weakly expressed. Moreover, association analysis showed that polymorphisms at both *KAP8.1* and *KAP1.3* loci were associated with fiber diameter ($P = 0.021$ and 0.001 , respectively). In detail, individuals with *KAP8.1* BC genotype were significantly smaller fibre diameter than those of individuals with genotype AB ($P < 0.01$) and BB ($P < 0.05$), individuals with *KAP1.3* AA genotype were significantly smaller fibre diameter than those of individuals with genotype AB ($P < 0.05$) and BB ($P < 0.01$). In addition, the combined genotypes of *KAP8.1*-*KAP1.3* genes seem to improve the association, and the optimal combined genotype of *KAP8.1*-*KAP1.3* genes was BC-AA.

Key words: Association analysis, Chinese Merino sheep, Expression pattern, *KAP8.1* gene, *KAP1.3* gene, Wool traits

The Chinese Merino sheep, a major wool and meat producing animal in China, has become a well-known Chinese native fine wool breed. It is predominantly found in north-western China and serves as one of the major components of agro-pastoral societies (Zhao 2002). However, the Chinese Merino sheep has a wide of variability in phenotypes, such as fibre diameter and staple length. Hence, improvement on wool traits is a major breeding goal for the Chinese Merino sheep in the north-western China. Study on wool production and quality genes (keratin and keratin-associated protein gene families) may help in improvement of wool traits through marker-assisted selection (MAS).

The keratins and keratin-associated proteins (*KAPs*) make up about 90% of the wool fiber (McLaren *et al.* 1997). The *KAPs* are classified into high sulphur, ultra-high sulphur and high glycine/tyrosine proteins categories based on their amino acid compositions (Yu *et al.* 2008, Zhao *et al.* 2008). These *KAPs* were further subdivided into 23 distinct families (Rogers *et al.* 2001, 2002, Zhao *et al.* 2008

and Gong *et al.* 2010).

The ovine *KAP8.1* is an important part of high glycine/tyrosine which are clustered together on ovine chromosome 1 (McLaren *et al.* 1997), and this chromosome region appears to be associated with wool fibre diameter in a Merino half-sib family (Parsons *et al.* 1994a, 1994b). Zhao *et al.* (2009) stated that *KAP8.1* gene had 2 silent SNPs in the cashmere goat. While the ovine *KAP1.3* gene located in OOV11 (McLaren *et al.* 1997). The genetic variation at *KAP1.3* gene might play an important role in determining various wool traits in sheep or goat (Liu *et al.* 2007, Zhang *et al.* 2007). These results further revealed that the *KAP8.1* and *KAP1.3* genes could be considered as important candidate genes for sheep breeding through marker-assisted selection (MAS).

In this study, Chinese Merino lambs at different age were selected to investigate the development expression levels of *KAP8.1* and *KAP1.3* genes in skin tissue. In addition, the polymorphisms of *KAP8.1* and *KAP1.3* genes and their associations with wool traits were also analyzed in adult Chinese Merino sheep.

MATERIALS AND METHODS

Xinjiang Junken type Chinese Merino lambs (36),

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randomly selected from 129 mission pasture in Kuitun city of Xinjiang province, were divided into 6 groups (3@& and 3B&) by different ages (0, 7, 14, 30, 60, 90 days). Skin samples from these animals were immediately frozen in liquid nitrogen and then stored at 6 to 8°C for total RNA analysis. Blood samples (197) were obtained from unrelated Chinese Merino ewes. Genomic DNA was extracted from blood samples using standard phenol-chloroform protocol (Liu *et al.* 1997).

Annual wool samples were obtained at shearing in June. The fibre diameter, nature staple length and stretch staple length were measured by fibre diameter analyzer and fibre length analyzer.

Total RNA isolation, cDNA synthesis and the developmental expression patterns of KAP8.1 and KAP1.3 genes: According to the manufacturer's instructions, total RNA of skin tissue was extracted individually using Trizol Reagent and dissolved in DEPC-treated water. Spectrophotometer was used to detect the RNA quality and concentration. For each sample, 2 µg of total RNA was treated with DNase I to remove contaminating genomic DNA and immediately reverse transcribed with oligo-dT primer and M-MLV reverse transcriptase. Meanwhile, the primers of β -actin and the gene of interest, KAP1.3 and KAP8.1 were designed by Primer 5.0 software according to the gene sequences of sheep β -actin (U39357), KAP1.3 (X02925) and goat KAP8.1 (AY510122) (Table 1). All the major PCR products were purified, subjected to cloning and sequenced to confirm the identity. The plasmid was isolated with plasmid kit.

The quantitative real-time PCR amplification was performed with PCR system. Quantitative real-time PCR assay program for thermal cycling was as follows: 2 min at 95°C for one cycle and 40 cycles at 95°C for 10 s, 60°C for 20 s, followed by 30 s at 72°C. β -actin served as an internal control under the same conditions, each sample was performed in triplicate and the standard curves were prepared for each gene by quantitative real-time PCR using the gradient diluted plasmid as template.

Polymorphisms of KAP8.1 and KAP1.3 genes: Based upon

the sheep high glycine/tyrosine keratin gene (X05639) and goat KAP8.1 mRNA (AY510122) sequences, one pair of primers (Table 1) was designed to amplify a 357 bp PCR product including the entire CDS region and its flanking region of the KAP8.1 gene. According to the report from Itenge-Mweza *et al.* (2007), a pair of primers (Table 1) was synthesized to amplify a 598 bp product of the KAP1.3 gene.

The PCR was performed in a 25 µl reaction mixture containing 10 pmol of each primer, 200 mM dNTPs, 1×polymerase buffer (including 1.5 mM MgCl₂), 1.5 units of Taq DNA polymerase and approximately 100 ng genomic DNA as template. The cycling protocol was 5 min at 95°C followed by 34 cycles of 94°C for 30 s, annealing for 30 s, 72°C for 30 s, with a final extension at 72°C for 10 min.

SSCP method was used to scan mutations within the amplified regions of KAP8.1 gene. Aliquots of 2 µl PCR products were mixed with 8 µl denaturing solution (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol), heated for 10 min at 98°C and immediately chilled in wet ice. Denatured DNA was subjected to 12% PAGE gel (180 mm×160 mm×0.75 mm) in 0.5×TBE buffer and constant voltage 140V for 14–16 h after a pre-run at 300 V for 5 min. The gel was stained by a silver staining method (Sanguinetti *et al.* 1994).

Aliquots of 10 µl PCR products of KAP1.3 gene were digested with 10 U *Bsr* I for 5–6 h at 65 °C following the supplier's directions. The digested products were detected by electrophoresis in 2.5% agarose gel stained with ethidium bromide. The PCR products representing different electrophoresis patterns of KAP8.1 and KAP1.3 genes were sub-cloned to pMD19-T and sequenced a commercial service.

Statistical analysis: Both genotypic and allelic frequencies were estimated directly. The effects of genotype on wool traits as well as their expression levels in different age groups were analyzed by the least-square method, as applied in the general linear model (GLM) procedure of statistical software SPSS (Version 13.0), with the mixed model report by Liu *et al.* (2010) and Zhang *et al.* (2007a). The sex and age of lambs had been included in the statistical model for gene expression levels in different age groups. The model analyses for the

Table 1. Primer sequences used in this study

Primer purpose	Primer name	Primer sequence (5'–3')	Sizes (bp)	Tm (°C)
Expression	KAP8.1	F: CTTCTCCAGCACCGTCTTCC R: AGTCCGCCTCGTGGGTTT	233	60
	KAP1.3	F: CGGCTGTGGCATTGGTGGC R: GGGTTGGGAGCAGGTGGGC	280	60
	β -actin	F: GCAGGTCATCACCATCGGCAATG R: TTGGCGTAGAGGTCTTTGCGGATGT	167	60
Polymorphism	KAP8.1	F: GAAATTCATTCCCTGCTC R: TTCCCTTGAGACTCTGGTG	357	57
	KAP1.3	F: GGGTGGAAACAAGCAGACCAAACTC R: TAGTTTGTGGGACTGTACACTGGC	598	58

wool traits at different genotypes included marker genotype and year as fixed effects. Data were presented as least squares means with associated standard error.

RESULTS AND DISCUSSIONS

Developmental changes of *KAP8.1* and *KAP1.3* mRNA expression: Previous studies have demonstrated that there are many genes important in skin and wool/hair. Adelson *et al.* (2004) also reported 2939 ESTs from fetal and adult sheep skin. Researchers have reported a great deal about the expression profile of keratins and KAPs gene in wool/hair follicles (Aoki and Ito 1997, Rogers *et al.* 2008, Shu *et al.* 2009, Yu *et al.* 2009, Jin *et al.* 2010). However, there is no report about the developmental expression of keratins and KAPs gene in skin of Chinese Merino lamb. In this study, the developmental changes of *KAP8.1* and *KAP1.3* mRNA expression levels were firstly surveyed by real-time PCR in skin.

Sequencing results showed that the amplified *KAP1.3*, *KAP8.1* and β -actin genes were 100% homologous to the original sequences used for primer design, indicating the PCR amplification fragments were specific *KAP8.1*, *KAP1.3* and β -actin cDNAs. The standard curve was achieved using the

gradient diluted plasmid as template. Standard curve showed a good linear relationship, correlation coefficient were 0.985, 0.991 and 0.998 for *KAP 8.1*, *1.3* and β -actin respectively.

The results showed that the developmental expression patterns of *KAP8.1* and *KAP1.3* genes were similar, presenting the highest levels at day 0 followed by decline (Fig. 1), implying that these genes will have similar functions in skin or wool of sheep. Significantly higher level of *KAP8.1* mRNA was detected at day 0 than that at the other days ($P < 0.01$). From 14 to 90 days, the expression level of *KAP8.1* mRNA maintained steady with a little fluctuant. For the expression of *KAP1.3* mRNA level, at day 0, the abundance of this gene was much significantly higher than that at the days of 14–90 ($P < 0.01$) and obviously higher than that at day 7 ($P < 0.05$); at day 7, it was higher than that at day 14 ($P < 0.05$) and significantly higher than that at the days of 30–90 ($P < 0.01$). It was worth noting that the expression levels of these genes were obviously different in skin tissues, the sheep *KAP1.3* was over expressed in the skin, while the *KAP8.1* was weakly expressed. The suitable explanation for this is that the biological activities associated with the functions of these genes are demanded to a different extent in different time.

Polymorphism of *KAP8.1* and *KAP1.3* genes: Many studies have reported polymorphism in members of the keratin and keratin-associated proteins (KAP) gene families. However, few studies on polymorphism of *KAP8.1* gene in sheep have been reported. In this study, it was demonstrated that there were 3 SSCP genotypes and 3 alleles in the *KAP8.1* CDS region (Fig. 2). Zhao *et al.* (2009) also confirmed the same PCR-SSCP polymorphisms in Inner Mongolia and Shaanbei white cashmere goat. However, there were monomorphic at this locus in Liaoning Cashmere goat, Licheng goat, and Liaoning Kelan crossed goat (Zhao *et al.* 2007). The alignment between nucleotide sequence of X05639 and sequencing results demonstrated that there were 2 novel mutations, and the SNPs were described as g.1026T>C and g.1130C>T in *KAP8.1* CDS region. The genotype distributions was in good agreement with Hardy-Weinberg equilibrium ($P > 0.05$) in analyzed population. It was worth noting that AA and CC genotypes were not detected in Chinese Merino population, which may due to the number of samples.

The *KAP1.3* *Bsr* I site polymorphism was detected in previous studies (Rogers *et al.* 1994, McLaren *et al.* 1997). Three genotypes of *KAP1.3* were generated by digesting PCR products with restriction enzyme *Bsr* I (Fig. 3) and

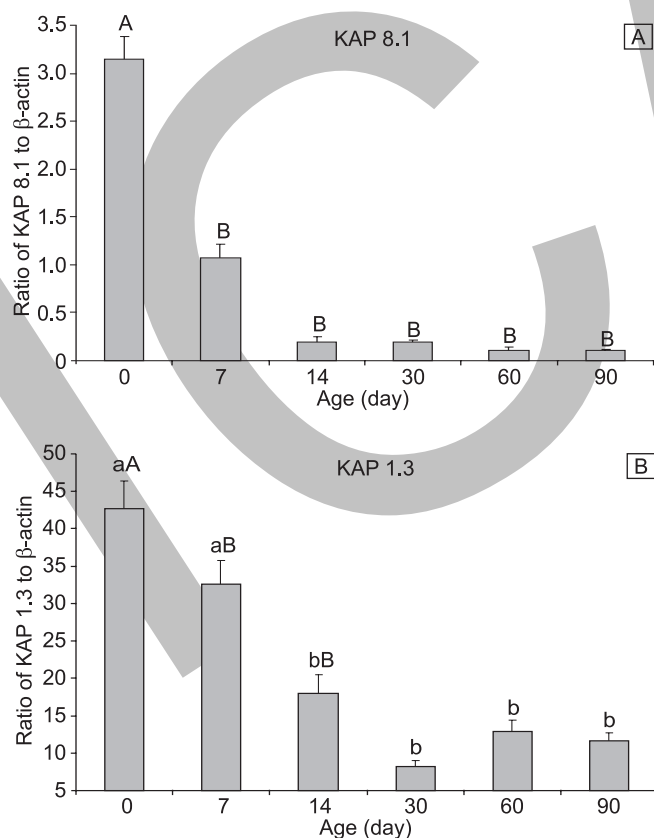


Fig 1. The developmental changes of *KAP8.1* and *KAP1.3* in the skin of Chinese Merino sheep. Values with different lower-case superscripts are significantly different at $P < 0.05$, with capital superscripts different at $P < 0.01$.



Fig. 2. PCR-SSCP analysis of the ovine *KAP8.1* gene.

Table 2. The effect of different genotypes in *KAP8.1* and *KAP1.3* genes on wool traits in Chinese Merino sheep

Traits	KAP8.1			P value	KAP1.3			P value
	AB (14)	BB (163)	BC (13)		AA (116)	AB (52)	BB (29)	
Fiber diameter (μm)	24.41 $\pm$ 0.74 ^a	23.58 $\pm$ 0.28 ^{aA}	21.48 $\pm$ 0.46 ^{bA}	0.021	22.72 $\pm$ 0.31 ^a	23.41 $\pm$ 0.40 ^{aA}	25.19 $\pm$ 0.69 ^{bA}	0.001
Nature staple length (cm)	8.54 $\pm$ 0.28	7.95 $\pm$ 0.14	8.52 $\pm$ 0.53	0.175	8.01 $\pm$ 0.19 ^a	7.95 $\pm$ 0.22 ^a	8.87 $\pm$ 0.36 ^b	0.066
Stretch staple length (cm)	12.29 $\pm$ 0.12	11.61 $\pm$ 0.27	11.89 $\pm$ 0.19	0.409	11.67 $\pm$ 0.25 ^a	11.74 $\pm$ 0.25 ^{ab}	12.70 $\pm$ 0.40 ^b	0.105

Values with different lower-case superscripts are significantly different at $P < 0.05$, with capital superscripts different at $P < 0.01$.

frequencies of alleles A and B were 0.721 and 0.279, respectively, in the population, agree with Hardy-Weinberg equilibrium ($P > 0.05$). The result showed that the AA genotype and A allele were dominant in detected population, which goes against previous research in Chinese Merino sheep (Xu *et al.* 2008). The reason might be that the same breed but belonging to different farms or different lines were detected in two studies. In addition, the *KAP1.3* PCR-SSCP polymorphisms have been studied in several studies (Itenge-Mweza *et al.* 2007, Liu *et al.* 2007, Zhang *et al.* 2007a, b).

Association of genotypes with wool traits: In this study, the relationships between the polymorphisms of *KAP8.1* and *KAP1.3* genes and 3 wool traits (fibre diameter, nature staple length, stretch staple length) were done in 197 Chinese Merino ewes. The *KAP8.1* SSCP polymorphism showed significant association with fiber diameter ($P = 0.021$) (Table 2). Multiple comparisons results indicated that individuals

al. 2007, Xu *et al.* 2008, Zhang *et al.* 2007b), these results were a good authentication for *KAP1.3* gene having a positive effect on wool traits, the *KAP1.3* AA genotype could be regarded as molecular marker for superior fibre diameter, and *KAP1.3* BB genotype could be regarded as molecular marker for superior staple length.

Effect of combined genotypes on wool traits: Such studies on complex traits should lead to the identification of a great number of genetic factors responsible for the heritable variation of these traits. In addition, taking into account the gene-gene interactions, it is necessary to analyze the effect of combined genotypes of a series of target genes. Gene combination was first proposed by Yadav *et al.* (1990) and has already proven its usefulness in several crop breeding programs to improve the disease and insect pest resistance of crops (Jiang *et al.* 2004, Barloy *et al.* 2007). Jiang *et al.* (2008) has reported that gene combination was feasible in animal breeding and the interaction between genes might affect the final results. However, in domestic animals, gene combination has not been practiced yet, because of the low fertility and long generation intervals.

Table 3. Comparison of fiber diameter for combined genotypes

KAP8.1-KAP1.3	Fiber diameter (μm)
AB-AA (4)	22.82 $\pm$ 0.26 ^{bcd}
AB-AB (4)	23.98 $\pm$ 0.97 ^{ab}
AB-BB (4)	27.17 $\pm$ 1.50 ^a
BB-AA (98)	22.85 $\pm$ 0.37 ^{bd}
BB-AB (41)	22.97 $\pm$ 0.39 ^b
BB-BB (24)	24.84 $\pm$ 0.75 ^{ac}
BC-AA (6)	21.80 $\pm$ 0.60 ^d
BC-AB (3)	22.93 $\pm$ 0.33 ^{bcd}

Values with different lower-case superscripts are significantly different at $P < 0.05$.

In this study, the relationships between different combined genotypes of *KAP8.1*-*KAP1.3* genes and 3 wool traits were also done in 197 Chinese Merino ewes. Genotype BC-BB was removed from statistical analysis because of the low frequency. Significant statistical association of different combined genotypes of *KAP8.1*-*KAP1.3* with fibre diameter was found in the analyzed population ($P = 0.014$), and the results were presented in Table 3. The combined genotype BC-AA presented a favorable association with fibre diameter,

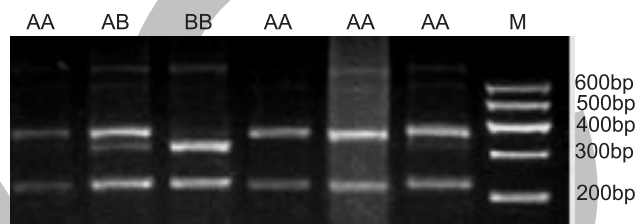


Fig 3. Agarose gel electrophoresis of *Bsr I* PCR-RFLP of ovine *KAP1.3* gene.

with *KAP8.1* BC genotype were significantly smaller fiber diameter than those of individuals with genotype AB ($P < 0.01$) and BB ($P < 0.05$), which implied that *KAP8.1* gene had a positive effect on fibre diameter and it is reported firstly that *KAP8.1* BC genotype could be regarded as molecular marker for superior fibre diameter.

The relationship between *KAP1.3* genotypes and 3 wool traits are also presented in Table 2. The results showed that *KAP1.3* *Bsr I* polymorphism was significantly associated with fiber diameter ($P < 0.01$), and the individuals carrying *KAP1.3* AA genotype had significantly lower fibre diameter, lower nature staple length and stretch staple length than those carrying BB genotype. The individuals carrying AB genotype had also lower fibre diameter and nature staple length than those carrying *KAP1.3* BB genotype. The previous association studies have found genetic association of *KAP1.3* gene with fibre diameter and cashmere weight trait (Liu *et*

the individuals with genotype BC-AA were significantly smaller than those of individuals with genotype BB-BB, BB-AB, AB-BB and AB-AB in fibre diameter ($P < 0.05$).

Combination effect of multiple genes is not the simple sum of the genotype effects and may be higher or lower than the effect of a single genotype (Chang *et al.* 2009, Han *et al.* 2009, Liu *et al.* 2010). The samples of Chinese Merino has an excellent combined genotype BC-AA only 6 individuals, accounting for 3.28%, indicating that these major genes for possession of sheep wool traits have a greater potential of choice.

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