

The *FABP4* gene polymorphism is associated with meat tenderness in three Chinese native sheep breeds

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ABSTRACT: The aim of this study was to assess the association of single nucleotide polymorphisms (SNP) of sheep fatty acid binding protein 4 (*FABP4*) gene with *longissimus thoracis* muscle (LT) meat quality traits in sheep. The *FABP4* cDNA was cloned by RT-PCR method, and the sequence analysis showed that the open reading frame of sheep *FABP4* is 399 bp and codes 132 amino acids. A mutation (A/G) detected in intron 1 of *FABP4* gene was studied in 286 lambs of three Chinese native sheep breeds by PCR-SSCP procedure. Significant statistical association results revealed that AA genotype conferred higher tenderness ($P < 0.05$), muscle marbling score ($P < 0.05$) and intramuscular fat content (IMF; $P < 0.05$). Thus we suggested that the genotype AA could be regarded as a molecular marker for LT meat tenderness and IMF content in sheep.

Keywords: sheep; *FABP4* gene; polymorphism; intramuscular fat

For decades, consumers have considered tenderness as the most important meat quality attribute (Ouali et al., 2006; Erkens et al., 2009). To increase meat tenderness by traditional selective breeding is a difficult task, since meat quality is controlled by polygenes (Bendixen, 2005). However, the marker-assisted selection based on investigating the associations of candidate gene polymorphism and traits has advantages for selection of meat quality traits (Wimmers et al., 2005). The intramuscular fat content (IMF) or marbling improves meat tenderness by reducing bulk density and decreasing the strength of the connective tissue (Savell and Cross, 1988), and has beneficial effects on the taste quality and juiciness (Stupka et al., 2008). Therefore the genes involved in fatty acid metabolism are usually considered as potential candidate genes for meat tenderness.

Fatty acid binding proteins play an important role in the regulation of lipid and glucose homeostasis by interaction with peroxisome proliferator acti-

vated receptors (*PPARs*) (Adida and Spener, 2006). Specifically, the fatty acid binding protein 4 (*FABP4*) fatty acid complex activates the peroxisome proliferator-activated receptor- γ (*PPAR- γ*) isoform, which in turn regulates transcription of *FABP4* (Damcott et al., 2004). *FABP4* was proposed as a potential candidate gene for obesity as it was located within a quantitative trait locus (QTL) region for serum leptin levels in mice (Ogino et al., 2003). In addition, *FABP4* protein content could be a marker of *longissimus thoracis* muscle (LT) IMF content accretion in pigs (Gerbens et al., 2001; Damon et al., 2006; Mercadé et al., 2006). Obviously, *FABP4* has become a strong candidate gene for fat metabolism. However, the effect of *FABP4* on fat deposition and meat quality traits in sheep has not been reported yet.

Here, we cloned the cDNA of *FABP4* gene and applied the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) assay to assess the association of the single nucleotide polymorphisms (SNP) of the *FABP4* gene with the LT

IMF, meat tenderness and other important carcass quality traits in three Chinese native sheep breeds.

MATERIAL AND METHODS

Sheep populations

Three Chinese native sheep breeds, Small-tailed Han sheep (STH, 96 lambs), Tan sheep (TS, 94 lambs) and Inner Mongolia sheep (IMS, 96 lambs), were obtained from several farms. There was no pedigree relationship among lambs within the breed, and no genetic communication history in three sheep breeds. Sixty-days-old lambs were collected, vaccinated, branded and maintained on the laboratory farm of the College of Animal Science and Technology, Northwest A and F University with the uniform feeding and management conditions.

Meat quality traits and sampling

At 90 days of age, all lambs were slaughtered after overnight fasting. Body weight, hot carcass weight, loin-eye area, net meat percentage and fat thickness were recorded at slaughter by the methods described by Dickerson et al. (1972). Subcutaneous white adipose tissue samples were frozen in liquid nitrogen and stored at -80°C for the *FABP4* gene isolation. Blood samples (10 ml) were collected in heparin at exsanguination and stored at -80°C . Fresh

samples of LT muscle between the 4th and 6th rib were removed from left carcasses. LT meat colour and shear force value were determined according to Fiems et al. (2000). The pH values of LT meat were measured with a pH meter after slaughter within 45 min. Drip loss rate was determined using the method described by Honikel (1998). Meat marbling score was measured according to Xing and Deng (1999). IMF was extracted from about 10 to 20 g of LT according to the method described by Bligh and Dyer (1959) using chloroform and methanol.

FABP4 gene isolation and sequencing

A putative sheep *FABP4* cDNA sequence was identified using the *Bos taurus FABP4* complete coding sequence (accession No. NM_174314) to search *Ovis aries* expressed sequence tags (EST) database. Based on the putative sequences, the primer pair FAp1 (Table 1) was designed to amplify the *FABP4* cDNA coding sequence.

Total RNA was extracted from subcutaneous white adipose tissue with Trizol reagent according to the manufacturer's instructions (Invitrogen Inc., Carlsbad, CA). Then, 2 μg of total RNA, 10 pmol of oligo(dT) 18 primer (TaKaRa, Dalian, China) and 200 U of MMLV reverse transcriptase (Invitrogen Inc., Carlsbad, CA) were used to synthesize the cDNA first strand. The amplification of *FABP4* gene was carried out according to the conditions listed in Table 1 with FAp1 primers. The obtained

Table 1. The primer sequences and amplification conditions

Primers	Sequences ^a	Regions	Products	PCR cycling conditions ^b
FAp1	F: 5'-ATGTGTGATGCATTTGTAGG-3' R: 5'-ACAGCACATCCA ACAGAA -3'	cDNA	~480 bp	1(95-5 min)-35(94-30 s-53-30 s-72-45 s)- 1(72-10 min)
FAp1n	F: 5'-ATGTGTGATGCATTTGTAGG-3' R: 5'-TCCTGGCCCAATTTGAAG-3'	intron 1	~2500 bp	1(95-5 min)-35(94-30 s-53-30 s-72-3 min)- 1(72-10 min)
FApli	F: 5'-TGGTTATTCAAGAAGCAA-3' R: 5'-ATCTAAGAAAGAGCAGGC-3'	partial intron 1	407 bp	1(95-5 min)-35(94-30 s-49-30 s-72-45 s)- 1(72-10 min)
M13	F: 5'-TGTAACGACGGCCAGT-3' R: 5'-CAGGAAACAGCTATGACC-3'			

^aF = forward; R = reverse

^bcycles (temperature-time)-cycles (denaturation temperature-time-annealing temperature-time-elongation temperature-time)-cycles (elongation temperature-time)

PCR reaction mix in a total volume of 25 μl contained: 25 ng genomic DNA (2 μl cDNA), 0.4 μM primer F, 0.4 μM primer R, 0.4 mM dNTPs, 10 $\times$ PCR buffer and 1.5 U Taq DNA polymerase (TaKaRa, Dalian, China)

PCR products were cloned into pMD18-T plasmid vectors (TaKaRa, Dalian, China) and positive clones were sequenced with M13 forward and reverse primers (Table 1). Plasmid transformation, isolation and other standard molecular biology techniques were performed as described earlier (Sambrook et al., 1989).

Genotyping

The intron 1 fragment of *FABP4* was amplified with FAp1n primer pair (Table 1), which was designed according to the sequence of sheep cDNA and *Bos taurus* genomic DNA (accession No. NC_007312). The protocols for cloning and sequencing of FAp1n amplicon were the same as for the cDNA isolation.

DNA was extracted by the phenol/chloroform purification method from blood samples (Sambrook et al., 1989). To amplify partial intron 1 of *FABP4* gene, the FAp1i primers (Table 1) were designed based on the sequence of intron 1. The PCR products were then analyzed by electrophoresis for SSCP according to the standard protocol (Sambrook et al., 1989). Silver staining was carried out as described by Bassam et al. (1991). Several PCR products showing different band patterns on SSCP gel were subcloned to T-vector and sequenced from both directions.

Statistical analyses

Genotype distribution for Hardy-Weinberg equilibrium was tested by the method described by Falconer and Mackay (1996). The statistical software SAS 8.0 (GLM procedure, SAS 8.0 software, SAS Institute, Cary, NC) was used to analyze the relationship between genotypes and meat quality traits by means of the least-squares method. Allele frequencies were tabulated and compared by χ^2 analysis using PROC FREQ.

The model used to analyze the data was:

$$Y_{ijk} = \mu + S_i + B_j + G_k + bx + E_{ijk}$$

where:

Y_{ijk} = observation value of the trait

μ = population mean

S_i = fixed effect of sex

B_j = effect of j -th breed

G_k = effect of k -th genotype

b = regression coefficient of slaughter day

E_{ijk} = random error

The effects of sire, farm, feeding and age were not built into the linear model, because all lambs were raised on the same farm from 60 to 90 days of age.

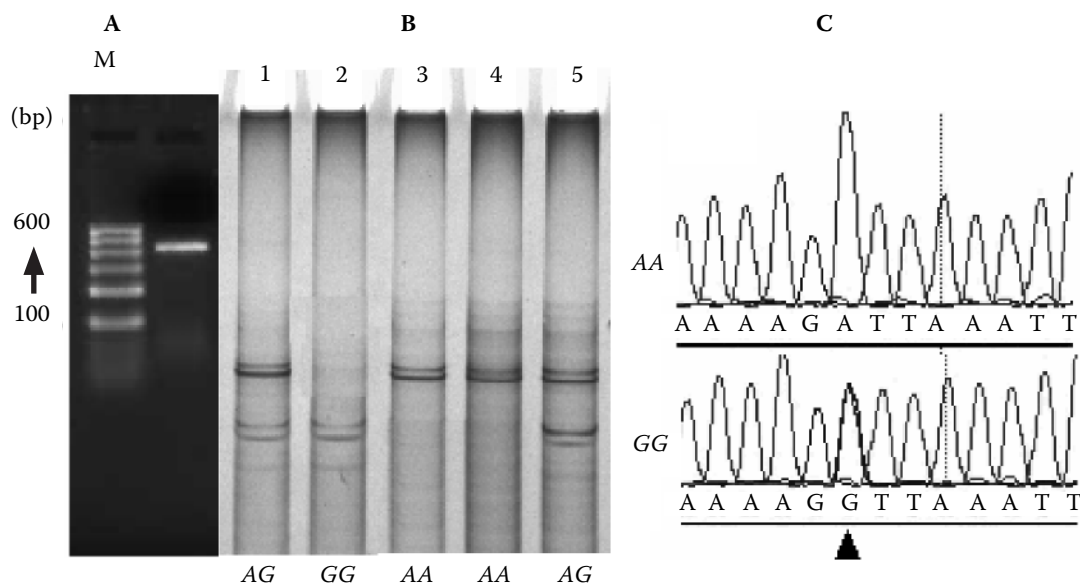


Figure 1. The Polymorphisms analysis of FAp1i fragment. Panel A is the electrophoresis examination of amplification product 407 bp of FAp1i primers. Panel B is the PCR-SSCP band pattern of FAp1i amplicons; four bands were separated on the gel, and three haplotypes were detected based on the bands mobility and the sequenced results; from lane 1 to 5, the genotypes are AG, GG, AA, AA and AG, respectively. Panel C is the demonstrations of sequenced results; the arrow indicated the mutation sites

Table 2. *FABP4* gene Genetic diversity of *FAP1i* loci in the three sheep populations

Index	STH	TS	IMS
Individuals	96	94	96
Individuals of <i>AA</i> genotype (frequency)	50 (0.52)	38 (0.40)	48 (0.50)
Individuals of <i>AG</i> genotype (frequency)	30 (0.31)	32 (0.34)	20 (0.21)
Individuals of <i>GG</i> genotype (frequency)	16 (0.17)	24 (0.26)	28 (0.29)
Frequency of allele <i>A</i> ¹	0.68	0.57	0.60
Frequency of allele <i>G</i> ¹	0.32	0.43	0.40
<i>P</i> value (locus equilibrium χ^2 test)	< 0.01 (disequilibrium) < 0.01 (disequilibrium) < 0.01 (disequilibrium)		

¹allele frequency was not significantly different between breeds ($P = 0.988$, by χ^2 tested)

RESULTS

that the RT-PCR product in this study is authentic sheep *FABP4* cDNA.

Sequence analysis of cDNA of sheep *FABP4*

Sequencing demonstrated that the RT-PCR amplicon of *FAP1* primer is 467 bp, containing an open reading frame of 399 bp and coding 132 amino acids. The sequence was submitted to Genbank (accession No. EU301804). The putative amino acid sequence of *Ovis aries* *FABP4* protein showed 86%, 86%, 93%, 84%, 72% and 74% identity, with the *FABP4* amino acid sequences of the *Mus musculus*, *Homo sapiens*, *Bos taurus*, *Sus scrofa*, *Gallus gallus*, *Anas platyrhynchos*, respectively, confirming

Polymorphism of sheep *FABP4*

In the *FAP1i* amplicons, a SNP in intron 1 was found which was located at nt 209 (accession No. HM061165: 282 A > G) counted from the first nucleotide of intron 1, but no restriction site for endonuclease was created or abolished. Three genotypes were detected in all individuals of the three Chinese native sheep breeds (Figure 1). *AA* genotype frequencies were 0.52, 0.40 and 0.50 and *A* allele fre-

Table 3. The associations of *FABP4* genotypes with LM meat quality and hot carcass traits in the sheep populations

Meat quality/hot carcass traits	<i>FABP4</i> genotypes (LSM $\pm$ SE) ¹			<i>P</i> value
	<i>AA</i> (<i>n</i> = 136)	<i>AG</i> (<i>n</i> = 82)	<i>GG</i> (<i>n</i> = 68)	
90 days-body weight (kg)	11.60 $\pm$ 1.31	10.98 $\pm$ 2.15	10.67 $\pm$ 0.83	0.205
Hot carcass weight (kg)	5.88 $\pm$ 0.57	5.56 $\pm$ 1.12	5.46 $\pm$ 1.87	0.131
Loin-eye area (cm ²)	10.70 $\pm$ 2.53	10.36 $\pm$ 2.70	10.01 $\pm$ 2.33	0.422
Net meat percentage (%)	57.14 $\pm$ 3.86	58.07 $\pm$ 2.58	59.29 $\pm$ 3.66	0.197
Intramuscular fat (IMF, %)	4.71 $\pm$ 0.99 ^B	4.45 $\pm$ 0.35 ^B	3.77 $\pm$ 0.25 ^A	0.018
Shear force (kg)	2.24 $\pm$ 0.45 ^A	2.78 $\pm$ 0.44 ^B	2.88 $\pm$ 0.31 ^B	0.031
Drip loss rate (%)	8.86 $\pm$ 1.33	9.48 $\pm$ 1.76	9.39 $\pm$ 2.11	0.062
Muscle marbling score	2.24 $\pm$ 0.42 ^A	2.10 $\pm$ 0.26 ^A	1.77 $\pm$ 0.32 ^B	0.015
Meat color score (<i>L</i> [*])	40.76 $\pm$ 2.33	41.32 $\pm$ 3.65	40.00 $\pm$ 2.98	0.433
pH value	6.5 $\pm$ 0.2	6.3 $\pm$ 0.1	6.5 $\pm$ 0.4	0.085
Fat thickness in 7 th rib ² (mm)	1.2 $\pm$ 0.5	1.3 $\pm$ 0.3	1.3 $\pm$ 0.4	0.100

¹least square means (LSM) estimated for each polymorphism was indicated with its standard error (SE)

²subcutaneous fat thickness was measured at the 7th rib interface perpendicular to the outside surface of the fat organism at the point three-fourths the length of the *longissimus muscle* from its chine bone end

A-B within row, mean with no common superscript differ significantly ($P < 0.05$).

quencies were 0.68, 0.57 and 0.60 in STH, TS and IMS, respectively (Table 2).

Effects of genotypes on meat quality traits

As seen in Table 3, the *FABP4* A allele had a significant effect on IMF ($P < 0.05$), shear force ($P < 0.05$) and marbling score ($P < 0.05$). Compared with the GG genotype, the AA genotype conferred significantly higher IMF and marbling score but lower shear force. Significant differences were also observed between AG and GG genotypes in IMF and marbling score. However, the *FABP4* A allele had no significant effect on 90-day body weight, hot carcass weight, loin-eye area, net meat percentage, drip loss rate, meat colour score, pH value and fat thickness on the 7th rib ($P > 0.05$).

DISCUSSION

This study reported the isolation of 467-bp cDNA of the sheep *FABP4* gene, and a SNP in intron 1 (A > G) was detected in three Chinese sheep breeds. This SNP was significantly associated with LT IMF, marbling score and tenderness in sheep.

There are many papers concerned with *FABP4* as a candidate gene for fat traits of meat quality in pigs, and *FABP4* shows high nucleotide variability (Nechtelberger et al., 2001; Mercadé et al., 2006; Ojeda et al., 2006). A mutation of the sheep *FABP4* gene, which is located in intron 1 at nt 209 was associated with meat quality traits in our study. The A allele had a positive effect on IMF, marbling score and tenderness of LT, which is consistent with the results documenting that higher IMF content was related with higher tenderness (Fiems et al., 2000) and higher marbling score was related with lower shear force (Huff-Lonergan et al., 2002). However, no correlation was found between tenderness and subcutaneous fat content (Riley et al., 1983). Statistical results also indicated that the genotypes had no significant association with hot carcass traits including hot carcass weight, loin-eye area, and net meat percentage (Table 3). It indicates that the genetic marker *FABP4* cannot be recommended for selection for growth and carcass traits in sheep breeding. The same result was obtained in the study of the *FABP4* polymorphism effect on porcine meat production traits (Nechtelberger et al., 2001).

Most SNPs in introns or silent mutations do not have a direct impact on production traits. To the best of our knowledge, the SNP of *FABP4* intron 1 was first detected in the sheep population in the present study, and this SNP was associated with meat quality traits. These results could be due to the following reasons: (1) other mutations occurred within 3' or 5' flanking regulatory regions and these mutations have not been detected yet; (2) the SNP indirectly affected meat quality traits by being in linkage disequilibrium with another polymorphism that directly influenced the quantitative traits. This hypothesis is supported by the following facts: the porcine *FABP4* is closely associated to the *FAT1* locus on chromosome 4, and the *FAT1* has an important effect on meat marbling and growth (Mercadé et al., 2006). Furthermore, integrated analyses of both genetic map and radiation hybrid (RH) map indicated that the bovine *FABP4* gene falls into the QTL interval for marbling on chromosome 14 (Michal et al., 2006). Interestingly, the same functional QTL has been detected on sheep chromosome 9, which is homologous to bovine chromosome 14 (Barillet et al., 2005). Hence, future studies should focus on mapping the sheep *FABP4* gene and define the genetic basis of *FABP4* effects on sheep meat quality traits.

In conclusion, we cloned the full coding region of the sheep *FABP4* gene and studied the relationships between *FABP4* polymorphism and 11 meat production traits in 286 lambs. The A allele of *FABP4* had a positive effect on sheep meat tenderness. Hence we suggest that the AA genotype could be regarded as a molecular marker for meat tenderness and IMF in sheep.

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