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Bovine *Angptl6* gene effects on growth traits in three Chinese native cattle breeds

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ABSTRACT

As a circulating orphan peptide secreted by liver, *Angptl6* gene might have great effects on growth and development of animals. The objective of this study was to explore sequence variants (SVs) of *Angptl6* and to analyze their associations with growth traits in 737 individuals belonging to 3 Chinese native cattle. Herein, 3 variants were revealed by DNA pools sequencing and PCR-RFLP (or ACRS-PCR-RFLP) methods. These SVs were located at nt 2359, nt 2403 and nt 3258 (viz. SV1, SV2, SV3) respectively. All the analyzed populations were at Hardy-Weinberg equilibrium by χ^2-test. The linkage disequilibrium (LD) and haplotype analyses of 3 SVs revealed 8 different haplotypes. Thereinto, 2 dominant haplotypes (TCG and CCG) occurred with a frequency of 63.17% and 18.67% respectively. Association analysis showed the SV1 was significantly associated with BLI-6, and the SV2 and SV3 were significantly associated with CGI-24 and BHI-6 of individuals of Nanyang breed respectively. Further, combined SVs analysis revealed the ADG-18 of individuals with diplotype TC-CC-GG was higher than other combined genotypes in SV1-SV2-SV3 locus. Moreover, CG-18 with TC-GG and ADG-18 with TT-GT were preponderant in SV1-SV3 locus. Thus, 3 SVs showed great comprehensive effects on growth traits of Nanyang cattle, which indicated that they could be contributed to genetic and breeding programmes in cattle. Although there was no sufficient evidence these SVs greatly effect on the growth traits of cattle, the significant effects of these variants with these parameters could not be ignored.

Key words: *Angptl6* gene, Cattle, Growth traits, Polymorphism

As a very important symbol of measuring the level of modern agricultural development, animal husbandry plays a crucial role in the production and development of agriculture. As we know, growth traits mainly affect the husbandry not only in animal quantity but also in animal quality, which are controlled by polygene with pleiotropic effects. As it needs a long time for breeders to improve animal growth traits by traditional breeding method, the modern molecular breeding such as marker-assisted section (MAS) is greatly emphasized and carried out to study effect of a few genes on growth and development of animals, which will be candidate gene for MAS.

It is well known that angiopoietin-like protein 6 is encoded by the gene *Angptl6* (also named as angiopoietin growth factor, *AGF*) as a member of the Angptl family (including Angptl1–7), as well as it is a circulating orphan peptide secreted by liver (Hato *et al.* 2008). Angptls are structurally similar to angiopoietins, which are characterized by a coiled-coil domain in the N-terminus and a fibrinogen-like domain in the C-terminus (Gale and Yancopoulos 1999, Oike *et al.* 2004). Interestingly, angiopoietins have a signal sequence in the N-terminus for protein secretion, and secreted angiopoietin function to maintain the vascular system and hematopoietic stem cells through the endothelial-cell tyrosine kinase receptors (Tie2) (Hato *et al.* 2008). However, Angptls do not bind to either Tie2 or the related protein Tie1, suggesting that these orphan ligands function differently from angiopoietins (Kadomatsu *et al.* 2011). *Angptl6* gene is abundantly expressed in liver, and expressed at relatively low levels in other tissues (Hato *et al.* 2008). In functions, the early research concentrated in angiogenesis, proliferation of skin cells and wound healing (Oike *et al.* 2003, Oike *et al.* 2004, Oike *et al.* 2004). In recent years, a lot of studies are carried out for insulin sensitivity and related metabolic diseases in *Angptl6* gene, and have shown that it is probable as a potential target for developing attractive pharmacological interventions counteracting related metabolic diseases (Oike *et al.* 2005). *Angptl6* null mice showed marked obesity because of decreased energy expenditure and insulin

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resistance, but *Angptl6* transgenic mice exhibit a lean phenotype with enhanced energy expenditure (Ebert *et al.* 2009). Furthermore, *Angptl6* levels have been positively correlated with fasting serum glucose levels (Ebert *et al.* 2009).

Angptl6 gene not only has an important role in angiogenesis and wound healing, but also in counteracting obesity resulted from high lipid diet and improving insulin sensitivity during growth and development of animals. Nevertheless, little is known of the physiology of *Angptl6*. The mechanisms by which it stimulates angiogenesis and wound healing and the nature of the tissues targeted by the protein have yet to be established. In mice, *Angptl6* gene is located on chromosome 9, which is expressed predominantly in the liver, but also in haematopoietic cells (Oike *et al.* 2003). In humans, the *ANGPTL6* gene is located on chromosome 19p13.2, which is expressed restrictively in liver (Zandbergen *et al.* 2006). In bovine, almost nothing is known about its expression and function of *Angptl6* gene. For this reason, we aimed to establish whether genetic variability of *Angptl6* could be associated with growth traits of cattle. Accordingly, we used DNA sequencing, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and artificial created restriction site PCR-RFLP (ACRS-PCR-RFLP) methods to investigate sequence variants (SVs) within *Angptl6* gene (including 52 UTR, all exons and introns, 32 UTR region) in a total of 737 individuals belonging to three Chinese cattle, and analyzed their associations with growth traits of Nanyang cattle in this study. Further, we report associations of combined genotypes with growth traits of Nanyang cattle, which possibly contributed to molecular genetics and breeding programme in local Chinese cattle breed.

MATERIALS AND METHODS

DNA samples: A total of 737 genomic DNA samples were obtained from three domestic cattle breeds in China, namely Nanyang (n=211), Jiaxian (n=287) and Caoyuan (n=239). They were from Nanyang cattle breeding base in Henan province, Jiaxian cattle breeding center in Henan province and Caoyuan cattle breeding field in Jilin province, China respectively. After weaning at 6 months, all cows were fed on an *ad lib.* concentrate and straw up to 24 months.

DNA samples were extracted from blood samples with standard procedures (Sambrook and Russell 2002). The data of birth weight (Birth-W), body weight (Body-W), body length (BL), body height (BH), chest girth (CG), hucklebone width (HW), average daily gain (ADG), body hindquarters index (BHI), body length index (BLI) and chest girth index (CGI) from different growth periods (at the age of 6, 12, 18 and 24 months) in Nanyang cattle breed were recorded accurately for statistical analysis.

Primer sequences: Based on bovine *Angptl6* gene sequence (GenBank: NM_003104015), six pairs of primer

Table 1. Primer sequences for scanning sequence variations within bovine *Angptl6* gene

Loci	Primer sequences (5'-3')	Sizes (bp)	Tm (?)	Notes (GenBank: NW_003104015)
P1	F: -CCTTCCCGTTCATTCTCTGCCC- R:-GGGAGGGTGATAGTTTCGGAGT-	634	60.0	(nt 1- 634) 5?UTR region; exon 1; partial intron 1
P2	F: -CAGCAGCAGGTAACTCCG- R: -ACTGAAGCCTCCCAAAGC-	746	58.0	(nt 601- 1346) partial exon 1; partial intron 1
P3	F: -TATTGGGAGTGGGTTGCC- R: -CCACGCCCTTGACTTACC-	726	60.5	(nt 1197- 1922) partial intron 1; exon 2; partial intron 2
P4 (Ang6-ScaI)	F: -ACCCTGCTGTTCCTCCTAA- R: -ATCTTGCCGCCTCTGAAT-	731	60.0	(nt 1879- 2609) partial exon 2; intron 2; exon 3; partial intron 3
P5	F: -GCTCCACATTTGCTCTACTGA- R: -TTTCCTCCATCCTTTCTGC-	1069	59.0	(nt 2257- 3325) partial intron 2; exon 3; intron 3; exon 4; partial intron 4
P6	F:-AATGAGGAAATGAAAGAGGGTG- R: -GGACCTGAGGGACAGATGGA-	663	55.5	(nt 3049-3711) partial intron 4; exon 5; 3?UTR region
Ang6-VspI	F:-GGACCAGTGAGAATCAAGACTATTA- R:-ATCTTGCCGCCTCTGAAT-	232	57.0	(nt 2378- 2609) partial intron 2; exon 3; partial intron 3
Ang6-Csp6I	F:-ATTCTCTAATGAGTCAATAAGT- R:-CCTTTACCAGAAAAAACAACCTGT-	128	54.0	(nt 3155- 3282) partial intron 4

Notes: VspI-F: ATTA-, A is the mismatch; Csp6I-R: GT-, G is the mismatch.

(P1-P6) were designed to scan SVs of *Angptl6* gene by constructing genomic DNA pools that used as template for PCR amplification and PCR products were sequenced (Norton *et al.* 2004). The results revealed three SVs within the *Angptl6* gene were found in three local Chinese cattle breeds. Then three pairs of primer (Ang6-*Sca*I, Ang6-*Vsp*I and Ang6-*Csp6*I) were designed to genotype these SVs of analyzed populations according to bovine *Angptl6* gene sequence (GenBank: NM_003104015). All information of eight pairs of primer was given in Table 1.

PCR amplification: The three pairs of primer were used to amplify partial DNA fragments of bovine *Angptl6* gene containing these SVs. The 25 µL volume included 50 ng genomic DNA, 10 pmol of each primer, 2×*Taq* PCR MasterMix (including $MgCl_2$, dNTPs and Buffer and *Taq* DNA polymerase. The PCR protocol was 95°C for 5 min followed by 32–34 cycles of 94°C for 40s, annealing at Tm (°C) for 30–40s, 72°C for 20–50s and a final extension at 72°C for 10 min.

Genotype analyzed by PCR-RFLP (or ACRS-PCR-RFLP) and DNA Sequencing: Using the above pairs of primer protocol, PCR amplification was carried out to detect all cattle individuals in this study. Then the volumes of 6–8 µL PCR products were digested by 5 U restriction endonucleases for whole night. The digested products were detected by electrophoresis in 2.0%–3.0% agarose gel and taking pictures following staining with ethidium bromide by UV transilluminator and documented using gel documentation system. Further, the PCR products of different electrophoresis patterns were sent to Sequencing Company for sequencing completely in order to validate authenticity.

Statistical analysis: Genotypic and allelic frequencies were directly calculated, as well as Hardy-Weinberg equilibriums. Population genetic diversity indexes, namely gene homozygosity (Ho), effective allele numbers (Ne) and polymorphism information content (PIC), were also calculated by Nei methods (Nei and Li 1979). The formulas were as follows:

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

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

(*Pi* is the frequency of the *i* allele, "*n*" is the number of alleles)

The software SPSS (Version 17.0) was used to analyze the associations between different genotypes and growth traits in female Nanyang cattle. The linear model was used to assess genetic effects: $Y_{ijk} = \mu + M_i + G_j + e_{ijk}$, where Y_{ijk} was observations on the traits, μ was the mean of the animal populations, M_i was the fixed effects of observations for j month, G_j was the fixed effects of SNPs marker genotypes, e_{ijk} was the random errors (Gilbert *et al.* 1993).

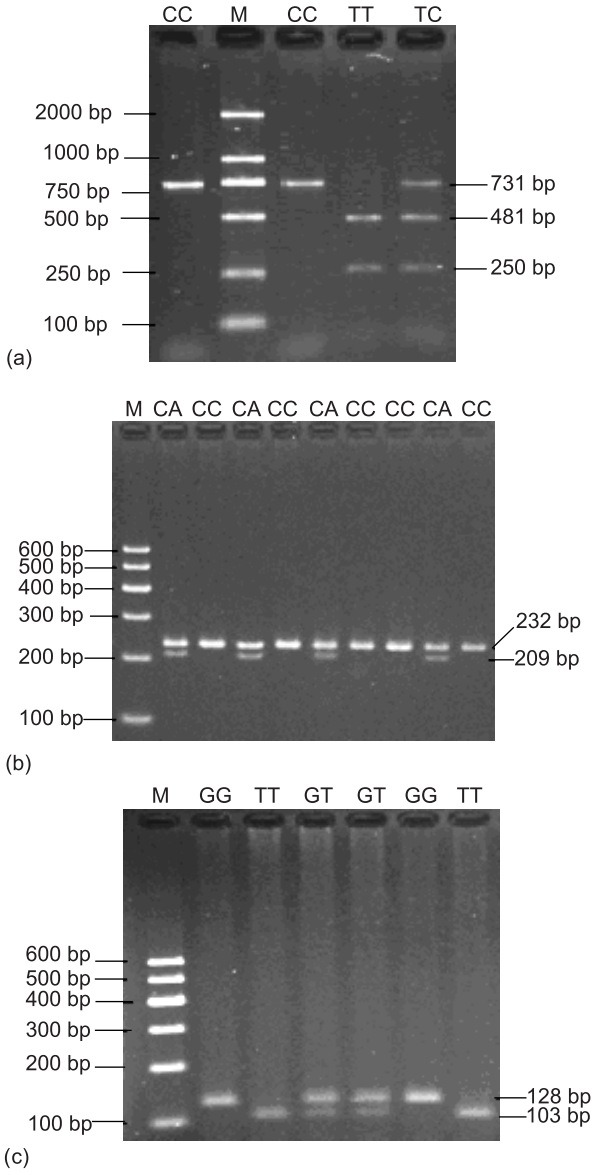

Fig. 1. The electrophoresis patterns of RFLP analysis at three loci.

Note: Fig. 1a, PCR products digestion with *Sca*I demonstrate three genotypes (CC: 731bp; TT: 481bp, 250bp; TC: 731bp, 481bp, 250bp); M: D2000 DNA ladder (2000bp, 1000bp, 750bp, 500bp, 250bp, 100bp). Fig. 1b, PCR products digestion with *Vsp*I demonstrate two genotypes (CC: 232bp; CA: 232bp, 209bp, 23bp); M: marker É DNA ladder (600bp, 500bp, 400bp, 300bp, 200bp, 100bp). The 23 bp fragment was invisible on the gel. Fig. 1c, PCR products digestion with *Csp6*I demonstrate three genotypes (GG: 128bp; TT: 103bp, 25bp; GT: 128bp, 103bp, 25bp). M: marker É DNA ladder (600bp, 500bp, 400bp, 300bp, 200bp, 100bp). The 25 bp fragment was invisible on the gel.

RESULTS AND DISCUSSION

Genotyping by PCR-RFLP (or ACRS-PCR-RFLP): The results showed that three sequence variation (SVs) (NW_003104015: g.2359T>C, NW_003104015: g.2403C>A

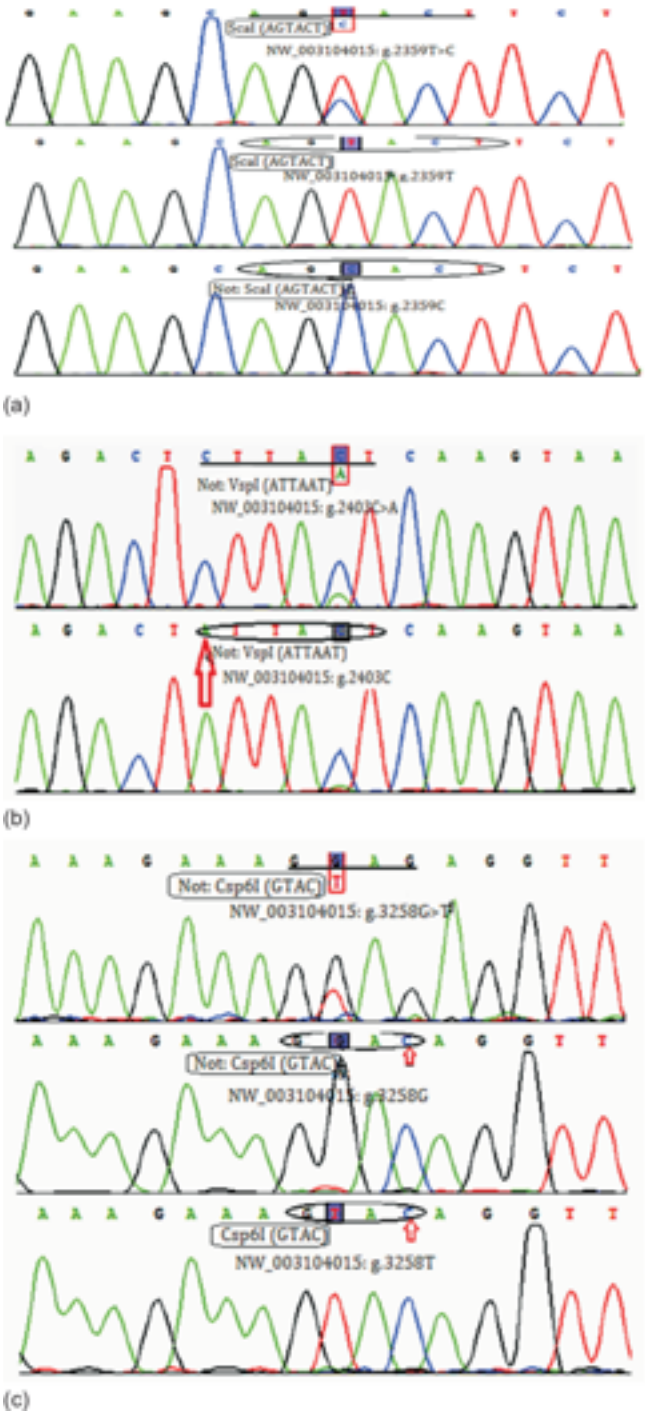

Fig. 2. The sequence chromatograms of the fragment at three SVs loci.

Note: Fig. 2a, the sequence chromatograms of different genotypes (TC, TT and CC) at SV1 locus; Fig. 2b, the sequence chromatograms of different genotypes (CA and CC) at SV2 locus; Fig. 2c, the sequence chromatograms of different genotypes (GT, GG and TT) at SV3 locus. The red arrows mean mismatch base pair for creating restriction sites.

and NW_003104015: g.3258G>T, namely SV1, SV2, SV3, respectively) had been found, and the sequences containing those SVs had been deposited in GenBank database (Acc. No.: JF261096 and JF261097). According to the sequence of bovine *Angptl6* gene (GenBank: NW_003104015), SV1 and SV2 were in intron 2, SV3 was in intron 4. The "T" mutation of intron 2 was defined as T allele, while called the "C" mutation for C allele. Similarly, another "C" mutation of intron 2 was also named as C allele, "A" mutation was named as A allele, and "G" mutation of intron 4 was G allele, "T" mutation was T allele. Interestingly, the T allele of intron 2 has a AGTACT (*Sca*I) restriction site between nt 479 and nt 484 of 731 bp PCR amplification products by primer Ang6-*Sca*I (P4), while the allele C has no AGTACT (*Sca*I) site at SV1. However, SV2 and SV3 have no appropriate natural restriction site, but they could be identified by creating a *Vsp*I or *Csp6*I restriction site (ACRS-PCR-RFLP method). As SV2 locus, the A allele has an ATTAAT (*Vsp*I) restriction between nt 22 and nt 27 of 232 bp PCR amplification products by primer Ang6-*Vsp*I, while the C allele has no *Vsp*I site. Similarly, the T allele of SV3 locus has a GTAC (*Csp6*I) restriction site between nt 103 and nt 106 of 128 bp PCR amplification products by primer Ang6-*Csp6*I, while the G allele has no *Csp6*I site. Therefore, these three novel mutations were identified for the first time by PCR-RFLP (or ACRS-PCR-RFLP) method. The electrophoresis patterns of RFLP analysis and sequence chromatograms of the fragment at three loci were listed in Fig. 1 and Fig. 2, respectively.

Genetic diversity and haplotype analysis: However, genotypes (TT, TC and CC) and (GG, GT and TT) are found at SV1 and SV3 loci in analyzed populations respectively. At the SV2 locus, only two genotypes (CC and CA) were observed. An independent chi-square test suggested that all the analyzed populations are at Hardy-Weinberg equilibrium (P>0.05). In order to evaluate genetic diversity of analyzed populations, Ho, Ne and PIC were calculated at the three loci. The PIC of all populations in SV1 locus were between 0.2702 and 0.2970 (0.25<PIC<0.5), which imply SV1 was a moderate genetic diversity in all analyzed populations. Contrary, SV2 showed a low genetic diversity (PIC<0.25). At SV3 locus, it appeared a moderate genetic diversity in Nanyang and Jiaxian cattle breeds, but a low genetic diversity in Caoyuan cattle breed. In addition, the linkage disequilibrium and haplotypes analysis were analyzed among the three loci in the three cattle breeds by online software (http://analysis.bio-x.cn/myAnalysis.php). The results showed that they were little linked (r^2<0.33) in all the analyzed populations. The results of haplotype analysis of three SVs showed eight different haplotypes. And two dominant haplotypes (TCG and CCG) occurred with a frequency of 63.17% and 18.67% in the study population, respectively.

Associations with growth traits of individuals of Nanyang breed: We analyzed the associations of these SVs with growth traits in Nanyang breed. As could been seen from Table 2

Table 2. Associations of single SV of *Angptl6* gene with growth traits in Nanyang breed.

Loci	Indexes	Genotypes (Mean ± S.D.)	Age (months)			
			6	12	18	24
SV1	BLI	TT (n=111)	99.40^{a}±1.98	102.50±4.37	106.10±3.66	110.20±4.95
		TC (n=66)	100.10^{ab}±3.06	101.70±3.92	106.60±7.67	109.20±4.97
		CC (n=12)	102.70^{b}±4.48	104.60±6.02	110.60±5.05	110.50±5.62
		P values	0.041*	0.409	0.295	0.661
SV2	CGI	CC (n=170)	121.60±3.79	124.00±5.18	128.40±7.02	134.70b±6.69
		CA (n=41)	122.30±7.83	118.30±27.36	128.40±4.79	130.50a±6.80
		P values	0.610	0.110	0.989	0.022*
SV3	BHI	GG (n=134)	121.80^{a}±3.58	120.70±5.29	120.60±4.82	121.90±4.73
		GT (n=62)	121.90^{ab}±4.91	121.70±5.00	120.50±3.58	122.80±4.82
		TT (n=8)	129.50^{c}±9.30	125.80±6.18	119.90±1.69	127.20±11.18
		P values	0.048*	0.318	0.970	0.274

Notes: Means of index with different superscript letters (e.g. a and b) show significantly difference (*P<0.05). BLI: body length index = body length / body height × 100%; CGI: chest girth index = chest girth / body height × 100%; BHI: body hindquarters index= chest girth / body length × 100%.

and Table 3, the C allele of SV1 was significant association with body length index at 6 months old (BLI-6), and body length index of individuals of genotype CC (mutant type) were higher than individuals of genotypes TT and TC. Genotype CC was significantly associated with chest girth index of 24 months old (CGI-24) at SV2 locus. Genotype CA, and TT (mutant type) of SV3 locus was significantly related to body hindquarters index of 6 months old (BHI-6) (Table 2). Further, associations of combined genotypes among two or three SVs with growth traits were also analyzed in Nanyang breed. The results showed that the average daily gain of 18 months old (ADG-18) of the individuals with genotype TC-CC-GG were higher than the individuals of other combined genotypes in SV1-SV2-SV3 combined site. Moreover, chest girth (CG-18) with TC-GG and average daily gain (ADG-18) with genotype TT-GT of 18 months old of the analyzed individuals were preponderant in SV1-SV3 site (Table 3).

Table 3. Associations of combined SVs of *Angptl6* gene with growth traits in Nanyang breed.

Combined sites	Combined genotypes	Traits	
		CG-18 (cm) (Mean±S.D.)	ADG-18 (kg) (Mean±S.D.)
SV1-SV3	TT-GG (n=36)	152.20a±8.50	0.268a±0.209
	TC-GT (n=25)	156.40ab±8.01	0.488b±0.173
	TT-GT (n=27)	157.10ab±7.50	0.519b±0.168
	TC-GG (n=17)	161.10b±6.82	0.479b±0.273
	P values	0.032*	0.015*
SV1-SV2-SV3		ADG-18 (kg) (Mean±S.D.)	
	TT-CC-GG (n=16)	0.276a±0.204	–
	TT-CA-GT (n=10)	0.398ab±0.090	–
	TC-CA-GT (n=15)	0.403ab±0.072	–
	TT-CC-GT (n=25)	0.479b±0.273	–
	TC-CC-GT (n=8)	0.538b±0.199	–
	TC-CC-GG (n=14)	0.575b±0.169	–
	P values	0.017*	–

Notes: Means of traits with different small or capital superscript letters (e.g. a and b) show significantly difference (*P<0.05). CG-18: chest girth of 18 month old; ADG-18: average daily gain of 18 month old. The combined genotypes (n<6) were neglected in this

Angptl6 gene is both a crucial modulator of angiogenesis and a regulator of energy metabolism. It could produce an important effect on growth and development of animals. In previous literatures, genetic variability of human *ANGPTL6* gene was explored and a total of 17 SNPs were revealed, which were all located non-coding region, related to metabolic syndrome-related phenotypes in the French MONICA (Legry *et al.* 2009). And our studies also showed three SNPs of bovine *Angptl6* gene were situated in intron region. These conclusions suggest the proteins encoding by the *Angptl6* gene are conservative, which imply *Angptl6* gene could be an important functional gene.

In conclusion, *Angptl6* gene had an important role in growth and development of animals. Nevertheless, only gene and mRNA sequences of *Angptl6* gene can be searched, and other studies still have not been reported about *Angptl6* gene in bovine. Herein, we firstly reported sequence variants, haplotypes and combined genotypes within *Angptl6* gene and analyzed their effects on growth traits in three Chinese native breeds. The results demonstrated that the growth traits of individuals of mutant genotypes were higher than individuals of other genotypes at both SV1 and SV3 loci. Further, combined effects of these SVs were remarkable rather than single SV on growth traits of Nanyang cattle breed. These are coincided with the conclusion that growth traits are complex quantitative traits that not only are affected by single SNPs, but also by SNP-SNP interaction of candidate genes (Orozco *et al.* 2009). Therefore, three SVs of the *Angptl6* gene could be used as candidate genetic molecular markers

for breeding and improving Chinese cattle breeds. Specifically, this study provided some helpful information for molecular breeding of Nanyang cattle.

Although there was no sufficient evidence that variants of bovine *Angptl6* had significant influence on the growth traits of cattle in this study, the potential effects of these SVs on these parameters cannot be excluded either. Further association studies are needed in order to receive the definite conclusions.

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