ONLINE RESOURCES

Haplotype combination of the bovine *PCSK1* gene sequence variants and association with growth traits in Jiaxian cattle

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Introduction

Prohormone convertase subtilisin/kexin type 1 gene (*PCSK1*) plays a role in body mass control. Recent association studies have shown that three common nonsynonymous SNPs are linked to increase risk of obesity and therefore it has been the focus of this study. Hence, in this study, polymorphisms of the bovine *PCSK1* gene were detected in 374 individuals from Chinese Jiaxian cattle by DNA pooling, forced PCR-RFLP, PCR-SSCP and DNA sequencing methods. The results showed that only seven SNPs were identified which resulted in two missense mutations and four silent mutations. In the tested individuals, six common haplotypes were identified, in which ACACAAT with frequency of 34.6% were more prevalent. Additionally, association analysis between mutations of bovine *PCSK1* gene and growth traits in Jiaxian cattle for up to two years was performed. The results indicated that the polymorphisms were significantly associated with bovine body length and chest circumference at 24 months old, and no convincing associations were identified at 6, 12 and 18 months old.

Obesity is a modern epidemic strongly influenced by both genetic and environmental factors. The discovery of genes causing monogenic forms of obesity such as the *PCSK1* gene has greatly improved our understanding of the pathophysiology of obesity (Ramachandrappa and Farooqi 2011). Recently, the associations of the nonsynonymous rs6232 (N221D) and rs6235 (S690T) SNPs in the *PCSK1* gene with the risk of obesity were investigated and the results showed that highly significant differences were identified in a meta-analysis, comprising more than 13,000 individuals of European ancestry (Benzinou *et al.* 2008). In addition, Jackson *et al.* (1997, 2003) and Farooqi *et al.* (2007) have also characterized the genomic sequence of the human *PCSK1* gene mapped to chromosome 5q15-q21 and determined that mutations have been found to cause monogenic obesity. Further, a mouse model of heritable N222D mutation in the catalytic domain of *PCSK1* was generated by Lloyd *et al.* (2006). Consistent with the human phenotype, $PCSK1^{N222D/N222D}$ mice develop maturity-onset obesity and have lower lean mass than wild-type littermates. Interestingly, in contrast to human *PCSK1* deficiency, $PCSK1^{-/-}$ mice exhibit smaller birth weight and stunted growth; they also suffer from gastrointestinal dysfunctions as manifested by a moist texture of their stools (Zhu *et al.* 2002). The distinct phenotypes of $PCSK1^{N222D/N222D}$ and $PCSK1^{-/-}$ suggest that PCSK1 can operate with different efficiencies on different prohormones. Reduced PCSK1 activity (as in $PCSK1^{N222D/N222D}$) leads to apparently normal processing of proGHRH, but deficient processing of proinsulin and POMC, whereas ablation of PCSK1 activity (as in $PCSK1^{-/-}$) leads to deficient processing of all three prohormones (Lloyd *et al.* 2006).

As mentioned earlier, several reports have suggested that the *PCSK1* was an obesity susceptibility gene. However, the related information in cattle is not available. Hence, in this paper, we studied in 374 Chinese individuals from Jiaxian cattle to scan the SNPs in the exons and exon/intron junctions of bovine *PCSK1* gene using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), PCR-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing analysis, and an attempt was made to establish an association between detected polymorphisms and performance traits.

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Keywords. growth traits; SNP association; *PCSK1* gene.

Materials and methods

Cattle populations, genomic DNA isolation and data collections

In this study, 374 Jiaxian cattle were from the breeding farm of Jiaxian (Jiaxian, Henan, China). Blood samples were obtained from these cattle and genomic DNA was extracted from the blood samples using standard methods (Sambrook and Russell 2001). Additionally, the animals were weaned at an average age of six months and raised from weaning to slaughter on a corn–corn silage diets. Records of growth traits and body size (height, length, chest circumference, hucklebone width and average day gain) in Jiaxian breed were collected for statistical analysis. The study was conducted according to the ethical guidelines of the declaration of the College of Animal Science and Technology, Northwest A&F University.

Oligonucleotides and PCR condition

Based on the nucleotide sequence of the bovine *PCSK1* gene (GenBank accession number NC_007305), six pairs of PCR primers (table 1) were designed to amplify the exons and intron/exon boundaries using Primer ver. 5.0 software. Each amplification reaction was carried out in a 15 μL reaction mixture containing 50 ng genomic DNA, 10 pM of each primer, 1× buffer (including 1.5 mM $MgCl_2$), 0.2 mM dNTPs (dATP, dTTP, dCTP and dGTP) and 0.60 U *Taq* DNA polymerase (MBI Fermentas, Pittsburgh, USA). The cycling protocol was 5 min at 94°C, 32 cycles of denaturing at 94°C for 45 s, annealing at X°C for 1 min, extension at 72°C for 45 s, with a final extension at 72°C for 10 min (X values are presented in table 1).

DNA pooling, PCR-RFLP, PCR-SSCP and DNA sequencing

In an effort to discover sequence mutations by a cost-effective manner, variation discovery was implemented by sequencing pooled PCR products, which were amplified from DNA pools of different sizes ranging from 50 to 100 randomly chosen individuals (Bansal *et al.* 2002).

PCR-RFLP method was used to detect missense mutation g.44686T>G in *PCSK1* gene. Aliquots of 10 μL PCR products of bovine *PCSK1* gene were digested with 10 U *Hinf* I (MBI Fermentas,) for 5 h at 37°C following the manufacturer's instructions, and the digested products were detected by electrophoresis in 10% polyacrylamide gel electrophoresis gels (PAGE) stained with 0.1% silver nitrate and visualized with 2% NaOH solution (containing 0.1% formaldehyde). In addition, the PCR-SSCP method was applied to scan P_1, P_3, P_{10} and P_{11} loci, respectively. Aliquots of 4 μL PCR products were mixed with 6 μL denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene–cyanole and 0.025% bromophenol blue), heated for 10 min at 98°C and chilled in ice immediately. Denatured DNA was subjected to 10% PAGE analysis, then gels were stained with silver nitrate and visualized with NaOH solution. Finally, PCR products of different electrophoresis patterns were sequenced by the DNA sequencer from both directions and the results were analysed by DNAMAN software (ver. 5.2.2, Lynnon Biosoft, Quebec, Canada).

Data analyses

Linkage disequilibrium (LD) and inferred haplotype across SNPs were estimated by the expectation maximization (EM) algorithm, as determined by the Haploview program (Barrett *et al.* 2005). The pattern of pairwise LD between SNPs was measured by LD coefficient (D') and correlation coefficient (r^2) and visualization of LD measures was carried out using the program Haploview (http://www.broad.mit.edu/mpg/haploview). The relationships between the variations of the *PCSK1* gene, growth traits and body sizes in Jiaxian cattle were analysed by analysis of variance (ANOVA) (SPSS software GLM procedure) using the following model: $Y_{ij} = \mu + Age_i + Marker_j + e_{ij}$, where Y_{ij} is the observation of the trait, μ the least square mean, Age_i the effect of age, $Marker_j$ the effect of marker genotype and e_{ij} is the residual effect.

Results and discussion

Analysis of sequence variants in bovine PCSK1 gene

In this study, genomic DNAs were successfully amplified using primer pairs of the *PCSK1* gene. To better understand the detailed genetic variation, pooled DNA amplification fragments were sequenced and the results revealed seven novel mutations. On comparing with the previously reported sequences (Ref. NC_007305.3 and NM_174412.2),

Table 1. Primer pairs information of bovine *PCSK1* gene.

Locus	Primer sequences (5′–3′) (fwd primer seq/rev primer seq)	Size (bp)	T_m (°C)	Primer position Ref. no. NC_007305
P_1	ACTCATTTCCATTCCTCCTCG/CATTCATTCACAAACTGCCTCTT	341	63	192–532
P_3	TGAACAGACTAAGAAGAAAGGG/GCAAGCACAAAGAAAGCAGA	351	63	3215–3565
P_{10}	ACAGTGGGAGGACTTCAT/ACTTTGCTTCCAGGGCTA	356	60	24434–24534
P_{11}	AATTACAGAGGCACAGTTGC/ATTCCTGGAGACGCTTACC	380	63	33841–34074
P_{14}	TCAACTACTCGTTTTCATCGCAA/GTCCACCACCTTCTCCACGCCTC	338	64	41954–42115
Hinf I	TCAAAGCAATCACCAAAGAAG/ACAAAACACCCACTTCAGACA	306	62	44581–44886

g.81G>A was in the 5′-UTR, g.130G>C (exon 1), g.3448G>A (exon 2), g.33844C>A (exon 10), g.33985A>G (exon 10) and g.44686T>G (exon 14) were in the coding region, and g.24431DelC was in the intron, respectively. The SNP EX2_50G>A and EX14_262T>G were the missense mutations p.R77Q and p.S716A, while EX1_130G>C, EX10_4C>A and EX10_145A>G resulted in three synonymous mutations p.L15L, p.P400P and p.K447K, respectively. In addition, the sequences of SNPs were submitted in GenBank, and the nature and distribution of *PCSK1* variations are shown in table 2.

In the P_1 fragment, three unique SSCP banding patterns (BPs) were observed which were denoted as AA_1, BB_1 and AB_1, respectively (figure 1a). The sequencing analysis of the three SSCP BPs revealed two SNPs: 5′UTR_-5G>A and EX1_130G>C, and they formed two consistent haplotypes: A_1(AC) and B_1(GG). In the P_3 fragment, polymorphic information with three unique SSCP BPs were also identified which were designated as electrophoresis patterns AA_3, BB_3 and AB_3, respectively (figure 1b). Then, the polymorphic DNA amplification fragments were sequenced and only one novel mutation was revealed, EX2_50G>A. Further analysis of the mutation showed that individuals with BP-AA_3 represent as genotype AA, BP-BB_3 individuals as genotype GG, while BP-AB_3 individuals as genotype AG, respectively. Through SSCP analysis, exon 9 with intron/exon boundaries of bovine *PCSK1* gene, amplified with P_{10} primer pairs showed polymorphisms and were named as BP AA_{10}, BB_{10} and AB_{10}, respectively (figure 1c). The sequencing analysis revealed one SNP IVS8+2475delC, and two different alleles IVS8+2475del for A_{10} and IVS8+2475C for B_{10}. In the P_{11} fragment, four unique SSCP BPs were observed which were denominated as AA_{11}, BB_{11}, CC_{11} and AB_{11}, respectively (figure 1d). The sequencing analysis of the four SSCP BPs and two novel SNPs were revealed as EX10_4C>A and EX10_145A>G. The four two-SNP combined genotypes were characterized as genotype CCAA, CCGG, AAAA and CCAG; BP-AA_{11} individuals represent for genotype CCAA, BP-BB_{11} for CCGG, BP-CC_{11} for AAAA and BP-AB_{11} for CCAG, respectively. In the P_{14} fragment, the mutation showed a transversion T>G at position 44686, which resulted in a missense mutation S>A at position 716aa according to NM_174412.2. Further, *Hinf* I PCR-RFLP was first used to detect the SNP g.44686T>G in exon 14 of bovine *PCSK1* gene, i.e., the 306 bp PCR products digestion with *Hinf* I demonstrated one fragment (306 bp) for *PCSK1* AluI-TT, two fragments (203 and 103 bp) for *PCSK1* AluI-GG and three fragments (306, 203 and 103 bp) for *PCSK1* AluI-TG. Three unique RFLP BPs were marked as TT, GG and TG (figure 1e).

Linkage disequilibrium and haplotype analysis of the bovine PCSK1 gene

With Haploview programe, LD between polymorphism pairs and haplotype structure analysis of the *PCSK1* gene in Jiaxian cattles are shown in figure 2 and table 3. The standardized measure of LD denoted as r^2 was first calculated for all pairs of SNPs in Jiaxian cattle population, and numbers in each cell stood for pairwise r^2 values between the corresponding SNPs. Three distinct LD blocks within *PCSK1* gene were obtained (r^2 values were 100%). The first LD block included g.81G>A and g.130G>C which were separated by 49 bp between 5′ UTR and exon 1, while the second LD block included g.3448G>A and g.44686T>G which were separated by 41238 bp between exon 2 and exon 14, respectively. The third LD block included g.24431DelC and g.44686T>G which were encompassed by intron 8 and exon 14. Accordingly, haplotypes were established in all the three blocks, and six common haplotypes were identified based on the seven SNPs, with the most common haplotype ACACAAT occurring at a frequency of 34.6% in Jiaxian cattle (table 3). Interestingly, the degree of LD was significantly different in different regions of the gene, which maybe due to selection. Selection during domestication and improvement was found to influence the LD level of a gene (Saunders *et al.* 2005), and selection aiming at alleles of structural gene could increase the LD level significantly in the target gene region

Table 2. SNP information of the bovine *PCSK1* gene.

Locus	SNP	Mutation site	Allele	AA coded	$DPS_{(nt)}$	NCBI_ss#
P_1	g.81G>A	5′UTR_5G>A			0	503727005
	g.130G>C	EX1_130G>C	ct[g]/ct[c]	p.L15L	49	503727008
P_3	g.3448G>A	EX2_50G>A	c[g]g/c[a]g	p.R77Q	3318	503727010
P_{10}	g.24431DelC	IVS8+2475delC			21083	503727422
P_{11}	g.33844C>A	EX10_4C>A	cc[c]/cc[a]	p.P400P	9413	503727020
	g.33985A>G	EX10_145A>G	aa[a]/aa[g]	p.K447K	141	503727022
Hinf I	g.44686T>G	EX14_262T>G	[t]ct/[g]ct	p.S716A	701	503727025

$DPS_{(nt)}$, distance from previous sequence variants (nt). The framed ([ ]) bases show mutations and Refs NC_007305.3 and NM_174412.2.

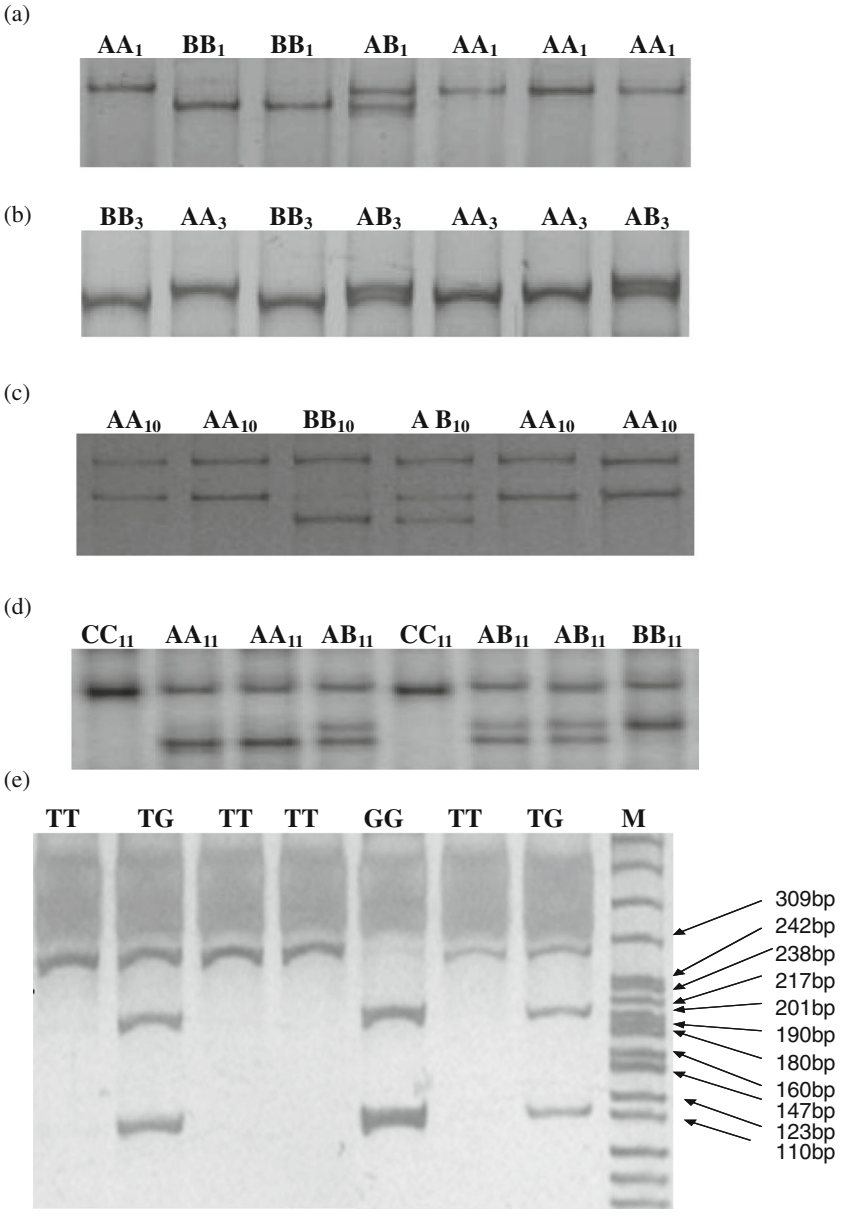

Figure 1. The electrophoresis patterns of PCR-SSCP for (a) P_1, (b) P_3, (c) P_{10} and (d) P_{11} loci, and the electrophoresis patterns of PCR-RFLP for (e) *Hinf* I locus, respectively. M, 622 bp, pBR322DNA/*Msp* I markers.

(Clark *et al.* 2004). In Chinese indigenous breeds, Jiaxian cattle have been undergoing selection during the long-term studies of cattle breeding, which might increase the LD.

Combined genotypes identified in bovine PCSK1 gene and their association with growth traits

PCSK1 is particularly abundant in the arcuate and paraventricular nuclei of the hypothalamus (Dong *et al.* 1997), which control appetite and satiety (Wynne *et al.* 2005). Mouse models of heritable PCSK1 deficiency have supported a role of PCSK1 protein in body mass control (Zhu *et al.* 2002; Lloyd *et al.* 2006; Mbikay *et al.* 2007). Cases of monogenic obesity associated with PCSK1 deficiency have been reported in humans (Jackson *et al.* 1997, 2003; Farooqi *et al.* 2007). Recent association studies have also shown that minor alleles of three common nonsynonymous SNPs at the *PCSK1* locus: rs6232, rs6234 and rs6235, are linked to increased risk of obesity (Benzinou *et al.* 2008; Kilpelainen *et al.* 2009; Chang *et al.* 2010; Qi *et al.* 2010). Therefore, further investigation of structure, function and gene polymorphisms of *PCSK1* gene would be essential and meaningful. In this study, seven mutations have been identified in the bovine *PCSK1* gene which resulted in two missense mutations and

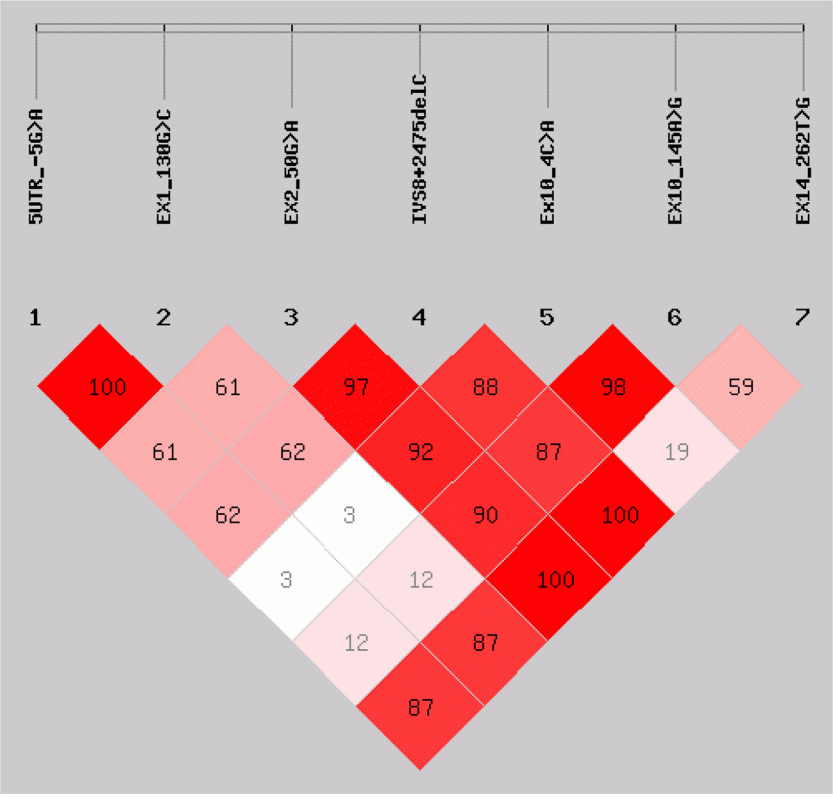

Figure 2. LD plot in Jiaxian cattle. Colour scheme is according to Haploview r^2 scheme. Numbers in each cell denote pairwise r^2 values (%) between the corresponding SNPs.

four silent mutations. In addition, through sequence comparison, it was found that a total of seven primary combined genotypes were identified in the tested population, and genotype G2 was more prevalent (table 4). Thereby, we proposed the hypothesis that the polymorphic sites identified within the bovine *PCSK1* gene might be associated with growth traits. Concomitantly, the relationships between genotypes and body sizes of the Chinese Jiaxian cattle in 6, 12, 18 and 24 months old were analysed using SPSS software (ver. 17.0, SPSS, Chicago, USA) (table 4). Regrettably, none of the significant differences between combined genotypes and growth traits of younger individuals showed convincing associations in the 374 Jiaxian population-based studies ($P > 0.05$). At 24 months, the animals with G4, G6 and G7 genotypes had significantly greater body length than those with G1 genotypes, while the animals with G3, G4 and G7 genotypes showed greater chest circumference than those with G1 genotypes, respectively. Hence, we suggest that *PCSK1* gene could be regarded as a molecular marker for superior body length and chest circumference in the older individuals.

In conclusion, seven polymorphisms in the Jiaxian bovine *PCSK1* gene were observed in this study, which resulted in two missense mutations and four silent mutations. We have also defined the LD and haplotypes in the Jiaxian cattle and preponderant haplotype ACACAAT was identified, which would provide a background for more extensive

Table 3. Haplotypes of *PCSK1* gene and their frequencies in Jiaxian cattle breed.

	Position of sequence variants							Frequency
Haplotype	g.G81A	g.G130C	g.G3448A	g.24431DelC	g.C33844A	g.A33985G	g.T44686G	(%)
1	A	C	A	–	C	G	T	9.3
2	A	C	A	C	A	A	T	34.6
3	A	C	A	C	A	A	G	3.6
4	A	C	A	C	C	A	T	16.5
5	G	G	G	C	A	A	G	19.2
6	G	G	G	C	C	A	G	8.1

Frequency <0.03 has been dropped.

Table 4. Association of genotypes with growth traits in Jiaxian cattle.

Age	G (N)	BW (kg)	BH (cm)	BL (cm)	ChC (cm)	HW (cm)	ADG (g)
6 M	G1 (23)	163.3 ± 4.9	106.4 ± 1.1	105.8 ± 1.2	129.6 ± 1.4	18.8 ± 0.3	742.0 ± 27.0
	G2 (70)	157.4 ± 2.2	104.9 ± 0.5	104.7 ± 0.7	127.1 ± 0.9	18.2 ± 0.2	715.0 ± 12.6
	G3 (15)	168.2 ± 5.1	108.3 ± 1.3	107.7 ± 1.7	130.9 ± 1.9	18.5 ± 0.4	769.6 ± 27.3
	G4 (16)	157.1 ± 5.0	105.5 ± 1.3	104.8 ± 1.4	128.3 ± 2.0	17.9 ± 0.3	705.6 ± 27.1
	G5 (12)	150.7 ± 6.5	103.7 ± 1.9	103.3 ± 2.1	126.1 ± 2.2	17.8 ± 0.3	685.0 ± 34.3
	G6 (30)	160.7 ± 3.6	105.9 ± 1.1	105.3 ± 1.2	128.7 ± 1.2	18.4 ± 0.2	731.7 ± 18.7
	G7 (35)	160.3 ± 2.7	107.0 ± 0.5	106.6 ± 0.8	130.2 ± 1.0	18.4 ± 0.2	724.4 ± 14.5
	G1 (23)	233.5 ± 5.8	115.3 ± 0.7	119.7 ± 1.8	143.2 ± 1.6	20.4 ± 0.3	390.1 ± 28.2
	G2 (70)	223.5 ± 2.3	113.5 ± 0.4	116.1 ± 0.9	139.7 ± 1.0	20.5 ± 0.2	366.9 ± 12.4
	G3 (15)	220.9 ± 9.5	114.1 ± 1.3	118.3 ± 2.4	143.1 ± 2.5	21.0 ± 0.4	292.6 ± 33.7
12 M	G4 (16)	226.8 ± 5.9	114.4 ± 1.2	117.6 ± 2.1	140.9 ± 1.9	20.3 ± 0.4	386.8 ± 28.0
	G5 (12)	217.6 ± 2.8	113.8 ± 0.8	115.3 ± 1.9	138.8 ± 1.4	20.6 ± 0.2	371.3 ± 39.2
	G6 (30)	224.7 ± 3.8	114.8 ± 0.8	117.4 ± 1.4	141.5 ± 1.1	20.7 ± 0.3	355.0 ± 20.9
	G7 (35)	222.6 ± 3.3	114.1 ± 0.5	118.7 ± 1.1	143.1 ± 1.1	20.7 ± 0.2	346.2 ± 18.2
	G1 (23)	294.5 ± 3.4	120.5 ± 0.9	127.3 ± 1.4	153.3 ± 1.4	22.9 ± 0.3	338.9 ± 45.4
	G2 (70)	294.2 ± 3.9	121.0 ± 0.9	127.2 ± 0.8	154.6 ± 1.1	23.0 ± 0.2	392.9 ± 26.0
	G3 (15)	303.9 ± 6.7	122.1 ± 0.9	128.9 ± 1.6	157.4 ± 2.7	24.0 ± 0.4	461.1 ± 72.7
18 M	G4 (16)	300.1 ± 5.0	121.8 ± 0.8	132.1 ± 1.3	156.8 ± 1.5	22.8 ± 0.5	406.9 ± 34.5
	G5 (12)	298.1 ± 8.5	119.4 ± 0.8	127.0 ± 2.3	154.7 ± 2.9	22.6 ± 0.5	447.2 ± 45.7
	G6 (30)	292.9 ± 4.8	123.1 ± 1.9	128.7 ± 1.2	154.4 ± 1.4	22.9 ± 0.3	379.1 ± 34.4
	G7 (35)	301.2 ± 6.5	122.6 ± 1.7	130.0 ± 2.7	157.7 ± 1.6	23.3 ± 0.4	436.6 ± 39.6
	G1 (23)	348.8 ± 7.7	124.2 ± 0.8	132.6 ± 1.5^{A}	161.6 ± 2.3^{A}	24.5 ± 0.4	301.7 ± 37.5
	G2 (70)	364.5 ± 4.2	126.0 ± 0.5	135.8 ± 0.9^{AB}	166.2 ± 1.2^{AB}	24.7 ± 0.2	390.6 ± 35.2
	G3 (15)	360.5 ± 15.2	124.9 ± 1.1	134.9 ± 1.9^{AB}	169.7 ± 3.1^{B}	25.3 ± 0.6	314.4 ± 95.2
24 M	G4 (16)	370.6 ± 7.0	127.1 ± 0.5	139.4 ± 1.5^{BC}	171.3 ± 2.1^{B}	25.4 ± 0.6	392.0 ± 45.1
	G5 (12)	355.0 ± 8.3	125.3 ± 1.2	135.3 ± 2.2^{ABC}	164.4 ± 2.1^{AB}	25.5 ± 0.5	316.2 ± 56.8
	G6 (30)	366.0 ± 6.0	126.6 ± 0.7	136.7 ± 1.4^{BC}	166.2 ± 1.7^{AB}	25.0 ± 0.3	405.9 ± 47.3
	G7 (35)	366.9 ± 41.3	127.5 ± 0.8	139.9 ± 1.1^{C}	169.8 ± 1.6^{B}	25.6 ± 0.4	364.9 ± 44.4

M, months; G (N), genotype (observed number); BW, body weight; BH, body height; BL, body length; ChC, chest circumference; HW, hucklebone width; ADG, average day gain. ^{A,B}Means differ significantly at $P < 0.01$. G1 = AACCGACCCGATT, G2 = AACCGG CCAAAATT, G3 = AACCGGCCAAAAGT, G4 = AACCGGCCCCAATT, G5 = AGCGGTCACCGAGT, G6 = AGCGGGCCAAAAGA, G7 = GGGGGGCCAAAAGG. The number of genotypes <10 are not listed in table. ^{A,B,C} Significant differences at $P < 0.01$.

characterization of the bovine *PCSK1* gene. In addition, association analyses of combined genotypes of *PCSK1* gene among younger individuals indicated no convincing associations with any of the studied traits, at least in Chinese Jiaxian cattle. Genotype G7 were obviously associated with higher values than the others with regard to bovine body length and chest circumference in 24 months. In the future, samples of other ethnicities should be looked at, and more variants should also be considered.

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