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Haplotype combination of the caprine *PC1* gene sequence variants and association with growth traits in Chinese Haimen breed

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[Sun J., Xue J., Zhang C., Lan X., Lei C. and Chen H. 2012 Haplotype combination of the caprine *PC1* gene sequence variants and association with growth traits in Chinese Haimen breed. *J. Genet.* **91**, e54–e59. Online only: http://www.ias.ac.in/jgenet/OnlineResources/91/e54.pdf]

Introduction

Proprotein convertase 1 (PC1) is an endopeptidase involved in proteolytic processing of peptide hormone precursors in granules of the regulated secretory pathway of endocrine cells. Mutations in *PC1* gene are related to obesity-related traits which may influence the performance of animals, and therefore it has been the focus of this study. In this study, polymorphisms of the caprine *PC1* gene were detected in 407 individuals from three breeds by PCR-SSCP and DNA sequencing methods. The results showed that only 12 novel SNPs were identified, which included three in the 5′-UTR, eight in the introns and only one in the coding region, resulting in a synonymous mutation p.S(TCT)143S(TCC). In Haimen individuals, nine common haplotypes were identified, in which GGGCC and TGGCC with frequency of 18.8% and 17.7% were more prevalent haplotypes, while these SNPs fell into two linkage disequilibrium (LD) blocks with strong multi-allelic D' ($D' = 1$). Additionally, association analysis between mutations of caprine *PC1* gene and growth traits in adult Haimen breed was performed, and we observed no convincing associations with any of the studied traits in the tested population.

Proprotein convertase 1 (PC1, also known as PCSK1, PC1/3, PC3 or SPC3) (Steiner 1998) is an endopeptidase involved in proteolytic processing of peptide hormone precursors, and belongs to the serine proteases recognized as the proprotein convertases subtilisin kexin/type (PCSK) enzymes. In mouse, the *PC1* gene is located on chromosome 13, and is transcribed into two major mRNA isoforms of 2.8 and 4.4 kb differing in their untranslated regions (Seidah *et al.* 1991; Ftouhi *et al.* 1994). These transcripts are primarily found in endocrine cells of several mammalian tissues, including, but not limited to, the pituitary (Marcinkiewicz *et al.* 1993), the hypothalamus (Nilaweera *et al.* 2003), the pancreas (Marcinkiewicz *et al.* 1994), and the gut (Gagnon *et al.* 2009). Recently, the associations of the nonsynonymous rs6232 (N221D) and rs6235 (S690T) SNPs in the *PC1* 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 13000 individuals of European ancestry (Benzinou *et al.* 2008). In addition, Jackson *et al.* (2003) and Farooqi *et al.* (2007) have also characterized the genomic sequence of the human *PC1* 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 PC1 was generated by Lloyd *et al.* (2006). Consistent with the human phenotype, $PC1^{N222D/N222D}$ mice develop maturity-onset obesity and have lower lean mass than wildtype littermates. Interestingly, in contrast to human *PC1* deficiency, $PC1^{-/-}$ 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 $PC1^{N222D/N222D}$ and $PC1^{-/-}$ suggest that PC1 can operate with different efficiencies on different prohormones. Reduced PC1 activity (as in $PC1^{N222D/N222D}$) leads to apparently normal processing of proGHRH, but deficient processing of proinsulin and POMC, whereas ablation of PC1 activity (as in $PC1^{-/-}$) leads to deficient processing of all three prohormones (Lloyd *et al.* 2006).

As mentioned above, several reports have suggested that the *PC1* was an obesity susceptibility gene. However, the related information in goat is meagre. Hence, in this study,

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Keywords. goat; growth traits; haplotype combination; *PC1* gene; SNP association.

we studied 407 individuals from three goat breeds (Boer goat, Chinese Xuhuai white goat and Chinese Haimen goat) to scan the potential variations in the exons and exon/intron junctions of caprine *PC1* gene, using PCR-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing analysis, and attempted to establish an association between the haplotypic diversity and performance traits.

Materials and methods

Genomic DNA samples and data collections

Genomic DNA samples were obtained from 407 individuals (without genetic relationships) belonging to three genetic types: Boer goat (BE, $N = 85$), Chinese Xuhuai white goat (XH, $N = 111$), and Chinese Haimen goat (HM, $N = 211$) reared in the province of Jiangsu (China). Records of growth traits and body sizes (height, length, chest circumference and cannon circumference) in adult HM breed were collected for statistical analysis, and DNA samples were isolated from 1 mL whole blood.

Primer design, single-stranded conformation polymorphism (SSCP) and DNA sequencing

Based on the nucleotide sequence of the bovine *PC1* gene (GenBank accession number NC_007305), five pairs of polymerase chain reaction (PCR) primers were designed to amplify the exons with intron/exon boundaries and 5′-flanking regions of the caprine *PC1* gene using Primer v5.0 software (PREMIER Biosoft International, California, USA) (table 1). SSCP method was used to scan mutations within the amplified regions. Aliquots of 4 μL PCR products were mixed with 6 μL denaturing solution, 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 0.1% silver nitrate and visualized with 2% NaOH solution (containing 0.1% formaldehyde). After the polymorphism was detected, the PCR products of different electrophoresis patterns were sequenced by the DNA sequencer from both directions.

Statistical analysis

Gene frequencies were determined from observed genotype counts, while Hardy–Weinberg equilibrium (HWE) had been tested based on likelihood ratio for different locus-population combinations by POPGENE software (http://www.ualberta.ca/fyeh). LD and inferred haplotype across SNPs in Haimen breed 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 performed using Haploview (http://www.broad.mit.edu/mpg/haploview). Statistical analysis was performed on records of growth traits in Chinese Haimen goat breed. The relationships between variations of the *PC1* gene and growth traits were analysed by ANOVA using the following model: $Y_i = \mu + Marker_i + e_i$ where Y_i is the observation of the trait, μ is the least square mean, $Marker_i$ is the effect of ith marker genotype and e_i is the residual effect.

Results and discussion

Analysis of sequence variants in caprine PC1 gene

In the present study, genomic DNAs of all three goat breeds were successfully amplified using five primer pairs for the *PC1* gene. To better understand the detailed genetic informations, the DNA amplification fragments were sequenced and the results were analysed using online program (http://rulai.cshl.org/software/index1.htm), which predicted that the caprine *PC1* gene consists of five exons and four introns, and the nucleotide sequence was deposited in GenBank with accession number JF693490. Through SSCP analysis, the five loci of the caprine *PC1* gene were detected and all the loci showed polymorphisms. The number of bands and their positions in the gel clearly showed the occurrence of DNA sequence variations, while the amplification fragments of different SSCP variants were sequenced and 12 novel SNPs were identified. Compared with submitted sequence (GenBank accession number JF693490),

Table 1. Primer pairs information of caprine *PC1* gene.

Loci	Primer sequences (5′–3′)	Size (bp)	T_m(°C)	Primer position ref. JF693490
P_1	F: ACTCATTTCCATTCCTCCTCG R: CATTCATTCACAAACTGCCTCTT	341	62.5	5′UTR and CDS 1 g.1–341
P_2	F: TCTCGCTCCCCTCCCTCCCCACT R: CGCCCAGCCCAAGCAATAAA	415	64.5	Exon 2 with intron/exon Boundaries g.342–756
P_3	F: TGAACAGACTAAGAAGAAAGGG R: GCAAGCACAAAGAAAGCAGA	351	66.5	Exon 3 with intron/exon Boundaries g.757–1107
P_4	F: CAATCTCAGCAGATGATAACAGA R: ACTTCCCAATACATACCCACC	418	65.5	Exon 4 with intron/exon Boundaries g.1108–1525
P_5	F: ACAGTGGGAGGACTTCAT R: ACTTTGCTTCCAGGGCTA	356	64.0	Exon 5 with intron/exon Boundaries g.1526–1883

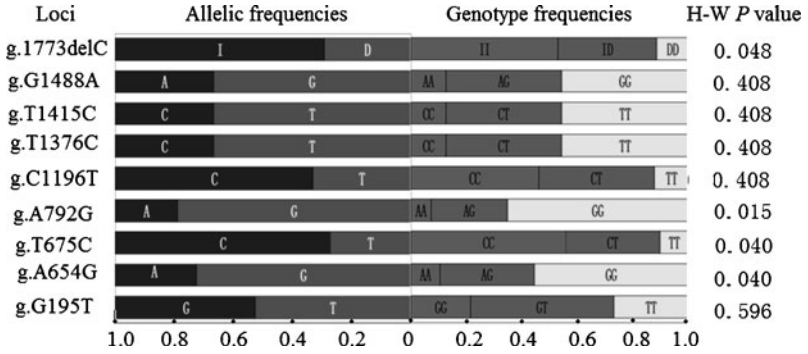

Figure 1. The allelic and genotype frequencies of the *PC1* gene in Chinese Haimen goat. Numbers at the right are the probability values for the test of the Hardy–Weinberg equilibrium.

in detail, g.A168G, g.G195T and g.G221A were in the 5′-UTR, g.A654G, g.A792G, g.A682G and g.T675C were in intron 2, g.C1196T was in exon 4, and g.T1376C, g.T1415C, g.G1488A and g.1773delC were in intron 4, respectively. In addition, the mutation g.C1196T showed a transversion C>T at position 1196, which resulted in a synonymous mutation p.S(TCT)143S(TCC). Surprisingly, g.A682G, g.G221A and g.A168G SNPs were only found in the BE breed. Concomitantly, based on SSCP and responsive sequence variations, the genotype distribution and allelic frequencies of *PC1* gene in HM breed were analysed (figure 1).

Linkage disequilibrium and haplotype analysis of the caprine PC1 gene in HM population

With Haploview program, LD between polymorphism pairs and haplotype structure analysis of the *PC1* gene in HM breed were performed (figure 2; table 2). The standardized measure of LD denoted as r^2 was first calculated for all pairs of SNPs in HM population, then two distinct LD blocks within *PC1* gene were yielded with strong multiallelic D' ($D' = 1$). The first LD block included IVS2+86A>G and IVS2+107T>C, which were separated by 20 bp in intron 2, while the second LD block included EX4_39C>T, IVS4+6T>C, IVS4+45T>C and IVS4+118G>A, which encompassed exon 4 and intron 4, respectively. Accordingly, haplotypes were established on the first and the second blocks, and nine dominant haplotypes were identified based on nine SNPs of caprine *PC1* gene in Haimen breed, with the most common haplotype GGGCC and TGGCC occurring at a frequency of 18.8% and 17.7%, respectively. Interestingly, the degree of LD was significantly different in different regions of caprine *PC1* gene, which may be resulted from selection. Selection during domestication and improvement could influence the LD level of a gene (Saunders *et al.* 2005), and selection aiming at alleles of structural gene could increase the LD level in the target gene region significantly (Clark *et al.* 2004). In China, indigenous Haimen and Xuhuai breeds have been undergoing selection during relatively long improvement, which might increase the LD.

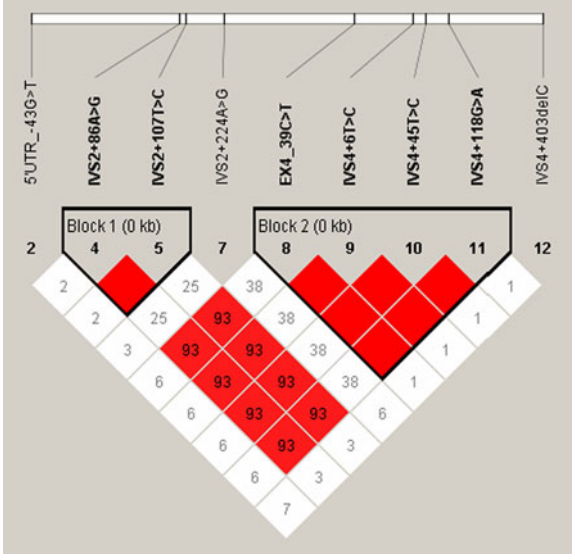

Figure 2. Linkage disequilibrium plot in Chinese Haimen goat. Colour scheme is according to Haploview r^2 scheme. Numbers in each cell stand for pairwise r^2 values (%) and empty cells mean pairwise r^2 equals to 1 between the corresponding SNPs.

Polymorphisms of caprine PC1 gene and their association with growth traits in HM population

Mutations in the 5′-UTR have been reported to influence mRNA stability (Stefanovic *et al.* 1999), the efficiency of translation of mRNA (Woodman *et al.* 1996). Meanwhile, Capon *et al.* (2004) and Nackley *et al.* (2006) have provided evidence that synonymous SNPs can affect protein expression (and thus function) by alteration or increase in the stability of the mRNA. Therefore, we hypothesized that the polymorphic site identified within the caprine *PC1* gene might be associated with growth traits. Concomitantly, the relationships between different single-locus genotypes and four growth traits of HM were analysed by the SPSS software v17.0 (SPSS, Illinois, USA) (table 3). The results

Table 2. Haplotypes of *PC1* gene and their frequencies in Chinese Haimen goat breed.

Haplotype	SV_2	$SV_{4(5)}$	SV_7	$SV_{8(9,10,11)}$	SV_{12}	Frequency
1	G	G	G	C	C	0.188
2	T	G	G	C	C	0.177
3	G	A	G	T	C	0.081
4	G	G	G	C	T	0.076
5	T	A	G	T	C	0.073
6	T	G	G	C	T	0.063
7	T	G	A	C	C	0.056
8	T	A	G	T	T	0.055
9	G	G	A	C	C	0.040

Frequency <0.03 has been ignored in analysis; SV, sequence variants; SV_4 and SV_5 are in complete linkage disequilibrium, thereby SV_4 and SV_5 of the genotyping results are same, as well as $SV_{8,\ 9,\ 10,\ 11}$.

Table 3. Association analysis of mutations in caprine *PC1* gene with growth traits in the Chinese Haimen goat breed.

SNP	Genotype	Sample size	Mean values ± standard error (cm)			
			Body height	Body length	Chest circumference	Cannon circumference
SV_2	TT	57	60.09 ± 1.12	72.79 ± 1.35	75.46 ± 1.75	9.53 ± 0.21
	TG	109	61.47 ± 0.73	77.44 ± 1.36	77.77 ± 1.32	9.56 ± 0.15
	GG	45	60.76 ± 0.98	75.16 ± 2.09	77.02 ± 1.98	9.51 ± 0.22
	P		0.533	0.095	0.581	0.981
$SV_{4(5)}$	AA	21	60.05 ± 1.67	74.29 ± 2.14	76.14 ± 3.12	9.76 ± 0.33
	AG	69	61.07 ± 0.88	74.96 ± 1.24	76.46 ± 1.36	9.72 ± 0.17
	GG	121	60.95 ± 0.53	76.37 ± 1.38	77.43 ± 1.33	9.40 ± 0.14
	P		0.852	0.685	0.856	0.304
SV_7	AA	15	60.13 ± 1.32	73.53 ± 3.19	75.73 ± 2.56	9.53 ± 0.40
	AG	58	60.62 ± 1.17	75.36 ± 1.91	75.03 ± 1.74	9.49 ± 0.21
	GG	138	61.17 ± 0.62	76.08 ± 1.09	77.94 ± 1.19	9.56 ± 0.13
	P		0.823	0.762	0.363	0.958
$SV_{8(9,10,11)}$	TT	25	60.76 ± 1.50	72.88 ± 1.79	76.96 ± 2.91	9.62 ± 0.28
	CT	84	61.43 ± 0.80	74.88 ± 1.14	76.94 ± 1.32	9.65 ± 0.16
	CC	102	60.58 ± 0.79	77.06 ± 1.57	77.02 ± 1.43	9.43 ± 0.16
	P		0.749	0.284	0.999	0.576
SV_{12}	II	112	60.08 ± 0.71	73.90 ± 1.01^{b}	75.46 ± 1.31	9.31 ± 0.14^{B}
	ID	76	61.51 ± 0.81	76.72 ± 1.80^{ab}	78.38 ± 1.50	9.53 ± 0.15^{B}
	DD	23	63.26 ± 2.04	81.09 ± 3.04^{a}	79.83 ± 2.68	10.70 ± 0.38^{A}
	P value		0.138	0.042	0.197	<0.001

Superscript letters indicate significant differences at $P < 0.05$ (lower case a, b) or at $P < 0.01$ (upper case A, B).

showed that significant statistical differences were found only in g.1773delC locus, and individuals with genotype DD had greater body length than those with genotype II ($P < 0.05$), while individuals with genotype DD had 14.93% and 12.28% greater cannon circumference than those with genotypes II and ID, respectively ($P < 0.01$). Regrettably, the results indicated that the combined genotypes were insignificantly associated with caprine growth traits (table 4). The differences observed between our study and previous studies may have been because of differences in study design (population-based compared with control subjects) or differences in samples (human beings compared with HM goat).

In conclusion, 12 novel polymorphisms in the caprine *PC1* gene were observed in this study. We have also defined the LD and haplotypes in the caprine *PC1* gene of the Chinese Haimen breed, the results showed that two distinct LD blocks and nine dominant haplotypes were identified, which would provide a background for more extensive characterization of the caprine *PC1* gene. In addition, the study identified that g.1773delC mutation was significantly associated with caprine body height and chest circumference, at least in Chinese Haimen breed. However, these results are preliminary ones, in future samples of other local origins should be looked at, and potential rare variants should also be considered.

Table 4. Associations between combined genotypes of *PC1* gene and growth traits in the Chinese Haimen goat breed.

Combined genotypes	Sample size	Mean values ± standard error (cm)			
		Body height	Body length	Chest circumference	Cannon circumference
TTAAAACTDD	6	58.50 ± 3.19	69.17 ± 4.27	71.00 ± 6.67	9.50 ± 0.56
TTAGAGCTID	6	61.50 ± 5.80	72.17 ± 3.19	73.50 ± 2.72	9.67 ± 0.56
TTAGAGCCID	9	60.67 ± 2.61	77.67 ± 5.09	76.44 ± 3.30	10.17 ± 0.53
TTGGGGCTII	12	59.17 ± 2.67	72.58 ± 2.99	74.58 ± 5.05	9.46 ± 0.64
TTGGGGCCID	6	59.50 ± 2.08	71.17 ± 2.74	70.50 ± 5.57	9.50 ± 0.50
TTGGGGCCII	11	60.73 ± 8.93	72.18 ± 3.15	79.09 ± 4.23	9.00 ± 0.45
TGAAAACCDD	10	62.90 ± 2.53	77.90 ± 2.37	82.10 ± 4.14	10.15 ± 0.42
TGAGAGTTID	6	58.50 ± 2.26	69.67 ± 4.81	72.00 ± 4.53	9.83 ± 0.78
TGAGAGCTID	6	60.17 ± 3.90	73.33 ± 3.39	72.83 ± 5.96	9.00 ± 0.52
TGAGAGCCID	14	62.18 ± 1.67	79.00 ± 2.67	78.43 ± 3.52	9.57 ± 0.45
TGAGAGCCII	6	59.33 ± 2.50	78.83 ± 4.32	75.00 ± 4.68	10.33 ± 0.67
TGGGGGCTII	14	60.79 ± 2.51	80.36 ± 5.72	75.82 ± 4.13	9.29 ± 0.37
TGGGGGCCID	11	63.55 ± 2.58	74.91 ± 3.09	81.45 ± 4.84	9.55 ± 0.56
TGGGGGCCII	29	61.69 ± 1.60	78.19 ± 3.27	78.55 ± 2.92	9.43 ± 0.28
GGAGAGCCID	11	61.09 ± 1.82	74.91 ± 2.54	78.27 ± 4.07	9.73 ± 0.36
GGGGGGCTII	8	60.13 ± 2.89	71.75 ± 5.23	72.75 ± 4.62	9.50 ± 0.73
GGGGGGCCII	14	61.14 ± 1.52	80.64 ± 4.59	79.64 ± 3.99	9.57 ± 0.40
P value		0.999	0.884	0.996	0.931

Genotypes with number <5 are not listed in table.

Acknowledgements

This work was supported by Research Fund for the Doctor Programme of Higher Education of China (no. 200807120012). National Natural Science Foundation of China (no. 31172184); Natural Science Foundation of Shaanxi province of China (no. 2011JQ3009), Natural Science Foundation of Jiangsu province (no. BK2008120).

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Received 25 September 2011, in revised form 9 January 2012; accepted 10 January 2012
Published on the Web: 5 April 2012