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The polymorphism of bovine *FIT* gene and its associations with cattle (*Bos taurus*) growth traits*

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Fat-inducing transcript (*FIT*) are endoplasmic reticulum-resident membrane proteins that induce lipid droplet accumulation. It plays a crucial role in the fundamental process of storing fat. In this study, applying the PCR-SSCP and DNA sequencing methods, polymorphism of the *FIT* gene were detected. A total of 708 individuals from four Chinese cattle breeds were examined. The results showed that only P_5 *locus* had two SNPs, resulting in a synonymous mutation (NM_001103095: m.199G > T resulting in L124L) and a missense mutation (NM_001103095: m.434G > T resulting in V176L). The associations between polymorphic *loci* and selected growth traits of indigenous Nangyang cattle were analysed, and significant associations were found in body weight at the age of 12 months and mean daily live weight gain. The body weight at month 12 of life and mean daily live weight gain of individuals with genotype AA were by 3.75% and 4.88% higher than of those with genotype AB, respectively. Hence, it was suggested for the first time, that genotype AA could be regarded as molecular marker for superior body weight and daily live weight gain in Chinese Nanyang cattle.

KEY WORDS: association analysis / FIT gene / gene polymorphism / SSCP / SNP

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Generally, a lipid droplet – a unified view of a dynamic organelle [Martin *et al.* 2006] – is composed of a core of neutral lipids, primarily triglycerides (TGs), surrounded with a monolayer of phospholipids and lipid droplet-associated proteins [Brasaemle *et al.* 2004, Liu *et al.* 2004, Mullner *et al*. 2004, Beller *et al.* 2006, Chang *et al.* 2007]. Under conditions of extreme lipid droplet acquisition, the risk for acquiring common debilitating diseases such as obesity, type 2 diabetes and cardiovascular diseases is increased [Spiegelman *et al.* 2001]. Although much is known about the composition and catabolism of lipid droplets, the molecular components necessary for their biogenesis remain obscure. Recently, Kaderei *et al.* [2008] have reported the characterization of a conserved gene family important for lipid droplet formation named fat-inducing transcript (*FIT*). *FIT1* and *FIT2* are endoplasmic reticulum-resident membrane proteins that induce lipid droplet accumulation in cell culture. Putative 292 and 262 amino acid proteins are encoded, respectively, with a 50% similarity to each other. *FIT1* and *FIT2* contain multiple potential transmembrane domains that are highly expressed in heart and skeletal muscle according to the gene array studies (by Novartis Gene Atlas). Interestingly, shRNA silencing of *FIT2* in 3T3-LI adipocytes prevents accumulation of lipid droplets. Moreover, the knockdown of *FIT2* in zebrafish embryos dramatically reduces the accumulation of lipid droplets, but increases TGs levels in mammalian cells.

Given the evidence that *FIT* is involved in the regulation of lipid droplet accumulation, and associated with obesity, we hypothesized that *FIT* is an obesity-susceptibility gene and its mutations may influence the performance of animals. However, only very limited relevant information has been reported. In the case of bovine, very few nucleotide sequences of *FIT1* (GenBank accession NC_007308, NM_001105351) and *FIT2* gene (GenBank accession NC_007311, NM_001103095) are described, so far. Hence, in the present paper, exons and exon/intron junctions of the bovine *FIT* gene were scanned for the mutations in 708 individuals from four Chinese indigenous cattle breeds (Nanyang, Qinchuan, Jiaxian and Jinnan) using PCR-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing analysis. The association between detected mutations and performance traits were analysed.

Material and methods

Genomic DNA samples and data collection

Genomic DNA samples were obtained from 708 genetically non-related individuals belonging to four cattle breeds reared in China: Nanyang (n=208), Qingchuan (n=255), Jiaxian (n=137) and Jinnan (n=108). Animals were weighed at birth and at the age of 12 months. On this basis the mean body live weight gain was calculated. Moreover, at the age of 12 months the following body measurements of Nanyang group were taken for statistical analysis: height at withers, trunk length, heart girth and width at hook bones. DNA was extracted from leukocytes according to Mullenbach *et al.* [1989].

Primer design and polymerase chain reaction (PCR) amplification

According to the GenBank, the bovine *FIT1* gene (accession number: NC_007308) and *FIT2* gene (accession number: NC_007311), six pairs of polymerase chain reaction (PCR) primers (Tab. 1) using the Primer V5.0 software were designed to amplify the exons and intron/exon boundaries. The amplification fragments covered almost the entire coding region, so all the mutations in this region could be detected. The PCR was performed in a 15 μl reaction mixture containing 50 ng genomic DNA, 10 pmol of each primer, 1× buffer (including 1.5 mmol/l MgCl2), 200 μmol dNTPs (dATP, dTTP, dCTP and dGTP), and 0.60 U Taq DNA polymerase (*MBI*). 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 shown in Table 1).

Table 1. Primer sequences for six *loci* of the *FIT* gene

Locus	Primer pairs	Product size (bp)	T_m (X°)	Region
P_1	F 5′-GCAGAGCAAGCAGTTAGCG-3′ R 5′-CAGAAACCTGGAGGGAGC-3′	369	63.5	Partial exon of NC_007308 (Cover the whole CDS) (488th to 856th)
P_2	F 5′-AGAGCCAGGCTGGGAACA-3′ R 5′-TGTGGGAGGAGACCGTGTAT-3′	368	64.6	Partial exon of NC_007308 (1081th to1448th)
P_3	F 5′-GATACACGGTCTCCTCCC-3′ R 5′- GACGTGCCTTTATTCCTTT-3′	359	58.7	The remaining part of exon of NC_007308 (1428th to 1786th)
P_4	F 5 -CGGATGAAGGGCAAGACGG-3 R 5 -GCCACGATGCGGAGAAGGA-3	366	62.2	Partial exon of NC_007311 (Cover the whole CDS) (-15 th to 350th)
P_5	F 5 -TCCTCCTGCCTTTCATCG-3 R 5 -GTGGTTCCTGTCCGTCTT-3	333	60.4	Partial exon of NC_007311 (2735th to 3070th)
P_6	F 5 -GTCTCCACGCAGCCATCA-3 R 5 -CCTTCCCGAAGCAGGATC-3	343	65.5	The remaining part of exon of NC_007311 (3071th to 3413th)

Single-stranded conformation polymorphism (SSCP) and DNA sequencing

SSCP method was used to scan mutations within the amplified regions. Aliquots of 4 μl PCR products were mixed with 6 μl denaturing solution (95% formamide, 25 mMEDTA, 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 (polyacrylamide gel electrophoresis) analysis which was run with 1× TBE buffer for 15 h at a constant temperature of 4°C and under a constant voltage (150V). The gels were stained with 0.1% silver nitrate and visualized with 2% NaOH solution (containing 0.1% formaldehyde). After the polymorphism was detected, PCR products of different genotypes in the *FIT* gene were sent to Shanghai

Sangon Biological Engineering Technology & Services Co., Ltd, and automatically sequenced in an ABI377 sequencer (APPLIED BIOSYSTEMS, USA). The sequence was analysed using the DNAMAN5.2.2.

Statistical

Genotypic frequencies and haplotype frequencies were determined by direct counting for each breed. Population genetic indices, *i.e*. gene heterozygosity, gene homozygosity, and effective allele numbers were calculated using the PopGen software (version 3.2). The polymorphic information content (PIC) was calculated with the method of Botstein *et al.* 1980]. A chi-square test [Mead and Curnow, 1985] was applied to assess statistical significance by the SPSS (software version 17.0). The relationships between the variations in the *FIT* gene and growth traits were analysed by ANOVA (SPSS software GLM procedure) using the following model:

$$Y_{ijk} = \mu + Breed_i + Marker_k + e_{ijk}$$

where:

Y_{ijk} – is the observation of the trait;

μ – is the least squares mean;

$Breed_i$ – is the effect of breed;

$Marker_k$ – is the effect of marker genotype;

e_{ijk} – is the residual effect.

Results and discussion

Based on SSCP analysis and DNA sequencing, only partial exon of the bovine *FIT2* gene, amplified with P_5 primer pairs, showed polymorphism. At this *locus*, polymorphic information was observed with six unique SSCP banding patterns, designated as AA, AB, BB, AC, BC and CC genotypes (Fig. 1). In order to characterize the sequence polymorphism detected the polymorphic DNA amplification fragments were sequenced and two novel SNPs: EX2_218G>T and EX2_353G>T were revealed. Compared to previously reported sequence (GenBank accession number

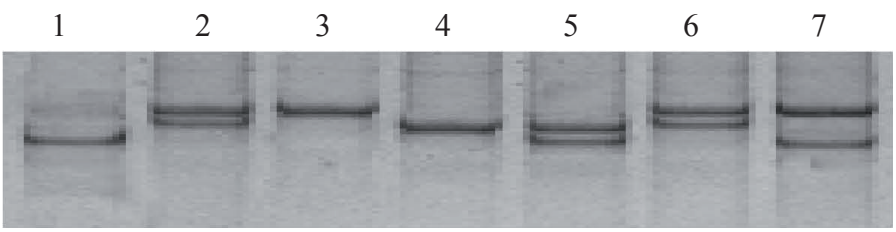

Fig. 1. The electrophoresis patterns of PCR-SSCP for P_5 *locus*. 1 – genotype CC; 2, 6 – genotype AB; 3 – genotype AA; 4 – genotype BB; 5 – genotype BC; 7: Genotype AC.

NC_007311), EX2_218G>T mutation showed a transversion G>T at position 2814, resulting in a synonymous mutation p.L97L. In addition, EX2_353G>T mutation also showed a G>T transversion at position 3049, resulting in a missense mutation p.V176L (Fig. 2). Through sequence comparison, three haplotypes were described as: A (T-G), B (G-G) and C (G-T), with their sequences submitted in GenBank with accession numbers GQ213994, GQ213995 and GQ213996, respectively. Accordingly, six respective genotypes can be conflated: AA(T-T/G-G), AB(T-G/G-G), BB(G-G/G-G), AC(T-G/G-T), BC(G-G/G-T) and CC(G-G/T-T). Remarkably, genotype AA and

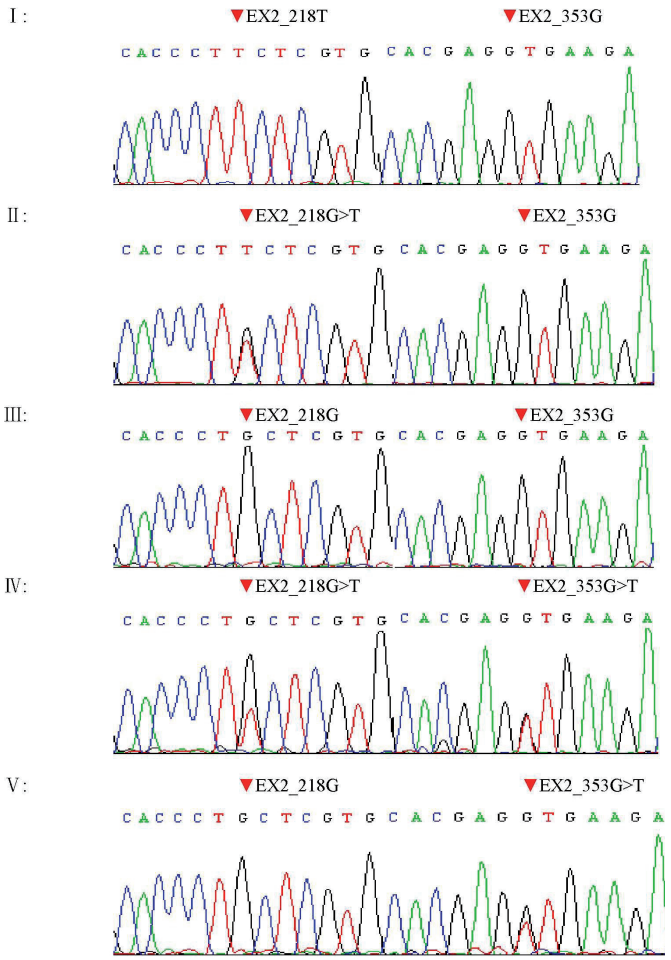

Fig. 2. The sequencing maps of two novel SNPs for P5 locus, SNPs are indicated by arrows. I – genotype AA; II – genotype AB; III – genotype BB; IV – genotypeAC; V – genotype BC. Synonymous mutation located NM_001103095: m. G1199T L97L. Missence mutation located NM_001103095: m.G434T V176L.

haplotype A were dominant in all four breeds, while the frequencies of genotype AC, BC and CC and haplotype C were obviously lower than the others. Additionally, the genotype distributions in four breeds were all significantly deviated from the Hardy-Weinberg equilibrium (P<0.05) – Table 2. This may be due to selection, as genotype AA and haplotype A were selected largely in the long-term artificial selection process, which tended to be homozygous and then fixed gradually.

With the PopGen software (version 3.2) and according to Botstein method, the population genetic indices (gene homozygosity, gene heterozygosity, effective allele numbers [Ne] and PIC) were estimated (Tab. 3). Hence, gene homozygosity varied from 0.6667 to 0.7644 and Ne from 1.5308 to 1.8180. According to the classification of PIC (low polymorphism if PIC <0.25, median polymorphism if 0.25<PIC <0.5, and high polymorphism if PIC>0.5), Jinnan cattle presented the highest PIC value (0.3951), and four tested breeds all belonged to middle genetic diversity class, being suitable as marker in molecular breeding [Vaiman *et al.* 1994]. Genotype frequencies for the various polymorphisms at FIT-P_5 *locus* were found to be significantly different among the four analysed breeds based on a chi-square test (chi-square = 35.470; df = 15; P=0.002). Significant differences for haplotype frequencies among breeds were also revealed (chi-square = 39.886; df = 6; P<0.001). Moreover, significant differences for the genotype frequencies between Nanyang and Jinnan (P=0.012), between Qinchuan and Jiaxian (*P*=0.002) and between Qinchuan and Jinnan (P=0.001), as well as significant for the haplotype frequencies among all analysed breeds occurred, except for the Nanyang and Qinchuan, or Jiaxian and Jinnan cattle (P<0.05 or P<0.001) – Table 4. Obviously, the breed factor significantly affected the distribution of genotype and haplotype frequencies at P_5 *locus* of the bovine *FIT2* gene.

Table 2. The distribution of genotypic and allelic frequencies at P_5 *locus* of the bovine *FIT* gene in four Chinese indigenous cattle breeds

Breed	Genotypic frequencies (n)						Allelic frequencies			Chi-square[1]
	AA	AB	BB	AC	BC	CC	A	B	C	
NY	0.6683(139)	0.2115(440	0.0962(20)	0.0144(3)	0.0096(2)	0.0000(0)	0.7812	0.2067	0.0120	24.6146
QC	0.6039(154)	0.2784(71)	0.1059(27)	0.0078(2)	0.0000(0)	0.0039(1)	0.7471	0.2451	0.0078	99.6803
JX	0.6643(93)	0.1643(23)	0.0714(10)	0.0357(5)	0.0214(3)	0.0214(3)	0.7750	0.1750	0.0500	37.9761
JN	0.5556(60)	0.2593(28)	0.0741(8)	0.0370(4)	0.0370(4)	0.0370(4)	0.7037	0.2222	0.0741	29.3071

[1]Hardy-Weinberg equilibrium chi-square value.
n– number of animals.
NY – Nanyang cattle; QC – Qingchuan cattle; JX – Jiaxian cattle; JN – Jinnan cattle.

Table 3. Genetic diversity at P_5 *locus* in four Chinese indigenous cattle breeds

Breed	Number of animals	Gene homozygosity (Ho)	Gene heterozygosity (He)	Effective allele numbers (Ne)	Polymorphic information content (PIC)
NY	208	0.7644	0.2356	1.5308	0.2945
QC	255	0.7137	0.2863	1.6175	0.3146
JX	137	0.7571	0.2429	1.5779	0.3263
JN	108	0.6667	0.3333	1.8180	0.3951

NY – Nanyang cattle; QC – Qingchuan cattle; JX – Jiaxian cattle; JN – Jinnan cattle.

Table 4. Chi-square and P values differences for genotypic (above the diagonal) and allelic (below the diagonal) frequencies among four China indigenous cattle breeds

Breed	NY	QC	JX	JN
NY		6.647 (*P*=0.248)	8.484 (*P*=0.132)	14.696 (*P*=0.012)
QC	2.243 (*P*=0.326)		18.861 (*P*=0.002)	20.596 (*P*=0.001)
JX	10.463 (*P*=0.005)	19.666 (*P*<0.001)		4.862 (*P*=0.433)
JN	17.771 (*P*<0.001)	24.883 (*P*<0.001)	3.867 (*P*=0.145)	

Total genotypic chi-square = 35.470, df=15, *P*=0.002 and total allelic chi-square = 39.886, df=6, *P*< 0.001).
NY – Nanyang cattle; QC – Qingchuan cattle; JX – Jiaxian cattle; JN – Jinnan cattle.

In this paper two novel mutations are described in the bovine *FIT2* gene. The synonymous EX2_218G>T mutation (NC_007311:g. 2814G>T, NM_001103095:m. 199 G>T) resulted in p.L97L and the missense EX2_353G>T mutation (NC_007311: g. 3049G>T, NM_001103095:m. 434 G>T) resulted in p.V176L. As it is known, the missense NM_001103095:m. 434 G>T SNP of the bovine *FIT2* gene resulting in p.V176L may change the amino acid properties and affect the encoded protein structure. However, in contrast to the missense mutation, numerous studies in the past few decades have clearly shown that synonymous single-nucleotide polymorphisms (SNPs) do not produce altered coding sequences, and are not, therefore, expected to change the expression and function of the protein. Recently, however, Nackley *et al.* [2006] and Capon *et al.* [2004] have provided evidence that synonymous SNPs can affect protein expression (and thus function) by alteration or increase of the mRNA stability. Additionally, Kimchi-Sarfaty *et al.* [2007] have reported that a "Silent" Polymorphism changes substrate specificity. Therefore, we hypothesized

that synonymous NM_001103095:m. 199 G>T mutation resulting in p.L97L might have similar biological effect on missense mutation, including alteration of mRNA stability, modulation of the efficiency of translation of mRNA, and in consequence, affection of the encoded protein structure and properties, which might influence directly or indirectly the production traits of animals. Concomitantly, the relationships between genotypes at P_5 *locus* and body conformation traits and growth rate were analysed in Nanyang cattle by the SPSS software (version 17.0) and are presented in Table 5. Multiple comparison results indicated that the body weight and mean daily live weight gain of individuals with genotype AA were by 3.75% and 4.88% higher than of those with genotype AB, respectively. However, no significant differences were identified between genotype AA and BB, BC or AC, and between BB and BC or AC. No significant differences were identified within each of the four body conformation traits in relation to the P_5 *locus*.

In conclusion, SSCP analysis and DNA sequencing methods revealed two novel SNPs in the *FIT* gene: a synonymous EX2_218G>T SNP and a missense EX2_353G>T SNP resulting in V176L change. In addition, the results of association analysis between the *FIT* gene polymorphisms and body conformation and growth rate performed for Nanyang cattle, indicated that the two SNPs detected were significantly associated with body weight and mean daily live weight gain. Hence, we suggest for the first time that genotype AA could be regarded as molecular marker for superior body weight and mean daily live weight gain in Chinese Nanyang cattle. It might be useful in improving meat production and growth. However, the results presented are preliminary and further investigations are essential.

Table 5. Least squares means (LSM) and standard errors (SE) for body conformation and growth rate traits across P_5 *loci* in Nanyang indigenous Chinese cattle breed

Trait	LSM±SE					P Value
	AA (n=139)	AB (n=44)	BB (n=20)	AC (n=3)	BC (n=2)	
Wither height at month 12 (cm)	113.94±0.313	113.93±0.575	113.70±0.901	113.00±2.082	110.00±2.000	0.664
Trunk length at month 12 (cm)	116.53±0.592	117.73±1.276	118.15±1.674	117.33±2.963	117.50±1.500	0.827
Width at huck bones at month 12 (cm)	20.580±0.134	21.010±0.278	20.780±0.291	20.830±0.441	19.500±0.500	0.475
Heart girth at month 12 (cm)	140.93±0.630	141.59±1.288	142.30±7.292	142.67±3.528	138.50±0.500	0.899
Body weight at birth (kg)	29.633±0.189	30.659±0.471	29.800±0.459	31.333±3.245	28.000±0.000	0.099
Body weight at month 12 (kg)	230.13±1.290^{A}	221.82±3.308^{B}	226.25±23.204^{AB}	220.00±5.859^{AB}	220.50±1.500^{AB}	0.076
Mean live weight gains for month 0-12 (g)	556.93±3.458^{A}	531.00±8.435^{B}	545.69±13.900^{AB}	524.07±8.574^{AB}	534.72±4.167^{AB}	0.021

n = number ofanimals.
^{AB}Within rows means bearing different superscripts differ significantly at P≤0.01.

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