RESEARCH NOTE

Two novel single nucleotide polymorphisms (SNPs) and 4-bp deletion mutation of *RBP4* gene in Chinese cattle

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Introduction

Retinol binding protein 4 (RBP4), a newly discovered adipocytokine, has been implicated in insulin resistance and obesity (Graham *et al*. 2006). RBP4 is produced by peripheral tissues, including liver and adipose tissues (Yang *et al*. 2005). Injection of RBP4 decreases insulin signalling in muscle and induces the expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in the liver of mice (Yang *et al*. 2005). RBP4 was seen to contribute to glucose and lipid metabolism in several clinical studies in Takashima cattle (Janke *et al*. 2006; Lee *et al*. 2007). A recent study described that concentrations of RBP4 were elevated in plasma of diabetic patients and were higher in those with microalbuminuria (Raila *et al*. 2007). The result suggests that plasma RBP4 levels in patients with type 2 diabetes mellitus are affected by nephropathy.

To date, no polymorphism in the *RBP4* gene has been reported in Chinese cattle breeds. Therefore, we analysed the genetic variations of *RBP4* gene in about 818 cattle. Herein, we are the first to identify the novel genetic variation of bovine *RBP4* gene by PCR-SSCP and DNA sequencing methods, which will possibly contribute to conducting association analysis and evaluating them as genetic markers in meat production and other performance for animal breeding and genetics.

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Materials and methods

Cattle and DNA sources

Genomic DNA samples were obtained from 818 cattle from four cattle breeds: Nanyang cattle (NY, $n = 283$, farming and meat type) were from the breeding centre of Nanyang cattle (Nanyang, P. R. China); the Jiaxian cattle (JX, $n = 134$, farming and meat type) were from the breeding protection region of Jiaxian cattle (Jiaxian, P. R. China), the Qinchuan cattle (QC, $n = 306$, farming and meat type) were from the protection region of Qinchuan cattle (Weinan, P. R. China), and the breeding farm of Qinchuan cattle and the fineness breeding centre of Qinchuan cattle (Fufeng, P. R. China), and the Chinese Holstein animals (CH, $n = 95$, milk type) were from the breeding farm of milk breed (Xi'an, P. R. China).

PCR amplification

Primers used to amplify bovine *RBP4* gene exon 3 were designed from a published gene sequence (GenBank accession NC_007327). The sequences of the primers: forward 5′-GGCGCTAAGTTTCCCTGAC-3′ and reverse 5′-TGCCTCTGCCGCACGATT-3′. The size of expected PCR products was 232–236 bp, containing the whole exon 3 and part of the intron 3 regions. Each PCR was performed in a 15 μL reaction volume containing: 50 ng genomic DNA, 10 pM of each primer, 1× buffer (including 1.5 mM $MgCl_2$), 200 μM dNTPs and 0.5 units of *Taq* DNA polymerase (MBI, Michigan, USA). The cycling protocol was 5 min at 95°C, 33 cycles of 94°C for 35 s, 52°C annealing for 35 s, 72°C for 40 s, with a final extension at 72°C for 10 min.

Variation in the RBP4 gene detected by PCR-SSCP, DNA sequencing and agarose electrophoresis analysis

PCR products (5 μL) were mixed with 5 μL denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylenecyanole and 0.025% bromophenol blue), heated for 10 min at 98°C and chilled on ice. Denatured DNA samples were subjected to PAGE (80 × 73 × 0.75 mm) in 1× TBE buffer at constant voltage (200 V) for 3.0 h. The gel was stained with 0.1% silver nitrate. After polymorphisms were detected, the PCR products of different electrophoresis patterns were sent to sequence in both directions in ABI 377 DNA analyzer (Applied Biosystems, Foster City, USA) and the sequences were analysed with BioXM software version 2.6 (Msight, Guangzhou, P. R. China).

Statistical analysis

Based on the genotype number of *RBP4* exon 3 and its flanking region in analysed breeds, genotypic and haplotype frequencies were directly calculated. Differences for genotypic frequencies at bovine *RBP4* exon 3 and its flanking region among/between analysed populations were analysed using χ^2 test, which was performed by SPSS software version 13.0 (SPSS, chicago, USA). Population genetic indexes; namely, gene heterozygosity, gene homozygosity, effective allele numbers were calculated using PopGen software version 3.2 (http://www.ualberta.ca/nfyeh/index.htm) and PIC (polymorphism information content) was calculated by the method of Botstein *et al*. (1980).

Results and discussion

In this paper, the polymorphisms of bovine *RBP4* gene were detected by PCR-SSCP and DNA sequencing methods. The results showed that a 4-bp (TCTG) deletion was detected in intron 3 and two mutations (C>G and C>T) were located in the exon 3 in four Chinese indigenous bovine breeds. The SSCP results showed polymorphic information with three unique SSCP banding patterns that were observed in four Chinese bovine populations. In order to better understand the detailed genetic variation within Chinese bovine *Rbp4* gene, the polymorphic DNA amplification fragments between exon 3 and its flanking region were sequenced. The DNA sequences were deposited in GenBank database (bankit1189296).

The comparison between nucleotide sequence of bovine *RBP4* gene (GenBank accession no. NC_007327) and the above sequences revealed a 4-bp deletion and two novel SNPs: NC_007327:g. 3486–3489 deletion (TCTG), 3571C>G, T. Especially, the base g.3571 C was in close linkage with the g.3486–3489 (TCTG). The 4-bp deletion occurred when mutation occurred in this area. The g.3571 C>G, T mutation led to the 15th amino acid of the *Rbp4* exon 3 being changed: C-C-G (Pro) >C-G-G (Arg) ,C-T-G (Leu).

The 4-bp deletion mutation observed genotype was described as *WW, WD* and *DD*, and linkage with the two novel SNPs can put the gene into the following genotypes: *WW* (*CC*), *WD* (*CG & CT*) and *DD* (*GG & GT*). Frequencies of allele *Rbp4-W (C), G* and *T* allele in the analysed population separetely were: 0.728, 0.780, 0.786, 1.000; 0.191, 0.123, 0.137, 0.000; 0.081, 0.097, 0.077, 0.000 for Nanyang, Jiaxian, Qinchuan, and Chinese Holstein, respectively. The χ^2 test showed that the genotype distributions of NY, JX and QC breeds were in agreement with Hardy–Weinberg equilibrium ($P > 0.05$) (table 1). In present populations, the population genetic parameters (homozygosity, heterozygosity, effective allele numbers (N_e) and PIC were calculated. Value of homozygosty estimate varied from 0.6039 (NY) to 1.0000 (CH) and N_e ranged from 1.0000 (CH) to 1.6559 (NY). PIC values varied from 0.0000 (CH) to 0.3177 (NY).

Recent studies show that RBP4 is not only a carrier of retinol, but also a new adipokine. Injection of purified *RBP4* or transgenic overexpression of *RBP4* in mice decreased insulin sensitivity in muscle and increased gluconeogensis by activation of phosphoenolpyruvate carboxykinase in liver (Lee *et al*. 2007). Elevated *RBP4* levels have been reported in people with type 2 diabetic and obesity (Janke *et al*. 2006). Moreover, Graham *et al*. (2006) discovered that serum RBP4 levels were increased even before the development of frank diabetes, and appeared to identify insulin resistance and associated cardiovascular risk factors in human (Graham *et al*. 2006). These suggest that *RBP4* gene is very likely to be a candidate gene for growth traits in cattle.

Therefore we investigated the association of 4-bp deletion and two novel SNPs polymorphisms in Rbp4 with growth traits (body height, body length, heart girth, hucklebone width, body weight, birth weight and average daily gain) in Nanyang cattle at birth weight 6, 12, 18, and 24 months old were analysed (tables 2 and 3). Individuals with

Table 1. Genotype distribution and allelic frequencies at the bovine *RBP4* intron 3.

	Observed genotypes				Allelic frequencies		
Breeds	*WW* (*CC*)	*WD* (*CG&CT*)	*DD* (*GT&GG*)	Total	*W* (*C*)	*D* (*G&T*)	χ^2 (HWE)
NY	185	42 (18&24)	56 (22&34)	283	0.728	0.272 (0.191&0.081)	0.00035
JX	92	25 (9&16)	17 (10&7)	134	0.780	0.220 (0.123&0.097)	0.00065
QC	205	71 (37&34)	30 (13&17)	306	0.786	0.214 (0.137&0.077)	0.00028
CH	95	0	0	95	1.000	0.000	0.00000

Table 2. Association of *WW*, *WD* and *DD* genotypes of the *RBP4* gene with growth traits in Nanyang cattle.

Traits	*WW* (Mean ± s.e.)	*WD* (Mean ± s.e.)	*DD* (Mean ± s.e.)
Birth weight (kg)	30.727±0.332^{A}	29.824±0.598	28.719±0.436^{B}
Body weight 6 months (kg)	166.145±2.393^{Aa}	148.000±4.305^{B}	156.094±3.138^{b}
Average daily gain 6 months (kg)	0.752±0.013^{Aa}	0.657±0.023^{B}	0.708±0.017^{b}
Body weight 12 months (kg)	226.327±3.094	221.095±5.564	218.500±4.056
Average daily gain 12 months (kg)	0.334±0.016^{a}	0.406±0.028^{b}	0.347±0.020
Body weight 18 months (kg)	296.927±4.202	299.235±7.559	299.344±5.509
Average daily gain 18 months (kg)	0.392±0.029	0.434±0.052	0.449±0.038
Body weight 24 months (kg)	365.182±7.034	358.529±12.652	366.281±9.222
Average daily gain 24 months (kg)	0.969±0.408	0.329±0.735	0.372±0.536

(A,B) values with different superscripts within the same line differ significantly at $P < 0.01$ and (a, b) $P < 0.05$. SE, standard error of means.

Table 3. Association of *CC*, *CG*, *CT*, *GG* and *GT* genotypes of the *RBP4* gene with growth traits in Nanyang cattle.

Traits	*CC* (Mean ± s.e.)	*CG* (Mean ± s.e.)	*CT* (Mean ± s.e.)	*GG* (Mean ± s.e.)	*GT* (Mean ± s.e.)
Birth weight (kg)	30.727±0.332^{A}	30.750±0.871	29.000±0.821	28.737±0.565^{B}	28.692±0.683^{B}
Body weight 6 months (kg)	166.145±2.378^{Aa}	148.5000±6.235^{b}	147.556±5.878^{B}	160.789±4.046	149.231±4.891^{B}
Average daily gain 6 months (kg)	0.752±0.013^{A}	0.654±0.034^{Bb}	0.659±0.032^{B}	0.734±0.022^{c}	0.670±0.026^{B}
Body weight 12 months (kg)	226.327±3.043	232.500±7.978	210.889±7.522	222.579±5.177	212.538±6.258
Average daily gain 12 months (kg)	0.334±0.015^{A}	0.467±0.040^{Bb}	0.352±0.038^{c}	0.343±0.026^{c}	0.352±0.036^{c}
Body weight 18 months (kg)	296.927±4.2218	290.875±11.060	306.667±10.428	301.158±7.177	296.692±8.676
Average daily gain 18 months (kg)	0.392±0.029	0.324±0.075^{a}	0.532±0.071^{b}	0.437±0.049	0.468±0.059
Body weight 24 months (kg)	365.182±6.957	368.875±18.242	349.333±17.198	380.684±11.837	345.231±14.310
Average daily gain 24 months (kg)	0.969±0.413	0.433±1.082	0.237±1.020	0.442±0.702	0.270±0.848

(A,B) values with different superscripts within the same line differ significantly at $P < 0.01$ and (a, b, c) $P < 0.05$. SE, standard error of means.

genotype *WW* had greater birth weight, 6 months body weight and average daily gain (6 months and 12 months) compared with those with genotypes *WD* and *DD* ($P<0.01$, $P<0.05$ respectively). Also, Individuals with genotype *CC* had greater birth weight, 6 months body weight and average daily gain (6 months and 12 months) compared with those with genotypes *CG, GT, CT* and *GG*, ($P<0.01$, $P<0.05$ respectively). In theory, the *DD (TT)* genotype should exist in four Chinese indigenous bovine breeds, but was not found. Perhaps the *DD* (*TT*) genotype of the *RBP4* is an early-acting lethal in cattle. Other growth traits in the records had no significant association with genotypes studied. Therefore, the presence of 4-bp deletion and the two novel SNPs (G and T) in *RBP4* might negatively influence the birth weight, bodyweight and average daily gain at birth weight, 6-month and 12-month-old cattle.

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