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Lack of association of single nucleotide polymorphisms of the bovine *Flt-1* gene with growth traits in Chinese cattle breeds

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ABSTRACT. We analyzed 20 exons, with their intron-exon boundaries, of the bovine *Flt-1* gene, using a strategy combining PCR amplification and single-strand conformational polymorphism analysis (PCR-SSCP), followed by nucleotide sequence analysis, in 675 cattle. We then looked for associations between polymorphisms and growth traits. Twelve novel SNPs (ss#184956516, ss#184956517, ss#184956518, ss#184956519, ss#251343993, ss#251343994, ss#251343995, ss#251343996, ss#251343997, ss#251343998, ss#251343999, and ss#251344000) were detected in the bovine *Flt-1* gene in all three breeds. We observed no significant associations between these polymorphisms and birth weight, body weight and average daily gain during different growth periods (6, 12, 18, and 24 months old) ($P > 0.05$), or in body height, body length, heart girth, or height at the hip in Nanyang cattle breeds.

Key words: Single nucleotide polymorphism; Chinese cattle; *Flt-1* gene

INTRODUCTION

Increased knowledge of the molecular basis has indicated that mutations in some genes play a major role in animal development and growth. Altered forms of some of these genes have already been shown to be potential prognostic markers in growth traits. Thus, in addition to basic research, analysis of genetic polymorphism has also become more important for animal molecular breeding.

Flt-1 (fms-like tyrosine kinase) is a transmembrane receptor in the tyrosine kinase family for vascular endothelial growth factor (VEGF) (De Vries et al., 1992). The VEGF gene family consists of seven members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor (Ferrara et al., 2003; Alitalo et al., 2005). Research conducted for almost two decades has established that VEGF (also referred as VEGF-A) is a key positive mediator of developmental angiogenesis associated with various physiological conditions including skeletal muscle capillarity (Tang et al., 2004), bone formation during normal bone development (Peng et al., 2005), and regulation of obesity (Cao et al., 2008). In previous studies, we reported that the polymorphisms of the *VEGF* gene had a positive effect on bovine growth traits and that the single nucleotide polymorphism (SNP; ss130456744) in intron 2 may be a molecular breeding marker in breeding strategies through MAS (marker-assisted selection) in Chinese domestic cattle (Pang et al., 2010). VEGF binds two highly related receptor tyrosine kinases (RTK), Flt-1 (VEGFR1) and KDR/Flk-1 (VEGFR2). Flt-1 was the first RTK to be identified as a VEGF receptor (De Vries et al., 1992). Increased Flt-1 expression was reported in endothelial cells during development (Quinn et al., 1993). Combined with *Flt-1* gene knock-out and receptor-blocking studies, these data suggest that the Flt-1 signaling system may have important regulatory roles during postnatal development (Autiero et al., 2003). However, the variations in the *Flt-1* gene have not been investigated. The aim of this study was to evaluate variation in the *Flt-1* gene and to test the association of these polymorphisms with body growth in 675 samples in three Chinese cattle breeds. We also try to understand whether these polymorphisms are genetic markers in cattle breeding traits and whether they can be used in breeding strategies through MAS.

MATERIAL AND METHODS

DNA samples

Blood samples were obtained from 675 individuals belonging to three native Chinese cattle breeds (without genetic relationships): Nanyang (N = 275), Jiaxian (N = 143), Qinchuan (N = 257). Many records of growth traits and body size traits (body height, body length, chest girth, hucklebone width, body weight, and average daily gain) for different growth periods (6, 12, 18, and 24 months) in the Nanyang breed were collected for statistical analysis. Genomic DNA was extracted from blood samples according to standard procedures (Sambrook and Russell, 2001).

PCR amplification

The bovine *Flt-1* gene is organized into 31 exons, separated by 30 introns, and its coding region spans approximately 14 kb. It has been assigned to chromosome 12 (http://www.ncbi.nlm.nih.gov).

Table 1. Primer pairs and optimized conditions for PCR amplification.

Production	Locus NC_007310	Length (bp)	Annealing temperature (X°C)	Primer sequence
C1	1..130	344	59	5' ACAGAGAACCAAGTCCCCAG 3' 5' TGTAAAGCAATTTTCCTCCAAC 3'
C2	2250..2346	250	57	5' CAACTAAGACCTGGAACA 3' 5' CCATCTACCTGTCTGCTC 3'
C3	2940..3169	334	60	5' GACGGTGTTTTGTTTTCTC 3' 5' CTATGGACCAGGTGGATTG 3'
C4	32155..32279	259	61	5' TGGGAAGGTGTATGGTGGC 3' 5' GCGGTGAAGAGCAGGTGAG 3'
C5	36118..36280	385	51	5' GCAAAGAAAACTAAAGAAC 3' 5' TCATTAAGTAGATTAACCCT 3'
C6	36386..36522	320	49	5' ACAAGTCAAACTATCTCAT 3' 5' AAAACTAAATACTGTCCTA 3'
C7	39579..39753	271	59	5' GAGTGGGTTTGCCTCTTAG 3' 5' GAAGGGTTGCTCACCATAT 3'
C8	41140..41257	312	63	5' GGTTTTGGTTCGGTTTTGTT 3' 5' GACCCTAGTCTTCCACTGTTGT 3'
C9	43540..43709	312	55	5' GTTATCTAAGAGGTGGTATGA 3' 5' TTATTTACTGGCTTTTGAC 3'
C10	44327..44486	330	67	5' CCAGTGAGAACGTGGTCCTT 3' 5' AGTGCCAACTCCCATCTGTG 3'
C11	68767-69087	321	56	5' GGAAAGTAAGAAAAATGTG 3' 5' CTGACTCTAATAAACCCA 3'
C12	78001-78327	327	53	5' GACCTGTTTTCCTGTTGTT 3' 5' ACTGGGCTGTCTTCCTAA 3'
C13	86043-86394	352	53	5' AGGTTGAGACACTGATTT 3' 5' ACTTCTTTCTTCTGGAGG 3'
C14	91261-91553	293	51	5' TGCTGCTTTTCTATTTTC 3' 5' GGGATTTTAAGAGGCTGA 3'
C15	113163-113476	314	61	5' CCGAAGGCAGAAAGATAGG 3' 5' GGAAAGGAACATACTGGAGG 3'
C16	128684-128936	253	61	5' TCGCCCTGATGAAGCACT 3' 5' GCCAAAAGGAGCATAGAAT 3'
C17	133861-134159	299	50	5' GCCAAGTATTGTTTTACA 3' 5' GTTACTTCTGCTTCCCTC 3'
C18	140117-140451	335	59	5' ATTTGGGTCTGTTCACT 3' 5' GCCTCTGTCTGCTGCTAG 3'
C19	144008-144259	252	64	5' TGTCCCGTCGTCCTAACTCT 3' 5' ACAGCAGGCCGTCTTCCAG 3'
C20	145703-146026	324	55	5' CCCAGAACTTGCTAACAG 3' 5' ATCAGTTCTTTGGTGCTC 3'

Polymerase chain reaction (PCR) was used to amplify the *Flt-1* gene fragments from bovine genomic DNA. The exon-specific oligonucleotide primers were designed according to the nucleotide sequence of the bovine *Flt-1* gene reported by GenBank NC_007310. The location and sequences of the primers and the length of the amplified DNA fragments are shown in Table 1. PCR amplification was carried out in a T-gradient thermal cycler (Biometra, Germany) in a volume of 25 µL, using 0.5 U Taq DNA polymerase, 1X PCR buffer with 2.5 mM $MgCl_2$, 10 pmol of each primer, each dNTP at 200 µM (all TaKaRa, Dalian, China) and 50 ng DNA template.

PCR was performed under the following conditions: 94°C for 5 min followed by 35 cycles of 94°C for 40 s, annealing at X°C (see Table 1) corresponding to 10 different primer pairs for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min.

SSCP analysis

For single-strand conformational polymorphism (SSCP) analysis, the PCR products were mixed with an equal volume (5 µL) of formamide loading dye (98% deionized formamide, 0.05% bromophenol blue and 0.05% xylene cyanol). The mixture was heated at 98°C for 10 min to denature the DNA. After denaturation, the sample was chilled on ice for 10 min, and immediately followed by loading on a gel. Electrophoresis was carried out at 50 mA and 130 V for 17 h at 4°C. DNA fragments were visualized by silver staining method (Tebbe et al., 2001).

Nucleotide sequence analysis

SSCP method was used to scan mutations within the amplified regions. Confirmation of the presence of mutation was done by DNA sequencing. The PCR fragments from different SSCP patterns in different breeds were cleaned up and sequenced in both directions in an ABI PRISM 3730 DNA analyzer (Applied Biosystems, USA).

Statistical analysis

The linear model was applied to analyze the association of the variations in the *Flt-1* gene with growth traits in the Nanyang breed. The following model for the PCR-SSCP marker effect was used for analysis: Y = l + age + marker + e, where *Y* is the phenotype of the animal, *l* is the mean of the population, *age* is the age effect, *marker* is the marked haplotype effect, and *e* is the stochastic error. Descriptive data for continuous variables were reported as means ± SD unless otherwise stated. Two-sided P values below 0.05 were considered to be statistically significant. Data were analyzed with the SPSS software (version 16.0).

RESULTS

SNP of *Flt-1* gene in three Chinese cattle breeds

The SSCP analysis indicated that there was no polymorphism in the C1, C3, C5, C6, C7, C8, C15, C18, and C19 loci of *Flt-1* gene and that mutations occurred in the C2, C4, C9, C10, C11, C12, C13, C14, C16, C17, and C20 of the *Flt-1* gene, respectively, in the ampli-

fied regions. Compared with the sequence of the bovine *Flt-1* gene (GenBank accession No. NC_007310), 12 novel SNPs (NC_007310: g.2260A>G, 32255C>A, 43654G>C, 44409C>G, 68859G>A, 68872G>A, 78152C>G, 86197A>T, 91417G>C, 128783C>G, 133972A>G, and 145849C>T) were detected in the three breeds. Twelve new SNPs were named FLT1-SNP2260, FLT1-SNP32255, FLT1-SNP43654, FLT1-SNP44409, FLT-SNP68859, FLT-SNP68872, FLT-SNP78152, FLT-SNP86197, FLT-SNP91417, FLT-SNP128783, FLT-SNP133972, and FLT-SNP145849, respectively, and submitted to the GenBank database (ss#184956516-ss#184956519 and ss#251343993-ss#251344000). The SNP locations in the gene, GenBank accession numbers and positions (intron/exon, etc.) are listed in Table 2.

Table 2. Single nucleotide polymorphism (SNP) name and location.

SNP name	Location NC_007310	Base position	NCBI_ss#	SNP
FLT1-SNP2260	Exon2	2260	184956516	A/G
FLT1-SNP32255	Exon4	32255	184956517	C/A
FLT1-SNP43654	Exon9	43654	184956518	G/C
FLT1-SNP44409	Exon10	44409	184956519	C/G
FLT-SNP 68859	Intron10	68859	251343993	G/A
FLT-SNP 68872	Intron10	68872	251343994	G/A
FLT-SNP78152	Intron11	78152	251343995	C/G
FLT-SNP86197	Exon13	86197	251343996	A/T
FLT-SNP91417	Exon14	91417	251343997	G/C
FLT-SNP128783	Intron15	128783	251343998	C/G
FLT-SNP133972	Exon17	133972	251343999	A/G
FLT-SNP145849	Exon20	145849	251344000	C/T

NCBI = National Center for Biotechnology Information.

Being located in the intron regions, four SNPs did not cause amino acid (aa) substitution. Eight SNPs were detected in exon regions and may cause eight synonymous mutations (Table 3): TCA (Ser)>TCG (Ser) at position 47th aa, CCC (Pro)>CCA (Pro) at position 186th aa, CTG (Leu)>CTC (Leu) at position 430th aa, ACC (Thr)>ACG (Thr) at position 476th aa, ATA (Ile)>ATT (Ile) at position 610th aa, ACG (Thr)>ACC (Thr) at position 701st aa, TCA (Ser)>TCG (Ser) at position 819th aa, AAC (Asn)>AAT (Asn) at position 942nd aa of FLT-1 (1359 aa, NCBI Reference Sequence: XP_001249769.2) in the three cattle breeds.

Table 3. Eight synonymous mutations in exon regions of *Flt-1* gene.

NCBI_ss#	SNP	Synonymous mutation	Amino acid position (1359 aa) NCBI : XP_001249769.2
184956516	A/G	TCA (Ser)>TCG (Ser)	47
184956517	C/A	CCC (Pro)>CCA (Pro)	186
184956518	G/C	CTG (Leu)>CTC (Leu)	430
184956519	C/G	ACC (Thr)>ACG (Thr)	476
251343996	A/T	ATA (Ile)>ATT (Ile)	610
251343997	G/C	ACG (Thr)>ACC (Thr)	701
251343999	A/G	TCA (Ser)>TCG (Ser)	819
251344000	C/T	AAC (Asn)>AAT (Asn)	942

NCBI = National Center for Biotechnology Information; SNP = single nucleotide polymorphism.

Genotype frequencies

A total of 675 animals were genotyped at all 12 SNPs; however, successful genotype assignment was not possible for all animals, in particular for the SNPs 184956517, 184956518, 251343993, 251343994, 251343995, and 251344000, where animals were missing genotypes in Qinchuan and Jiaxian Red breeds. The genotype frequency of 12 SNPs varied differently (Table 4).

Table 4. Genotype frequencies of each polymorphism.

NCBI_ss#	SNP	Genotype	Genotype frequency					
			NY (N = 275)		JX (N = 143)		QC (N = 257)	
184956516	A/G	AA	206	0.7491	103	0.7203	222	0.8638
		AG	53	0.1927	35	0.2448	32	0.1245
		GG	16	0.0582	5	0.0350	3	0.0117
184956517	C/A	CC	222	0.8073	128	0.8951	236	0.9183
		CA	53	0.1927	15	0.1049	21	0.0817
		AA	0	0	0	0	0	0
184956518	G/C	GG	212	0.7709	126	0.8811	247	0.9611
		GC	63	0.2291	17	0.1189	10	0.0389
		CC	0	0	0	0	0	0
184956519	C/G	CC	121	0.4400	57	0.3986	210	0.8171
		CG	91	0.3309	66	0.4615	37	0.1440
		GG	63	0.2291	20	0.1399	10	0.0389
251343993	G/A	GG	241	0.8764	143	1.0000	257	1.0000
		AG	34	0.1236	0	0	0	0
		AA	0	0	0	0	0	0
251343994	G/A	GG	241	0.8764	143	1.0000	257	1.0000
		AG	34	0.1236	0	0	0	0
		AA	0	0	0	0	0	0
251343995	C/G	CC	252	0.9164	143	1.0000	257	1.0000
		CG	23	0.0836	0	0	0	0
		GG	0	0	0	0	0	0
251343996	A/T	AA	97	0.3527	55	0.3846	105	0.4086
		AT	104	0.3782	68	0.4755	90	0.3502
		TT	74	0.2691	20	0.1399	62	0.2412
251343997	G/C	GG	80	0.2909	40	0.2797	98	0.3813
		GC	98	0.3564	76	0.5315	147	0.5720
		CC	97	0.3527	27	0.1888	12	0.0467
251343998	C/G	CC	110	0.4000	42	0.2937	27	0.1051
		CG	128	0.4655	45	0.3147	182	0.7082
		GG	37	0.1345	56	0.3916	48	0.1868
251343999	A/G	AA	29	0.1055	3	0.0210	37	0.1440
		AG	154	0.5600	55	0.3846	113	0.4400
		GG	92	0.3345	85	0.5944	107	0.4163
251344000	C/T	CC	252	0.9164	143	1.0000	257	1.0000
		CT	23	0.0836	0	0	0	0
		TT	0	0	0	0	0	0

NCBI = National Center for Biotechnology Information; SNP = single nucleotide polymorphism.

Lack of association of SNPs of the *Flt-1* with growth traits in Nanyang cattle breed

The relationship between the different genotypes of each SNP and the various traits recorded was evaluated using a single-marker mixed-model association analysis. Genotypes for the 12 SNPs tested did not significantly influence any of the 6 traits measured. The data containing genotype means, standard errors, P values, estimates of additive and dominance effects for SNP with significant trait associations were not shown.

DISCUSSION

Nanyang, Qinchuan and Jiaxian Red breeds have been important cattle breeds in China for thousands of years. People have long been using a conventional breeding method to improve multiple traits of beef cattle, which calls for a long cycle and causes low efficiency. In recent years, with the development and application of molecular biotechnology, MAS can complement the traditional breeding method. MAS has the potential to significantly increase the rate of genetic improvement in some traits (MacNeil and Grosz, 2002), using markers linked to economically relevant traits, which can be used to predict the genetic merit of an animal. Several such markers have been identified in the last decade. These markers included polymorphisms in the *LEP* (*leptin*) gene, involved in the control of appetite and energy metabolism, which have been shown to be associated with body weight and body size indexes (Yang et al., 2007). The polymorphisms of the *GHRHR* gene (growth hormone-releasing hormone receptor) was associated with average daily gain in Chinese bovine breeds (Zhang et al., 2008). Variations detected in the *PRKAB1* (protein kinase, AMP-activated, beta 1 non-catalytic subunit) gene underpin the development of gene markers for bovine energy balance and in glycogen metabolism (Zhang et al., 2009).

There is a considerable complexity in VEGF signaling. VEGF and its two transmembrane tyrosine-kinase receptors, Flt-1 (VEGFR1) and KDR/Flk-1 (VEGFR2), constitute a ligand-receptor signaling system that is crucial for developmental angiogenesis. Flt-1 was the first RTK identified as a VEGF receptor (De Vries et al., 1992), but the precise function of this molecule is still debated in the field (Ferrara, 2004). The functions and signaling properties of Flt-1 appear to vary with the developmental stage and the cell type, e.g., endothelial versus non-endothelial cells. In spite of the uncertain role of Flt-1 as a signaling receptor, knockout studies have demonstrated its essential role during embryogenesis. *Flt-1*$^{-/-}$ mice die in utero between day 8.5 and 9.5 (Giantonio et al., 2007), because endothelial cells develop but fail to organize in normal vascular channels. Excessive proliferation of angioblasts has been reported to be responsible for such disorganization and lethality (Yang et al., 2003), lending support to the hypothesis that, at least during early development, Flt-1 is a negative regulator of VEGF action.

In a previous study, we reported a significant association between the *VEGF* gene and cattle growth traits in three Chinese cattle breeds (Pang et al., 2010). In this subsequent study, we aimed to investigate the VEGF receptor (Flt-1) as a candidate gene affecting growth traits in Chinese cattle. Given that Flt-1 is a member of the VEGF signal transduction pathway and that it is expressed in the muscle, bone and fat, it was chosen for the investigation of its association with growth traits. Statistical results, however, showed no significant relationship between the *Flt-1* polymorphism system and growth traits in the Nanyang breed in different growth periods. Therefore, we cannot suggest that the C2, C4, C9, C10, C11, C12, C13, C14, C16, C17, and C20 loci had a positive or negative effect on bovine growth traits, although C2, C4, C9, C10 C11, C12, C13, C14, C16, C17, and C20 loci within the bovine *Flt-1* gene were not considered as a DNA marker for bovine growth traits in MAS. A larger sample size (including Qinchuan and Jiaxian Red cattle breeds) and other SNPs of bovine *Flt-1* gene (from exon 20 to exon 30) are needed to confirm our findings. The novel SNPs identified by using PCR-SSCP and DNA sequencing methods has extended the spectrum of genetic variation in the bovine *Flt-1* gene, which may contribute to a better understanding of genetic variation in animal resources.

CONCLUSIONS

This study is the first to investigate the association of these polymorphisms of *Flt-1* gene with body growth in three Chinese cattle breeds. We have reported 12 novel SNPs. These sequence data have been submitted to the GenBank databases under accession Nos. ss#184956516-ss#184956519 and ss#251343993-ss#251344000. Statistical results showed no significant relationship between the *Flt-1* polymorphism system and growth traits in the Nanyang breed in different growth periods. We conclude that the identified SNPs of the *Flt-1* gene cannot be considered as a DNA marker for bovine growth traits in MAS. Other SNPs of *Flt-1* gene should be further studied.

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