Polymorphism of the VEGF gene and its association with growth traits in four goat breeds

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Abstract

The vascular endothelial growth factor (*VEGF*) is a regulator of angiogenesis which is an important physiological adaptation to increased metabolic demand. Thus, mutations of this gene may exert a significant influence on animal growth. We screened the exons of the caprine *VEGF* gene using PCR-SSCP and DNA sequencing methods in 459 individuals from four goat breeds to identify sequence variations that may have an effect on protein structure and function, and might be related to different phenotypes of growth traits. Two single nucleotide polymorphisms (SNPs) (*GU014696:g.49 G>C* and *GU014696:g.270G>A*) were identified in the P3 locus of the caprine *VEGF* gene. Significant associations were observed between the genotypes of the P3 locus and body length, body height and chest circumference. Individuals with genotype P3-B had a significantly longer body length and higher body height than individuals containing genotype P3-AB. Thus, animals of genotype P3-AB should be culled in a selection programme for fast growth. It is suggested that P3-B could be used as a molecular marker in marker-assisted selection (MAS).

Keywords: *VEGF* gene, caprine, single nucleotide polymorphism (SNP), genetic variation, PCR-SSCP
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Introduction

Domestic animals have been subjected to a long history of breeding desirable phenotypic traits slowly into their populations. Classical Mendelian genetics were used to select desirable traits. However, these breeding programmes do not allow for optimal control over specific phenotypic characteristics. As a consequence many breeders now-a-days focussed mainly on DNA markers for animal selection and breeding through marker assisted selection (MAS). This field is strongly focusing on gene loci and polymorphisms that have been discovered to be specifically associated with desirable traits related to eating quality of meat and growth efficiency. The focus in this study is on the caprine *VEGF* gene as the most plausible candidate gene affecting economically important traits in livestock.

The vascular endothelial growth factor is also called *VEGF-A*, following the identification of several *VEGF*-related factors (*VEGF-B, VEGF-C, VEGF-D, VEGF-E*). The human *VEGF* gene is located on chromosome 6p21.3 (Vincenti *et al.*, 1996) and consists of eight exons exhibiting alternate splicing to form a family of proteins (Tischer *et al.*, 1991). The splice forms of *VEGF* differ in biological properties such as the receptor types that they recognize and their interaction with heparan sulfate proteoglycans (Houck *et al.*, 1991; Tischer *et al.*, 1991). The vascular endothelial growth factor is a highly specific mitogen for vascular endothelial cells (Gerber *et al.*, 1998). It significantly influences vascular permeability and also plays a role in neovascularisation (Mukhopadhyay *et al.*, 1998). A potent synergism between *VEGF* and *bFGF* in the induction of angiogenesis has been observed (Cross & Claesson-Welsh, 2001). Angiogenesis is an important physiological adaptation to increased metabolic demand. Vascular endothelial growth factor is important for bone formation and is essential for normal growth plate morphogenesis, which includes blood vessel invasion and cartilage remodelling (Gerber *et al.*, 1999; Zelzer *et al.*, 2002). The *VEGF* has also been implicated in bone repair (Street *et al.*, 2002). During bone repair, *VEGF* is required not only for blood vessel formation, but also for normal callus volume and mineralization (Peng *et al.*, 2002; Colnot *et al.*, 2003). The *VEGF* is a major inducer of angiogenesis during embryogenesis and has been implicated in neuronal survival, neuroprotection, regeneration, growth, differentiation and migration (Sun *et al.*, 2003; Ferrara, 2004; Greenberg & Jin, 2004). Several studies have shown that polymorphisms within the *VEGF*

gene could influence the etiology of a variety of pathological conditions such as diabetic retinopathy (Awata *et al.*, 2002), prostate cancer (Lin *et al.*, 2003) and breast cancer (Krippl *et al.*, 2003). Thus, variations in this gene may exert vast influences on animal growth. However, to date, no studies on the effects of the polymorphisms of the VEGF gene on the economically important traits in livestock have been published. We therefore screened the entire exons of the caprine *VEGF* gene to identify sequence variations that might have an effect on protein structure and function and might be related to different phenotypes of growth traits.

Material and Methods

Genomic DNA samples were obtained from 459 unrelated goats belonging to four breeds: the Boer goat (BE, n = 111), Chinese Xuhuai white goat (XH, n = 108), Chinese Haimen goat (HM, n = 136) and a crossbred population (BE×XH, n = 104). They were reared in the Jiangsu Province of China. DNA samples were extracted from leucocytes according to Mullenbach *et al.* (1989). Four growth traits (body height, body length, cannon circumference and chest circumference) were measured when the goats were 18 months old (Luo & Wang, 1998). Body length index (%, body length/body height×100), chest circumference index (%, chest circumference/body height×100), cannon circumference index (%, cannon circumference/body height × 100) and trunk index (%, chest circumference/body length × 100) were calculated as described by Luo & Wang (1998). Data including eight growth traits for the four breeds were summarized by descriptive statistics and are presented in Table 1.

As there was no available sequence information of the caprine *VEGF* gene, comparative alignments of amino-acid sequences and nucleotide sequences from cattle and sheep were used to design PCR primers that would amplify complete exons and some introns of the caprine *VEGF* gene (Table 2).

For all assays processed in this study, the same conditions were used: 50 ng genomic DNA, 0.5 μM of each primer, 1 × buffer (including 1.5 mM $MgCl_2$), 200 μM dNTPs and 0.625 units of Taq DNA polymerase (MBI). The cycling protocol was 5 min at 94 °C, 35 cycles at 94 °C for 30 s, annealing for 35 s at 72 °C, with a final extension at 72 °C for 10 min.

The SSCP method was used to scan mutations within the amplified regions (Zhang *et al.*, 2007). Aliquots of 5 μL PCR products were mixed with a 5 μL denaturing solution (95% formamide deionized, 25 mM EDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), heated for 10 min at 98 °C and chilled immediately in ice. Denatured DNA was subjected to 10% polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer and a constant voltage (150 V) for 15 h at a constant temperature of 4 °C. The gel was stained with silver nitrate and visualized with a 2% NaOH solution (containing 0.1% formaldehyde) (Zhang *et al.*, 2007). The PCR products which represent different PCR-SSCP genotypes, including both homozygous and heterozygous genotypes, were purified with the GenElute PCR DNA Purification Kit (Sigma-Aldrich Corporation, USA) and sequenced using the ABI 377 sequencer according to the manufacture's instructions (Applied Biosystems, USA). Sequences were aligned using web based CLUSTAL-W (http://www.ebi.ac.uk/clustalw/index.html) software.

Population genetic indices (e.g. observed and expected heterozygosity and effective allele numbers) were calculated by the Nei methods (Nei & Li, 1979). The polymorphism information (PIC) content was calculated using the Botstein′s methods (Botstein *et al.*, 1980). The software SPSS (Version 17.0) was used to analyze the relationship between genotypes and growth traits in the goats. The adjusted linear model is $Y_{ijklmn} = \mu + B_i + S_j + D_{jk} + A_l + G_m + (SG)_{jm} + e_{jklmn}$, where Y_{ijklmn} is the trait measured on each animal, μ - the overall population mean, B_i - the fixed effect associated with the i^{th} breed, S_j - the fixed effect associated with the j^{th} sire, D_{jk} - the fixed effect associated with k^{th} dam with sire j, A_l - fixed effect due to the l^{th} age, G_m - the fixed effect associated with m^{th} genotype, $(SG)_{jm}$ - interaction between the j^{th} sire and the m^{th} genotype and e_{ijklmn} - the random error. The least square means estimates (LSM) with standard errors and multiple range tests for different genotypes and growth traits were used.

Results

According to the strong similarity between cattle, sheep and goats, six pairs of primers were designed to detect polymorphisms in exons and some introns of the caprine *VEGF* gene. The primers amplified the desired sized fragments from different regions of the caprine *VEGF* gene.

SSCP polymorphisms were detected in the P3 locus of the caprine *VEGF* gene. The number of bands

and their positions in the gel clearly showed the occurrence of DNA sequence variations (Figure 1). The PCR products of the different SSCP variants were sequenced and the partial sequence of the caprine *VEGF* gene was deposited in GenBank with accession number GU014696. The exon-intron boundaries of the caprine *VEGF* gene were predicted by aligning the goat and cattle DNA sequences with the known structure of the bovine *VEGF* gene.

Table 1 Descriptive statistics of the recorded growth traits for four goat breeds

Breeds (n)	Traits	Mean	s.e.	Min	Max
BE (111)	BH (cm)	65.55	0.69	45.00	98.00
	BL (cm)	77.00	0.75	52.00	99.00
	CaC (cm)	10.30	0.12	6.50	14.00
	ChC (cm)	82.61	1.03	50.00	99.00
	BLI (%)	118.57	1.52	84.69	177.55
	ChCI (%)	126.96	1.78	80.95	182.22
	CaCI (%)	15.83	0.21	10.87	22.92
	TI (%)	108.43	1.70	57.95	167.31
XH (108)	BH (cm)	61.36	0.51	49.00	73.00
	BL (cm)	70.63	0.74	53.00	84.00
	CaC (cm)	8.22	0.06	7.00	9.50
	ChC (cm)	77.09	0.80	59.00	93.00
	BLI (%)	115.06	0.71	133.33	115.06
	ChCI (%)	125.79	1.01	101.61	150.88
	CaCI (%)	13.47	0.12	11.29	16.67
	TI (%)	109.48	0.83	92.65	145.28
HM (136)	BH (cm)	56.80	0.31	50.00	67.00
	BL (cm)	65.43	0.49	54.00	84.00
	CaC (cm)	8.47	0.08	6.00	11.00
	ChC (cm)	75.35	0.39	65.00	85.00
	BLI (%)	115.21	0.62	93.44	130.77
	ChCI (%)	132.94	0.75	115.25	152.94
	CaCI (%)	14.91	0.12	10.91	17.24
	TI (%)	115.64	0.68	96.34	140.00
BE×XH (104)	BH (cm)	65.12	0.41	54.00	74.00
	BL (cm)	73.73	0.69	60.00	88.00
	CaC (cm)	8.63	0.08	7.50	11.00
	ChC (cm)	79.23	0.79	62.00	100.00
	BLI (%)	113.23	0.80	94.37	133.33
	ChCI (%)	121.67	0.94	94.29	143.75
	CaCI (%)	13.26	0.10	11.43	16.92
	TI (%)	107.62	0.69	92.86	125.00

BE = Boer goat; XH = Xuhuai goat; HM = Haimen goat; BE×XH = Boer goat×Xuhuai goat;
BH = body height; BL = body length; CaC = cannon circumference; ChC = chest circumference; BLI = body length index; ChCI = chest circumference index; CaCI = cannon circumference index; TI = trunk index.
n = number of observations; s.e. = standard error.

Table 2 The primer pair sequences and their information on the VEGF gene in goats

Loci	Sequence	Annealing temperature	Size (bp)
P1	F:5'- ACTTCTGGGCTGTTCTCGTTCC -3' R:5'- AGAGGTTGAGATGGCTGGATGG -3'	61.0 °C	350
P2	F:5'- CTGCCGCTGCCCATTCTT -3' R:5'- CCAACAGACCTTCCCACTCATC -3'	59.5 °C	184
P3	F:5'- CCTTTCCCGTGGTGGTTAC -3' R:5'- CACCTGCATGGTGATGTTGA -3'	58.0 °C	320
P4	F:5'- TCACCATCTGAACGCCTCT -3' R:5'- CTCCATCCCACTGCTGCTA -3'	59.0 °C	245
P5	F: 5'- TCTTGTCTTCCGCTGTGGCAT -3' R: 5'- CTCTGACTTGCTCGCCCTCTG -3'	65.0 °C	327
P6	F:5'- TGGAGGCTAGGACTGTGCTTT -3' R:5'- GCGGCTATGGGTAGTTCTGTG -3'	60.0 °C	241

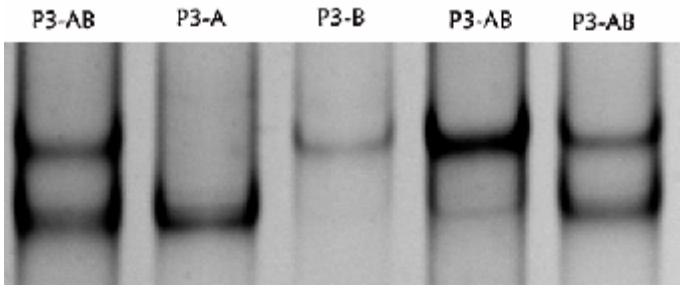

Figure 1 Genotypes at P3 locus in the caprine *VEGF* gene.

Table 3 Genotype and haplotype frequencies for the sequence variations observed in the study

Breed	Observed genotype (P3 locus)			Total	Haplotypes frequencies	
	P3-A (CC-AA)	P3-AB (CG-AG)	P3-B (GG-GG)		C-A	G-G
BE	3	16	92	111	0.099	0.901
XH	6	19	83	108	0.144	0.856
HM	10	25	101	136	0.165	0.835
BE×XH	5	13	86	104	0.111	0.889

BE - Boer goat; XH - Chinese Xuhuai white goat; HM - Chinese Haimen goat; BE×XH - crosses.

The comparisons among these sequences revealed two mutations. In detail: *GU014696:g.270G>A* caused a predicted amino acid change from Glu to Lys. The other one, *GU014696:g.49 G>C*, was located in the intron of the caprine *VEGF* gene. Three genotypes identified in the SSCP analyses for the P3 locus showed homozygous P3-A and P3-B, and heterozygous P3-AB by haplotypes C-A and G-G, respectively.

Based on SSCP and responsive sequence variations, the genotype and allelic frequencies were analyzed (Table 3). Differences in genotypic and allelic frequencies at the P3 locus among the four goat breeds were analyzed using a $\chi 2$-test, which was performed by SPSS software (version 17.0) (Table 4). There was no significant distribution difference of genotype and allele ($P < 0.05$). With PopGen software (version 3.2) and according to the Botstein's methods (Botstein *et al.* 1980), the population genetic indices (i.e. gene homozygosity, gene heterozygosity, effective allele numbers (Ne) and polymorphism information content (PIC)) were calculated (Table 5).

Table 4 P value differences for genotypic and allelic frequencies between breeds at P3 locus

Breeds	BE	HM	XH	BE×XH
BE		0.432	0.159	0.675
HM	0.363		0.831	0.550
XH	0.112	0.518		0.296
BE×XH	0.872	0.462	0.163	

P values in the upper triangle of the table represent differences in genotypic frequencies between two breeds; In the lower triangle of the table, P values represent differences in allelic frequencies between two breeds.
BE - Boer goat; XH - Chinese Xuhuai white goat; HM - Chinese Haimen goat; BE×XH - crosses.

Table 5 PIC, He, Ho and Ne at two polymorphic loci of caprine *VEGF* gene

Locus	Breeds	Gene homozygosity (Ho)	Gene heterozygosity (He)	Effective allele numbers (Ne)	Polymorphism information content (PIC)
P3	BE	0.8559	0.1441	1.217	0.162
	XH	0.8241	0.1759	1.326	0.216
	HM	0.8162	0.1838	1.382	0.238
	BE×XH	0.8750	0.1250	1.245	0.178

BE - Boer goat; XH - Chinese Xuhuai white goat; HM - Chinese Haimen goat; BE×XH - crosses.

Boer goats produced the highest means for body height, body length, cannon circumference and chest circumference, while Haimen goats produced the lowest mean values for the four growth traits (Table 1).

To investigate the effects of the P3 locus, the relationships between genotypes for the effects on variation in body height, body length, cannon circumference, chest circumference and body length index in 459 goats (Table 6) were analysed. The results indicated a significant effect of P3 locus on body length, body height and chest circumference. Individuals with genotype P3-AB were associated with a decreased body length and body height compared with genotype P3-B.

Discussion

Body height, body length, chest circumference and cannon circumference are four main growth traits which have important impacts on the production of goat meat and pelt. Therefore, breeding for optimal growth traits and higher gains is a major consideration in goat breeding programmes. Most genetic variation is represented by single nucleotide polymorphisms and many of them are believed to cause phenotypic differences between individuals. Identification of causative mutations that affect growth traits will greatly enhance the progress towards this goal.

Table 6 Association analysis of the P3 locus with growth traits in goat breeds (Boer goat, Xuhuai white goat, Haimen goat, and Boer goat×Xuhuai goat)

Growth trait (n = 459)	P3 Locus(means ± standard error of means)			
	P3-A (CC-AA)	P3-AB(CG-AG)	P3-B (GG-GG)	P-value
BH (cm)	59.88 ^{ab} ± 0.99	59.76 ^{a} ± 0.73	62.43 ^{b} ± 0.33	0.001
BL (cm)	69.96 ^{ab} ± 1.82	69.19 ^{a} ± 1.04	71.85 ^{b} ± 0.43	0.031
CaC (cm)	8.67 ± 0.23	8.64± 0.15	8.95 ± 0.07	0.105
ChC (cm)	75.54 ± 1.83	76.84 ± 1.00	78.90 ± 0.45	0.041
BLI (%)	116.76 ± 2.19	115.92 ± 1.21	115.38 ± 0.55	0.773
ChCI (%)	126.26 ± 2.45	129.24 ± 1.66	126.92 ± 0.68	0.359
CaCI (%)	14.52 ± 0.38	14.50 ± 0.21	14.40 ± 0.10	0.881
TI (%)	109.02 ± 2.69	111.99 ± 1.53	110.46 ± 0.59	0.466

Within columns means with different superscript, a and b, are significantly different (P <0.05);
BH = body height; BL = body length; CaC = cannon circumference; ChC = chest circumference;
BLI = body length index; ChCI = chest circumference index; CaCI = cannon circumference index;
TI = trunk index.
n = number of observations.

The *VEGF* gene was investigated because of the important role of *VEGF* in angiogenesis which is an important physiological adaptation to increased metabolic demand. Several studies have shown that polymorphisms within the *VEGF* gene could influence the etiology of a variety of pathological conditions (Awata *et al.*, 2002; Krippl *et al.*, 2003; Lin *et al.*, 2003). Because of its biological function, the *VEGF* gene represents an excellent candidate gene that may influence growth traits in goats.

The exons of the caprine *VEGF* gene were amplified and screened for polymorphisms. In a sample consisting of 459 goats, two DNA sequence polymorphisms based on SSCP were identified, and sequencing *GU014696:g.270G>A* demonstrated a predicted amino acid change from Glu to Lys, and *GU*014696:g.49 G>C, was located in an intron of the caprine *VEGF* gene. Comparisons of genetic diversity of the four goat breeds demonstrated that BExXH had the highest homozygosity and BE the lowest PIC in two loci. Gene heterozygosity and the PIC of the indigenous breeds (HM and XH) were higher than in the foreign breed (BE).

The association between the different genotypes and growth traits in the 459 goats was assessed. In an associated study, indications were found that support a trait relationship of the two SNPs in the P3 locus with body length, body height and chest circumference of goats. Individuals with Genotype P3-B displayed a significantly (P <0.05) higher body length and body height compared to Genotype P3-AB individuals. Furthermore, there was a tendency (P >0.05) that P3-B genotype individuals had a higher cannon circumference and chest circumference than those possessing genotype P3-AB, although no statistical differences are presented. *GU014696:g.270G>A* caused a predicted amino acid change from Glu to Lys. Thus, it might cause a functional difference to the caprine *VEGF* gene that caused phenotypic differences between individuals. Moreover, the two SNPs could be in linkage disequilibrium with another SNP in the caprine *VEGF* gene with greater effects on the traits. Introns have also been revealed to affect transcriptional efficiency of numerous genes in a variety organism.

It must be mentioned that the sample size within each genotype was not very large. If a larger number of goats was analysed, differences would probably have been clearer. However, these results provide useful information on genes that should be studied.

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