

## Comparative Mapping and 3'UTR SNP Detection of *ANGPTL4* Gene in Beef Cattle

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**Abstract:** Angiotensin-like 4 (ANGPTL4), a member of the angiotensin-like gene family, encodes a glycosylated, secreted protein with a fibrinogen C-terminal domain which is induced under hypoxic conditions in endothelial cells and is the target of peroxisome proliferation activators. ANGPTL4 is a serum hormone directly involved in regulating angiogenesis, lipid metabolism, glucose metabolism, cancer and metabolic diseases. In this study, Radiation Hybrid (RH) cloning board technique, PCR-SSCP and DNA sequencing methods were employed to map bovine *ANGPTL4* gene and examine the single nucleotide polymorphisms (SNPs) at the 3'UTR of this gene in 281 cattle from seven breeds. Here, the bovine *ANGPTL4* gene was located between the framework marker CA006 and DIK4204 on BTA7 with a map distance of 2.33 cR to the former and an interval of 8.54 cR to the latter. A novel mutation (NC\_007305.3: g.C6640T) was revealed which constructed three genotypes (CC, CT and TT). This locus proved to be significantly associated with Average Daily Gain (ADG), Rib-eye Area (REA), Intramuscular Fat (IMF) ( $p < 0.05$ ) and Beef Performance Index (BPI) ( $p < 0.01$ ) in the analyzed populations. Meanwhile, individuals with genotype TT were significantly higher than those with CC in BPI, ADG and REA while it was reversed for genotype TT and CC in the IMF which proved that bovine *ANGPTL4* gene had a positive effect on production traits. Hence, genotype TT and CC could be regarded as molecular markers for BPI, ADG and REA and IMF, respectively.

**Key words:** Cattle, *ANGPTL4* gene, RH, SNP, IMF, China

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### INTRODUCTION

Angiotensin-like 4 (ANGPTL4), originally identified as a peroxisome proliferator-activated receptor alpha and gamma target gene is an important functional gene with stimulating angiogenesis (Zhu *et al.*, 2002a, b; Hermann *et al.*, 2005; Cazes *et al.*, 2006). ANGPTL4 protein (also known as FIAF, PGAR, HARP, ARP4 and pp518) is predominantly expressed in adipose tissue and is strongly up-regulated by fasting in white adipose tissue and liver which is fatty acid-induced oxidative stress, acting on the native conformation in an unusual fashion *in vitro* (Kersten *et al.*, 2000; Sukonina *et al.*, 2006; Georgiadi *et al.*, 2010). ANGPTL4 regulates circulating triglyceride levels during different nutritional states and therefore plays an important role in inhibiting Lipoprotein Lipase (LPL) activity and hepatic cholesterol uptake

(Koster *et al.*, 2005; Shan *et al.*, 2009; Lu *et al.*, 2010). Then ANGPTL4 may provide a link between hypoxia-induced angiogenesis and metabolic disorders (e.g., diabetes and hyperlipidemia) (Le Jan *et al.*, 2003; Ge *et al.*, 2004; Lee *et al.*, 2009; Goh *et al.*, 2010) and is a candidate tumor suppressor gene which prevents the metastatic process through inhibiting vascular activity as well as tumor cell motility and invasiveness and taken to possess a potential therapeutic effect in neoplastic diseases (Zhu *et al.*, 2002a; Ito *et al.*, 2003; Galaup *et al.*, 2006; Nakayama *et al.*, 2010).

All reports about *ANGPTL4* gene concentrated on its expression, regulation, function, metabolic mechanism and application for disease therapy in human and mouse while only few reports investigated domestic animals. Moreover, the chromosome mapping information of *ANGPTL4* gene has been reported in human and pig (Zhu *et al.*, 2002b;

Feng *et al.*, 2006). Even, only one report investigated bovine *ANGPTL4* gene which indicated that liver and adipose tissue were key sources of *ANGPTL4* while the protein was highly abundant in ruminal epithelium in cattle (Mamedova *et al.*, 2010). In this study, bovine *ANGPTL4* gene was mapped by SUNbRH-PCR method on BAT 7 and a novel SNP was identified at the 3'UTR of *ANGPTL4* gene in cattle using PCR-SSCP and DNA sequencing methods.

Furthermore, the novel mutation of *ANGPTL4* gene was significantly associated with BPI, ADG, REA and IMF in cattle. Therefore, *ANGPTL4* possibly contributed to conducting association analysis and be evaluated as a genetic marker in production traits for cattle breeding and genetics.

**MATERIALS AND METHODS**

**Animals and DNA samples:** Genomic DNA samples were obtained from 281 cattle (bullock, 6 months old) belonging to seven breeds: Simmental (n = 108), Angus (n = 46), Qinchuan cattle (n = 27), Luxi cattle (n = 27), Jimnan Cattle (n = 22) and hybrids (Charolais and Limousin crossbred with indigenous female yellow cattle in China) (n = 51), selected from commercial farms. Each animal was housed in a single lattice inside of the concrete-floored cowshed during a 195 days trial with standard feed and water *ad libitum*. The amount of feed each animal consumed was recorded at 22:00 everyday while empty body weight was measured at 8:00 on 75 and 195 days following 12 h fasting. At the termination of the trial, blood samples were collected from each individual and then animals were slaughtered at a processing plant (KeErQing Beef Cattle Co., Ltd.). Records of production traits (back fat thickness, REA, marbling, IMF, tenderness, high-grade meat proportion, BPI and ADG) were collected for statistical analysis with all protocols following the Canadian Council on Animal Care (CCAC) guidelines. DNA samples were isolated from blood according to the phenol chloroform extraction method (Sambrook *et al.*, 1989).

**PCR amplification:** Two pairs of PCR primers (P1 and P2 showed in Table 1) were designed using Primer 5.0 software to amplify the bovine *ANGPTL4* gene (Gen Bank Accession No: NC\_007305.3) while P1 was used for mapping information and P2 for SNPs detection

of bovine *ANGPTL4* gene. The 15 µL PCR solution contained 25 ng DNA template, 2 µM each primer, 1.5 mM MgCl<sub>2</sub>, 0.30 mM dNTP and 0.3 U Immolase™ DNA polymerase (Bioline, London, UK). The PCR was performed using the following program: 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, annealing for 40 sec, 72°C for 40 sec and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 2% agarose gels using 1×TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na<sub>2</sub>EDTA), visualized and photographed with AlphaImager™ 2200 (Alpha Innotech Corp., San Leandro, CA, USA).

**Radiation hybrid typing:** The bovine *ANGPTL4* gene was mapped using 7000 rad SUNbRH panel of the cattle-rat-hybridization, consisting of 92 hybrids (Itoh *et al.*, 2005). The hamster and mouse genome were taken as negative control while the bovine genome as the positive control. PCR reactions were performed in 96-well Techne Touchgene thermocyclers (Burlington, NJ, USA). PCR data for the SUNbRH panel were subjected to the RHMAPPER program (Table 2) and then the vector was evaluated with 3216 framework markers (Van der Wind *et al.*, 2004; Itoh *et al.*, 2005).

**SSCP and sequencing:** SSCP method was used to scan mutations within the amplified region. The 4 µL PCR products were mixed with 8 µL denaturing solution (95% formamide deionized, 25 mM EDTA, 0.025% bromophenol blue and 0.025% xylene-cyanole), heated for 10 min at 98°C and chilled in ice rapidly. Denatured DNA was separated by 10% PAGE (polyacrylamide gel electrophoresis) in 1×TBE buffer and stable voltage (250 V for 20 min followed by 200 V for 4 h) at a constant temperature of 4°C and then gels were stained with 0.1%

Table 1: Primers used for mapping and PCR-SSCP for bovine *ANGPTL4* gene

Symbol	Primer sequence (5'-3')	Binding region	Temp. (°C)	Fragment size (bp)
A	F:CATTCCTGC	-	-	-
	CTCTCCGAATC			
	R:TGTGCCA	5'UTR-E1	58	190
B	GACGTTACCTC			
	F:CTCCAACCT	-	-	-
	GAACGCCAGTATTT			
	R:CAGTCATGGGC	E7-3'UTR	67.5	280
	ATCTTCTCTGTCT			

A and B: Names of the primer pairs; E: Exon; F: Forward primer; R: Reverse primer

Table 2: H mapping for bovine *ANGPTL4* gene

Assignment in bovine chromosome							
Gene	Location in human	Chromosome	LOD score	Marker 1	Distance from maker 1 (cR)	Marker 2	Distancefro maker 2 (cR)
<i>ANGPTL4</i>	19p13.3	7	29.40	CA006	2.33	DIK4204	8.54

silver nitrate (Kim *et al.*, 2005; Zhou *et al.*, 2006). The PCR products representing different electrophoresis patterns in different breeds were subcloned to T-vector (Promega) and then sequenced in both directions with ABI PRIZM 377 DNA sequencer (Perkin-Elmer).

**Statistical methods:** Genotypic and allelic frequencies in seven breeds were calculated using the SPSS software (Version 13.0, SPSS Inc.). Differences between allelic and genotypic frequencies were compared using the Chi-square ( $\chi^2$ ) test. Population genetic indexes (e.g., Polymorphism Information Content (PIC)) were analyzed by Nei method (Nei and Li, 1979). Relationship between genotypes and production traits in cattle were also analyzed by the SPSS software (Version 13.0, SPSS Inc.) with the following mixed linear model:

$$Y_{ijkl} = \mu + G_i + Y_{sj} + B_k + (G Y s)_{ijk} + e_{ijkl}$$

Where:

- $Y_{ijkl}$  = The trait measured on each of the ijklth animal
- $\mu$  = The overall population mean
- $G_i$  = The effect of genotype
- $Y_{sj}$  = The effect of cattle maturity
- $B_k$  = The effect of breed
- $(G Y s)_{ijk}$  = The interactions between every two effects of the model
- $e_{ijkl}$  = The random error

Difference tests in the means were performed by Duncan test for significant levels ( $p \leq 0.05$  and  $p \leq 0.01$ ).

**RESULTS AND DISCUSSION**

**Mapping information of bovine *ANGPTL4* gene:** PCR reactions for the cattle x hamster radiation hybrid panel were carried out which indicated that a 190 bp fragment

could be amplified with P1 primer pairs for bovine *ANGPTL4* gene but not for the hamster (Fig. 1). The statistical analysis of PCR data revealed that at a 2-point LOD score of 29.4, the bovine *ANGPTL4* gene was located between the framework marker CA006 (2.33 cR) and DIK4204 (8.54 cR) on BTA7 (Table 2).

**Analysis of polymorphism of bovine *ANGPTL4* gene:** A 280 bp fragment for 3'UTR of the bovine *ANGPTL4* gene was amplified with P2 primer pairs and then the bidirectional sequencing of PCR products with different patterns (CC, CT and TT) revealed that there was one mutation (NC\_007305.3: g.C6640T) (Fig. 2 and 3).

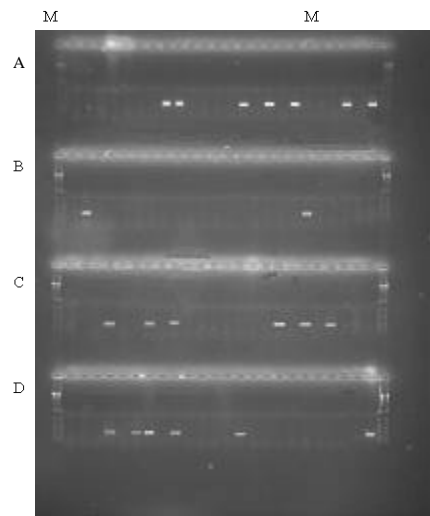


Fig. 1: PCR results of cattle x hamster SUNbRH panel for bovine *ANGPTL4* gene. M: DNA Marker; A-D: 1-24, 25-48, 49-72, 73-96 hybrids, respectively; 93 and 94 hybrids were hamster controls; 95 hybrid was water control; 96 hybrid was positive control

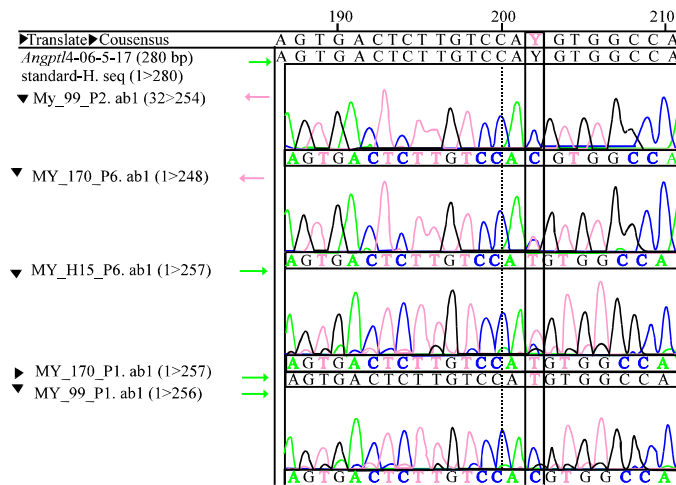


Fig. 2: Alignment of individuals with different PCR-SSCF bands displaying 3'UTR of bovine *ANGPTL4* gene. PCR products amplified by P2 primer were sequenced and a mutation (C-T) was marked in the block

Table 3: Genotype and allele frequencies within 3'UTR of bovine *ANGPTL4* gene in seven different populations

Genotypes	N	Qinchuan	Charolais	Luxi	Limousin	Jinnan	Angus	Simmental
CC	116	0.889 (24/27)	0.448 (13/29)	0.963 (26/27)	0.864 (19/22)	0.591 (13/22)	0.722 (13/18)	0.276 (8/29)
CT	10	0.074 (2/27)	0.069 (2/29)	0.037 (1/27)	0.045 (1/22)	0.091 (2/22)	0.000 (0/18)	0.069 (2/29)
TT	48	0.037 (1/27)	0.483 (14/29)	0.000 (0/27)	0.091 (2/22)	0.318 (7/22)	0.278 (5/18)	0.655 (19/29)
C(T)	-	0.926 (0.074)	0.483 (0.517)	0.982 (0.018)	0.887 (0.113)	0.637 (0.363)	0.722 (0.278)	0.311 (0.689)

T is the superior allele for Simmental and Charolais while C is the superior allele in Qinchuan, Luxi, Jinnan, Limousin and Angus cattle

Table 4: Genetic diversity within 3'UTR of bovine *ANGPTL4* gene

Bread/ index	Qinchuan	Charolais	Luxi	Limousin	Jinnan	Angus	Simmental
PIC	0.128	0.375	0.035	0.180	0.356	0.321	0.337
He	0.137	0.499	0.035	0.200	0.462	0.401	0.429
Ne	1.316	1.998	1.037	1.251	1.860	1.671	1.750

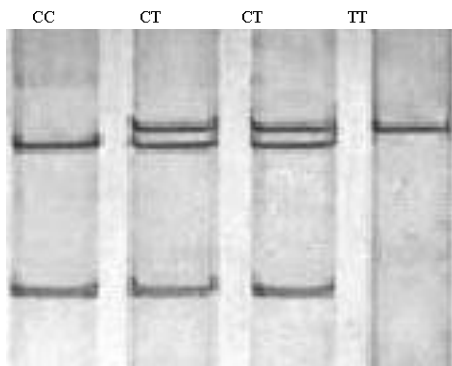


Fig. 3: PCR-SSCP of 3'UTR of bovine *ANGPTL4* gene. CC, CT and TT represent three different patterns

Genotypic and allelic frequencies and indexes of genetic polymorphism at 3'UTR of bovine *ANGPTL4* gene in seven cattle breeds were calculated (Table 3 and 4) which showed that the frequencies of allele T and genotype TT were dominant in Simmental and Charolais breeds while frequency of allele C was in dominant in the other breeds. Difference of genotypic frequency between different breeds was significant ( $p < 0.01$ ).

According to the classification of PIC (low polymorphism if PIC value  $< 0.25$ , median polymorphism if  $0.25 < \text{PIC value} < 0.5$  and high polymorphism if PIC value  $> 0.5$ ) (Botstein *et al.*, 1980), Charolais, Jinnan, Angus and Simmental populations were similar belonging to a median polymorphism level while the other breeds belonged to a low polymorphism level. At the same time, the Heterozygosity (He) and effective number of alleles (Ne) value of the former populations were noticeable  $> 3$  other breeds, especially for Luxi cattle.

The association analysis between genotypes and eight production traits indicated that significant differences were found in BPI, ADG, REA, IMF ( $p < 0.05$ ) (Table 5). Individuals with genotype TT were significantly higher than those with genotype CC in BPI ( $p < 0.01$ ) but

not for genotype CT ( $p > 0.05$ ). Meanwhile, individuals with genotype TT were significantly higher than genotype CC in ADG, REA ( $p < 0.05$ ) while individuals with genotype CC were significantly higher than genotype TT in IMF ( $p < 0.05$ ). No significant effect on the other production traits was found among the three genotypes of this locus ( $p > 0.05$ ).

ANGPTL4 protein regulates glucose homeostasis, lipid metabolism and insulin sensitivity and also acts as an apoptosis survival factor for vascular endothelial cells which has been shown to prevent the metastatic process by inhibiting vascular activity as well as tumor cell motility and invasiveness (Oike *et al.*, 2005; Nettleton *et al.*, 2008; Lichtenstein and Kersten, 2010; Clement *et al.*, 2011). However, there is hardly any report about bovine *ANGPTL4* gene.

**Radiation hybrid method for mapping bovine *ANGPTL4* gene:** Among various physical mapping techniques, the somatic hybridization technique is a more convenient method, especially used for the RH panel with 92 hybrid cells.

The mapping task could be completed in 1 day with reasonable primers, stable amplification condition and rapid detection technology. DNA markers mapped by RH panel were additive, enabling a crop of typing data of DNA markers to be analyzed at the same time.

Furthermore, the conclusion acquired could be compared conveniently among different labs, enormously facilitate the process of mapping areas. The radiation hybrid panel used in this study was got after 7000 Rads radiation which was more precise than the 3000 and 5000 Rads to obtain more precise location information and the most frequently method used for gene mapping is LOD. The larger LOD value is the linkage between undetermined gene and definite markers is more possible.

If LOD value is  $> 3$ , it is generally considered to exist gene linkage so, the location of undetermined gene could be gained accordingly. Therefore, bovine *ANGPTL4* gene was closely linked to the CA006 sign on bovine chromosome seven and at the LOD value of 29.40 which supported the mapping result of bovine *ANGPTL4* gene was reliable.

Table 5: Polymorphism analysis of 3'UTR within bovine *ANGPTL4* gene on production traits

Genotypes	Back fat thickness (cm)	Marbling (rank)	IMF (%)	Tenderness (kg)	High-grade meat (%)	BPI (kg cm <sup>-1</sup> )	REA (cm <sup>2</sup> )	ADG (kg day <sup>-1</sup> )
CC	1.153±0.490	2.043±1.219	5.756±3.714 <sup>a</sup>	4.791±1.450	0.127±0.009	4.052±0.524 <sup>A</sup>	75.310±14.819 <sup>a</sup>	0.512±0.231 <sup>a</sup>
CT	1.160±0.406	1.600±1.075	5.047±2.745	4.593±1.061	0.125±0.004	4.336±0.669	74.800±11.905	0.520±0.247
TT	1.075±0.538	2.250±0.957	4.390±2.132 <sup>b</sup>	4.784±1.520	0.124±0.006	4.530±0.470 <sup>B</sup>	81.208±12.634 <sup>b</sup>	0.687±0.419 <sup>b</sup>
M±SD	1.132±0.498	2.075±1.148	5.338±3.342	4.778±1.444	0.126±0.008	4.198±0.558	76.908±14.276	0.561±0.304
p value	0.654	0.234	0.055	0.917	0.080	0.000	0.048	0.003

Data with a different letter (A-C) and (a-c) within the same column differ significantly at p<0.001 and 0.01<p<0.05, respectively

**The identification of the SNPs:** The polymorphism of 3'UTR of bovine *ANGPTL4* gene in 281 individuals from seven different breeds were identified and characterized. While numbers of breeds Luxi, Qinchuan and Jinnan were not individually large enough to allow a meaningful statistic analysis, 281 individuals were taken as a whole to analyze the relationship between polymorphism and bovine production traits in the present study, providing a sufficient power for the mixed linear mode analysis of SPSS software (13.0).

According to the analysis of genetic polymorphism if PIC, He and Ne are larger, the number of genetic variation of this locus will be more in this breed so, it revealed Charolais>Jinnan>Simmental>Angus>Qinchuan>Limousin>Luxi in this study which approximately accord with the frequency of allele T.

The SNP can be used to construct bovine haplotype map and applied in new strategies for quality trait locus mapping. To date, few polymorphisms in bovine *ANGPTL4* gene were reported and there is identified a novel SNP (NC\_007305.3: g.C6640T) within 3'UTR by PCR-SSCP and DNA sequencing methods.

### CONCLUSION

The results indicated that genotype CC and allele C were predominant in the studied populations; significant statistical differences were found in BPI, ADG, REA and IMF according to the analysis between different genotypes and production traits in seven breeds. Hence, genotype TT and CC could be regarded as molecular markers for superior BPI, ADG and REA and IMF, respectively which accorded with the preliminary function of *ANGPTL4* gene in previous reports. Meanwhile, *ANGPTL4* gene is highly homophylic in nucleotide sequence and amino acid sequences among different species such as the human, mouse, pigs and cattle and has been identified to express abundantly in adipose tissue, relating directly with metabolic disturbance of carbohydrate and fat.

Therefore, bovine *ANGPTL4* gene seemed to be a promising candidate for its crucial roles in production

traits and fat metabolism which would possibly contribute to evaluating them as genetic markers for cattle breeding and genetics.

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