Associations of Single SNP and Q-PCR of *Leptin* Gene with Growth Traits in Chinese Luxi Cattle

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Abstract: Leptin is encoded by *obsese* gene is an adipocyte-secreted cytokine. The main function of it is to regulate the balance of energy through the signal transduction pathway and it has a strong relationship with the function of reproduction, organism immune. PCR-SSCP and SYBGREEN Q-PCR was used to analyze the polymorphism of *leptin* gene and expression difference in 139 samples of Luxi (LX) cattle breeds. The association of variations of *leptin* gene with growth traits was analyzed. Some indexes of the individuals with genotype BB were higher than that with genotype AA and AB in LX breed such as the indexes of body length, heart length, body weight, hucklebone width, body height. Leptin express high in the white adipose tissue but there is little expression in the organ or the tissue such as heart, liver, kidney, spleen, lung and muscle. These results may be applied to Marker-Assisted Selection (MAS) of Chinese LX cattle breeds improving the economic traits of cattle.

Key words: Chinese Luxi cattle, *leptin* gene, PCR-SSCP, Q-PCR, polymorphism, growth traits

INTRODUCTION

The mRNA molecule as the link between DNA and proteins is of central interest in bioscience and medicine. In recent years, the profiling of mRNA transcription has become a popular research field. Changes in mRNA transcription levels are crucial during cattle developmental processes, different tissues and environmental conditions. qRT-PCR has become the most emerging method for quantification of mRNA transcription levels due to its outstanding accuracy, broad dynamic range and high sensitivity (Zhang *et al.*, 1994).

Leptin is a 16 kDa protein expressed in adipose tissue and is involved in the regulation of feed intake, energy balance, fertility and immune functions. Leptin binds to its receptor localized mainly on neuropeptide Y-neurons which results in a reduction of feed intake and an increase of energy expenditure (Sommer *et al.*, 1992). Many researches indicated that leptin plays an important role in the regulation of growth, development and feed conversion efficiency (Kononoff *et al.*, 2005; Liu *et al.*, 1997; Schenkel *et al.*, 2005). When compared to foreign beef cattle breeds, Chinese indigenous cattle breeds have slower growth rate and lower ability of beef production because of the lower milk-producing capacity but have a relatively favorable meat quality. To meet the increasing demand of the consumers for the beef with high quality thus experiments were carried out to increase the yield and improve the quality of meat. Genetic improvement of Chinese indigenous cattle has been made using traditional methods of selection based on phenotypic information. Although, the nature of genes influencing the economically important traits is vaguely known, the genetic selection by this method has been very successful.

However, the effects of polymorphisms and expresion level within *leptin* gene on LX cattle have not been reported until now. The objectives of this study were to detect the Single Nucleotide Polymorphism (SNP) and expression level of *leptin* gene and to investigate their associations with the growth traits in 139 samples in LX cattle breeds. It is expected that the results of this study will provide basic data for marker-assisted selection related to the early growth traits and theory base for the improvement of genetic characters of the Chinese indigenous cattle.

MATERIALS AND METHODS

Sample collection and growth traits measurements: The study was conducted on a total of 139 individuals in Chinese Luxi cattle (LX) all of which were female and reared in the province of Shandong Province. Both maintenance and feeding were similar for all animals and

remained in accordance with obligatory standards. Blood samples were collected from the aforementioned cattle and genomic DNA was isolated from leukocytes as recommended by Sambrook and Russell (2001). In addition, the phenotypic data on growth traits such as body weight, body height, body length, chest girth and average daily gain was measured and collected for statistical analysis.

For the quatitive real time PCR, researchers collected the spleen, heart, liver lung kideny and muscle for every cattle, all samples were frozen in liquid nitrogen and kept at -80°C until use. The 109 individues cattle's tissue were collected in total in the slaughter house.

RNA isolation, quality control and cDNA synthesis: Total RNA were isolated from samples using RNA Total RNA kit extractions (Takara). RNA quality was confirmed by gel electrophoresis. In general, the 28S band was stronger than the 18S rDNA band indicating high RNA quality. Total RNA concentration and integrity were checked with a nucleic acid analytic apparatus (bio rad) before and after Dnase I digestion. Only RNA samples with 260/280 wavelength ratio between 1.9 and 2.1 and 260/230 wavelength ratio >2.0 before and after Dnase I digestion were used for cDNA synthesis. The first-strand of cDNA was synthesized using the M-MLV reverse transcriptase (Takara, Japan) according to the manufacturer's protocol and diluted 1:10 before use in qRT-PCR assays.

Primer design, q RT-PCR and PCR-SSCP: *β-actin* a widely used reference gene candidate was selected from reverse-transcribed cDNA in an Expressed Sequence Tag (EST) library. Primers were designed using Primer Express 2.0 Software (PE Applied Biosystems, USA) under default parameters. The primer sequences are given in Table 1. All RT-PCR reactions were viewed on 1.2% agarose gels and revealed single bands with a predicted molecular size and then the band was purified and prepared the standard curves by q RT-PCR.

Quantitative RT-PCR was performed on a Light Cycler with the Light Cycler-RNA Amplification kit SYBR

Green I (Takara, Japan) and carried out in 25 μL reaction volumes containg 1 μL of each cDNA. The thermal profile comprised three segments: 95°C for 2 min; 40 cycles of 20 sec denaturation at 95°C, 20 sec annealing at 55°C and 20 sec extension at 72°C (amplification data collected at the end of each extension step); dissociation curve consisting of 1 min incubation at 95°C, 30 sec incubation at 55°C, a ramp up to 95°C. Three technical replicates were analysed for each biological replicate. Gene expression levels were recorded as Ct values which are inversely related to the initial DNA concentration (Sejrsen *et al.*, 2000). All the data from one gene were recorded at the same time and have the same threshold (0.2). Average Ct values of each sample were calculated via the standard curve (which takes into account primer effciency) (Paolacci *et al.*, 2009). A standard curve generated using a fourfold dilution series of one sample over at least five dilution points that were measured in triplicate which is According to the slopes of the standard curve by using the equation $E_s = 10^{(-1/slope)}-1$ (Ramakers *et al.*, 2003).

PCR was carried out in 25 μL reaction volume containing 20 ng of DNA, 0.4 μM of each primer, 50 μM of each dNTP (Tiangen Biotech Co., Ltd. China), 0.6 U of Taq DNA polymerase (Sino-American Biotechnology Co., China), 1×PCR buffer (10 mM Tris-HCl [pH 9.0 at 25°C]; 1.5 mM MgCl$_2$; 50 mM KCl; 0.1% Triton X-100). PCR was carried out on a PTC-200 thermocycler (MJ Research Inc.) under the following conditions: 97°C for 2 min, followed by 35 cycles of 95°C for 45 sec, 63°C for 1 min and 72°C for 1 min. After 35 cycles, reactions were finished by an extension of 5 min at 72°C described in the earlier research (Deprez *et al.*, 2002; Zhao *et al.*, 2004).

Single-Strand Conformation Polymorphism (SSCP) Method was used to screen for mutations within the amplified region. The reaction mixture which included 10 μL of digested PCR product, 10 μL of ddH$_2$O and 12 μL of loading dye was denatured at 95°C for 5 min and placed in ice for 10 min. The samples were then loaded on 10% nondenaturing polyacrylamide gels with 10% urea or 10% formamide to improve the resolution of the DNA bands on the gel.

Data analysis and statistical analysis: The Microsoft Excel file of average expression Ct values and Standard Deviation (SD) for the *leptin* gene in 6 different tissues in 129 samples.

Genotypic and allelic frequencies were calculated for each polymorphism. Hardy-Weinberg equilibrium was estimated through χ^2-test performed by PopGene Software V 3.2. Heterozygosity (H) and Polymorphism Information Content (PIC) were determined by the HET Program (Ott, 1997). Linkage Disequilibrium (LD) of the SNPs in

Table 1: Selected candidate reference genes used in the real-time PCR assays indicating gene name, symbol, accession number, gene description, primer sequence 5'-3', PCR efficiency, amplicon size and Tm

Gene name	Primer sequence 5'-3' (forward and reverse)	Amplicon size	Tm	R^2	PCR efficiency
β-actin	GTCGACACCGCAACCAGT AGGGTCAGGATGCCTCTCTT	160	60	0.999	96.5%
Leptin	AAAACCCTCATCAAGACAA GTGGAGCCCAGGGATTGAA	180	60	0.998	107.7%
Leptin	GGGAAGGGCAGAAAGATAGG AGGCAGACTGGTGAGGATC	330	60	-	-

five cattle breeds was analyzed by the Expectation Maximization (EM) algorithm as obtained through the Haploview Software (Barrett *et al.*, 2005). Haplotype frequencies were analyzed by PHASE V 2.1.1 (Stephens and Donnelly, 2003).

The association analysis was carried out on the growth traits using the General Linear Model (GLM) procedure by SPSS V 17.0 Software. The traits and leptin genotypes and the referring diplotypes were statistically analyzed using the following model:

$$Y_{ijkl} = \mu + BF_i + A_j + G_k + e_{ijkl}$$

Where:

Y_{ijkl} = The observation of the growth traits

μ = The overall mean of each trait

Bf_i = The fixed effect associated with ith breed and farm

A_j = The effect due to jth age

G_k = The fixed effect of kth genotype of each SNP or diplotype of the three SNPs and

e_{ijkl} = The random residual error $N \sim (0, \sigma_e^2)$

RESULTS AND DISCUSSION

Genetic polymorphism of the *leptin* gene and χ^2-test: χ^2-test showed that the polymorphism of the leptin locus in the population of LX was not at Hardy-Weinberg equilibrium. The genetic diversity of the locus was then calculated. Table 2 showed that the frequencies of allele A/B of LX populations was 0.523/0.477. These results showed that the population had high heterozygosity and high polymorphism information content, suggesting that it had a high level of genetic variation and information content.

Effect analysis of ANOVA on the growth traits of Chinese LX cattle: Effect analysis of ANOVA showed that genotypes had a significant effect on height at hip cross. The Linear Model was then applied to analyze the birth season effect, genotype effect and the cooperative effect of the two factors. The growth traits (body weight, withers height, body length, heart girth, hucklebone width and height at hip cross) were analyzed and the results are shown in Table 2. Table 3 shows the following results comparing to AB and BB individuals, AA individuals had higher height at cross (p<0.01). Although, there was no significant difference with respect to other parameters, there was always a trend towards favoring allele A; there was no significant difference between the individuals with genotype AB and the individuals with genotype BB (p>0.05). Table 2 shows that in height at hip cross with genotype AA in LX population were higher than that of genotype AB and BB in LX population.

Table 2: Distribution of gene and genotype frequencies in the *leptin* gene in LX cattle $\chi^2_{\alpha=0.05}(2) = 5.991$, $\chi^2_{\alpha=0.01}(2) = 9.210$

P_{AA}	P_{BB}	Jk	Hk	Ne	PIC	χ^2
0.591	0.409	0.517	0.483	1.936	0.367	1.35

Value with ** and ** differ significantly at p<0.01 and p<0.05, respectively

Table 3: Least square analysis between leptin and physical measure of Qinchuan cattle

Growth traits	AA	AB	BB
Body weight (kg)	122.8±5.795	116.01±8.884	117.22±3.548
Withers height (cm)	93.075±1.146	91.792±1.096	99.104±1.106
Body length (cm)	94.917±1.586	89.156±5.604	91.98±3.285
Heart girth (cm)	112.94±1.753	113.43±1.63	109.88±1.592
Hucklebone width (cm)	9.53±0.309	9.74±0.467	9.06±0.293
Height at hip cross (cm)	97.219±1.243[a]	95.1±1.153[b]	94.917±1.586[b]
Waist corner width (cm)	24.875±0.671	25.556±0.827	25.172±0.716
Rump width (cm)	32±0.443	31.06±1.921	31.54±0.497

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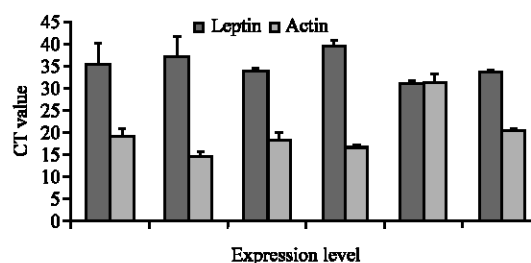

Fig. 1: Leptin expression level of different tissues in Luxi *Bos taurus*

Leptin expression difference of *Bos taurus* through the method of Q-PCR showed that *Bos taurus* leptin locus on the fourth chromosome include 3 exons and 2 introns. The full-length of leptin is 18.9 kb and the length of mRNA is 2.93 kb. Leptin express high in the white adipose tissue but there is few expression in the organ or the tissue such as heart, liver, kidney, spleen, lung and muscle (Fig. 1).

Results showed that genotypes were the main reason for the diversity of the growth traits in Chinese cattle. Leptin is a hormone affecting the regulation of body composition, energy balance and meat quality in mammals. Tian *et al.* (2013) found that polymorphisms in leptin might be one of the important genetic factors that influence carcass yield and meat quality in beef cattle and it may be a useful marker for meat quality traits in future marker-assisted selection programs in beef cattle breeding and production.

The results showed that the allele A should be associated with better growth traits of LX cattle. But for the withers height genitype AA had lower than genotype BB and AB. It may suggest that there are either multiple mutions or levels of LD in different ages and populations and species. The association of the leptin polymorphism

with growth traits of Chinese cattle revealed from this study suggests its feasibility as a molecular breeding marker. This information could help animal scientists to develop genetic markers or biomarkers in cattle breeding. According to the biological function of the leptin, it is worthy to investigate the associations between these genotype and the meat quality traits in the next study.

CONCLUSION

For the different tissue expression, the result showed that leptin has lower expression in the liver, lung, spleen, heart, kidey and muscle. With the whole genome research development, SNP and gene ontology in the LX cattle will be studied to investigate the new SNPs and their functions.

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