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Full Length Research Paper

Association between A59V polymorphism in exon 3 of leptin gene and reproduction traits in cows of Iranian Holstein

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We used the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique to screen for DNA polymorphisms of the leptin gene in 255 cows of Iranian Holstein. Amplified region is located in exon 3 of leptin gene. The genomic bovine leptin sequences, which consist of three exons, were obtained from GeneBank (Accession number U50365). Genotype frequencies in all herds were 0.588, 0.388 and 0.024 for AA, AB and BB, respectively, and allelic frequencies were 0.782 and 0.218 for A and B, respectively. We investigated effect of A59V polymorphism in the leptin gene on three reproduction traits. Significances of the genotype effects were tested using approximated F-statistic provided by SAS (v.8, GLM procedure). This study showed that genotype had no effect on open days and calving interval (NS) but had significant effect on length of pregnancy (P < 0.01). Animals with the AA genotype had higher length of pregnancy than other genotypes.

Key words: Leptin, Iranina Holstein, polymerase chain reaction-restriction fragment length polymorphism, reproduction trait.

INTRODUCTION

Leptin is a 16-kDa polypeptide hormone synthesized and secreted predominantly by adipose tissue. It functions in regulating body weight, food Intake, energy expenditure, reproduction and immune system. Leptin was first identified as gene product found deficient in obese (ob/ob) mice. A single base mutation of the leptin gene at the codon 105, as observed in the ob/ob mouse involved C/T mutation and replacement of arginine by a premature stop codon and a subsequent production of an inactive form of leptin (Zhang et al., 1994). The genetically obese ob/ob mouse exhibits obesity, infertility, hyperglycemia, impaired thyroid function and hyperinsulinemia with insulin resistance (Dubuc, 1976). Treatments of the ob/ob mice with recombinant leptin reduce feeding and body weights (Halaas et al., 1995). The gene encoding leptin was mapped to bovine chromosome 4 and it consists of 3 exons and 2 introns of which only two exons are translated into protein. The coding region of the leptin gene (501 nucleotides in length) is contained in exon 2 and 3, which are separated by intron of approximately 2 kb.

The gene encoding leptin was mapped to bovine chromosome 4 (Stone et al., 1996) and it is considered as a candidate gene for milk performance related traits in cattle. QTLs for milk performance on bovine chromosome 4, close to the leptin gene, were described (Lindersson et al., 1998). There were numerous polymorphic sites within the leptin gene. However, research on associations between leptin gene polymorphism and performance traits in dairy cattle is rather scanty (Buchanan et al., 2003; Liefers et al., 2002; Zwierzchowski et al., 2002), while literature data on associations between leptin combined genotypes and those traits are absent.

Several polymorphisms in this gene have been found (Liefers et al., 2002). In exon three, A59V polymorphism,

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Abbreviations: PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism; **QTLs,** quantitative traits loci; **NBTA,** nutrient bromothymol blue agar; **LH,** luteinizing hormone.

Table 1. Genotype and allele frequencies of the A59V polymorphism.

Herd	N	Genotype			Allele	
		AA	AB	BB	A	B
1	58	0.552	0.414	0.034	0.759	0.241
2	54	0.648	0.352	0.000	0.824	0.176
3	40	0.650	0.325	0.025	0.813	0.188
4	103	0.553	0.417	0.029	0.762	0.238
Total	255	0.588	0.388	0.024	0.782	0.218

causes amino acid change from alanine to valine. These amino acids both belong to the group of aliphatic amino acids, but valine is more hydrophobic. Expression of leptin receptor mRNA transcripts in the rat ovary, uterus, hypothalamus and anterior pituitary further demonstrate the potential of reproductive tissues for leptin responsiveness (Barash et al., 1996). Importantly, leptin-associated mechanisms appear to be conserved across species, as leptin dose-dependently attenuated insulin-induced steroid production by bovine ovarian granulosa (Spicer and Francisco,1997) and theca cells (Spicer and Francisco, 1998). This, in addition to reports that leptin stimulates gona-dotropin release in rhesus macaques (Finn et al., 1998), identifies the polypeptide as a mediator of reproduction in multiple species, from rodents to primates. In the human, mRNA transcripts for both leptin and its receptor are expressed in preovulatory follicles (Cioffi et al., 1997). The aim of this study is to consider association between A59V polymorphism in exon 3 of leptin gene and some reproduction traits in dairy cows.

MATERIALS AND METHODS

The study covered a total of 255 Holstein cows kept on four different farms in central part of Iran. The animals were kept in identical conditions: house feeding in compliance with the feeding standards. Genomic DNA was extracted from whole blood. Genotypes analyses of A59V polymorphism were performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Amplified region is located in exon three of leptin gene. The genomic bovine leptin sequences, which consist of three exons, were obtained from GeneBank (Accession number U50365). The polymerase chain reaction (PCR) was used to amplify the 331 bp DNA fragments from genomic DNA. The PCR reaction contained 100 ng of genomic DNA, 0.3 µM of each primer, 1.5 mM $MgCl_2$, 200 µM dNTP, 10mM Tris HCl, 50 mM KCl and 1 U Taq-polymerase in total volume of 20 µl. Sequences of primers that were used in PCR were reported previously by Haegeman et al. (2000). The sequences of the forward and reverse primers, respectively, were: 5´-GGG AAG GGC AGA AAG ATA G-3´ and 5´-TGG CGA ACT GTT GAG GAT C-3´. Conditions for PCR were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 30 s. Followed by final extension for 15 min at 72°C. Digestion of PCR product of 331 bp with 5 U of H*ph*I (Fermentas) in 20 µl of reaction volume at 37°C for 8 h and analysed on 8% non-denature polyacrylamide gel. Allele A in the A59V polymorphism was the allele not digested by restriction enzyme, allele B was the restriction enzyme-digested PCR product. Digestion revealed 3 genotypes, AA (331 bp), AB (331, 311 and 20 bp) and BB (311 and 20 bp). The PCR reaction resulted in an artefact band of 600 bp which did not interfere and was not digested by the enzyme. Products of amplification were recognized by electrophoresis on 1.5% agarose gel stained with ethidium bromide. The allele and genotype frequencies of A59V polymerphism were examined for deviation from Hardy-Weinberg equili-brium using χ^2 test. Data were analyzed using PROC GLM of SAS (SAS Institute Inc. year. 8th edition. SAS User Guide, Version 8, Cary, NC) using the following model:

$$Y_{ijk} = \mu + G_i + H_j + b_1 (X_{ijk} - \overline{X}) + b_2 (Z_{ijk} - \overline{Z}) + e_{ijk}$$

Y_{ijklmn} = Dependent variable; µ = Overall mean; G_i = Effect of genotype; H_j = Effect of herd; X = Effect of dry period; B_1= Linear regression for dry period trait; Z = Effect of lactation period; b_2 = Linear regression for lactation period trait; e_{ijk}= Residual error; Birth weight, calving interval and open days = Fix effect, herd and genotype Random effect.

The differences between mean values of the traits were analyses with the Duncan test.

RESULTS AND DISCUSSION

Genotype and allele frequencies of the A59V polymerphism are listed in Table 1. Genotype frequencies in all herds were 0.588, 0.388 and 0.024 for AA, AB and BB, respectively, and allelic frequencies were 0.782 and 0.218 for A and B, respectively. Allelic frequency analyses has shown that frequencies ranged from 0.759 to 0.824 for allele A and 0.176 to 0.241 for allele B in all herds. In the second herd, we did not find BB genotype. The genotype frequencies were distributed according to Hardy-Weinberg equilibrium proportions in every four herd but were not in all herds ($P < 0.01$, Table 2). Open days and calving interval did not differ among genotypes. Results also showed that genotype effect on length of pregnancy were significant ($P < 0.01$). Animals with genotype AA had higher length of pregnancy than others ($P < 0.01$, Table 2).

Our findings for A59V polymorphism in bovine leptin gene are similar to those of Kulig (2005) who reported A and B allele frequencies of 0.760 and 0.240, respectively. Liefers et al. (2002) found a frequency of 0.747 for the A allele and 0.254 for the B allele. The results showed that allele B has lower frequency in all studies. Nassiry et al. (2008) reported that allele C in Sarabi, Taleshi, Sistani, Golpayegani, Brown Swiss and Holstein cattle with 68,

Table 2. Effect of the A59V polymorphism on reproductive traits.

Trait	Genotype (LS means ± SE)		
	AA	AB	BB
Calving interval	407.51 ± 0.6[a]	416.05 ± 0.8[a]	394.3 ± 0.3[a]
Open days	128.15 ± 1.1[a]	135.72 ± 1.3[a]	119.71 ± 1.6[a]
Length of pregnancy	279.17 ± 0.47[b]	276.96 ± 0.57[a]	274.8 ± 2.2[a]

Least square means within a row without a common superscript letter differ ($P \leq 0.05$).

55, 69, 71, 55 and 57% value, respectively, were the most frequent alleles. Observed heterozygosities were highest in Golpayegani (57.89%). Buchanan et al. (2002) described a cytosine (C) to thymine (T) substitution (C T substitution) in intron 2 of the leptin gene of the *Bos taurus* breeds, that is Angus, Charolais, Hereford and Simmental, suggesting the existence of C and T alleles and therefore CC, TT and CT genotypes. Choudhary et al. (2005) identified three *Kpn*2I digestion patterns in the *B. taurus* and crossbreds indicating three genotypes: an intact 94 bp fragment as TT genotype; 75 and 19 bp fragments as CC genotype; and 94, 75 and 19 bp fragments as CT genotype. Buchanan et al. (2002) reported T allele frequencies of 0.58 in Angus cattle, 0.34 in Charolais cattle, 0.55 in Hereford cattle and 0.32 in Simmental cattle (all taurine cattle), while Konfortov et al. (1999) found the T allele frequency to be 0.41 in taurine cattle. In all the four breeds of indicine cattle, the C allele frequency was 1.0, possibly due to the absence of the V allele (valine) in these populations.

Woodside et al. (1998) showed that leptin influences the length of the anestrus period in rats suffering from severe negative energy balance due to food deprivation. As high producing cows also suffer from a negative energy balance due to lactation, leptin may influence the postpartum anestrus period in early-lactating cows. Two days of total feed restriction in 11 – 12-month-old heifers markedly reduced leptin mRNA in adipose tissue, as well as circulating concentrations of leptin IGF-I and insulin, and reduced the frequency of luteinizing hormone (LH) pulses compared to controls (Amstalden et al., 2000). In contrast to the prepubertal heifer, short-term fasting (60 h) did not attenuate pulsatile LH release in the mature cow. Central administration of leptin increased plasma LH in fasted but not in control-fed cows (Amstalden et al., 2002). Short-term (72-h) fasting and fasting-mediated reductions in LH pulse frequency were attenuated by peripherally administered recombinant leptin (Nagatani et al., 2000).

Intracellular mechanisms involved in the ability of leptin to regulate LH secretion at the adenohypophyseal level have not been thoroughly explored. However, there are several potential pathways through which these effects could occur, including effects on Ca^{2+} ion channels, an increase in the releasable pool of LH, and/or GnRH-receptor desensitization (McArdle et al., 2002). In porcine chromaffin cells, leptin caused a sustained increase of intracellular Ca^{2+} and activated inositol 1,4,5-triphosphate production (Takekoshi et al., 2001), intracellular factors known to be associated with GnRH-receptor signaling and release of LH (Zorec, 1996).

Conclusions

This polymorphism could be further evaluated for marker assisted selection. Polymorphisms had no effect on open days and calving interval (NS). The diversity data generated for Iranian native cattle may be utilized for characterizing the genetic relationships with *Bos indicus* and *B. Taurus* from other countries as well.

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