Chapter 3

Genetic Polymorphisms of Some Bovine Lactogenic Hormones

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1. Introduction

1.1. The role of quantitative and molecular genetics in animal breeding

To date, most genetic progress for quantitative traits in livestock, especially for dairy cows has been made by selection on phenotype or on estimated breeding values (EBV) derived from phenotype, without knowledge of the number of genes that affect the trait or the effects of each gene. In this quantitative genetic approach to genetic improvement, the genetic architecture of traits has essentially been treated as a 'black box'. Despite this, the substantial rate of improvements that have been and continue to be achieved in commercial populations is clear evidence of the power of these approaches. The success of this approach depends on accurate information concerning data or data structure and genetic evaluation methods (Tambasco et al. 2003). The traits have high heritability and the traits can be measured on all selection candidates (males and females) are ideal situation for quantitative selection methods because accurate EBV can be obtained on all animals (Dekkers, 2004).

However, genetic progress may be further enhanced if we could gain insight into the black box of quantitative traits. Molecular genetics allows for the study the genetic make-up of individuals at the DNA level and may provide the tools to make those opportunities a reality, either by direct selection on genes that affect traits of interest major genes or quantitative trait loci (QTL) - or through selection on genetic markers linked to QTL. The main reasons why molecular genetic information can result in greater genetic gain than phenotypic information are: a) Assuming no genotyping errors, molecular genetic information is not affected by environmental effects and, therefore, has heritability equal to 1. b)-Molecular genetic information can be available at an early age, in principle at the embryo stage, thereby allowing early selection and reduction of generation intervals. C)

Molecular genetic information can be obtained on all selection candidates, which is especially beneficial for sex-limited traits, traits that are expensive or difficult to record, or traits that require slaughter of the animal (carcass traits) (Dekkers, 2004).

For the last decade, molecular genetics has lead to the discovery of individual genes or candidate genes with substantial effects on the traits of economic importance. Candidate gene strategy has been proposed by direct search for quantitative trait loci (QTL) (Tambasco et al. 2003). In other words, the genetic variation in a gene affects the physiological pathways and phenotype. Moreover, the proportion of genetic and phenotypic variation would be likely to affect the breeding strategy for improvement of important traits in the future. Genetic markers associated with traits of interest can be searched directly by applying molecular biology techniques. These techniques can identify genetic variation at specific loci and analyze the relationship between genetic variation at QTL and production traits (Arendonk et al., 1994). Application of molecular genetics for genetic improvement relies on the ability to genotype individuals for specific genetic loci. The information utility from candidate genes in breeding programs has potential to substantially enhance the accuracy of selection and increasing selection differences (Missohou et al., 2006).

1.2. Importance of genetic polymorphisms studies in dairy cattle breeding

For more than 50 years, dairy breeders have used genetic evaluations to identify superior animals. Selective use of these animals improved phenotypic measures for milk production and milk components, especially in Holstein cattle. However, there are some limitations to selecting on predicted breeding values. Most breeding schemes do not account for population effects on genetic diversity, and selection is optimized for genetic response in the next generation rather than the highest long-term response (Meuwissen, 1997). This selection approach also has limited ability to improve lowly heritable traits without adversely affecting production. Lowly heritable traits often include those associated with disease resistance, reproduction, duration of productive life, and some conformation traits correlated with fitness. Information from genetic markers that identify desirable alleles of economically important traits could be used with breeding values to guide mating decisions, resulting in genetic gains over a broader range of traits. In addition, marker-assisted selection (MAS) could be used to select the most desirable phenotypes affected by nonadditive gene action or epistatic interactions between loci. Soller and Beckmann (1983) proposed that MAS can also reduce the costs of the artificial insemination industry incurs using progeny test evaluations as the sole method for screening candidate bulls.

The most genetic improvement in dairy cattle industry through BLUP methodology has been made by selection of merited bulls. For this target, the recording of milk production traits is done for all industrial dairy cows, and breeding values are annually estimated for them. Thereafter, bulls are firstly selected based on parent EBVs, then some of them are proofed based on progeny test (at least 50 daughters) for using in wide artificial insemination system. The problems associated with phenotypic data recording such as long

time and high expense and also low cooperation of some dairymen cause to less accurate estimation of breeding values and thereafter in the selection process. Thus, collection of genotypic data by molecular methods in addition to phenotypic data is necessary to improve the selection procedure.

1.3. Objectives of this chapter

Many studies have reported that the candidate genes influence milk traits in cows. In addition, some genes control more than one trait. For instance, the growth hormone (*GH*) gene influences expression of growth and milk traits. The important candidate genes in bovine somatotropic axis play a key role in productivity, metabolism, reproduction and disease resistance. Therefore, The objective of this chapter is to review and evaluate the relationships between the polymorphisms of some candidate genes related with bovine somatotropic axis consist of prolactin (PRL), growth hormone receptor (GHR) and insulin like growth factore-1 (IGF-I) which have an influence on milk production traits such as milk yield, milk fat yield, milk protein yield, milk fat percentage and milk protein percentage in cows. A study of the candidate genes for significant economic traits could be applied for a direct search of QTL in order to plan a breeding program in the future.

2. Literature review

2.1. Prolactin (PRL)

2.1.1. Prolactin introduction

Prolactin is a versatile polypeptide hormone that was first identified as a product of the anterior pituitary in 1933. It is synthesized and secreted not only in the anterior pituitary gland but also produced by numerous other cells and tissues, including the mammary gland (extrapituitary prolactin). In various classes of vertebrates more than 300 actions and activities of this multifunctional hormone have been reported (Bole-Feysot et al. 1998). Based on its genetic, structural, binding and functional properties, prolactin belongs to prolactin/growth hormone/placental lactogen family group (group I of the helix bundle protein hormones) (Boulay and Paul, 1992; Horesman , Yu-Lee, 1994).

2.1.2. Structure of prolactin hormone

The prolactin molecule is arranged in a single chain of amino acids with three intramolecular disulfide bonds between 6 cystein residues (Cooke et al., 1981). In cattle, the prolactin chain consists of 199 amino acids with a molecular mass of ~23 KDa (Wallis, 1974). Prolactin is synthesized as a prohormone. Following cleavage of the signal peptide, the length of the mature hormone is between 194 and 199 amino acids, depending on species. The signal peptide contains 30 amino acids; thus the mature bovine prolactin is composed of 199 amino acids (Freeman et al., 2000).

2.1.3. Effects of prolactin on milk production

The varied effects of prolactin have been identified on the mammary gland include growth and development of the mammary gland (mammogenesis), synthesis of milk (lactogenesis), and maintenance of milk secretion (galactopoiesis). In the 1920's it was found that extracts of the pituitary gland, when injected into virgin rabbits, induced milk production. Subsequent research demonstrated that prolactin has two major roles in milk production: a) Prolactin induces lobuloalveolar growth of the mammary gland. Alveoli are the clusters of cells in the mammary gland that actually secrete milk. b) Prolactin stimulates lactogenesis or milk production after giving birth. Prolactin along with cortisol and insulin act together to stimulate transcription of the genes that encode milk proteins. The critical role of prolactin in lactation has been confirmed in mice with targeted deletions in the prolactin gene. Female mice that are heterozygous for the deleted prolactin gene (and produce roughly half the normal amount of prolactin) show failure to lactate after their first pregnancy (Freeman et al., 2000).

2.1.4. Bovine prolactin gene structure

The Bovine Prolactin gene (bPRL) found on the chromosome 23 (23q21 position) in the bovine genome (Hallerman et al., 1998). The bPRL gene is about 10 kb in size and is composed of 5 exons and 4 introns (Camper et al., 1984). This encodes the 199 amino acids mature protein in cattle (Cooke et al., 1981).

The exons of bPRL gene (GenBank: AF426315.1) consist of exon 1: 855 to 936 nt,, exon 2: 3661-3842 nt, exon 3: 6186-6293 nt, exon 4: 8321-8500 nt and exon 5: 9129-9388 That encode the 229 residues prolactin precursor (Protein ID: "AAL28075.1) (Cao et al., 2002).

All sequences of exons 2, 3 and 4 re coding sequences (CDS), but some sequences (not all) on the exon 1 (position 909 to 936 nt) and exon 5 (position 9129 to 9320 nt) are CDS. Nucleotides 909 – 936 (on exon 1) and 3661 to 3772 (on exon 2) encoded signal peptide of prolactin hormone that is separated of hormone in maturing. Matured section of hormone was encoded by CDS of exon 2 (3723-3842 nt), exon 3 (6186-6293 nt), exon 4 (8321-8500 nt) and exon 5 (9129-9317 nt) (Cao et al., 2002).

Transcription of the prolactin gene is regulated by two independent promoter regions. The proximal 5,000-bp region directs pituitary-specific expression, while a more upstream promoter region is responsible for extrapituitary expression (Berwaer et al., 1991). The Bovine prolactin cDNA is 914 nucleotides long and contains a 687-nucleotide open reading frame encoding the prolactin prohormone of 229 amino acids (Cao et al., 2002). In the 5' flanking region of the bovine prolactin gene, a distal regulatory element was found, which enhances the basal level of expression of the gene fivefold and functions independently of position and orientation. The postulated enhancer region extends from –1175 to –996 and displays considerable sequence similarity to equivalent regions in human and rat prolactin gene promoters (Brym et al., 2007).

2.1.5. Polymorphisms of bPRL gene associated with milk production

Extensive genetic polymorphism studies were carried out, finding more than 20 SNPs within the bovine PRL structure gene sequence, although all of them were silent mutations or located within introns (Sasavage et al. 1982; Brym et al. 2005). The most important polymorphism is located on exon 4 that identified by RsaI endonuclease. This SNP (A/G) has studied by many researchers. Nevertheless, a few independent groups confirmed statistically significant associations between this SNP variants and milk production traits in dairy cattle (Chung et al. 1996; Dybus, 2002; Dybus et al. 2005; Brym et al. 2005; Mehmannavaz et al., 2010). Based on Chung et al. (1996) study, it was shown the association between RsaI- PRL polymorphism with milk fat percentage. Study of this polymorphism in Jersey cattle showed a significant association with milk fat yield and milk fat percentage (Dybus, 2002). Study of RsaI- PRL polymorphism in 125 Russian Red Pied cows (Alipanah et al., 2007) and 186 Black-and-White cows and in 138 Jersey cows (Brym et al., 2005) confirmed the relationships of this SNP with milk yield, fat yield and milk fat percentage.

In the study of Li et al. (2006) Holstein dairy cows, 5′ regulation region of bovine prolactin (bPRL) gene was screened by PCR-RFLP and PCR-SSCP techniques and two mutation sites were discovered for the first time. Analysis of the association between the polymorphisms of PRL gene and milk traits showed that the XbaI-RFLP locus significantly affected milk protein and milk fat in first parity. The SSCP locus significantly affected the milk fat yield on parity 1 and milk protein yield on parity 4 ($p<0.05$).

The association analysis of the G/T SNP in position -485 of prolactin gene promoter in 649 Chinese Holstein cows with the milk performance traits indicated that the SNP in the promoter was significantly associated with milk yield, fat yield and protein yield and protein percentage (Feng et al., 2006).

2.3. Growth Hormone Receptor (GHR)

2.3.1. Growth Hormone Receptor introduction

Growth hormone (GH), also known as somatotropin, is a major stimulator of postnatal growth and milk production in cattle (Etherton and Bauman, 1998). At the tissue level, the GH action is mediated by a specific cell membrane receptor, the growth hormone receptor (GHR).

GHR is a member of the class I hematopoietin or cytokine/growth hormone/prolactin receptor superfamily. Members of this family include receptors for erythropoietin (EPO), granulocyte colony-stimulating factor (GCSF), granulocyte macrophage-colony stimulating factor (GM-CSF), The β-chain of interleukin (IL)-2 through (IL)-9, (IL)-11, (IL)-12, thrombopoietin, and leukemia inhibitory factor (LIF). Receptors for interferon (IFN) a/b, IFNg, and IL-10 are more distantly related and considered class II receptors in the family. The class I cytokine receptors span the membrane once and contain an extracellular region, a single hydrophobic transmembrane domain of 24 amino acids, and an intracellular region.

The extracellular and intracellular regions vary in length. The overall sequence homology of the class I hematopoietin receptors is low; however, there is 14–25% identity in approximately 200 amino acids of the extracellular domain. The family also contains a WSXWS (tryptophan, serine, any amino acid, tryptophan, serine) motif in the membrane proximal region of the extracellular domain that is present in all members except GHR. This motif in the GHR has the conserved substitutions YXXFS (where X is glycine, serine, lysine, or glutamic acid). There is also the presence of two pairs of cysteines usually found in the N-terminal region. GHR contains seven extracellular cysteins. The cytoplasmic domains of these receptors share some common motifs. There exists a membrane proximal proline rich motif referred to as Box 1. Box 1 is present in all members and consists of eight amino acids (c-X-X-X-AL-P-X-P, where c represents hydrophobic residues, X any amino acid, AL aliphatic residues, and P proline). In GHR, this sequence reads ILPPVPVP. Another motif, termed Box 2, is present in most of these receptors. It is characterized as a cluster of hydrophobic amino acids ending with one or two positively charged residues. In GHR, Box 2 is located approximately 30 amino acids toward the C-terminal from Box 1 and spans 15 amino acids (Kopchick & Andry, 2000).

Binding of GH to GHR activates the Janus kinase 2 (JAK2); activated JAK2 in turn activates signal transducer and activator of transcription 5 (STAT5) through phosphorylation; phosphorylated STAT5 translocates from the cytoplasm to the nucleus, where it binds to specific DNA regions and activates transcription (Herrington and Carter-Su, 2001). A well-known gene controlled by GH through this JAK2–STAT5 pathway is IGF-I, which is believed to mediate most of the growth-stimulating and at least part of the milk production-stimulating effect of GH (Etherton and Bauman, 1998). In addition to STAT5, GH-activated JAK2 also phosphorylates insulin receptor substrate 1, phospholipase C, and SHC protein, leading to changes in gene expression, enzymatic activity, or metabolite transport (Herrington and Carter-Su, 2001).

2.3.2. Growth Hormone Receptor structure

As predicted from its cDNA sequence (Hauser et al., 1990), the bovine GHR protein is a single-chain polypeptide of 634 amino acids, composed of an 18-AA signal peptide (not present in mature GHR protein) encoded by exon 2 of the GHR gene, a 242-AA extracellular domain encoded by exons 3 to 7, a 24-AA single transmembrane domain encoded by exon 8, and a 350-AA intracellular domain encoded by exons 9 and 10. The bovine GHR mRNA is heterogeneous in the 5′-untranslated region, due to initiation of transcription from different leader exons (or alternative exon 1) and alternative splicing (Jiang and Lucy, 2001).

2.3.3. Bovine Growth Hormone Receptor gene structure

The bovine GHR gene has been mapped to the proximal long arm of chromosome 20 in region 20q 7.1 (Viitala et al., 2006). The gene coding for bovine GHR consists of 9 exons (from 2 to 10) in the translated part and of a long 5′-noncoding region, that includes 9 untranslated exons – 1A, 1B, 1C, 1D, 1E, 1F, 1G, 1H, 1I (Chrenek et al., 1998). Exons from the

untranslated region are spliced alternatively and each of them has its own transcription start site. Long of exons of 2 to 10 are 72, 66, 130, 161, 179, 166, 91, 70 and 1432, respectively. All nocleotides of exons of 3 to 9 are coding sequences, but some of sequences of 2th and 10th exons are coding. Exon 2 encodes the last 11 bp of the 59-UTR, the 18-amino-acid signal peptide, and the first 5 amino acids of the extracellular domain. Exons 3–7 encode the majority of the amino acids that make up the extracellular region. Exon 8 encodes the final 3 extracellular amino acids, a 24-amino-acid hydrophobic (transmembrane) domain, and the first 4 amino acids of the intracellular domain. Exons 9 and 10 encode the remaining 346 amino acids of the intracellular domain. Exon 10 also encodes for a 2-kb 3'-UTR.

A LINE-1 element from the family of retrotransposons, about 1.2 Kbp-long, was found upstream from exon 1A (Lucy et al., 1998). Heterogeneity in the 5′ untranslated region (UTR) of the growth hormone receptor gene has been shown in different species of mammals. Nine variants of GHR mRNA have been identified in humans (V1–V9; [10]) and cattle (1A–1I; (Jiang and Lucy, 2001). In cattle, variant 1A is exclusively expressed in the liver and transcriptionally controlled by the liverenriched factor, hepatocyte nuclear factor-4 (HNF-4); (Jiang and Lucy, 2001).

2.3.4. Polymorphisms of GHR Gene in Relation with Milk Production Traits

The association of TaqI-RFLP on GHR gene with milk protein percentage in italian Holstein cows was reported by Falaki et al (1996). Study of genetic markers on GHR gene for milk traits in 128 holstein cows indicated that polymorphism GHR-AluI in 5'UTR region of GHR is associated with milk yield (Aggrey et al., 1998).

Aggrey et al. (1999) studied the association of three polymorphism of 5' UTR region of GHR (GHR-AluI, GHR-StuI and GHR-AccI) with breeding values of milk production traits in 301 Canadian Holstein bulls. They resulted that only GHR-AluI has significant effect on breeding values of milk fat yield.

The T to A substitution in exon 8 of bGHR gene results in the nonconservative replacement of a neutral phenylalanine with an uncharged but polar tyrosine residue (F279Y). The corresponding phenylalanine residue is located within the transmembrane domain 0f GHR and is conserved among all analyzed mammals. This SNP associated with a srong effect on milk yield and composition in the cows (Blott et al., 2003). The effects of this polymorphism were studied by Vittala et al., (2006) in Ayershire dairy cows and its association with milk fat percentage and milk protein percentage was showed.

A 286 bp fragment of exon 10 of bGHR in 365 Hungarian Holstein cows was amplified and genotyped by RFLP-AluI. The significant effects of this polymorphism with milk yield, milk fat percentage and milk protein percentage were shown (Kovacs, 2006).

Polymorphism analysis of A/G substitution in exon 6, detected by AluI enzyme has not any association with milk production traits (Hradecka et al., 2006) and breeding values of milk production traits (Hradecka et al., 2008).

2.4. Insulin Like Growth Factor 1 (IGF-I)

2.4.1. IGF-I introduction

Insulin-like Growth Factor I (IGF-I), also known as somatomedin C, is a member of the insulin superfamily. It was originally discovered as a mediator of growth hormone actions on somatic cell growth, but has also been shown to be an important regulator of cell metabolism, differentiation and survival (Werner et al., 1994). IGF-I is produced primarily by the liver as an endocrine hormone as well as in target tissues in a paracrine/autocrine fashion. It is found in blood and other body fluids as a complex with specific high affinity IGF binding proteins (IGFBP-1 to -6). The IGFBPs are modulators of IGF actions, which control IGF bioavailability to specific cell-surface receptors. IGF-I actions are mediated by two type I transmembrane receptor tyrosine kinases: the IGF-I receptor (IGF-I R), and the insulin receptor (INS R) that exists in two alternatively spliced isoforms (INS R-A and -B) (O'Dell and Day, 1998).

2.4.2. Protein structure of IGF-I

IGF-I is synthesized as a preproprotein that is proteolytically cleaved to generate the mature protein linked by three disulfide bonds. Mature IGF-I is highly conserved among mammals, with 100% sequence identity between the human, bovine, porcine, equine and canine proteins. Mature mouse IGF-I is a non-glycosylated, 70 amino acid (aa) residue secreted polypeptide that is derived from either a 153 aa or a 159 aa preproproteins. It shares 99% and 94% aa sequence identity with rat and human IGF-I, respectively (Rinderknecht and Humbel, 1978).

Sequences of bovine and human IGF-1 is the same form and bIGF-I is a variant of hIGF-I, deleted three N terminal peptides (glycine - proline - glutamine) of it and then bIGF-I is showed as (- 3N: IGF-1) (Francis et al. 1988).

IGF-I is synthesized in the liver and multiple other tissues. It is found in blood and other body fluids as a complex with specific high affinity IGF binding proteins (IGFBP-1 to -6). The IGFBPs are expressed in specific patterns during development. They are modulators of IGF actions, which control IGF bioavailability to specific cell-surface receptors. Their functions are further regulated by IGFBP proteases, which proteolytically cleave the IGFBPs to lower the affinity with which they bind IGFs and increase IGF bioavailability. Some IGFBPs also have IGF-independent effects on cell functions. IGF-I circulates primarily as a ternary complex with IGFBP-3 or IGFBP-5 and the acid-labile subunit (ALS). Some IGF-I is also present in binary complexes with other IGFBPs. Whereas the ternary complexes are generally restricted to the vasculature, the binary complexes freely enter the tissues.

IGF-I actions are mediated by two type I transmembrane receptor tyrosine kinases: the IGF-I receptor (IGF-I R), and the insulin receptor (INS R) that exists in two alternatively spliced isoforms (INS R-A and -B). Both IGF-I R and INS R share a highly homologous structure and are ubiquitously expressed. Each receptor is derived from a precursor that is proteolytically cleaved into two disulfide-linked subunits: The extracellular and the transmembrane-

subunits. Functional IGF-I receptors are tetrameric glycoproteins composed of two disulfide-linked IGF-I Rs or disulfide-linked hybrids of one IGF-I R and one INS R. Whereas IGF-I binds with high-affinity to homodimeric IGF-I R and heterdimeric IGF-I R:INS R-A or –B hybrids, high-affinity binding of insulin is observed only with dimeric INS R or IGF-I R:INS R-A hybrid but not with IGF-I R:INS R-B hybrid. The signaling responses from the various receptors are different depending whether insulin or IGF-I is used as the activating ligand. This kit demonstrates significant cross-reactivity with rat IGF-I and has been validated for the determination of relative mass values for natural rat IGF-I in cell culture supernates, rat serum and plasma. The amount of natural rat IGF-I measured is expressed as mouse IGF-I equivalent.

2.4.3. The role of IGF-I in milk production

Insulin-like growth factor I (IGF-I) is known as regulator of mammary gland development. This factor regulate the milk production through the stimulation of mitogenesis of mammary glands, prevention of apoptosis and mediatory of growth hormone function (GH) on milk synthesis (Lactogenesis) (Monaco et al, 2005).

Nutrient partitioning for lactogenesis is mediated and sustained by alterations in the growth hormone-insulin-like growth factor (GH-IGF) axis. Under physiological conditions, pituitary derived GH induces hepatic IGF-I synthesis via receptor mediated signalling (Bichell et al., 1992) and consequently systemic IGF-I negatively regulates GH production (Le Roith et al., 2001). However in situations of high nutrient demand, such as during NEB, the GH-IGF axis uncouples in the liver (Thissen et al., 1994) and this is associated with a reduction in total circulating IGF-I and elevated GH concentrations (Etherton and Bauman, 1998). The actions of GH vary considerably in different physiological states (Bell et al., 1995), however the net effect of this uncoupling during early lactation supports a facilitatory role for the indirect actions of GH on lipolysis and gluconeogenesis (Thissen et al., 1994) and attenuated growth promoting actions and support by IGF-I in peripheral tissues (Fenwick et al., 2008). In the dairy cow, the periparturient reduction in IGF-I synthesis is associated with a concomitant reduction in the liver-specific GH receptor type 1A (GHR1A) (Jiang et al., 2005).

2.4.4. Gene structure of IGF-I

The gene coding IGF-I in human is located on chromosome 12 at position 12q23 (Daughaday and Rotwein, 1989) and in the mouse and cow, it has been mapped on chromosome 10 (Shimatsu and Rotwein, 1987) and 5 (Miller et al., 1991), respectively. In humans the *IGF-1* gene contains 6 exons and is about 90 kbp-long (Steenbergh *et al*. 1991). Due to an alternative splicing of exons 1 and 2, two different transcripts are formed: the one with exon 1 containing 1155 nucleotides (nt), while the other one, with exon 2, is shorter and contains 750 nt. Production of these transcripts is controlled by two different promoters both containing canonical regulatory sequences – TATA-box and CCAAT-box (Jansen *et al*. 1991). It was shown that transcripts of both classes are differentially expressed in various tissues, being, however, most abundant in liver (Wang *et al*. 2003). In all bovine tissues tested, the

expression of IGF-1 class 1 transcript was higher than that of transcript 2. The expression of *IGF-1* was shown to be regulated both on the level of transcription and translation (Wang *et al*. 2003).

2.4.5. Polymorphisms of IGF-I Gene in relation with milk production traits

In cattle, a few polymorphisms has been identified in the nucleotide sequence of IGF-I gene that mainly associated with growth traits. But, there is only 3 studies, investigating the IGF-I polymorphisms with milk production traits. Hines et al. (1998) did not confirm any association of SnaBI-RFLP polymorphism on IGF-I gene with milk production traits. But, Siadkowska et al (2006) showed the significant association of the SNP with fat and protein of milk.

3. Methods and materials

3.1. Samples

Semen samples were collected from 282 progeny-tested Holstein bulls born from 1990 to 2006. They were obtained from Animal Breeding Center of Iran (Karaj, Iran). Genomic DNA from semen was extracted as previously described by Zadworny and Kuhenlein (1990).

3.2. Genotyping

3.2.1. Prolactin genotyping

The PCR was carried out according to Brym et al. (2005). Briefly, in 25 μL of a mix containing: 1.25 μL 20x PCR buffer; 1.3 μL dNTP (2 mM each); 70 pmol of each primer: forward 5' CCAAATCCACTGAATTATGCTT 3', reverse 5' ACAGAAATCACCTCTCTCATTCA 3'; 1.2 mM MgCl2; 0.8 unit TaqI DNA polymerase; 100-600 ng of genomic DNA; and H2O up to 25 μL. The PCR reaction was carried out in an Eppendorf thermocycler under the following conditions: initial denaturation (94ºC/3 min), 35 cycles of denaturation 94ºC/30 s), annealing (58.5ºC/30 s) and extension (72ºC/30 s), and final synthesis (72ºC/5 min). In order to genotyping of Bulls, 10 μL of PCR product was digested with 10 unit of RsaI restriction endonuclease and analysed by electrophoresis in 8% acrilamid gel with ethidium bromide.

3.2.2. Growth hormone receptor genotyping

A 836 bp fragment of 5'-flanking region of GHR (position -1866 to -1031) was amplified according to Aggrey et al. (1999). Briefly, the sequences of the forward and reverse primers were 5'-TGCGTGCACAGCAGCTCAACC-3' and 5'-AGCAACCCCACTGCTGGGCAT-3', respectively. The PCR amplification was carried out in 37 cycles at 95°C for 35 s, 66°C for 45 s, and 72°C for 60 s. The amplified DNA was digested for 12 hours at 37°C with 5 units of AluI restriction endonuclease. The digested DNA fragments were separated by electrophoresis in 8% acrylamid in 1X TAE buffer and visualized under UV light (UVIDOC).

3.2.3. Insulin like Growth Factor-I Genotyping

Detection of IGF-1 polymorphism was carried out according to Ge et al. (2001). Briefly, the 249-bp fragment of the IGF-1 gene was amplified using following primers: (Forward): 5'-ATTACAAAGCTGCCTGCCCC-3', and (Reverse): 5'-ACCTTACCCGTATGAAAGGAATATACGT-3'. The PCR amplification cycles were: 94°C for 1 min, 64°C for 45 s and 72°C for 1 min (31 cycles). The PCR amplified DNA fragment of the IGF-1 was digested at 37°C for 12 hours with 5 units of SnaBI nuclease. The digestion products were separated on 2% agarose gels in 1X TAE buffer and visualized in UVIDOC Imager.

3.3. Statistical analysis

The allele frequencies were calculated by simple allele counting according to the Hardy-Weinberg equilibrium (Falconer and Mackay 1996); the possible deviations of genotype frequencies from expectations were tested by chi-square ($\chi 2$).

The effect of genotypes of each gene on milk production traits, namely, milk yield (kg), fat content (%), fat yield (kg), and protein yield (kg) and protein content (%) were analyzed by GLM procedure of SAS (2002). The following statistical model was used:

$$EBV_{ijk} = \mu + year_i + G_j + e_{ijk}$$

Where, EBV_{ijk} ,the estimated breeding value for milk related traits adjusted for number of daughters; μ ,the overall mean, yeari ,the fixed effect of birth year of bulls (for genetic trends), G_j , the fixed effect of genotypes in each gene; e_{ijk} , the residual effect.

Breeding values of the bulls for milk production traits were obtained from the September 2008 Iranian Animal Breeding Center evaluations, which were based on an animal model. The model included animal effect as random effect, age of calving as covariate factor and fixed effects of herd-year-season. The reliabilities of EBVs for all the bulls were high and on average 92 %.

Average effect of allele substitution was determined by coding genotypes as 0 for low frequent homozygote genotype, 1 for hetrozigote genotype, and 2 for high frequent genotype in each gene. As described by Falconer and Mackay (1996), the regression coefficient estimates the average effect of allele substitution, or the average effect of replacing a high frequent allele with low frequent allele in each gene:

$$EBV = b_0 + b_1 (year) + b_2 (Genotypecode) + e$$

Where, EBV, the estimated breeding values as dependent variable, b_0, b_1 and b_2 representing the intercept, genetic trend and Average effect of allele substitution, respectively; year , the effect of birth years of bulls as independent variable (genetic trends), Genotypecode, assigned codes for genotypes.

For study of change trend of allelic frequencies in 18 years, the yearly Ratio of frequency of low frequent allele to high frequent allele for any gene was estimated. Then, the regression coefficient (b_1) of these yearly ratios on birth years of bulls was estimated:

$$Ratio=b_0+b_1\ (year)+e$$

4. Results and discussions

4.1. Paternal genetic trends for milk related traits

Genetic trends in the Iranian Holstein bulls were significant ($p<0.05$) for all traits and progressive for milk, fat and protein yield but it was diminishing for fat and protein content (Table 1). The progressive and diminishing trends were resulted from two reasons, very high economic importance of milk regarding fat and protein content in the Iranian Holstein selection indices; and negative correlations between milk yield, and fat or protein content.

Traits	b	SE	P-value
Milk yield (kg)	+63.7608	5.0534	<0.0001
Fat yield (kg)	+1.5073	0.1419	<0.0001
Fat content (%)	-0.0110	0.0014	<0.0001
Protein yield (kg)	1.4181+	0.1120	<0.0001
Protein content (%)	-0.0057	0.0049	<0.0001

Table 1. Paternal Path genetic trends for milk related traits in the Iranian Holstein bulls

4.2 *Rsa*I-RFLP in Bovine PRL Gene

The transition of G into A in position 8398 creates a restriction site for RsaI endonuclease. Digestion of the 294 bp PCR product with the enzyme resulted in two restriction fragments of 162 and 132 bp for AA homozygotes, one uncut fragment of 294 bp for GG homozygotes, and all three fragments for AG heterozygotes (Fig. 1).

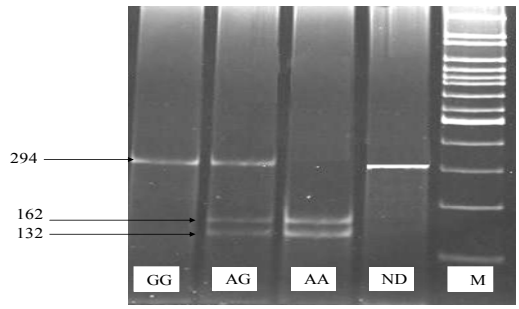

Figure 1. The 294 bp PCR products of PRL/exon4 were digested with RsaI and electrophoreses on 8% acrilamid gel. GG, AG and AA are the different genotypes of PRL/exon4. ND= Nondigested 294-bp PCR product. M= marker (100bp).

Allele frequencies were estimated in 268 bulls (0.069 and 0.931 for A and G, respectively). The frequencies of AA, AG and GG genotypes were 0.007, 0.123 and 0.870, respectively. Predicted genotype frequencies were similar to observed ones suggesting that genotype distributions were in the Hardy-Weinberg equilibrium ($\chi 2=0.477<3.82$).

Similar frequency of allele G (0.887) for Black-and-White cows and no similar frequency of allele G (0.294) for Jersey cows were reported by Brym et al. (2005). It can be explained by different history of the breeds, long-term geographical isolation, and selection towards high fat and protein contents of milk. It also indicated that PRL/exon4 SNP may be a marker of a linked SNP or locus involved in variation of milk composition.

Based on Table 2, the average allele substitution was negative and significant for milk and protein yield ($p<0.05$) i.e. allele G was an unfavorable allele for milk and protein yield. Brym et al. (2005) showed that PRL/exon4 SNP had a significant effect on milk yield and fat content in the first lactation. The result of present study for milk yield concurred with brym et al (2005), but results for fat content and protein yield were not similar. The mentioned authors showed that allele G is a favorable allele for fat content and unfavorable for milk yield. Similarly, the results of this study confirmed that allele G has negative effect on milk, fat and protein yield; and positive effect on fat and protein content, but it was significant only for milk and protein yield.

Traits	α	SE	P value
Milk yield (kg)	-203.4924	94.687	0.0325
Fat yield (kg)	-4.0107	2.5198	0.112
Fat content (%)	+0.027119	0.02376	0.2547
Protein yield (kg)	-4.3019	2.0935	0.0409
Protein content (%)	+.01755	0.00903	0.0530

α: Average substitution effects of allele G

Table 2. Average allele substitution effects of PRL/exon4 polymorphism for milk related traits

The coefficient of correlation between yearly EBV means of bulls with frequency Allele "A" to "G" ratio in any year was 0.104 and non significant (Table 3). This correlation showed that traditional selection programs did not affect the frequency of the PRL/exon 4 SNP. It was expected that number of allele A (favorable allele) would be increased during years, but the increasing rate was not significant. In the future, marker assisted selection based on major genes may increase the favorable allele frequency.

The results in table 3 showed that PRL gene polymorphism has no significant effect on genetic trends of milk performance traits. Genetic changes during years occurs in selected populations such as the analyzed sample of Iranian Holstein bulls, and the rate of change depend on selection strategies (selection indices, the traits accounting in indices, their economic and breeding coefficients, accuracy of estimated breeding values and etc). The effects of candidate genes or major genes on quantitative trait phenotype may be more than other genes, thus the effects of these genes on genetic trends may be more than others. Genetic trends in molecular level depend on change of frequency of genes (especially major genes) through years and the rate of independent effect of each gene. The results of the present study indicated that no significant effect of PRL polymorphism on milk related traits trends, Because of no significant trend in favorable allele frequency.

	Birth year	Milk yield	Fat yield	Fat content	Protein yield	Protein content
Ratio (A to G)	0.1069	0.0300	0.1083	0.1367	0.0254	-0.0496
p-value	0.6730	0.9058	0.6689	0.5886	0.9202	0.8449

Table 3. Coefficients of correlations between yearly EBV means of milk related traits and yearly ratio of allele "A" to "G" frequencies

4.3. *Alu*I-RFLP in Bovine GHR Gene

There were three AluI sites in the 836 bp fragment of 5' flanking region of the bovine GHR gene. The digested AluI(-/-) PCR product exhibited three fragments of 747 bp, 75 bp, and 14 bp (not detected on the gel). For the AluI(+/+) PCR product, the 747 bp fragment was cleaved into 2 fragments of 602 and 145 bp (Fig. 2). The polymorphic AluI site revealed a mutation at position -1182 (A-to-T transversion).

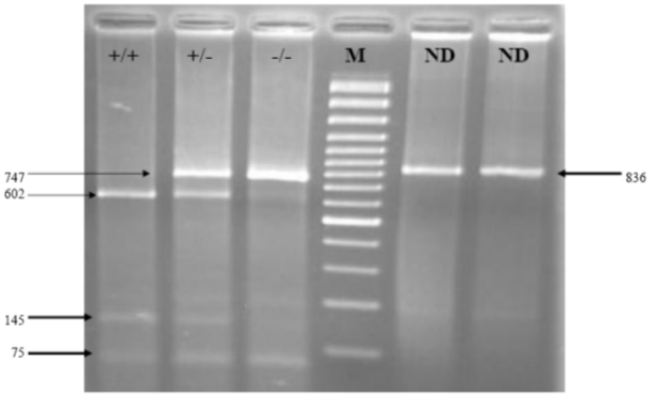

Figure 2. Digestion products of the 836 bp fragment in the 5'UTR region of growth hormone receptor gene with enzyme AluI, loaded on 8% acrilamid gel. The genotypes of AluI (-/-), AluI (+/+) and AluI (+/-) were shown in left side of gel, respectively. M: Marker (100bp), ND: undigested PCR product.

The genotype and allele frequencies at this SNP are shown in Table 4. The calculated $\chi 2$ was 1.282 and it was lower than critical value of χ^2 table ($\chi^2_{\alpha=0.05,df=1}$ =3.841), then the null hypothesis did not rejected, suggesting that genotypes distributions were in the Hardy-Weinberg equilibrium.

genotypes	AluI(+/+)	AluI (+/-)	AluI (-/-)
Number of animals	84	131	67
Observed frequency	0.298	0.465	0.237
Expected frequency	0.282	0.498	0.220
Allele frequencies	AluI (+)=0.531	AluI (-)=0.469	
Calculated $\chi 2$=1.282		Critical value of $\chi 2$ =3.841	

Table 4. The genotype and allele frequencies of GHR gene

Least square means of genotypes are presented in table 5. Bulls with AluI(+/+) genotype had best EBV for milk yield, fat yield and protein yield, but the differences between genotypes were not statistically significant (P>0.05). on the contrary, the highest fat percent and protein percent EBVs were observed in bulls with AluI(-/-) genotypes that its difference in compared to other genotypes was significant only for fat percent (P<0.05).

Trits	Genotypes			P-value
	AluI (-/-)	AluI (+/-)	AluI (+/+)	
Milk yield	-251.85	-210.34	-85.93	0.2828
Fat yield	-1.89	-2.76	-0.545	0.7411
Protein yield	-8.87	-1.73	-1.22	0.3869
Fat percent	0.113 [a]	0.057 [ab]	0.003 [b]	0.0155
protein percent	0.0488	-0.037	-0.0253	0.283

a,ab,b: Lsmeans were signed with the different letter within any row, were differ significantly (p<0.05).

Table 5. Least square means and p-value for estimated breeding values of milk related traits in Iranian Holstein bulls based on different GHR genotypes

The average allele substitution effect AluI (+) instead of allele AluI (-), estimated by Falconer and Mckay method (1996) was 80.62, 0.588, 1.772, -0.0548 and -0.007566 for EBVs of milk yield, fat yield, protein yield, fat percent and protein percent, respectively (table 6). These results showed that AluI (+) allele may be increased the milk, fat and protein yield; on the other hand, AluI(-) allele have increment effect on fat and protein percent that only the average effect of the AluI(-) allele on fat percent was statistically significant (P<0.05).

Change in ratio of AluI (+) to AluI (-) frequencies based on birth years of bulls (18 years) was studied by fitting of a linear regression. The estimated regression coefficient and its p-value were 0.01424 and 0.017, respectively. Therefore it can be said that frequency of allele AluI (+) has been increased averagely as 1.424 % per year. Based on obtained p-value (0.017) and lack of fit test, the linear relationship between allele frequency and birth years of bulls was confirmed in the Iranian Holstein bulls.

The AluI SNP was located within the 1.2-kb LINE-1 element, a retrotransposon of viral origin, inserted in the bovine GHR gene 5' region. The frequency of AluI(-) allele in present study was 0.469, that has been reported 0.473 in Canadian Holstein cattle (aggrey et al. 1999); 0.45 and 0.64 in Lithuanian Black & White and Lithuanian Red cattle, respectively (Skinkyte et al. 2005). Hetrozigosity rate (frequency of heterozygote genotype) in Iranian Holstein bulls was 0.465 and these rates were 0.45, 0.59 and 0.43 for Canadian Holstein bulls, Lithuanian Black & White and Lithuanian Red cattle, respectively. Comparison of allele frequencies and heterozygosity rate in Canadian and Iranian Holstein bulls showed these populations are similar that may be caused by similarity in selection programs of two populations. Observed differences between Iranian and Lithuanian cattle population may be due to the studied cows in the Iranian population included bulls that were used in the selection program, while the Lithuanian cows had been selected from commercial herds.

Traits	α	SE	P-value
Milk yield (kg)	80.62	53.204	0.1336
Fat yield (kg)	0.588	1.573	0.7096
Protein yield (kg)	2.022	2.870	0.4829
Fat percent (%)	-0.0548*	0.01838	0.0038
Protein percent (%)	-0.007566	0.00499	0.1338

α: Average allele substitution effects of allele AluI (+) instead of AluI (-).

Table 6. Average allele substitution effects of GHR polymorphism on EBVs of milk related traits

As reported by Aggrey et al. (1999), the AluI (+/+) bulls had higher fat EBV than the AluI (-/-) bulls. No significant differences in this study were shown between GHR genotypes for fat EBV, but the AluI(-) allele is a favorable allele for fat percent EBV. However, it seems that this polymorphism have marked effect on milk fat (fat percent and/or fat production), and observed differences between two results may be due to fat recording accuracy in Canada and Iran. The 5' region of the GHR gene contains regulatory sequences which control the expression of GHR and interact with a large number of cis-acting and trans-acting factors (Heap et al, 1995). Modulation of the affinity of binding of any of these factors may affect GHR transcription and consequently its binding ability with GH.

The significant relation between birth years and yearly ratio of allele frequencies may be resulted of association between AluI(+) allele and paternal genetic trends of economic important traits in Iranian Holstein selection program. Although, the significant correlation of allele AluI(+) with milk traits was not confirmed in this study, but its positive effect was observed, and also its likely associations with other important traits in dairy cattle breeding, such as body conformation traits is not impossible (under studding) that these reasons may be justifying of the allele frequency change during Holstein breeding program in Iran. The decreasing trend of allele frequency AluI (-) in selected years is probably due to fat percent trait is not included in the selection program of dairy cows in Iran.

One of the advantages of breeding values as the dependent variable in the association studies of polymorphism with traits is the possibility of investigation of major gene effect on genetic trends. It is recommended the various statistical models and further research for better understanding of molecular mechanisms of genetic trend.

4.4. SnaBI-RFLP in Bovine IGF-I gene

The C/T transition at position -472 in the 5'-noncoding region of the IGF-1was first reported in Angus cattle by Ge et al. (1997) as SSCP. This mutation is at position 512 bp upstream from the ATG codon. The C→T substitution creates a SnaBI restriction site and digestion of the 249 bp PCR product with the restriction SnaBI nuclease resulted in two DNA bands (223 and 26 bp) for homozygote (TT) and three bands (249, 223 and 26 bp) for the heterozygote. The DNA amplified from homozygous (CC) animals remained undigested with SnaBI restriction endonuclease (fig. 3).

Based on Table 7, the expected genotype frequencies were not similar to observed, suggesting that genotype distribution was not in the Hardy-Weinberg equilibrium ($p<0.05$).

Based on table 8, bulls with CT genotype had higher estimated breeding values of milk and fat yield compared to CC and TT genotypes ($P<0.1$). The heterozygous bulls had higher protein yield, fat and protein content (%), but the differences between genotypes for these traits were not statistically significant ($p>0.1$).

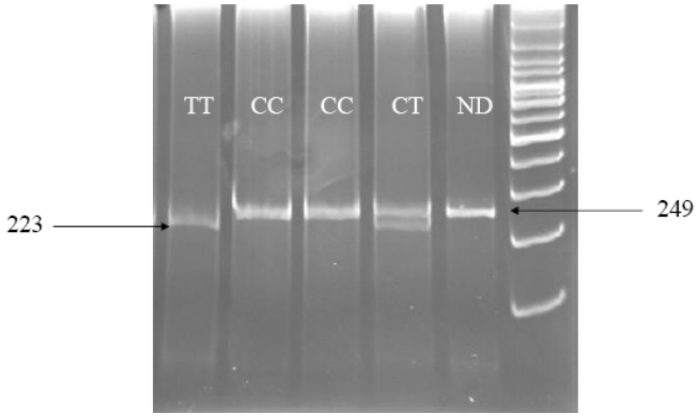

Figure 3. Acril amid gel (8%) electrophoresis showing RFLP-SnaBI in 5'-noncoding region of the bovine IGF-1 gene. TT=223 bp, CC= 249 bp, CT=249 and 223 bp, ND= undigested product and M=100 bp DNA marker. The 26 bp band was not seen in gel.

Genotypes	TT	CT	CC	Chi-square test
Number of animals	45	157	80	
Observed frequency	0.159	0.557	0.284	$\chi 2=4.878$
Expected frequency	0.192	0.492	0.316	Critical Value=3.841
Alleles	T=0.438		C=0.562	

Table 7. The observed and expected genotypic and allelic frequencies of IGF-1 gene polymorphism

Traits	Genotypes			
(EBV)	CC	CT	TT	P-Values
Milk Yield (Kg)	9.8355 [b]	118.8269 [a]	-46.819 [b]	0.072
Fat Yield (Kg)	1.5159[b]	5.4918 [a]	1.1288 [b]	0.092
Protein Yield (Kg)	2.0755	3.5566	1.2167	0.302
Fat Content (%)	0.0059	0.02815	0.02599	0.4779
Protein Content (%)	0.0150	0.0099	0.0181	0.4132

Table 8. Least square means for milk production traits in Iranian Holstein bulls with different IGF-1 genotypes

The average effect of the T allele substitution was not statistically significant and that was 28.88, 0.0962, -0.468 kg for EBVs of milk, fat and protein yields, and 0.014 and 0.0019 % for fat and protein content, respectively (table 9).

The regression coefficient of yearly frequencies of heterozygous genotype on birth years of bulls were -0.0048 and this coefficient was not statistically significant ($p<0.1$). So, the change trend of CT genotype frequencies was not linear. No significant relations were shown between yearly means of estimated breeding values of milk related traits and yearly frequencies of CT genotype i.e. no significant relation was seen between genetic trends and IGF-1 gene (Table 10).

Trait (EBV)	b	SE	P-Value
Milk Yield (Kg)	28.88	42.806	0.500
Fat Yield (Kg)	0.09622	1.1636	0.9358
Protein Yield (Kg)	0.46796	0.9269	0.6141
Fat Content (%)	-0.01406	0.01166	0.229
Protein Content (%)	-0.00196	0.00371	0.5984

b: Linear regression coefficient estimating average substitution effects of T allele

Table 9. Average allele substitution effects of IGF-1 polymorphism in EBVs of milk related traits

Trait	b	SE	P-Value
Milk yield (kg)	-0.00007	0.000087	0.4328
Fat yield (kg)	-0.00331	0.00377	0.3925
Protein yield (kg)	-0.00285	0.00382	0.4665
Fat content (%)	0.2852	0.0337	0.5366
Protein content (%)	0.7685	0.9638	0.4369

Table 10. Effects of IGF-1 gene on genetic trend in Iranian Holstein bulls

Similar frequencies of alleles C and T in bovine IGF-1 gene were found by Hines et al. (1998), who reported an estimate of 0.55 and 0.45 for the frequency of C and T alleles in a population of Holstein cattle. Also, Li et al. (2004) reported estimates of 0.56 and 0.44 in two commercial lines of dairy cattle; respectively. However, different estimates of frequencies (0.64 for (C) and 0.36 (T) alleles) were reported by Ge et al. (2001) in Angus cattle.

The association between RFLP-SnaBI of the IGF-1 gene and milk traits was studied by Siadkowska et al. (2006), using 262 polish Holstein-Friesian cows. They did not find any differences between genotypes in daily milk yield, but CT cows yielded significantly more daily fat (+20 g) and protein (+14.5 g) than the cows with CC genotype ($P<0.05$). The CT genotype also appeared favorable for fat and protein content of milk. Hines et al. (1998) reported no association between IGF-1 gene RFLP-SnaBI and dairy production traits in Holstein dairy cattle. No other papers were found in the literature concerning effects of IGF-1 polymorphism on milk production traits. The effects of this SNP have been generally tested in relation to meat production traits in previous studies.

5. Conclusion

The analysis of this study confirmed that PRL, GHR and IGF-I could be a strong candidate for application in marker-assisted selection. This study did not prove a significant effect of PRL polymorphism on paternal path genetic trends for milk production traits in Iranian Holsteins. The effects of the SNP on selection indices or other traits especially conformation traits and semen related traits of bulls should be the subject of further research.

The results proved a significant effect of IGF-1 polymorphism on EBVs for milk production traits. However, the important role of IGF-1 in the meat production process is well known, thus its polymorphism effects on other traits specially conformation traits of bulls should be the subject of further research. Also, the association of gene polymorphism with genetic trend was studied in first time in this study, and understanding of molecular mechanism of genetic trend needs to additional researches.

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