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Polymorphisms in the 5′ Flanking Region and First Exon of Insulin-Like Growth Factor 1 Gene in Southern Populations of Buffalo

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ABSTRACT

The Insulin-like Growth Factor 1 (IGF-1) system plays a critical endocrine role controlling nutrient metabolism in animals and it is a group of structurally-related polypeptides that regulate the growth of many types of mammalian cells. In liver, IGF-1 is dynamically regulated by lactation and energy balance. Less is known about the regulation of IGF-1 and IGF-binding protein mRNA in reproductive tissues and plays an important role in bovine follicular growth, acquisition of oocyte competence and embryo viability. The present study describes Polymorphisms in the IGF-1 Gene of Buffalo population in south of Iran. In order to characterization polymorphism, blood sample were collected from 95 buffalos from Ahvaz, Dezful, Shushtar, Shadegan and Susangerd cities. DNA extraction was based on Boom method and exon 1 of the IGF-1 gene was amplified to produce a 250 bp fragment. The amplified fragment were digested with *Eco105*I (*SnaB*I) restriction endonuclease. Also, the 265 bp of IGF-1 promoter in the 5′ Flanking Region was amplified and subsequently subjected to Single Strand Conformation Polymorphism (SSCP). The results were revealed one pattern (B) in digestion and all the five populations were monomorph. But, the populations exhibited three different SSCP patterns. It is possible that this gene has not exposed to selection and inbreeding is estimated high.

Keywords: Buffalo, Polymorphism, IGF-1 Gene, Flanking Region, SSCP

1. INTRODUCTION

Genetic evaluation of animal performance has been accomplished by quantitative approaches which depend on molecular technology for identifying genes of economically interesting traits and analysis of the polymorphism of genes whose products are key enzymes in the metabolic pathways of important physiological processes and are related to phenotypes (Beuzen *et al*., 2000). Candidate genes have known biological functions related to the development or physiology of an important trait (Rothschild *et al*., 1997). Such genes can encode structural proteins or a member in a regulatory or biochemical pathway affecting the expression of the trait (Bryne and McMullen, 1996) and can be tested as putative QTLs (Yao *et al*., 1996). The Growth Hormone (GH) and Insulin-like Growth Factor 1 (IGF-1) genes are candidates for growth in bovine, since they play a key role in growth regulation and development (Breier, 1999). Genetic polymorphism in native breeds is a major concern considering the necessity of preserving genetic resources. It is very important to characterize genetically indigenous breeds (Bastos *et al*., 2001). Insulin-like growth factors 1 and 2 (somatomedins-IGF-1 and IGF-2) are structurally related proteins, playing a key role in cell differentiation, embryogenesis, growth and

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regulation of metabolism. IGF1 and IGF2 were first identified by Salmon and Daughaday (1956; 1957) and designated 'sulphationfactor' due to their ability to incorporate sulphate into ratcartilage in vitro. They were also known as Non-Suppressible Insulin-Like Activity (NSILA) I and II (Froesch *et al*., 1963). A decade later, the terms sulphation factor and NSILA were replaced by the term 'somatomedin'(Daughaday *et al*., 1972) and subsequently they were renamed 'IGFs 1 and 2' due to their structural similarity with insulin and their growth-promoting activities (Rinderknecht and Humbel, 1976a; 1976b). IGF1 is one of two ligands of the IGF family (Hwa *et al*., 1999; Spicer, 2004). The established components of the IGF system also include two receptors, six high-affinity IGF-Binding Proteins (IGFBPs) and IGFBP proteases (Giudice, 1995; Hwa *et al*., 1999; Spicer, 2004). Furthermore, another group of low-affinity binding proteins, known as IGFBP-related proteins (IGFBP-rPs), belongs to the IGF family. However, no final nomenclature has been agreed for these proteins, as several research groups have identified the same protein and each group has used a different nomenclature (Hwa *et al*., 1999; Rosenfeld *et al*., 2001). Potential receptors for IGFBP(s) and IGFBP-RP (s) have also been reported to be part of the IGF family (Hwa *et al*., 1999) (**Table 1**). Due to similarities in structure and sharing intracellular signalling cascades with other members of the IGF system, insulin, its receptor and a hybrid insulin/IGF receptor are also considered to be part of the IGF superfamily (Jones and Clemmons, 1995; Monget and Martin, 1997; McCusker, 1998; Poretsky *et al*., 1999; Butler and Roith, 2001; Juul, 2003). Insulin-like Growth Factor I (IGF1) is known to play an important role in various aspects of muscle growth and development (Davis and Simmen, 2006). Due to the effect of IGF1 on the hypertrophy of muscle cells, muscle fibre diameter can be affected by IGF1 (Musaro *et al*., 2001). Since increasing muscle fibre diameter may decrease tenderness, it can be postulated that IGF1 may also affect tenderness by increasing the size of the muscle fibres (Koohmaraie *et al*., 1995). The aim of this study was to investigate relationship between DNA polymorphisms in the IGF1 gene and tenderness and muscle development. IGF-1 is a polypeptide of the molecular weight 7.5 kDa built of 70 amino acids (Daughaday and Rotwein, 1989). The amino acid sequence of IGF-1 is identical in humans, cattle, dogs and pigs (Nixon *et al*., 1999). In humans the IGF-1 gene contains 6 exons and is about 90 kbp-long (Rotwein *et al*., 1986; 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.

Table 1. The insulin-like growth factor superfamily (Velazquez *et al*., 2009)

Ligands		Receptors
IGF1		Type 1 IGF receptor
IGF2		Type 2 IGF or IGF 2/ mannose-6-phosphate
Binding protein	Binding protein proteases	(IGF2/M6P) receptor Binding protein-related proteins
IGFBP1	IGFBP2 proteases	IGFBP-rP1 a (IGFBP7/MAC25/TAF/PSF
IGFBP2	IGFBP3 proteases	IGFBP-rP2 a (CTGF
IGFBP3	IGFBP4 proteases	IGFBP-rP3 a (NovH)
IGFBP4	IGFBP5 proteases	IGFBP-rP4 a (CYR61)
IGFBP5		IGFBP-rP5 a (L56/HTRA1)
IGFBP6		IGFBP-rP6 a (ESM1
	Potential receptors	IGFBP-rP7 a
	IGFBP(s)	(rCOP-1/WISP2/CTGF-L)
	IGFBP-rP(s)	IGFBP-rP8 a (ELM1/WISP1)
		IGFBP-rP9 a (WISP3

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*., 2006). In vertebrates, the Insulin-Like Growth Factor 1 (IGF1) or somatomedin gene plays a key role in various physiological and metabolic processes, where IGF1 and growth hormone or somatotrophin is involved in the somatotropic axis. IGF1 is a mediator of many biological effects; for example, it increases the absorption of glucose, stimulates my genesis, inhibits apoptosis, participates in the activation of cell cycle genes, increases the synthesis of lipids, stimulates the production of progesterone in granular cells and intervenes in the synthesis of DNA, protein, RNA and in cell proliferation (Etherton, 2004). The bovine IGF1 gene was mapped on chromosome 5, in the centimorgan 73.5 (Grosse *et al*., 1999). The provisional nucleotide sequence is approximately 72 kb (ID number 281239). In humans, pigs, goats, rats and chickens, the IGF1 nucleotide sequence is about 70-90 kb (Rose, 2002). Exon number is different between species; for example, goats, pigs and sheep have 1-6 exons (Mikawa *et al*., 1995) and humans and rats 1-5 (Rotwein *et al*., 1986). The IGF-1 gene is extremely conserved among species and few polymorphisms are described. The presence of a microsatellite at the promoter region of this gene in bovine, human and horse allows to analyze genetic variations related to this locus (Kirkpatrick, 1992). Evidence of selection effects on allele frequencies at these two loci was found in the traditional lineage of Canchim (Regitano *et al*., 1999). Nucleotide sequence

polymorphisms were identified in the bovine IGF-1 gene and their correlations with animals' growth rate and meat performance traits were found. The Short Tandem Repeat (STR) polymorphism in the 5'-flanking region and the Single Strand Conformation Polymorphism (SSCP) in intron 3 of the IGF-1 were reported by Kirkpatrick (1992). In Hereford cattle the STR polymorphism was shown to be associated with body weight at birth and at weaning and with the growth rate (Moody *et al*., 1996). Such associations were not found in other beef breeds (Curi *et al*., 2005). The SSCP in the 5'-flanking region of IGF-1 was found by Ge *et al*. (1997) in Angus cattle. This polymorphism was then identified as T/C transition, also recognizable as RFLP-*SnaB*I (Ge *et al*., 2001). Two alleles and three genotypes were found. Allele A (with nt T at position-472) appeared significantly more frequent than allele B (with nt C) in a group of animals selected for the high IGF-1 content of blood. However, the BB genotype (with nucleotides CC) was found to be associated with higher body weight at weaning (Li *et al*., 2004). No association was found between IGF-1 RFLP-*SnaB*I and dairy production traits in Holstein cattle (Hines *et al*., 1998). Two polymorphisms in the IGF-1 were reported by Lien *et al*. (2000) in Norwegian cattle: The TTTG insertion/deletion (InDel) in intron 4 and the RFLP-DpnI in intron 5. However, the effects of these polymorphisms on beef or milk production traits were not investigated. The objective of the present study was to investigate in the 5´ Flanking Region and exon 1 of IGF-1 Gene in southern populations of Iranian Buffalo in Khuzestan province of IRAN using PBR (PCR Based RFLP) and SSCP technique.

2. MATERIALS AND METHODS

2.1. Animals and Sampling

In current study, Random blood samples were collected from 95 Buffalos from five populations involve: Ahvaz, Shadegan, Susangerd, Dezful and Shushtar cities in Khuzestan province of Iran (**Fig. 1**). Approximately, 3 ml blood sample was gathered from venom in EDTA tube and was transferred to-20°C freezer.

2.2. DNA Extraction and PCR Amplification

Genomic DNA was isolated by using DNA Extraction Kit (Diatom) and was based on Boom *et al*. (1989) method. Quantity was determined by measuring the absorbance at 260 nm and the concentration, purity and quality were determined by measuring the absorbance at 260/280 nm and 230/260 ratios using a NanoDropTM 1000 spectrophotometer (Thermo Scientific). DNA extractions were appropriately labeled and stored at-20°C for analysis. The study was concentrated on a 250 bp fragment of IGF-1 gene spanning over exon 1. Primer sequences for PCR were established by Ge *et al*. (1997; 2001) (**Table 2**). The PCR reaction volume of 25 µL contained approximately 100 ng of genomic DNA, 1unit Taq DNA Polymerase, 1×PCR Buffer, 1.5 mM MgCl2, 200 µM dNTPs and 0.3 pMol of each primer. Amplification conditions included an initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 63°C for 30 s and 72°C for 40 s, followed by a final extension at 72°C for 5 min.

2.3. Enzyme Digestion

The amplified fragment was digested with *Eco105*I (*SnaB* I). The digestion reaction contained by 5 µL of PCR product, 2 µL Buffer 10×, 5 U of *Eco105*I (*SnaB* I) and H_2O up to a total volume of 20 µL then were incubated at 37°C for 12-16 h. The digestion products were electrophoresed on 2% agarose gel in 0.5×TBE and visualized by ethidium bromide staining for 40 min at 100 V. The Statistical analysis was calculated using Popgene32 software (version 1.31).

Fig. 1. Geographical location of the populations studied

Table 2. Primer used in the amplification in the bovine IGF1 gene

Primer	Primer sequence	PCR product size (bp)	Reference
IGF677	ATTACAAAG CTGCCTGCCCC		
IGF897	ACCTTACCC GTATG AAAGGAATATACGT	250	Ge *et al*. (2001)
IGF677	GGGGCAGGC AGCTTTGTAAT TCACATCTGCTAA		
IGF917	TACACCTTACCCG	265	Ge *et al*. (1997)

2.4. Single-strand Conformational Polymorphism Analysis (SSCP)

For SSCP analysis, 5 ml of PCR product and 10 mL of diluting dye were denatured at 95°C for 10 min and immediately plunged into ice for 5 min. The dilution was loaded on to a 12% polyacrylamide gel with 10% glycerol. The gel was run at a constant voltage of 250 V at 20°C for 24 h and gels were silver stained.

3. RESULTS

The amplified of promoter in the 5′ Flanking Region and exon 1 of IGF-1 Gene resulted in a DNA fragment with 250bp and 265bp with PCR technique (**Fig. 2**). Analysis of exon 1: The results were revealed one pattern (B) in digestion, resulting in one genotype and all the five populations were monomorph. one allele (B) was observed, The *Eco105*I (*SnaB* I) indicated single restriction pattern in all the populations of buffalo and it didn't produce digestion fragments pattern (bands of approximately 226 and 23 bp) in southern populations of Iranian buffalo. This study had no impact which agrees with the results obtained in cattle with similar gene locus of IFG-1 (Laureano *et al*., 2006). The animals with one allele were assigned with BB genotype (**Fig. 3**). A and B allele frequencies were 0.0000 and 1.0000, respectively. The high frequency of the B allele suggests that this allele Variant might have been favored by selection for production traits. The observed Homozygosity and heterozygosity were 1.000 and 0.000, respectively (**Table 3**). Analysis of promoter in the 5′ Flanking Region: The PCR-SSCP analysis revealed three distinct patterns (**Fig. 4**).

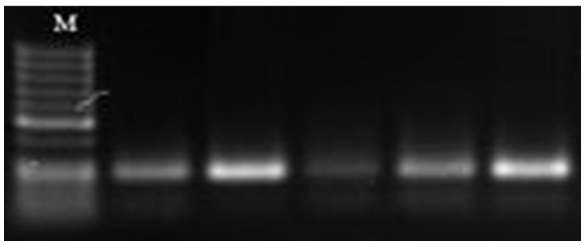

Fig. 2. PCR products of IGF-1 (Size obtained: 250 bp)

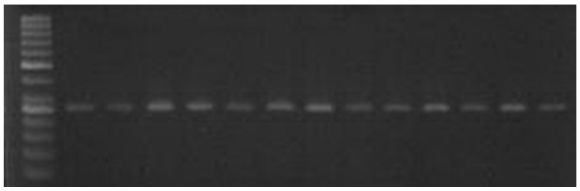

Fig. 3. IGF-1genotyping by PBR method (2% agaros gel)

All the five populations, the means of frequencies were 47.5% for pattern 1, 41% for pattern 2, 11.5% for pattern 3. We observed most frequency in pattern of 1 and low frequency in pattern 3. This study had impact which agrees with the results obtained in bufallo with similar locus of IFG-1 (Fatima *et al*., 2009).

4. DISCUSSION

Candidate genes have known biological functions related to the development or physiology of an important trait. Such genes can encode structural proteins or a member in a regulatory or biochemical pathway affecting the expression of the trait (Bryne and McMullen, 1996) and can be tested as putative QTLs (Yao *et al*., 1996). The study by Ge *et al*. (2001) characterizes a G→A transition polymorphism within an *Eco130*I site of intron 3 of the IGF1 gene in swamp buffaloes (*Bubalus b. bubalis kerebau*). Polymorphisms in the bovine IGF-I gene are associated with circulating IGF-I concentrations and growth traits. Growth in animals is controlled by a complex system, in which the somatotropic axis plays a key role. Genes that operate in the somatotropic axis are responsible for the postnatal growth, mainly GH that acts on the growth of bones and muscles mediated by IGF-1 (Sellier, 2000).

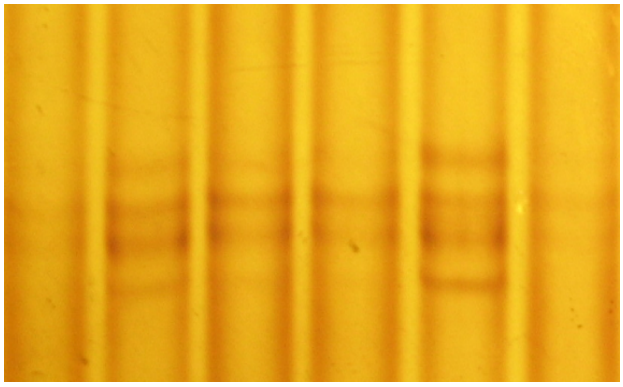

Fig. 4. The distinct patterns of SSCP analysis

Table 3. The Homozygosity and heterozygosity in different regions

Regions	Homozygosity	Heterozygosity
Ahvaz	1.000	0.000
Dezfol	1.000	0.000
Shushtar	1.000	0.000
Shadegan	1.000	0.000
Susangerd	1.000	0.000

5. CONCLUSION

The southern populations of Iranian Buffalo showed a low degree of genetic diversity for the IGF-1 locus. Also, random genetic drift can be one of the causes of the homozygosity. Although we observed the low variability for exon 1 but there was high degree of genetic diversity for the 5′ Flanking Region of IGF-1 gene. In the other hand, this data provide evidence that buffalo populations have a good polymorphism for some gene, which opens interesting prospects for future selection programs, especially marker assistant selection between different genotypes of different locus and milk, gain and meat traits. The genetic variation of 265 bp fragment used in Present Results showed that PCR-RFLP (PBR) is appropriate tools for evaluating genetic variability.

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