Identification of Novel SNPs in Bovine Insulin-like Growth Factor Binding Protein-3 (*IGFBP3*) Gene

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ABSTRACT : The insulin-like growth factors (IGFs), their receptors, and their binding proteins play key roles in regulating cell proliferation and apoptosis. Insulin-like growth factor binding protein-3 (*IGFBP3*, OMIM #146732) is one of the proteins that bind to the IGFs. IGFBP3 is a modulator of *IGF* bioactivity, and direct growth inhibitor in the extravascular tissue compartment. We identified twenty-two novel single nucleotide polymorphisms (SNPs) in *IGFBP3* gene in Korean cattle (Hanwoo, *Bos taurus coreanae*) by direct sequencing of full gene including -1,500 bp promoter region. Among the identified SNPs, five common SNPs were screened in 650 Korean cattle: one SNP in promoter (*IGFBP3 G-854C*), one in 5'UTR region (*IGFBP3 G-100A*), two in intron 1 (*IGFBP3 G-421T*, *IGFBP3 T+1636A*), and one in intron 2 (*IGFBP3 C+3863A*). The frequencies of each SNP were 0.357 (*IGFBP3 G-854C*), 0.472 (*IGFBP3 G-100A*), 0.418 (*IGFBP3 G+421T*), 0.363 (*IGFBP3 T-1636A*) and 0.226 (*IGFBP3 C-3863A*), respectively. Haplotypes and their frequencies were estimated by EM algorithm. Six haplotypes were constructed with five SNPs and linkage disequilibrium coefficients (|D'|) between SNP pairs were also calculated. The information on SNPs and haplotypes in *IGFBP3* gene could be useful for genetic studies of this gene. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 1 : 3-7)***

Key Words : Insulin-like Growth Factor Binding Protein-3 (*IGFBP3*), Single Nucleotide Polymorphism (SNP), Haplotype, Linkage Disequilibrium (LD)

INTRODUCTION

Over the last decade, the role of the IGF system in growth has been extensively studied. Although IGFBP3 can attenuate the effects of IGF-1 by sequestering it from its receptor, it may also act independently of IGF-1 via a putative receptor, inhibiting cellular proliferation (Clemmons, 1997). The gene encoding IGFBP3 is highly expressed in liver, where the bulk of the circulating protein originates, and it is also expressed in a highly regulated fashion in the various tissues in which it influences cell renewal kinetics (Phillips et al., 1998; FerryCerri and Cohen, 1999). Circulating IGFBP3 is derived primarily from hepatic Kupffer cells, under regulation by growth hormone; but IGFBP3 is also produced locally in many tissues, where it serves important paracrine and autocrine roles in modulating cellular growth and apoptosis (Ferry et al., 1999). IGFBP3 activity in the circulation and at the cellular level is regulated not only by its rate of synthesis but also by post-translational modification and proteolysis (Collett-Solberg and Cohen, 1996).

IGFs play pivotal roles in regulating fetal growth and development (D'Ercole, 1987; Stewart and Rotwein, 1996; Ryu et al., 2003). They are noncovalently bound to one of six insulin-like growth factor binding proteins, IGFBP-1, -2, -3, -4, -5 and -6 (Jones and Clemmons, 1995). Considerable evidences pointed to the essential role of the IGFBPs in controlling and regulating the biological activities of IGFs (Clemmons, 1993; Thissen et al., 1994). In addition, some of the IGFBPs, such as IGFBP3 may possess intrinsic biological activity independent of any interaction with IGF (Oh et al., 1993; Valentinis et al., 1995). IGFBP3, located in chromosome 4 (Kappes et al., 1997), is not only a modulator of IGF bioactivity and a direct growth inhibitor in the extravascular tissue compartment (Ferry et al., 1999), but also the major IGFBP in the circulation, accounting for the binding of >90% of the plasma IGFs in a trimeric 150 kDa complex containing IGF, IGFBP3 and an acid-labile subunit (Liu et al., 1990; Baxter, 1993).

In this study, 10 kb of *IGFBP3* gene, including -1,500 bp promoter region has been examined to identify additional single nucleotide polymorphisms (SNPs) in *IGFBP3* gene. Several SNPs were newly identified in various region of the gene. The allele frequency of each SNPs, linkage disequillibrium between SNPs and haplotypes were also described.

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Table 1. Primers for sequencing of bovine *IGFBP3* and primers for amplifying and extension for genotyping by single base extension (SBE) method

Region (locus)	Forward primer 5`→3`	Reverse primer 5`→3`	Extension primer 5`→3`
Promoter-1	actgcaaactgcaaaaggctcc	ttctcaatgccatgagggttcg	.
Promoter-2	aattacgcttcagcaatgctcg	gctactgcaagccccggcg	.
Exon1	ggccgccccggcttctat	ttcggcagcgcatctgg	.
Intron1-1	tgcgcgagggtcagcctt	gcccacattcccggtcca	.
Intron1-2	tactgtgtggcccaattccgat	tgaaagtctggccccaaatgat	.
Intron1-3	atcatttggggccagactttca	tggagaatgcttctggagggtt	.
Intron1-4	aaccctccagaagcattctcca	ggcactgtcctgggtaagtcct	.
Intron1-5	gtggcttccttcccagagtga	tggtactcccttggcctcaag	.
Exon2	cggaaaagctgagagcatgaaa	tgccataggggagggtcaatat	.
Exon3	atattgaccctcccctatggca	ttccctcgtccatgtcagtgg	.
Intron3-1	aaaaagcaggtgagcaccatcc	caggatggggccctctgatt	.
Intron3-2	ttcacagaacagcacagccatg	tttcccccttcacgtcgaagc	.
Exon4	ccatcctgggtgctgaccacc	ccaggtggcgcagtggtaaa	.
Intron4-1	gatcgaacccacatctcttgca	ttgctaaaacctggggctggta	.
Intron4-2	taccagccccaggttttagcaa	ccaggttcaccatgtggaagaa	.
3`UTR-1	tgcacaaaagactgccaagga	ttttccgcagtcatctggagag	.
3`UTR-2	accctttttgtcaggcagctt	tggaaagttccaactacagccg	.
*IGFBP3 G-854C**	aattacgcttcagcaatgctcg	gctactgcaagccccggcg	tcaatgatgatcaagtccctggttactaaagtggaccgg
*IGFBP3 G-100A**	caggagaccccgggacga	aaagccgcagcccagagc	gatgatgcagcagctattccaagctgagtgtc
*IGFBP3 G+421T**	tgcgcgagggtcagcctt	gcccacattcccggtcca	ggtgagctgcccgcgcca
*IGFBP3 T-1636A**	atcatttggggccagactttca	tggagaatgcttctggagggtt	atgatgataagtaagtcaaaagtttaagaccattatctgtggct
*IGFBP3 C+3863A**	atattgaccctcccctatggca	ttccctcgtccatgtcagtgg	ttggcctctgagtgtcctgg

* Number indicates the location of the SNP relative to translation start site.

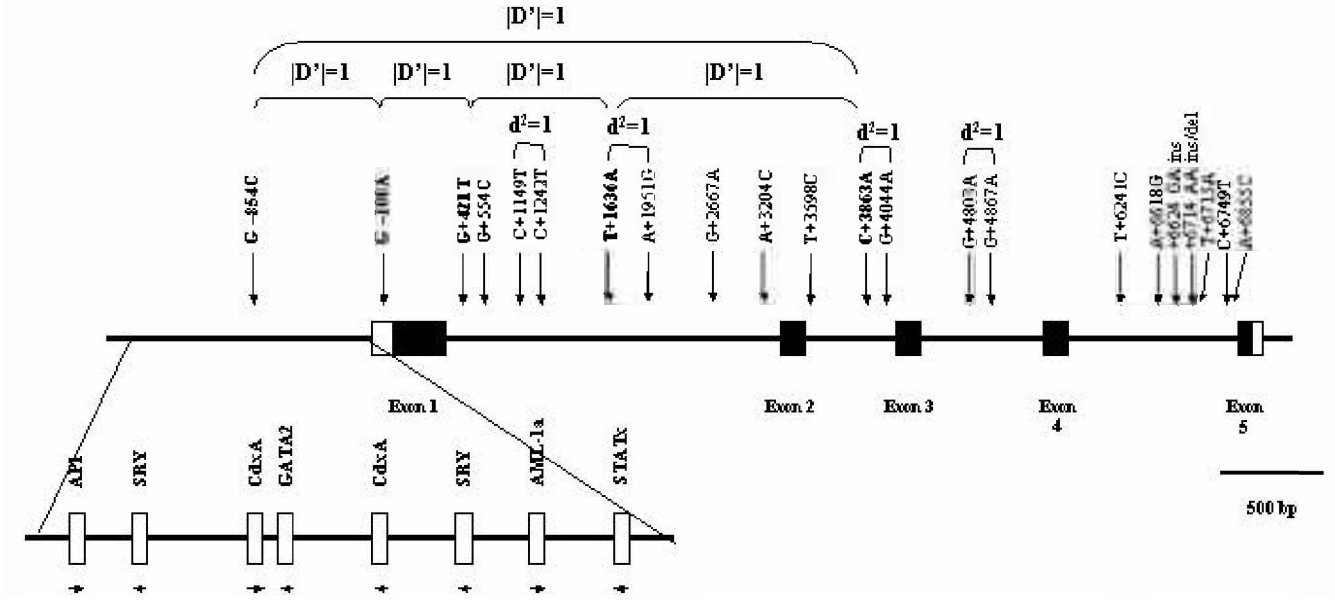

Figure 1. Map of *Bos taurus* insulin-like growth factor binding protein-3 (IGFBP-3) gene on chromosome 4. Coding exons are marked by shaded blocks and 5` and 3`UTR by white blocks. First base of translational start site is denoted nucleotide+1. IGFBP-3 [C+1149T: C=1242T], [T+1636A: A+1951G], [C+3863A: G+4044A] and [G+4803A: G+4867A] are in absolute LD (d^2=1). IGFBP-3 G-854C, G-100A, G+421T, T+1636A, and C+3863A are in complete LD (|D`|=1 and $d^2 \neq 1$).

MATERIALS AND METHODS

Sequencing analysis of the bovine *IGFBP3* gene

Genomic DNA for sequencing was isolated from twenty four unrelated Korean cattles. Full gene including -1,500 bp promoter region were PCR-amplified and directly sequenced using ABI PRISM 3700 genetic analyzer (Applied Biosystems. Foster City CA). Seventeen primer sets for the amplification and sequencing analysis were designed based on GenBank sequences (AF_305712) (Table 1). Sequence variants were verified by chromatograms.

Genotyping by Single base extension (SBE) and electrophoresis

DNA samples for genotyping were isolated from 650 Korean cattle in National Livestock Research Institute, Korea. Primer extension reactions were performed with SNaPSHOT ddNTP Primer Extension Kit (Applied Biosystems, Foster City, CA). To clean up the product of the primer extension reaction, one unit of SAP (shrimp alkaline phosphate) was added to the reaction mixture and the mixture was incubated at 37°C for 1 h, followed by 15 min at 72°C for enzyme inactivation. The DNA samples containing extension products and GeneScan 120 Liz size standard solution were added to Hi-Di Formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5 min, followed by 5 min on ice and then analyzed by electrophoresis in ABI Prism 3100 Genetic Analyzer. The results were analyzed using the software GeneScan and Genotyper (Applied Biosystems, Foster City, CA).

Statistics

The x^2 tests were used for estimation of Hardy-Weinberg equilibrium in population. Heterozygosity (H) for each locus with allele frequencies p and q=1-p was given by $H=1-p^2-q^2=2p$ (1-p). We also calculated two widely used measures of linkage disequillibrium between all pairs of biallelic loci; Lewontin's D' (|D'|) (Hedrick, 1987) and the measure d^2. Haplotypes and their frequencies were estimated from the EM algorithm.

RESULTS AND DISCUSSION

Sequence variants were verified by chromatograms. As a result, twenty two SNPs were identified: one in promoter, eight in intron 1, three in intron 2, two in intron 3, seven in intron 4 and one in 5'UTR (Figure 1 and Table 2). The allele frequencies of identified SNPs in twenty four unrelated Korean cattle are shown in Table 2. The one SNP reported in three cattle breeds (Holstein, Angus and Hereford) was corresponding to *IGFBP3 C3863A* in our study (Maciulla et al., 1997) and the SNP identified in Canadian beef cattle was also corresponding to *IGFBP3 T+3598C* (Thue and Buchanan, 2002). By pair-wise linkage analysis with SNPs, we have found that all SNPs in *IGFBP3* gene were in tight linkage disequilibrium (|D'|>0.9, data not shown) and four pairs of SNPs, [C+1149T: C+1242T], [T+1636A: A+1951G], [C+3863A: G+4044A], and [G+4803A: G+4867A] were in absolute LD (d^2=1) (Figure 1).

Among identified SNPs, five SNPs; *IGFBP3 G-854C* in promoter, *IGFBP3 G-100A* in 5'UTR, *IGFBP3 G-421T*, *IGFBP3 T-1636A* in intron 1 and IGFBP3 C+3863A in intron 2 were selected for large scale screening on the base of the location and frequency. And then these five common SNPs were screened in 650 Korean cattle (National Livestock Research Institute, Korea). The frequencies of those SNPs were 0.357 (G-854C), 0.472 (G-100A), 0.418 (G+421T), 0.363 (T+1636A) and 0.226 (C+3863A) in 650 Korean cattle, respectively (Table 2). Genotype

Table 2. Novel single nucleotide polymorphisms (SNPs) of *IGFBP3* and allele frequencies in Korean cattle (*Bos taurus coreanae*)

Locus	Region	No. of variant/No. of chr.	Frequency*	Heterozygosity	P for HWE
G-854C	promoter	426/1,194	0.36	0.46	0.46
G-100A	5'UTR	610/1,292	0.47	0.50	1.00
G+421T	intron 1	450/1,076	0.42	0.49	0.86
G+554C	intron 1	4/48	0.08	0.14	0.94
C+1149T	intron 1	1/48	0.03	0.05	0.99
C+1242T	intron 1	1/48	0.03	0.05	0.99
T+1636A	intron 1	378/1,042	0.36	0.46	0.28
A+1951G	intron 1	17/48	0.354	0.36	0.46
G+2667A	intron 1	4/48	0.083	0.09	0.16
A+3204C	intron 1	2/48	0.042	0.04	0.08
T+3598C†	intron 2	19/48	0.395	0.39	0.48
C+3863A‡	intron 2	199/880	0.23	0.35	0.63
G+4044A	intron 2	10/48	0.23	0.35	0.99
G+4803A	intron 3	2/48	0.05	0.09	0.97
G+4867A	intron 3	2/48	0.05	0.09	0.97
T+6241C	intron 4	3/48	0.06	0.12	0.95
A+6618G	intron 4	10/48	0.21	0.34	1.00
+6624GA ins	intron 4	2/48	0.10	0.17	2.75×10^{-5}
+6714AA ins/del	intron 4	18/48	0.38	0.47	0.31
T+6715A	intron 4	3/48	0.06	0.12	0.95
C+6749T	intron 4	4/48	0.17	0.28	6.14×10^{-6}
A+6855C	intron 4	3/48	0.13	0.22	6.14×10^{-6}

* Frequencies of rare alleles. † Reported by Thue & Buchanan (Thue and Buchanan, 2002).

‡ Reported by Maciulla et al. (MaciullaZhang and DeNise, 1997). The bald indicates SNPs screened in larger population (n=650).

Table 3. Six haplotypes constructed by five SNPs in bovine *IGFBP3* gene and their frequencies in Korean cattle

Single nucleotide polymorphisms in *IGFBP3*							
Haplotype	G -854C	G -100A	G +421T	T +1636A	C +3863A	N*	Frequency
ht1 (C-G-G-T-C)	C	G	G	T	C	369	0.46
ht2 (G-A-T-A-A)	G	A	T	A	A	181	0.23
ht3 (G-A-T-A-C)	G	A	T	A	C	107	0.13
ht4 (C-A-G-T-C)	C	A	G	T	C	93	0.12
ht5 (C-A-T-T-C)	C	A	T	T	C	41	0.05
ht6 (C-A-T-A-C)	C	A	T	A	C	13	0.02

* Number of chromosomes (for accurate haplotype construction, genotypes with missing data were omitted).

distributions of all loci were in Hardy-Weinberg equilibrium ($p>0.05$). All five SNPs showed complete linkage disequilibrium for each other ($|D'|=1$) (Figure 1). Six distinct haplotypes were identified among the thirty-two possible haplotypes (2^5) with the five SNPs by software Arlequin (Genetics and Biometry Laboratory) using EM algorithm. The most common haplotype accounted for 46% of contribution, and haplotype 1 and 2 are equivalent with *IGFBP3 G-100A* and *IGFBP3 C-3863A*, respectively (Table 3). There was no ambiguous haplotype phasing due to complete LD ($|D'|=1$ and $\hat{d}^2\neq1$) between five SNPs.

Two SNPs in *IGFBP3*, which were identified in other breeds of cattle, were also appeared in Korean cattle, e.g., T+3598C (nt 8069 in previous reports) and C+3863A (HaeIII polymorphism in intron 2) (Maciulla et al., 1997; Thue et al., 2002). The frequencies of T+3598C in Koran cattle (freq.=0.395, Table 2) was higher in Korean cattle than that in the Canadian Beef Cattle Reference Herd (freq.=0.3) (Schmutz et al., 2001), although no frequency data of the other SNP (HaeIII polymorphism in intron 2) was available in previous report (Maciulla et al., 1997).

The insulin-like growth factors, their receptors, and their binding proteins play key roles in regulating cell proliferation and apoptosis. Among the several roles of IGFBP3 are its function as the major carrying protein for IGF1 and IGF2 in the circulation, and its action as a modulator of IGF bioactivity and as a direct growth inhibitor in the extravascular tissue compartment, where it is expressed in a highly regulated manner {Ferry, 1999 #10;Ferry, 1999 #75}. IGFBP3 possesses both growth inhibitory and potentiating effects on cells that are independent of IGF action and are mediated through specific IGFBP3-binding proteins/receptors located at the cell membrane, cytosol, or nuclear compartments and in the extracellular matrix. Although it would be hard to explain the possible functions of identified SNPs in *IGFBP3* without any evidences from further association studies, the potential involvement of this gene and gene polymorphisms in quantitative and/or qualitative traits in cattle might be worth to follow-up in further experiments.

In summary, we have identified twenty-two novel SNPs, in Korean cattle (n=24) and five common SNPs were screened in a larger population (n=650). Six haplotypes were reconstructed in *IGFBP3* and linkage disequilibrium between all SNP pairs were also calculated ($|D'|$ and d^2). The information of SNPs and haplotypes of bovine *IGFBP3* gene could be useful for genetic studies of this gene and, therefore, further studies are needed to elucidate the roles of bovine *IGFBP3* polymorphisms.

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