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Research Article

Hypermethylation of *INS* Promoter in the Developing Liver of Cattle

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Insulin (*INS*) and insulin-like growth factor 2 (*IGF2*) are both important for the milk synthesis in human and dairy cattle. Liver is a source of growth factors that play an important role in the regulation of milk synthesis in the mammary gland in influence its composition. Occurrence of common *INS-IGF* transcript and imprinting regulation in some human tissues suggests close interaction between both genes. Here, we analyzed the DNA methylation pattern of the *INS* promoter and *IGF2* expression at six different pre- and postnatal developmental stages of bovine liver. We found stage-independent DNA hypermethylation (93%) of the *INS* promoter. In contrast, we observed a 4-fold decrease of the *IGF2* expression in 12-month-old adult animals compared to 2-month-old fetuses. We therefore suggest that DNA methylation at the *INS* promoter does not directly regulate the *IGF2* expression in the bovine liver.

1. Introduction

During the past few decades, advances in molecular genetics have led to identification of numerous genes that influence production traits in farm animals. Insulin-like growth factor 2 (*IGF2*) and insulin (*INS*) are localized within the milk production QTL on the chromosome 29 in cattle, and several studies have showed a statistical association between *IGF2* polymorphisms and milk production traits [1–4]. Pivotal role in the control of milk synthesis plays somatotropic axis with its major component—the growth hormone (GH) which acts via IGF to increase milk synthesis in the mammary gland. *INS* and *IGF2* are both found in the milk of human and dairy cattle [5, 6] and relatively highly expressed in the bovine liver [7].

IGF2 is a fetal growth-promoting peptide, mainly produced in liver and expressed from many alternatively spliced transcripts that are controlled by four different promoters. In the bovine genome, *IGF2* is localized in a large imprinted domain that includes tyrosine hydroxylase (*TH*), *INS*, *IGF2*, and *H19* (*H19*—imprinted maternally expressed transcript) genes. *IGF2* contains 10 exons, of which the first seven are noncoding leaders and exons 8–10 encode the 179 amino acid preproprotein. The alternatively spliced *IGF2* transcripts are expressed in a tissue—and developmental—stage specific manner, each containing the same coding exons 8–10 but different leader exons. Three transcripts, containing leader exons 1, 3, and 1–3, are exclusively expressed in the bovine liver [8].

IGF2 is flanked at its 5′ end by *INS*. *INS* is a polypeptide hormone regulating cell growth and survival and is encoded by three exons. Insulin is essential for the induction of milk protein synthesis. In dairy cows, the milk protein yield increases by approximately 15% after insulin administration [9].

While, *IGF2* is paternally expressed in most fetal tissues [10], the monoallelic paternal *INS* expression was found in the mammary gland and some developing liver samples in marsupials [11]. Biallelic *IGF2* expression was observed from P3 and P4 in the adult tissues including liver in cattle [7, 12]. Monk et al. [13] found a hypermethylation of the P1 promoter and hypomethylation of P2–P4 promoters of *IGF2* in human prenatal tissues. *INS* promoter, which is localized close to the P1 *IGF2* promoter, is differentially methylated in the liver and mammary gland in marsupials [11].

Occurrence of common *INS-IGF* transcript in some human tissues [13], as well as common imprinting regulation by the same imprinting control region (ICR) as *IGF2* and *H19* [14], suggests close interaction between both genes.

In this study, we describe the *INS* promoter methylation and *IGF2* expression profile at six different pre- and postnatal stages of the developing liver in cattle.

2. Material and Methods

2.1. Samples. Liver samples from 12 adult Holstein-Friesian (dairy breed) bulls at age of 6, 9, and 12 months ($n = 4$ per each stage) and from 9 male fetuses at three developmental stages (2, 5, and 8 months, $n = 3$) were analyzed.

All procedures carried out with the use of animals were approved by the Local Ethical Commission located at the Agricultural University in Warsaw; permission no. 23/2008.

2.2. Bisulfite DNA Methylation Analysis. The genomic DNA was prepared by proteinase K digestion and phenol-chloroform extraction.

Bisulfite treatment of genomic DNA was performed using methylSEQr Bisulfite Conversion Kit (Applied Biosystems) according to the manufacturer's protocol. Briefly, 300 ng of genomic DNA dissolved in 45 μL deionized water was mixed with 5 μL methylSEQr Denaturation Buffer and initially incubated at 37°C for 15 min. Following, 100 μL of conversion reagent was added to each sample and incubated in the dark at 50°C for 12 hours.

The mixture was purified using attached methylSEQr Purification Column. The final volume of 50 μL bisulfite-converted DNA in 1X TE buffer was stored at 4°C.

2.3. Sequencing of Bisulfite-Treated DNA. To determine the methylation status, the bisulphate-treated DNA was amplified by the use of nested PCR strategy and sequenced. The specific PCR primers were designed by the software of BiSearch (http://bisearch.enzim.hu/?m=param) and localized in promoter region of *INS*: forward—5′ GATATAGTAGGGGTTTTTTT 3′; reverse—5′ AACCTACTTACTAACAACCT 3′. PCR was performed under the following conditions: 35 cycles of 95°C for 40 s, 54°C for 1 min, and 72°C for 1 min. The obtained PCR products cloned into pJET vector (Fermentas). Three to four single colonies were sequenced and analysed using QUMA tool http://quma.cdb.riken.jp/.

2.4. IGF2 Expression Analysis

2.4.1. Total RNA Extraction and Reverse Transcription. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, USA) according to manufacturer's protocol. Isolated RNA samples were dissolved in diethylpyrocarbonate-treated water. Quantity and quality of total RNA were estimated by 2% agarose gel electrophoresis with ethidium bromide staining and confirmed with UV spectrometry. The equal amounts of 2 μg of total RNA were reverse-transcribed to cDNA with 200 units of MMLV Reverse Transcriptase (Promega corp., Madison, USA) according to the manufacturer's instruction.

2.5. RT-qPCR Analysis. The expression profile of the *IGF2* was studied using following primers: forward—5′ GACCGCGGCTTCTACTTCAG 3′; reverse—5′ AAGAACTTGCCCACGGGGTAT 3′, described by [15]. The amplification product (205 bp) spanned two exons (8 and 10) in highly conserved coding region detecting all known alternatively spliced mRNA variants. The *GAPDH* was used as a reference gene. Real-time qPCR amplification was performed in an ABI 7500 Real-Time PCR apparatus (Applied Biosystems) applying the SybrGreen technology. Amplification mix (25 μL) contained 100 ng of cDNA, 0.2 μL of each primer (5 μM finally), 12.5 μL SybrGreen PCR Master Mix (Applied Biosystems, Foster City, USA), and 11.1 μL water. The amplification programme was started with 10 min of initial denaturation at 95°C, followed by 40 cycles of 4-segment amplification with 15 s at 95°C for denaturation, 30 s at 58°C for annealing, and 40 s at 72°C for elongation. Finally, the emulation was selected, and dissociation stage was run.

The results were calculated using the comparative C_t method (delta-delta method) according to the instructions of manufacturer of the 7500 Real Time PCR System and were reported as relative mRNA level. The significance of the differences in the expression levels of *IGF2* was estimated using Duncan's new multiple range test using data from different stages (mean ± SD).

2.6. Bioinformatic Analysis. The genomic organization of *INS-IGF2* was predicted using GenomeThreader and viewed applying Apollo software. Sequence comparison was done with BLAST at http://www.ncbi.nlm.nih.gov/, and repetitive sequences were identified by RepeatMasker (http://ftp.genome.washington.edu/index.html). Conservation profile of promoter regions was analysed by the Consite tool (http://www.phylofoot.org/) and transcription factor binding sites with TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess) confirmed by the Cister (http://zlab.bu.edu/~mfrith/cister.shtml). Putative transcription start sites were identified by the Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html). Prediction of CpG islands was analysed by cpgplot (http://www.ebi.ac.uk/emboss/cpgplot).

3. Results and Discussion

Real-time RT-PCR was performed to detect and quantify *IGF2* mRNA expression. RT-PCR products showed single band (205 bp) of the predicted length. The abundance of *IGF2* mRNA was normalized relative to *GAPDH* mRNA. We found that *IGF2* is expressed at relatively high level at prenatal stages, which however suddenly decreases in the postnatal stages of the developing liver in cattle. The *IGF2* expression level in 2-month-old fetuses was 4-fold higher than in 12-month-old adult liver sample ($P < 0.001$; Figure 1).

Then we annotated a complete genomic sequence (28 kb) of the *INS-IGF2* cluster gene in cattle. The nucleotide sequence of the bovine *INS-IGF2* was deposited in GenBank accession no. EU518675. Three exons of *INS* and ten of

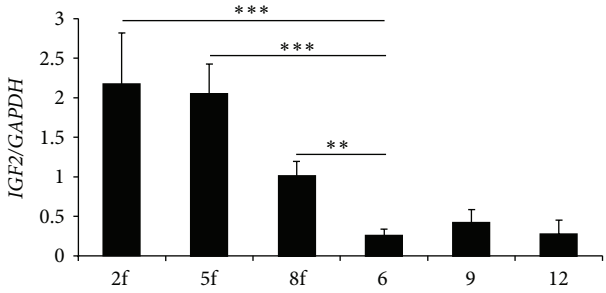

Figure 1: qPCR analysis of the *IGF2* expression in the bovine liver. The analysis was performed at three prenatal (2f, 5f, and 8f) and three of postnatal stages (6, 9, and 12 months of age). Results are presented as means of relative mRNA levels; error indicators show ± SEM. Values differ significantly at $^{**}P < 0.01$ or $^{***}P < 0.001$; qPCR values were normalized to *GAPDH*. qPCR, quantitative real-time RT-PCR; SEM, standard error of measurement or mean; *IGF2*, insulin-like growth factor 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; f, fetus.

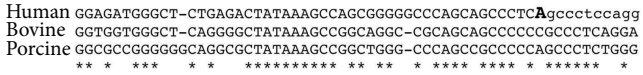

Figure 2: Comparative promoter organization of the *INS* gene. The predicted transcription start site (TSS) is shown as a bold letter. 50 bp of DNA sequence downstream of the predicted TSS was analyzed.

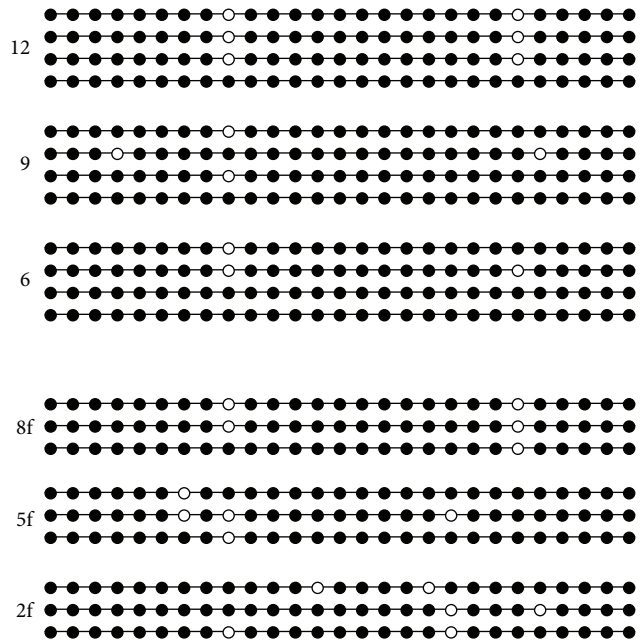

Figure 3: DNA methylation profile of the *INS* promoter. The bisulfite sequences of *INS* promoter region between 127 bp and 682 bp (according to the GenBank no. EU518675) were analyzed. Symbols for animals are as follows (from up): adult bulls—12 months old, adult bulls—9, adult bulls—6, fetus—8 months, fetus—5, fetus—2. Closed circles for methylated CpGs and open circle for unmethylated CpGs.

IGF2 were annotated. Afterwards, we identified the putative promoter region and putative transcription start site (TSS) for the *INS* gene (Figure 2). The *INS* promoter region, 500 bp upstream to the predicted TSS, contains high (up to 70%) GC bp content, which is similar to the GC bp content for the differentially methylated regions (DMRs). In the *INS* promoter, we identified highly conserved TATA-box element and 27 CpG sites (281 bp to 628 bp, GenBank accession no. EU518675). Bisulfite sequencing revealed development independent DNA hypermethylation (90–93%) of the *INS* promoter in the liver samples (Figure 3).

It is not yet clear whether *INS* and *IGF2* undergo common regulation. Occurrence of common *INS-IGF* transcript and ICR in some human tissues suggests close interaction between both genes. Both *INS* and *IGF2* are expressed in mammary gland and liver, but the expression patterns differ. In our previous study, we found a relatively high content of *IGF2* mRNA in adult liver and only trace amount in other tissues [7]. Therefore, we decided to analyze the regulation of *INS-IGF2* transcription in the liver as second important tissue for the milk metabolism. The decrease of the *IGF2* expression during the development of the liver observed in our study is similar to that found in other species [12, 16]. Monk et al. [13] reported similar imprinting status of both genes in some human tissues. However, in the adult liver mono- and biallelic expression of *INS* [11] and bi-allelic of *IGF2* [7, 12] have been reported. In the bi-allelic *IGF2* expression, an important issue for *IGF2* gene effect transmission is the equal representation of both alleles [17]. Moreover, the DNA hypermethylation of the bovine *INS* promoter is in line with the no-allele specific *INS* promoter methylation observed by marsupial [11] and is similar to the *IGF2-P1* methylation level [8]. While it has been suggested that major enhancers for *IGF2* transcription in human liver are located in the 5′ end of *INS-IGF2* locus [18], we suggest that DNA methylation at the *INS* promoter does not directly regulate the *IGF2* expression in bovine liver. Importantly, insulin plays crucial role in the transcription of another gene expressed in the mammary gland, the *STAT5A* [19]. The effect of the aberrant expression of the *STAT5A* in liver on the signaling network and the milk production has been reported [20–22].

4. Conclusions

The genomic interactions in the *TH-INS-IGF2-H19* imprinted domain have been broadly studied but are still not fully understood. We think that our results showing hypermethylation of *INS* promoter in the developing bovine liver will contribute in uncovering the complex regulatory architecture of the domain.

Conflict of Interests

The authors declare that no conflict of interests exists.

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