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 HI9 [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 IX 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’ GATATAG-TAGGGTTTTTTT 3’; reverse—5’ AACCTACTTAA-AACCT 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’ GAC-CGGCGGTTCTACTTCA-3’; reverse—5’ AAGAACTTG-CACGCGGTAT 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 emulsion was selected, and dissociation stage was run.

The results were calculated using the comparative Ct 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.cbl.uu.se/cgi-bin/tesst/tesst) 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
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 (281bp to 628bp, 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-PI 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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