An association of C/T polymorphism in exon 2 of the bovine insulin-like growth factor 2 gene with meat production traits in Polish Holstein-Friesian cattle

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ABSTRACT: The effect of the insulin-like growth factor 2 (*IGF*2) gene polymorphism – a g.292C>T transition in exon 2 on feed uptake and conversion, growth rates, and meat production traits in Polish Holstein-Friesian cattle was investigated. A total of 291 young bulls were genotyped using restriction fragment length polymorphism (RFLP-*BsrI*). Animals were slaughtered at the age of 11 or 15 months and carcass traits were examined. At the age of 11 months the association was shown of the CC genotype with higher cold carcass weight, daily gain and meat content (%) in valuable cuts. At 15 months of age the CC genotype bulls showed higher live body weight, whereas those with the CT genotype had more fat in valuable cuts. The TT genotype bulls appeared to consume more feed (dry matter and protein) and used more feed for maintenance and meat production as compared to the CC genotype. The imprinting status of the *IGF*2 gene was analysed using cDNA sequencing and RFLP-*BsrI*. In 15-months-old animals and in foetuses older than two months both *IGF*2 alleles, maternal and paternal, were equally expressed in liver. In a 2-months-old male foetus the *IGF*2 mRNA was primarily expressed from the paternal allele.

Keywords: IGF2; cattle; polymorphism; imprinting; meat traits

During the last few decades, advances in molecular genetics have led to the identification of genes which influence meat production and quality in farm animals (Mullen et al., 2006; Gao et al., 2007). Insulin-like growth factor 2 (IGF2) belongs to the family of structurally related peptides, which also includes IGF1, insulin, and relaxin. IGF2 is a potent cell growth and differentiation factor and is implicated in mammals' growth and development. IGF2 is synthesized mainly during the foetal and neonatal life. Therefore, it is thought to be a foetal growth and differentiation factor. However, a postnatal role of IGF2 has also been suggested in pigs (Jeon et al., 1999; Nezer et al., 1999; Wood et al., 2000). IGF2 plays a crucial role in the growth and differentiation of many tissues. IGFs, their receptors and binding proteins (BPs) play a crucial role in muscle growth and differentiation (Oksbjerg et al., 2004). Muscle cells produce *IGF*s and IGF-BPs: *IGF*1 and *IGF*2 stimulate proliferation and differentiation of myoblasts and satellite cells, thus suggesting their autocrine/paracrine mode of action

in muscle development. Due to the functions that IGF2 plays in muscle growth and development the *IGF*2 gene is considered a candidate for a molecular marker of meat production in farm animals.

In cattle genome the *IGF*2 gene is localized in chromosome 29 (Schmutz et al., 1996; Goodall and Schmutz, 2003), the same in which QTLs for milk and meat production were found (MacNeil and Grosz, 2002). The bovine *IGF*2 gene contains 10 exons, of which the first seven are non-coding leaders, while exons 8–10 encode the 179 amino *aci*d preproprotein. Sevral *IGF*2 transcripts containing different leader exons are expressed in cattle in a tissue- and developmental-stage specific manner (Goodall and Schmutz, 2007). Three transcripts, containing untranslated exons 1, 2, and 3, are exclusively expressed in liver. The full sequence of the bovine *IGF*2 gene was recently published (Flisikowski and Fries, 2008; GenBank Acc. No. EU518675).

The effect of the IGF2 gene polymorphism on meat traits was studied in pigs. Jeon et al. (1999) and Nezer et al. (1999) showed that a G/A transition in exon 2 of the porcine *IGF*2 gene (RFLP-*Nci*I) affected the rib-eye area and mean backfat thickness. As shown by Kolařikowa et al. (2003), this polymorphism had a significant effect on live weight in Large White pigs, while in the Black Pied breed a significant association was found with live body mass, lean content and backfat thickness (Horák et al., 2001). As shown by Van Laere et al. (2003), a G/A transition in *IGF*2 gene affected muscle growth, fat deposition and the heart size in pigs. This mutation occurs in an evolutionarily conserved CpG island that is hypomethylated in skeletal muscle. It abrogates the *in vitro* interaction with a nuclear factor, probably a repressor, and pigs inheriting this mutation from a sire have a threefold increase in the IGF2 messenger RNA expression in postnatal muscle. A significant effect of the porcine *IGF*2 gene g. 3072A>G polymorphism in intron 3 and g.162C>G (RFLP-*Nci*I) in intron 7 on backfat thickness and lean meat content was found in Large White and Landrace breeds (Vykoukalová et al., 2006). A paternally expressed QTL for muscle growth and backfat thickness has been identified near the *IGF*2 locus on the distal tip of pig chromosome 2 (SSC2p), and then a mutation in a regulatory element of the *IGF*2 gene was identified as the quantitative trait nucleotide (QTN) underlying this QTL.

Only few nucleotide polymorphisms have been found in the bovine IGF2 gene. A C/T transition

(RFLP-*Bsr*I) at position 292 was first reported by Goodall and Schmutz (2003). This polymorphism was associated with the rib-eye area and the carcass fat per cent in beef cattle (Godall and Schmutz, 2007). In another study, the g.292C>T polymorphism strongly affected LM (*musculus longissimus dorsi*) area, backfat, average daily gain, and feed conversion ratio (Sherman et al., 2008). Zhao et al. (2002) found a T/G transversion (RFLP-*Aci*I) in exon 9 of the *IGF*2 gene in Angus cattle; the GG genotype animals tended to have higher body weight and daily weight gain.

In most human and animal foetal tissues only the paternal allele of the *IGF*2 gene is expressed, but in adult tissues the expression is usually bi-allelic (Reik and Walter, 2001). In cattle, the preferential paternal expression of the *IGF*2 gene was reported first by Dindot et al. (2004). Curchoe et al. (2005) showed a promoter-specific loss of imprinting of the *IGF*2 gene in cattle after birth. The imprinting of growth factor genes may influence the foetal growth and growth after birth. Therefore, the imprinting of candidate genes is of a great interest for livestock breeders since it might influence phenotypic effects of genetic markers and QTLs.

The aim of the present study was to search for a possible association between the *IGF*2 gene g.292C>T polymorphism in exon 2 (RFLP-*Bsr*I) and meat performance of Polish HF bulls. In addition, the imprinting status of the *IGF*2 gene was determined in 15-months-old HF bulls and in bovine foetuses using cDNA sequencing and RFLP.

MATERIAL AND METHODS

Animals

A group of 291 Polish Holstein-Friesian (HF) bulls (Black-and-White type), a progeny of 24 AI Holstein sires, was included in the experiment. The study of feed consumption/conversion included 148 young Polish HF bulls. They were fed *ad libitum* silage, hay and concentrate. At the age of 8 months the growth rate and feed conversion were both investigated in a 28-day performance test, during which a concentrate offered ad libitum was the only feed. Two weeks of adaptation to feeding the concentrate preceded the test. Body weight at the start and at the end of the test (month 7 to 8) was recorded. Moreover, mean daily energy and protein, dry matter, INRA feed units for maintenance

and meat production (UFV), PDI (INRA; Jarrige, 1988), as well as net energy and protein intake per kg of live weight gain were calculated. For the whole animal's life the body weight was recorded monthly. After 24-hour fasting, 143 bulls were slaughtered at the age of 11 months and 148 bulls at the age of 15 months. The carcasses were chilled for 24 hours at 4°C. Valuable cuts (round, shoulder, tenderloin, best ribs + fore ribs) were obtained from the right carcass sides and dissected into lean, fat and bone (Oprządek et al., 2001). The data from both groups were processed separately. The full list of analysed traits was given in the previous paper (Maj et al., 2006).

All procedures carried out with the use of animals were approved by the Local Ethical Commission located at Agricultural University, Warsaw; permissions No. 3/2005 and 23/2008.

DNA isolation from blood

Blood samples for DNA genotyping were collected on K_2EDTA from the animals' jugular vein by an authorized veterinarian. Blood was stored frozen at −25°C for a few weeks or at −80°C up to several months. The isolation of DNA from the whole blood was performed according to Kanai et al. (1994).

*IGF*2 genotyping

The genotyping at the g.292C>T site was carried out using PCR-RFLP, with a modification of the method of Goodall and Schmutz (2003). A new pair of PCR primers was designed allowing avoidance of interference from a *Bsr*I cutting site at the 5' end of the amplified fragment of the *IGF*2 gene. The 184-bp fragment, from nucleotide (nt) 21 to nt 204 of the bovine *IGF*2 gene exon 2 (GenBank, Acc. No. AY237543), was amplified with primers designed using Primer3 program (http://frodo.wi.mit.edu/): IGF2F: 5'-TTGCCTCCCAGTCAAGCCTG-3'; IGF2R: 5'-GCTGTGTTGTCTCTGAAGCT-3'. The amplification reaction was performed in a 10 µl reaction mixture that contained approximately 50 ng of bovine genomic DNA, 0.30µM primers, 0.2µM dNTPs and 0.8 units of Taq polymerase (PolGen, Poland). The PCR amplification protocol was:

94°C–1 min, 64°C–1 min, 72°C–1 min (30 cycles). The PCR was carried out in MJ Research PTC-225 Thermal Cycler. The 184-bp amplicon was digested at 65°C for 3 hours with 5 units of *Bsr*I nuclease (New England BioLabs, USA). The digestion products were separated on 2% agarose (GIBCO-BRL, England) gels in TRIS-borate-EDTA (TBE) buffer. The gels were stained with ethidium bromide and then visualised and scanned in FX Molecular Imager (BioRad, CA, USA).

Allele-specific expression of *IGF*2

The *IGF*2 allele-specific expression was determined by sequencing of cDNA reverse transcribed to RNA derived from heterozygous bulls of known parentage. A HF cattle family was set up consisting of one sire and 15 dams of known IGF2 genotypes. Among fifteen male progenies eleven were CT heterozygotes. After slaughter at the age of 15 months liver samples were taken from the heterozygous bulls and used for RNA isolation. Moreover, liver samples were collected from 14 male HF foetuses at a commercial slaughterhouse in Morliny, Poland. Dams and foetuses were genotyped, and livers of heterozygous CT foetuses, a progeny of homozygous dams, were used for the measuring of allele-specific *IGF*2 expression.

Total RNA was isolated from frozen livers as described by Chomczynski and Sacchi, (1987), using TRIsol® Reagent (InvitrogenTM Life technologies, Carlbad, CA, USA), and treated with DNAseI (Sigma-Aldrich Inc., St. Louis, MO, USA, RNAse-free) to prevent DNA contamination. The reverse transcription was performed for 1 h at 42°C in 40 µl containing 2 µg RNA, 50mM Tris-HCl (pH 8.3), 75mM KCl, 3mM $MgCl_2$, 10mM DTT, 0.3mM dNTP mix, 40 U of RNasin, 0.5 µg oligo d(T)15 primer and 200 U of M-MLV reverse transcriptase, all from Promega (Madison, WI, USA). The IGF2 allele-specific expression was determined by sequencing of liver cDNA. PCR-amplification of the IGF2 cDNA was done using the IGF2F and IGF2R primers, and the identity of amplicons was checked by 2% agarose gel electrophoresis. PCR products were purified with GenElute PCR DNA Purification Kit (Sigma), and then sequenced in an ABJ377 sequencer (Applied Biosystem, CA, USA). Sequences were visually analysed for the presence

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[1]The 292th nucleotide in the bovine IGF2 exon 2; position 8656 relative to the transcription start site.

and height of specific nucleotide peaks at the g.292C>T SNP site. In addition, the allele identity of the PCR amplicons was determined using RFLP-*Bsr*I analysis with cDNA as a template.

Statistical procedures

For the association of feed consumption and conversion and carcass traits with the *IGF*2 genotypes the data were analysed by the SAS General Linear Model procedure as follows:

$$Y_{ijkl} = \mu + G_i + YSj + \beta(x_{ijk} - x) + e_{ijk}$$

where:

Y_{ijk}	= mean value of the trait
μ	= general mean
G_i	= fixed effect of genotype *IGF*2 (j = 1, 2, 3)
Y_{Sj}	= fixed effect of year and season (k = 1, .., 10)
$\beta(x_{ijk} - x)$	= regression on body weight at the age of 7 months[2] or at slaughter[3]
e_{ijk}	= random error

The differences between genotypes were tested by Duncan's test.

RESULTS AND DISCUSSION

The C/T transition in the *IGF*2 gene exon 2 was first reported by Goodall and Schmutz (2003). As the C/T substitution creates a new *Bsr*I restriction site, this polymorphism could be analysed using RFLP techniques. Digestion of the 184 bp amplicon with *Bsr*I restriction endonuclease resulted in three DNA bands (118, 58 and 8 bp) for TT homozygote, four bands (176, 118, 58 and 8 bp) for CT heterozygote, and two bands (176 and 8 bp) for CC homozygote.

Using RFLP-*Bsr*I a cohort of 291 young Polish Holstein-Friesian bulls was genotyped at g.292C>T polymorphism. The following genotype frequency was estimated: TT–0.27, CT–0.48, and CC–0.25. The frequency of alleles – C and T was 0.51 and 0.49, respectively. The distribution of genotypes and alleles followed the Hardy-Weinberg rule; no differences were found between expected and estimated values (data not shown).

Associations were estimated between *IGF*2 genotype and meat production traits in HF bulls slaughtered at the age of 11 or 15 months. Results for the animals maintained until the age of 11 months are shown in Table 1. No effect of the *IGF*2 genotype was shown on live weight, but the homozygous CC genotype appeared favourable for some carcass traits: cold carcass weight (+2.21 kg as compared to CT), weight of lean in valuable cuts (+0.79 kg), and average daily gain (+0.09 kg).

In 15-months-old bulls (Table 2) the CC genotype was significantly associated with higher body weight at slaughter (+13.0 kg against CT). However, the TT genotype animals seemed to grow faster (+0.09 kg daily) than those with the CT genotype.

The calculated associations between *IGF*2 genotypes and feed intake and conversion during the test period at the age of 7–8 months are shown in Table 3. Bulls carrying the CC genotype consumed significantly less feed, expressed as total protein (–0.05 kg) and dry matter (–33 kg), than those of the genotype TT. Moreover, the CC genotype animals digested less protein in the small intestine (–32 g) and used less feed units for maintenance and meat production (–0.31).

The effect of the g.292C>T polymorphism (RFLP-*Bsr*I) in the *IGF*2 gene exon 2 on growth and meat production traits was previously reported. In a Canadian beef cattle herd the CT animals had a significantly lower mean rib-eye area (REA) than those with the CC genotype but a significantly higher REA than those with the TT genotype (Godall and Schmutz, 2007). In a steer population, the TT genotype animals had a significantly lower REA mean than those with the CC and CT genotypes. The CC genotype bulls had significantly higher mean carcass fat content than those with the CT and TT genotypes. In the study by Sherman et al. (2007), the g.292C>T polymorphism significantly affected several traits related to meat production in the synthetic line of beef cattle. In steers the allele substitution effect for the final body weight was 6.67 kg, the TT animals being 17.66 kg heavier than those with the CC genotype, and 5.71 kg heavier than the CT animals. The TT genotype animals had a higher average daily gain and lower feed conversion ratio. In the study of Sherman et al. (2007) TT homozygous animals had larger REA, which was not consistent with the study of Godall and Schmutz (2007).

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[2]For the association of feed consumption and conversion.
[3]For the association of carcass traits.

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Table 1. Overall least-square means (LSM) and standard errors (SE) for growth rates and carcass traits across the g.292C>T (RFLP-*Bsr*I) *IGF*2 genotypes of Polish HF bulls slaughtered at the age of 11 months

Traits	CC (n = 33)		CT (n = 71)		TT (n = 39)	
	LSM	SE	LSM	SE	LSM	SE
Live weight at the age of 11 months (kg)	345. 74	7.45	338.35	4.89	332.04	6.66
Cold carcass weight (kg)	168.40[a]	1.06	166.19[a]	0.73	166.33	0.98
Carcass dressing percentage	50.37	0.32	49.75	0.22	49.85	0.29
Weight of lean in valuable cuts (kg)	35.65[a]	0.35	34.86[a]	0.24	34.94	0.32
Weight of fat in valuable cuts (kg)	5.21	0.17	5.20	0.12	5.14	0.16
Per cent of valuable cuts in carcass side	70.24	0.38	69.54	0.27	69.34	0.35
Per cent of fat in valuable cuts	10. 17	0.33	10.27	0.22	10.11	0.30
Average daily gain in the test period at 7–8 months (kg)	1.09[a]	0.03	1.00[ab]	0.02	1.06[b]	0.03

[a,b]within rows the means bearing the same superscript differ significantly at $P \leq 0.05$

The results of our study showed that the g.292C>T transition in the *IGF*2 gene (RFLP-*Bsr*I) influenced meat production traits in Polish HF bulls. For most traits the CC genotype appeared favourable. However, the effects are relatively small, thus suggesting that the mutation is not the causative one for the traits under study. The C/T transition is in leader exon 2 that encodes the 5' untranslated region (5'-UTR) in the *IGF*2 mRNA and therefore does not influence the amino *Aci*d sequence of the peptide. However, it might have a regulatory effect on IGF2 expression.

The gene encoding insulin-like growth factor 2 (IGF2) was shown to be paternally expressed in humans, mice and sheep (Reik and Walter, 2001; Wrzeska and Rejduch, 2004) and in cattle (Dindot et al., 2004). However, Goodall and Schmutz (2003), using the g.292C>T transition, demonstrated a bi-allelic expression of *IGF*2 gene in cattle. Also in their later study (Goodall and Schmutz, 2007), the expression of IGF2 in cattle was shown bi-allelic in foetal liver at day 275 of gestation and in adult animals, suggesting a loss of imprinting just before birth.

In this study, the allele-specific expresion of the *IGF*2 gene was investigated, using sequencing and RFLP-*Bsr*I of cDNA derived from livers of heterozygous male bovine foetuses and from

Table 2. Overall least-square means (LSM) and standard errors (SE) for growth rates and carcass traits across the g.292C>T (RFLP-*Bsr*I) *IGF2* genotypes of Polish HF bulls slaughtered at the age of 15 months

Traits	CC (n = 46)		CT (n = 69)		TT (n = 33)	
	LSM	SE	LSM	SE	LSM	SE
Live weight at the age of 15 months (kg)	445.3[a]	5.4	432.3[a]	4.2	440.8	5.9
Cold carcass weight (kg)	234.0	1.3	233.8	1.0	231	1.4
Carcass dressing percentage	52.8	0.2	52.9	0.2	52.3	0.3
Weight of lean in valuable cuts (kg)	49.2	0.4	49.1	0.3	48.7	0.4
Weight of fat in valuable cuts (kg)	9.4	0.2	9.5[a]	0.1	8.9[a]	0.2
Per cent of valuable cuts in carcass side	62.6	0.2	62.5	0.1	62.4	0.2
Per cent of fat in valuable cuts (%)	13.1	0.3	13.3[a]	0.2	12.6[a]	0.3
Average daily gain in the test period (7–8 months; kg)	1.04	0.02	1.00[a]	0.01	1.09[a]	0.02

[a]within rows the means bearing the same superscript differ significantly at $P \leq 0.05$

Table 3. Overall least-square means (LSM) and standard errors (SE) of daily feed uptake and conversion in Polish HF bulls during the feed intake test (at 7–8 months of age) across the IGF2 g.292C>T (RFLP-*Bsr*I) genotypes

Traits	CC (*n* = 46)		CT (*n* = 69)		TT (*n* = 33)	
	LSM	SE	LSM	SE	LSM	SE
Dry matter (kg)	4.07[a]	0.1	4.17	0.01	4.40[a]	0.1
Total protein (kg)	0.61[a]	0.01	0.63	0.01	0.66[a]	0.01
PDI – protein digested in the small intestine (g)	401[a]	11	411	0.5	433[a]	12
Feed unit for maintenance and meat production	4.23[a]	0.01	4.31	0.09	4.54[a]	0.17

[a]within rows the means bearing the same superscript differ significantly at *P* ≤ 0.05

15-months-old bulls. Total RNA was isolated from livers of heterozygous CT bulls, reverse transcribed to cDNA, and PCR amplified using primers matching exon 2, with the polymorphic g.292C>T site. These primers allowed the identification of liver-specific *IGF*2 mRNA, transcribed from IGF2 promoter 1 (bovine *IGF*2 gene has four alternative promoters – P1, P2, P3, and P4; Curchoe et al., 2005). Previously, while studying the *IGF*2 gene expression in different organs/tissues of adult, 15-months-old HF bulls by real-time PCR we found a relatively high content of IGF2 mRNA in kidney, liver and testis, but only trace amounts in bovine muscles (*longissimus dorsi* and *semitendinosus*) (Lisowski and Zwierzchowski, unpublished). Therefore, we decided to use liver to analyse the allele-specific *IGF*2 gene expression.

In all but one foetal liver both alleles – paternal and maternal – were equally represented in the transcripts. This demonstrated a bi-allelic expression and loss of imprinting after birth and in foetuses at the 5th month of gestation and older. In the liver of a male foetus from 2-month gestation the paternal allele was primarily expressed, as shown by both cDNA sequencing and PCR-RFLP. Therefore, in agreement with the previously reported data (Curchoe et al., 2005), our results showed that in bovine liver the paternal allele-specific expression of the *IGF*2 gene occurred only in early stages of development, i.e. in foetuses up to two months of gestation. In older foetuses and in adult bulls a loss of imprinting was observed and bi-allelic expression was seen in heterozygous animals.

Several imprinted QTLs have been found in the porcine genome (for references see Wrzeska and Rejduch, 2004). One of them appeared to be a G/A mutation in intron 3 of the paternally expressed *IGF*2 gene, causative for muscle growth (Jeon et al.,

1999; Nezer et al., 1999; Van Laere et al., 2003). A paternally expressed QTL for muscle growth and backfat thickness was identified near the *IGF*2 locus at the distal end of pig chromosome 2 (Jungerius et al. 2004).

No study has been carried out so far in cattle to investigate the relationship between the *IGF*2 gene imprinting status and its effect on phenotypic traits. Our results showed that in cattle the imprinting of the *IGF*2 gene might influence its effects on animal's growth and development only in the early foetal life.

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