Single nucleotide polymorphisms in the growth hormone and insulin-like growth factor-1 genes are associated with milk production, body condition score and fertility traits in dairy cows

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ABSTRACT. The somatotrophic axis (GH-IGF) is a key regulator of animal growth and development, affecting performance traits that include milk production, growth rate, body composition, and fertility. The aim of this study was to quantify the association of previously identified SNPs in bovine growth hormone (*GH1*) and insulin-like growth factor 1 (*IGF-1*) genes with direct performance trait measurements of lactation and fertility in Holstein-Friesian lactating dairy cows. Sixteen SNPs in both *IGF-1* and *GH1* were genotyped across 610 cows and association analyses were carried out with traits of economic importance including calving interval, pregnancy rate

to first service and 305-day milk production, using animal linear mixed models accounting for additive genetic effects. Two *IGF-1* SNPs, *IGF1i1* and *IGF1i2*, were significantly associated with body condition score at calving, while a single *IGF-1* SNP, *IGF1i3*, was significantly associated with milk production, including milk yield (means ± SEM; 751.3 ± 262.0 kg), fat yield (21.3 ± 10.2 kg) and protein yield (16.5 ± 8.0 kg) per lactation. Only one *GH1* SNP, *GH33*, was significantly associated with milk protein yield in the second lactation (allele substitution effect of 9.8 ± 5.0 kg). Several *GH1* SNPs were significantly associated with fertility, including *GH32*, *GH35* and *GH38* with calving to third parity (22.4 ± 11.3 days) (*GH32* and *GH38* only), pregnancy rate to first service (0.1%) and overall pregnancy rate (0.05%). The results of this study demonstrate the effects of variants of the somatotrophic axis on milk production and fertility traits in commercial dairy cattle.

Key words: Single nucleotide polymorphism; Fertility; *IGF-1*; Growth hormone; Dairy cattle

INTRODUCTION

Growth hormone (GH) and circulating insulin-like growth factor-1 (IGF-1) are major regulators of postnatal growth and metabolism and thus play critical roles in the control of lactation, mammary gland development, growth processes, and fertility in cattle (Jiang and Lucy, 2001; Renaville et al., 2002; Lucy, 2008). Anterior pituitary-derived GH stimulates the release of circulating IGF-1 from the liver and is critical to the control of nutrient utilization and partitioning (Chagas et al., 2007). Furthermore, in early lactation the somatotrophic axis is uncoupled in high-producing dairy cows so that the liver fails to respond to GH and produces less IGF-1 (Jiang and Lucy, 2001; Renaville et al., 2002; Lucy, 2008). This uncoupling has been implicated in the process of nutrient partitioning, enabling high milk production (Lucy, 2008). The action of GH is mediated through controlling the expression of many genes, including *IGF-1*. Hayhurst et al. (2009) reported a significant relationship between circulating concentrations of GH and calving interval, thereby concluding that GH may potentially be useful as a juvenile selection criterion for fertility. Furthermore, a positive association between systemic concentrations of IGF-1 in the early post-partum period and subsequent cow fertility has been reported (Patton et al., 2007). Such findings suggest that at least some variation of systemic GH and IGF-1 levels is potentially due to mutations within their encoding genes or proximal regulatory regions. Growth hormone (*GH1*) and insulin-like growth factor 1 (*IGF-1*) genes are therefore promising candidates for dissecting the genetics affecting performance, which may also identify genetic markers for improving fertility and milk production in cattle. Our group has identified significant associations between single nucleotide polymorphisms (SNPs) in *IGF-1* and *GH1* and production traits in Holstein-Friesian (HF) cattle (Mullen et al., 2010, 2011), where the dependent variable was genetic merit of the bull, based on progeny performance. However, there is no published information on the direct association or effects of SNPs in *GH1* and *IGF-1* with

HF cows exhibiting the performance traits. Thus, the aim of this study was to determine if associations exist between SNPs in *GH1* and *IGF-I* and fertility and milk production traits in lactating dairy cows.

MATERIAL AND METHODS

Animal material and phenotype recording

Blood samples were collected via the coccygeal vein from 610 Holstein-Friesian dairy cows from 10 herds (6 commercial and 4 research herds) across Ireland in 2008. All blood collection was conducted under license in accordance with the European Community Directive, 86-609-EC.

Experimental treatments within the four research herds were treated as separate herds and contemporary group was subsequently defined as herd-year-month of calving. In all cases, cows with calving interval <300 or >500 days were discarded. Furthermore, cows were excluded if insufficient information was available for a given trait. The number of cows, which were included for analysis of each trait, is given in the text. First, second and third 305-day milk, fat and protein yields were available on 392, 237 and 152 cows, respectively. Lactation 305-day yields were estimated using standard lactation curve methodology outlined by Olori and Galesloot (1999). Calving interval from first to second, second to third and third to fourth lactation were available on 369, 242 and 148 cows, respectively. Pregnancy rate was assessed by ultrasound scanning in 2006 on 362 cows. This was conducted initially at 30 to 50 days and subsequently at 100 days post-insemination, and service records and subsequent calving records were collected. This information was used to derive pregnancy rate to first service, overall pregnancy rate and calving to first service interval. Of these 362 cows, 241 had records of body condition score at calving. Each cow was scored within the first week of calving in each case using a 5-point scale (Wildman et al., 1982; Edmonson et al., 1989).

DNA extraction and genotyping

DNA was extracted from whole blood using a proteinase K/salting out/ethanol precipitation extraction method (adapted from Montgomery and Sise, 1990). Following extraction, the quality and quantity of DNA extracted were assessed using a Nanodrop® spectrophotometer (ThermoScientific, USA) and agarose gel electrophoresis.

A total of 16 SNPs, 10 SNPs spanning the intronic and 3' regions of *IGF-1* and 6 SNPs from the 5' region of *GH1* were genotyped across the 610 cows (Table 1). SNP genotyping was carried out commercially using the Sequenom MassArray® iPLEX Gold assay (Sequenom, San Diego, CA, USA).

Transcription factor binding site analysis

Bioinformatic analysis was carried out on SNP *GH33* located in the promoter region of *GH1* to examine the effects of allele substitution on transcription factor binding sites predicted by the MatInspector software package (Cartharius et al., 2005).

Statistical procedures

The association between each of the SNPs and performance was determined using animal linear mixed models in ASREML (Gilmour et al., 2009) accounting for the additive genetic relationship among animals. Analyses were undertaken within parity for milk production and calving interval. Heritability estimates for yield and calving interval were fixed at 0.35 and 0.03, respectively. Fixed effects included in the model were Holstein breed fraction and contemporary group. Only one record on calving to first service interval, pregnancy rate to first service, and overall pregnancy rate was available for all animals, and therefore, the association between each SNP and these fertility traits was determined across all parities in a model that included parity, contemporary group and Holstein breed fraction as fixed effects.

RESULTS

One of the *IGF-1* SNP frequencies in this study deviated from Hardy-Weinberg equilibrium (HWE); *IGF1i2* due to homozygote deficiencies. Eight of the 10 segregating *IGF-1* SNPs were segregating with minor allele frequencies (MAF) ≤5% (Table 1).

The G allele of *IGF1i3* was associated ($P < 0.05$) with increased milk yield in lactation one (means ± SEM; 398 ± 173.0 kg) and two (751.3 ± 262.0 kg), increased fat yield in lactation one (18.8 ± 6.8 kg) and two (21.3 ± 10.2 kg), and increased protein yield in lactation two (16.5 ± 8.0 kg). *IGF1i1* and *IGF1i2* were each associated ($P < 0.05$, $P < 0.001$, respectively) with body condition score at calving (Table 2).

Five of the six *GH1* SNPs were in HWE with MAF between 0.09 to 0.45. The remaining *GH1* SNP (*GH36*) was fixed in the population (Table 1).

Only one SNP, *GH33*, was associated with milk production, where the T allele was associated ($P < 0.05$) with increased milk yield in lactation two (9.8 ± 5.0 kg).

No SNP was associated ($P > 0.05$) with calving to first service. *GH32* and *GH38* were associated ($P < 0.05$) with calving to third parity and *GH32*, *GH35* and *GH38* were associated ($P < 0.05$) with pregnancy to first service and overall pregnancy rate (Table 2). Transcription factor site analysis showed that SNP *GH33* modulated a single predicted transcription factor binding sites (pTFBS), for the PAX-2 family of transcription factors (Table 3).

DISCUSSION

Apart from our own studies in HF sires (Mullen et al., 2010, 2011) there is little published information on *IGF-1* and *GH1* polymorphisms and their association with traits of economic importance in HF cattle. In particular, there is a dearth of information regarding direct associations between *IGF-1* and *GH1* polymorphisms and milk production and fertility traits in dairy cows. The present study describes the identification of previously reported SNPs in *IGF-1* and *GH1* in commercial dairy cows and novel associations with milk yield and composition, body condition score and fertility (Table 2).

IGF-1 SNPs

Eight of the 10 segregating *IGF-1* SNPs had an MAF of ≤5%, other studies from this

Table 1. Location and nomenclature of the 16 single nucleotide polymorphisms (SNPs) within *IGF1* and *GH1* genotyped across the 610 Holstein-Friesian cows in the present study.

SNP name	Location relative to each gene	Chr.	Nucleotide position on chromosome (bp)	SNP alleles with flanking nucleotide sequence	Genotype	Genotype frequency	MAF	HWE	Reference
IGF1i1	Intron	5	71176219	TCCTCTAGAT[T/A]GGCACATATT	A/A A/T T/T	0.14 0.47 0.39	0.38	0.930	Mullen et al., 2011 ss161151663[3]
IGF1i2	Intron	5	71175753	CTGTCAAGCC[C/T]GTAGTRGTTG	C/C C/T T/T	0.07 0.46 0.47	0.30	0.021	Mullen et al., 2011 ss161151664[3]
IGF1i3	Intron	5	71175747	AGCCYGTAGT[A/G]GTTGTGTGTA	A/A A/G G/G	0.90 0.10 0.00	0.05	0.627	Mullen et al., 2011 ss161151665[3]
rs29012855	Intron	5	71150007	GGAAGAAAGG[A/G]GGAGCATACC	A/A A/G G/G	0.91 0.09 0.00	0.04	0.244	Mullen et al., 2011 Islam et al., 2009 CH296968[2] rs29012855[1,3]
IGF1i5	Intron	5	71149996	GGAGCATACC[T/C]GCCCAGCTAG	C/C C/T T/T	0.00 0.07 0.93	0.03	0.397	Mullen et al., 2011 ss161151666[3]
IGF1i6	Intron	5	71149881	TTCAGTGCAA[A/G]GCGTGCAAGT	A/A A/G G/G	0.94 0.06 0.00	0.04	0.524	Mullen et al., 2011 ss161151667[3]
IGF1i7	Intron	5	71138849	GGGCGGGGGC[G/A]GGGGGGGAGTC	A/A A/G G/G	0.00 0.06 0.94	0.03	0.596	Mullen et al., 2011 ss161151668[3]
IGF1r8	3'	5	71124854	AAAACACTTT[G/T]CAAAATAACT	G/G G/T T/T	0.93 0.07 0.00	0.03	0.398	Mullen et al., 2011 ss161151669[3]
IGF1r9	3'	5	71124671	ACTAATAATC[A/G]AATAATTTGT	A/A A/G G/G	0.00 0.07 0.93	0.03	0.398	Mullen et al., 2011 ss161151670[3]
IGF1r10	3'	5	71124464	CACAGAGCCA[C/T]TGGGAAACC	C/C C/T T/T	0.93 0.07 0.00	0.03	0.387	Mullen et al., 2011 ss161151671[3]

Table 1. Continued.

SNP name	Location relative to each gene	Chr.	Nucleotide position on chromosome (bp)	SNP alleles with flanking nucleotide sequence	Genotype	Genotype frequency	MAF	HWE	Reference
GH32	5'	19	49693441	GAAGCTTGGAGAGA[C/T]GGGAGGCCCTAGT	C/C C/T T/T	0.11 0.48 0.41	0.35	0.143	Mullen et al., 2010 ss196301608[3]
GH33	5'	19	49693429	GGGAGCCCTAGT[T/A]TTTGATCTGCAT	A/A A/T T/T	0.01 0.17 0.82	0.09	0.844	Mullen et al., 2010 ss196003443[3]
GH34	5'	19	49693374	GTGGAAGAGTCC[G/C]TGTCCCCAGTGG	C/C C/G G/G	0.40 0.49 0.11	0.36	0.147	Mullen et al., 2010 rs41923521[1]
GH35	5'	19	49693328	CGCCTTGGTCAG[C/T]AGAGTCCTCTCS	C/C C/T T/T	0.19 0.52 0.29	0.45	0.283	Mullen et al., 2010 rs41923522[1]
GH36	5'	19	49693316	YAGAGTCCTCTC[G/C]TGCCCAAGCCTC	C/C	1.00	N/A	N/A	Mullen et al., 2010 rs41923523[1]
GH38	5'	19	49693278	GTAAASTGGGCA[C/T]GACTGTAGCACT	C/C C/T T/T	0.11 0.49 0.40	0.35	0.127	Mullen et al., 2010 rs41923525[1]

Sequences are written in the 5' to 3' direction of *IGF-1* and *GH1*, on the minus strand of the published Btau_4.0 chromosome 5 and 19, respectively. [1]Ensembl; [2]Genbank accession number; [3]dbSNP. MAF = minor allele frequency; HWE = Hardy-Weinberg equilibrium.

Table 2. Allelic substitution effects (standard errors in parentheses) between significant (P < 0.05) SNPs in *GH1* and *IGF-1* and calving interval (days), body condition score (BCS; scale 1 to 5 x 10), pregnancy to first service (PRFS; % x 100) and overall pregnancy rate (OPR; % x 100).

SNP	Allele substitution	Milk yield (kg)		Protein yield (kg)	Fat yield (kg)		Calving interval			BCS (1-5)	PRFS (% x 100)	OPR (% x 100)
		L1	L2	L2	L1	L2	Parity 1	Parity 2	Parity 3			
IGF1i1[i]	A → T									-1.3* (0.5)		
IGF1i2[i]	C → T									1.9*** (0.6)		
IGF1i3[i]	G → A	-398.0* (173.0)	-751.3* (262.0)	-16.5* (8.0)	-18.8* (6.8)	-21.3* (10.2)						
GH32	T → C								22.5* (11.4)		10.0* (4.2)	5.3* (2.5)
GH33	T → A			-9.8* (5.0)								
GH35	T → C										10.1* (4.1)	5.0* (2.4)
GH38	T → C								22.4* (11.3)		9.8* (4.2)	5.3* (2.5)

*P < 0.05; ***P < 0.001 significance from zero; [i]i = intronic; L1 = lactation one; L2 = lactation two.

Table 3. Effects of bovine *GH1* 5'UTR single nucleotide polymorphisms (SNPs) on predicted transcription factor binding sites[1].

SNP ID	Allele	Core similarity	Matrix similarity	Site sequence	Transcription factor binding sites
GH35	C	1.0	0.998	aggactctGCTGaccaaggcg	MAF and AP1-related factors
	C	1.0	0.866	cgccttGGTCagcagagtcctctcs	RXR heterodimer binding sites
GH38	C	0.978	0.721	aaactgctcttggagctgagaagtgctacaGTCGtgccc	General transcription factor IID (GTF2D)
	T	1.0	0.952	gagaagtgctacagtCATGccca	p53 tumor suppressor
GH33	T	1.0	0.785	tagaggatgcagatcaaAAACta	PAX-2 binding sites

[1]The "core sequence" of a matrix is defined as the (usually 4) consecutive highest conserved positions of the matrix (marked in capital letters). A perfect match between the consensus bovine sequence and the matrix gets a score of 1.00 (each sequence position corresponds to the highest conserved nucleotide at that position in the matrix), a "good" match with the matrix usually has a similarity of >0.80. MAF = musculoaponeurotic fibrosarcoma oncogene; AP1 = activator protein 1; RXR = retinol X receptor. Adapted from Mullen et al., 2010.

group have shown similar MAF for these 10 SNPs in a separate population of 848 HF sires (Mullen et al., 2010, 2011) validating their segregation, yet low MAF, within this breed.

Previous studies reporting association analysis of variants of *IGF-1* and milk production and growth traits in cattle include *IGF-1* SNPs [*AF017143* (Ge et al., 2001; Siadkowska et al., 2006; Islam et al., 2009) and *rs29012855* (Islam, 2009)]. In the present study, *IGF1i3* was associated with milk yield, milk fat yield and milk protein yield. In addition, *IGF1i3* was also associated with milk composition but not with milk yield in the previously mentioned cohort of sires based on daughter performance (Mullen et al., 2011). To our knowledge, this is the first reporting of a polymorphism in *IGF-1* associated directly with milk yield in cattle. Consistent with this, IGF-1 plays an important role in mammary gland growth and function by regulating several cellular processes (Akers, 2006), including the stimulation of protein synthesis in the epithelial cells of the mammary gland (Burgos and Cant, 2010). Furthermore, plasma IGF-1 correlates with milk production throughout lactation and particularly around mating start date and late lactation; circulating IGF-1 has been shown to be positively correlated with milk yield (Rose et al., 2005), and other studies have shown plasma IGF-1 to be positively correlated with milk fat concentrations (Moyes, 2004).

The results of the present study suggest that variants of *IGF-1* segregating at low frequency in HF cattle are associated with milk production. Low frequency and rare alleles have been speculated to contribute to the missing heritability described from large-scale genome-wide association studies of complex traits (Manolio et al., 2009). This reemphasizes the relevance of the candidate gene re-sequencing approach to help uncover rare variants, especially in genes with established physiological effects such as *IGF-1*.

It is well established that gene transcription is extensively and co-ordinately regulated. Although introns are known to carry regulatory sequences, they may not have a direct involvement in the regulation of transcription of highly expressed genes; however, systematic differences in motif distributions do suggest that introns play a role in the rate of their transcription (Zhang et al., 2008). Of the seven SNPs located within introns of *IGF-1*, two SNPs (*IGF1i1* and *IGF1i2*) were associated with body condition score, supporting a role of *IGF-1* in nutrient partitioning in cattle. Indeed, studies have described a relationship between systemic IGF-1 and carcass fat; Davis and Simmen (2000) reported that Angus bulls with lower plasma IGF-1 concentrations had higher marbling scores and

back fat thickness. Similarly, circulating IGF-1 was found to be negatively correlated with carcass fat percentage, fat accretion rate and fat thickness in Simmental crossbred bulls (Anderson et al., 1988). Furthermore, studies in transgenic mice have shown that the differentiation stage of precursor cells into mature fat cells is accompanied by enhanced expression of *IGF-1* (Rajkumar et al., 1999) indicating a role of IGF-1 in fat cell developmental processes.

GH1 SNPs

Studies involving *GH1* SNPs and production traits in cattle have mainly centered on two SNPs, *2141* (Lucy et al., 1993; Yao et al., 1996; Ge et al., 2003) and *2291* (Yao et al., 1996; Lagziel and Soller, 1999), located in exon 5. The importance and complexity of the regulatory elements within the 5'UTR and their effects on gene expression are well accepted (Cheung and Spielman, 2009). Studies have shown the relevance of polymorphisms of the 5'UTR of the bovine growth hormone receptor gene (*GHR*), with several significant associations with milk production and body size traits in HF cattle (Waters et al., 2011). In this study, we examined the 5' region of *GH1*, with all 6 SNPs located upstream of the first exon of *GH1*, according to assembly Btau 4.0 (http://www.ensembl.org; accessed April 14, 2010).

Only one SNP, *GH33*, was associated with milk production while several *GH1* SNPs were associated with fertility in the present study, as measured by calving interval, pregnancy rate to first service and overall pregnancy rate. Although no SNP was associated with calving to first service interval, *GH32* and *GH38* were associated with calving to third parity, and *GH32*, *GH35* and *GH38* with pregnancy to first service and overall pregnancy rate. To our knowledge, this is the first reporting of SNPs in the 5' region of *GH1*, associated with directly measured fertility traits in dairy cattle. Associations with indirect fertility indicators, such as survival, have been observed by our group for *GH35* (Mullen et al., 2010). Furthermore, associations between *GH32*, *GH35* and *GH38* and other production traits, including somatic cell count, survival (as mentioned above), carcass conformation, body depth, angularity, and stature, have previously been observed in HF cattle (Mullen et al., 2010). Although these traits were not examined in this study, coupled with the present evidence, these results support the argument that polymorphisms within or in linkage disequilibrium (LD) with the 5' region of *GH1* have direct effects on production traits in cattle.

Potential mechanisms for the observed effects of *GH32*, *GH35* and *GH38* polymorphisms have been previously examined *in silico* (Mullen et al., 2010), whereas novel mechanisms are discussed herein for the effects observed for *GH33* (Table 3). *GH35* and *GH38* both introduce differential pTFBS and may mediate their effects through modulation of the binding efficiency of *GH1* transcription factors. The C allele of *GH35* introduced pTFBS for musculoaponeurotic fibrosarcoma oncogene/activator protein 1 and retinoid X receptor (RXR) heterodimers. Activator protein-1 mediates many mammalian cell physiological processes and has been shown to be involved in transcriptional regulation of the chicken *IGF-1* gene (Umayahara et al., 1994), whereas RXR heterodimers modify chromatin structure within the promoter region and recruit transcriptional machinery to initiate transcription (Hebbar and Archer, 2003). The SNP *GH38*, altered 2 pTFBS; first, for general transcription factor IID (GTF2D), known to be involved in transcriptional regulation of hepatic genes in mice (Tatarakis et al., 2008), and second, for p53 tumor suppressor, which in part functions as a

transcriptional activator of many target genes, including *IGFBP3*, which modulates the mitogenic effects of *IGF-1* (Buckbinder et al., 1995). The SNP *GH33* modulated a single pTFBS, for the PAX-2 family of transcription factors. The PAX family of transcription factors is an ancient and remarkably conserved gene family, identified in many animal species (Vorobyov and Horst, 2006) and have been shown to play a key role in regulating a variety of genes involving growth and development in a tissue specific manner (Buckingham and Relaix, 2007). PAX-2, in particular, interacts with Pax transactivation domain-interacting protein (PTIP), which contains a carboxyl-terminal domain (BRCT) associated with active chromatin (Lechner et al., 2000), thereby potentially controlling access to the promoter region of *GH1*.

Although it is not predicted to affect TFBS directly, *GH32* may mediate its effects through LD with proximal causative variant(s), which could include *GH35* or *GH38* or both. While biological rationales for the possible mechanisms of the associations with *GH35*, and *GH38* and production traits have been proposed, the observed effects may also be due to other mutations in LD with these SNPs.

CONCLUSIONS

The present study has described novel associations of previously identified SNPs in both *IGF-1* and *GH1* with economically important traits in dairy cows, reaffirming the importance and established role of the somatotrophic axis in animal metabolism, fertility and milk production. Whether the associations observed in this study are due to functional effects of these SNPs is unknown and may be due to LD with caustive polymorphisms as yet undetected in bovine *IGF-1* or *GH1* or nearby regions of the genome. Despite the importance of the somatotrophic axis, there is a dearth of information on causative variants within this axis, and therefore, future study could include re-sequencing of the complete bovine *IGF-1* and *GH1* genes, alternate transcripts and transcriptional regulators in animals divergent for traits of economical importance. This approach could identify potential causative polymorphisms, which if augmented with functional genomic studies and validated in independent populations of cattle would yield greater insight into the influence of variants of *IGF-1* and *GH1* on performance.

ACKNOWLEDGMENTS

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