Association of the Protease Inhibitor Gene with Production Traits in Holstein Dairy Cattle

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ABSTRACT

Positional, comparative candidate gene analysis and previous quantitative trait loci linkage mapping results were used to search for candidate genes affecting milk production and reproduction traits in dairy cattle. The protease inhibitor (PI) gene was chosen for examination, and 5 single nucleotide polymorphisms were detected in coding regions of the gene by direct sequencing of reverse transcription-polymerase chain reaction products from a wide range of cattle tissues. A total of 6 different intragenic haplotypes were identified in North American Holstein population, and these were examined for associations with milk production traits in 24 half-sib families comprising 1007 sons utilizing a granddaughter design. One common haplotype was associated with increased milk and fat yields, increased productive life, and decreased somatic cell score. Another common haplotype was associated with decreased productive life and increased somatic cell score. One rare haplotype was associated with decreased milk, fat, and protein yields and increased milk protein percentage; another rare haplotype was associated with decreased milk yield, increased protein percentage, and decreased productive life. The observation that the PI gene is associated with analogous traits in humans demonstrates the effectiveness of the positional comparative candidate gene analysis that utilizes information about genes present in chromosomal regions with conserved synteny in other species.

(Key words: candidate gene, quantitative trait loci, intragenic haplotype, dairy cow)

Abbreviation key: DPR = daughter pregnancy rate, DYD = daughter yield deviation, PI = protease inhibitor, PL = productive life, QTG = quantitative trait gene, RT-PCR = reverse transcription-PCR, SNP = single nucleotide polymorphism.

INTRODUCTION

A major objective of genomic research in livestock species at present is to identify, map, and characterize individual QTL that affect production traits. A review of publications from the last decade demonstrates that a genome scan using anonymous markers has been used as the primary approach for QTL mapping in dairy cattle (Mosig et al., 2001; Bovenhuis and Schrooten, 2002; Khatkar et al., 2004). Such studies usually identify large confidence intervals for location of the QTL, typically of the order of 20 to 30 cM. Moreover, when applying such information in marker-assisted selection, the phase of marker-QTL associations must be established separately for each family. There is, however, an increasing interest in the candidate gene approach for identifying the actual genes corresponding to the QTL (Rothschild and Soller, 1997). The candidate gene approach ideally identifies the causative genes behind QTL, but more likely, it identifies markers that are close enough to the causative mutation that they are in linkage disequilibrium across the population (Dekkers, 2004). The candidate gene approach has been applied to different genes in cattle. Lien et al. (1995) studied the casein gene in a granddaughter design and showed an association of casein haplotypes with yields of milk and milk protein. Lagziel et al. (1996) used a single strand conformation polymorphism method for detection of polymorphisms and definition of intragenic haplotypes in the bovine growth hormone gene in the Israeli Holstein cattle population. One haplotype was found to have a highly significant positive effect on milk protein percentage. Grisart et al. (2002) reported the first positional cloning of a QTL in cattle that is associated with a significant increase in milk fat yield and a decrease in milk protein yield. These studies and others warrant exploiting the candidate gene approach to search for quantitative trait genes (QTG) in dairy cattle.

Investigation of the effect of the protease inhibitor (PI) gene on milk production and other traits was chosen because previous genome scans identified QTL affecting production and health traits on bovine chromosome 21. Heyen et al. (1999) reported a putative QTL affecting milk yield and protein yield in linkage with

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Table 1. Correlations among the daughter yield deviation (DYD) values of the 1007 sons and the average and the minimum reliabilities of the studied traits.

	Milk	Fat	Fat percentage	Protein	Protein percentage	PL ¹	SCS	DPR ²	Average reliability	Minimum reliability
Milk	1	0.59	-0.55	0.86	-0.40	0.14	-0.01	-0.17	85.2	53
Fat		1	0.36	0.69	0.10	0.14	-0.05	-0.14	85.2	53
Fat percentage			1	-0.27	0.57	-0.01	-0.04	0.05	85.1	53
Protein				1	0.12	0.19	-0.04	-0.15	85.1	52
Protein percentage					1	0.07	-0.04	0.06	85.1	52
PL						1	-0.36	0.36	65.9	35
SCS							1	-0.11	70.5	36
DPR								1	58.7	25

¹PL = Productive life.

²DPR = Daughter pregnancy rate.

microsatellite marker D21S27 at a position 56 cM from the centromere. Also, Rodriguez-Zas et al. (2002) reported that a QTL located at 56 cM was associated with variation in SCS and milk protein yield. Mosig et al. (2001) reported that a QTL at 67.3 cM was associated with milk protein percentage.

The PI gene belongs to the superfamily of serine proteinase inhibitors that includes C1 esterase, antithrombin, and α 1-antichymotrypsin, among others. The primary role of PI is to protect tissues against proteolytic digestion by neutrophil elastase. Possible roles of PI in the immune response include inhibition of lymphocyte toxicity and inhibition of chemotaxis (Blank and Brantly, 1994). Additionally, it has been reported that the PI protein is present in human milk and might increase survival of milk proteins by various mechanisms (Chowanadisai and Lonnerdal, 2002).

Based on the aforementioned studies, it was decided to further investigate the association of PI with quantitative traits in dairy cattle. In this paper, we describe identification of intragenic haplotypes in the PI gene in Holstein cattle and their effects on economic traits in the US Holstein population.

MATERIALS AND METHODS

Experimental Structure

Holstein semen samples were obtained from the Cooperative Dairy DNA Repository, which is maintained by the USDA Bovine Functional Genomics Laboratory. Semen samples of 30 half-sib families (Weller et al., 1990) composed of 1258 sons were obtained for QTG analysis. Daughter yield deviation (DYD) data for the traits of interest [milk protein yield, milk protein percentage, milk fat yield, milk fat percentage, milk yield, SCS, daughter pregnancy rate (DPR), and productive life (PL)] were obtained from the Animal Improvement Programs Laboratory (<http://www.aipl.arsusda.gov>). Correlations of the DYD values of the 1007 sons and

the average and minimum values of the reliability for each trait are shown in Table 1.

Detection of single nucleotide polymorphisms. Single nucleotide polymorphisms (SNP) were detected by direct sequencing of reverse transcription-PCR (RT-PCR) products from the cattle tissues. To detect polymorphisms in the exons of the PI gene (GenBank accession number X63129), 2 sets of primers, PI7-PI9 and PI3-PI6 (Table 2), were designed to amplify the total cDNA sequence of the gene. Total RNA was extracted from a wide range of tissues from 5 fetuses and 5 adult cows obtained from a local slaughterhouse. After dissection, tissues were immediately chilled on ice and submerged in an appropriate volume of RNeasy RNA stabilization reagent (QIAGEN, Valencia, CA). Total RNA was extracted using the RNeasy kit (QIAGEN). Reverse transcription was performed with the OneStep RT-PCR kit (QIAGEN) using cDNA-specific primers (Table 2). The temperature cycles were as follows: 50°C for 30 min (reverse transcription); 95°C for 15 min (initial PCR activation step); followed by 32 cycles of 94°C for 45 s, touchdown annealing from 65°C to 53°C for 45 s (–2°C per cycle), and 72°C for 45 s; and a final extension at 72°C for 7 min.

Products from PCR and RT-PCR were purified from the amplification solution using GFX PCR DNA Purifi-

Table 2. Primer sequences used in amplification of cDNA and genomic fragments.

Primer	Sequence	Product type	Product length (bp)
PI7-F	ATGGCACTCTCCATCACGCG	cDNA	1058
PI9-R	TTGGACACCTTCAGAGGCTG		
PI3-F	CCTCGCCAAGTTCTTGAAAA	cDNA	440
PI6-R	CCTTTAATGCCATGGAGGGA		
PI7-F	ATGGCACTCTCCATCACGCG	DNA	448
PI11-R	CCACTAGCTTTGCACTCTCA		
PI15-F	ACACCCCAGATCTCCAGGAG	DNA	362
PI9-R	TTGGACACCTTCAGAGGCTG		

cation Kit (Amersham Biosciences, Little Chalfont, UK). Sequencing reactions consisted of 2 μ L of BigDye Terminator mix (Applied Biosystems, Foster City, CA), 6 μ L of dilution buffer [200 mM Tris HCl (pH 9.0); 5 mM MgCl₂], 5 pmol of primer, and 0.04 to 0.1 μ g of template DNA in a final reaction volume of 20 μ L. Cycle conditions were an initial denaturation at 96°C for 3 min; then 50 cycles of 96°C for 10 s and 58°C for 4 min; followed by 7 min at 72°C. Excess dye terminators were removed using the CleanSeq magnetic bead sequencing reaction clean up kit (Agencourt Biosciences Corporation, Beverly, MA). The samples were eluted from the beads in 50 μ L of deionized H₂O. Ten microliters of each sample were electrophoresed on an Applied Biosystems 3730XL automated DNA sequencing instrument using 50-cm capillary arrays and POP-6 polymer. Data were analyzed using Applied Biosystems version 5.0 of Sequencing Analysis. The SNP were identified by visually inspecting each base in sequencing traces.

DNA Genotyping

Genomic DNA was extracted from semen samples with proteinase K and phenol/chloroform after the procedures of Kappes et al. (2000). The DNA concentration was measured using a spectrophotometer (Ultraspec 2100 Pro; Amersham Biosciences). A total of 24 Holstein sires and their 1007 Holstein sons were genotyped in this study. The 24 sires and 160 of their sons were genotyped by direct sequencing; 874 sons were genotyped by PCR-RFLP analysis using the restriction enzymes *Rsa*I and *Sfa*NI, as one of the detected SNP was in a *Rsa*I recognition site and 2 SNP were in the *Sfa*NI recognition sites. Two pairs of primers, PI7-PI11 and PI15-PI9, were designed to amplify genomic DNA flanking SNP that were detected in the cDNA sequence analysis. The PI7 and PI11 primers were used to amplify DNA fragments for sequencing and for PCR-RFLP with *Sfa*NI; primers PI9 and PI15 were used to amplify DNA fragments for PCR-RFLP analysis with *Rsa*I. The PI15 primer was designed in the intronic sequence (accession no. AY426761). Amplification of genomic DNA was performed in 25 μ L of reaction volume, which included 50 ng of genomic DNA, 50 ng of each primer, 200 μ M of each dNTP, 2.5 μ L of 10X PCR buffer (Promega, Madison, WI), and 0.3 U of Taq DNA polymerase (Promega). The temperature cycles were as follows: 95°C for 5 min; 32 cycles of 94°C for 45 s, touchdown annealing from 63°C to 50°C for 45 s (–2°C per cycle), 72°C for 45 s; and a final extension at 72°C for 7 min.

Inferring Haplotypes

Intragenic haplotypes can be inferred from SNP genotyping information (Lagziel et al., 1996). In this study,

haplotypes were inferred in one or more of the following ways.

- From homozygous individuals. For example, for an individual showing genotypes G/G, T/T, T/T, G/G, C/C, the haplotype GTTGC was inferred.
- From heterozygous individuals showing only a single heterozygous site. For example, for an individual having the genotype A/A, C/C, G/G, G/G, C/T, haplotypes ACGGC and ACGGT were inferred.
- From direct sequencing. Forward and reverse sequencing traces from all 24 Holstein sires were examined to determine haplotypes.
- From direct sequencing of 2 to 4 heterozygous and homozygous sons within each sire family. For example, for a sire having the genotypes G/A, C/T, G/T, G/C, C/T, haplotypes GTTGC and ACGCT were inferred based on one son of this sire being homozygous for the haplotype GTTCG and the other son being homozygous for the haplotype ACGCT.

Statistical Analyses

To test whether marker haplotypes have significant associations with the trait, the following allele substitution model was fitted to the DYD data (Batra et al., 1989; Weigel et al., 1990; Sharif et al., 1999):

$$Y_{ij} = \mu + \text{sire}_i + \sum_{k=1}^{M-1} \beta_k A_{ijk} + e_{ij}$$

where Y_{ij} is the DYD of the trait for son j of sire i ; μ is the mean; sire_i is the effect of sire i ; $A_{ijk} = 0, 1, 2$ is the number of copies of haplotype k present in the individual ij , where A_0 represents the most frequent of M marker haplotypes (in this case haplotype D) and the remaining haplotypes are denoted $A_1, \dots, A_{k-1}, \dots, A_{(M-1)}$; β_k are partial regression coefficients corresponding to effect of haplotype k as a deviation from the effect of the most frequent haplotype (A_0), which is set to zero to make the model have full rank; and e_{ij} is the random error associated with the individual ij . This model was fitted using weighted least squares with weights based on reliability (Israel and Weller, 1998). Significance of associations was determined for each trait separately by an F -test on the sum of squares explained by the combined effect of haplotypes. Then, for traits with significant associations, estimates of the effect of individual haplotypes, as a deviation from the effect of the most frequent haplotype (D), were evaluated for significance.

RESULTS

Intragenic Haplotypes in Holstein

Several methods can be used to uncover polymorphisms in candidate genes. Here, a direct sequencing

Table 3. Intragenic haplotypes at the protease inhibitor gene¹ in Holsteins.

Haplotype	Sequence ²	Frequency ³
A	2-1-1-2-2	0.276
B	1-2-2-1-2	0.061
C	1-1-1-1-2	0.012
D	1-2-2-1-1	0.390
E	1-1-1-1-1	0.265
F	2-1-1-2-1	0.005

¹Accession number X63129.

²Polymorphic sites at nucleotides 164, 269, 284, 407, 989; haplotypes show sequence according to the order of nucleotide sites.

³Allele frequencies were estimated based on the maternal alleles of sons.

method to determine intragenic haplotypes in the examined samples was used. In the search for polymorphisms in the PI gene in Holsteins, 5 SNP were identified, as were 6 intragenic haplotypes (A through F) (Table 3). The 6 haplotypes in Holstein may have derived from one another by point mutations that generated additional SNP plus some recombination among haplotypes. Based on genotyping Jersey, *Bos indicus*, and *Bison bison* individuals (data not shown), we assume that the consensus haplotype is GCGGC (denoted 1 to 1-1 to 1-1), which corresponds to haplotype E. Then, the C haplotype may have derived from E by a single point mutation: 1 to 1-1 to 1-2, and A from C by 2 further point mutations: 2 to 1-1 to 2-2. Haplotype D may have derived from E by 2 point mutations: 1 to 2-2 to 1-1. Haplotype B (1 to 2-2 to 1-2) may have derived by recombination between C (1 to 1-1 to 1-2) and D (1 to 2-2 to 1-1) and F (2 to 1-1 to 2-1) by recombination between A (2 to 1-1 to 2-2-) and D (1 to 2-2 to 1-1) or E (1 to 1-1 to 1-1).

Associations of PI Haplotypes with Production Traits in Holsteins

An allele substitution model was used to estimate associations of haplotypes with production traits. In this model, the most frequent haplotype (D) was set to have zero effect. Table 4 has the results of the analysis

Table 4. Significance of the associations of the protease inhibitor gene region with production traits.

Trait	P
Milk yield	0.02
Fat yield	0.04
Fat percentage	0.60
Protein yield	0.25
Protein percentage	0.03
Productive life	0.04
SCS	0.10
Daughter pregnancy rate	0.82

of the whole region level of the PI gene. This analysis integrates the information of all haplotypes together to test the significance of the region as a whole. Milk yield, fat yield, protein percentage, and PL showed strong associations with the PI region ($P < 0.05$), SCS showed a suggestive association ($P = 0.10$), and fat percentage, protein yield, and DPR did not show significant associations. Table 5 has the allele substitution effects associated with haplotypes A, B, C, E, and F for traits that were significant in the overall association. Estimates of associations with protein yield, although not significant in the overall test (Table 4), are included for completeness.

Haplotype D was associated with a significant increase in milk yield vs. haplotype B and a less significant increase vs. haplotype C. In addition, haplotype D was associated with a significant increase in fat and protein yield vs. haplotype B; haplotypes A, C, E, and F showed no significant differences from haplotype D. Haplotypes B and C were associated with an increase in milk protein percentage vs. haplotype D. For PL, haplotype D was associated with a significant increase vs. haplotype C and nonsignificant increases vs. haplotypes B, E, and F. On the other hand, haplotype A was associated with an increase in PL vs. haplotype D, although this difference was not significant. For SCS, haplotype D was associated with a significant decrease vs. haplotypes E and A. For milk fat percentage and DPR, haplotypes of the PI gene did not show significant associations.

DISCUSSION

In this paper, a combination of positional comparative candidate gene analysis (Rothschild and Soller, 1997) and previous linkage QTL mapping results (Heyen et al., 1999; Mosig et al., 2001; Rodriguez-Zas et al., 2002) were used to study the association of the PI gene with milk production traits in Holstein dairy cattle.

Five SNP, at positions 164, 269, 284, 407, and 989, were identified in the PI gene in Holsteins (Table 3). Genotyping of the 30 Holstein sires by direct sequencing revealed 6 sires homozygous for all 5 SNP and 24 sires that were heterozygous for at least one SNP. The 6 Holstein haplotypes were examined for association with milk production traits, PL, SCS, and DPR in the 24 grandsire families comprising 1007 sons.

Associations of PI Haplotypes with Milk Production Traits

In this study, by using samples from the North American Holstein population, haplotype D of the PI gene

Table 5. Estimates of substitution effects of haplotypes for the protease inhibitor gene for production traits as a deviation from the effect of the most frequent haplotype (D).

Haplotype	Milk yield (kg)	Milk fat yield (kg)	Milk protein yield (kg)	Protein percentage	PL ¹	SCS
A	-26.0 ± 20.7	-0.47 ± 0.7	-0.4 ± 0.5	0.003 ± 0.003	0.29.0 ± 0.15	0.02 ± 0.02†
B	-95.0 ± 33.6**	-3.4 ± 1.2*	-1.8 ± 0.8*	0.01 ± 0.01†	-0.11 ± 0.24	-0.004 ± 0.02
C	-85.0 ± 49.9†	-1.5 ± 1.8	-0.73 ± 1.2	0.02 ± 0.01†	-0.62 ± 0.36	-0.02 ± 0.04
D	0.00	0.00	0.00	0.00	0.00	0.00
E	-2.3 ± 19.6	-0.90 ± 0.7	-0.53 ± 0.5	-0.003 ± 0.003	-0.15 ± 0.14	0.04 ± 0.01**
F	48.0 ± 51	2.5 ± 1.8	0.9 ± 1.2	0.00 ± 0.01	-0.11 ± 0.37	0.01 ± 0.04

¹PL = Productive life (mo).†*P* < 0.10.*P < 0.05.**P < 0.01.

was associated with a significant increase in milk yield, increase in milk fat yield, and a decrease in milk protein percentage (Table 5). This inverse relationship mirrors the negative phenotypic and genotypic correlations among milk yield, milk fat yield, and milk protein percentage. The action of the PI gene may contribute to this antagonistic relationship. Thus, identification of genes associated with milk production traits using a positional candidate gene approach based on existing linkage mapping results is feasible in dairy cattle.

The PI protein is important in humans for its broad range of activities. Its primary role is to protect tissues from proteolytic destruction by neutrophil elastase. In addition, it has been reported that PI is produced by the human mammary gland and may affect survival of milk proteins such as lactoferrin and lysozyme (Chowanadisai and Lonnerdal, 2002). Consequently, the observation that bovine PI gene haplotypes were associated with milk production traits demonstrates the effectiveness of the positional comparative candidate gene analysis (Rothschild and Soller, 1997) that utilizes information on the genes present in chromosomal regions with conserved synteny in other species.

Associations of PI Haplotypes with PL

Productive life is a longevity trait that is measured as a cow's total months in the milking herd for the first 7 yr of life, with a limit of 10 mo per lactation (VanRaden and Wiggans, 1995). In a whole genome scan for QTL affecting health traits in dairy cattle, Heyen et al. (1999) reported a marker linked to a QTL affecting PL at 85 cM on chromosome 21. Our results for PI represent the first gene found to have an association with such a trait in dairy cattle. Analysis of the whole region at the PI gene showed a significant association with PL (Table 4). Haplotype D was associated with a significant increase in PL vs. haplotype C and nonsignificant increases vs. haplotypes B, E, and F; haplotype A was

associated with an increase in PL vs. haplotype D. Noting that estimates in Table 5 are of the effect of one copy of a given haplotype on mean performance of a sire's daughters, which reflects one-half of the sire's breeding value, a cow inheriting haplotype D is expected to have double the observed effects vs. inheriting haplotypes B, C, E, or F.

Associations of PI Haplotypes with SCS

Somatic cell score serves as an indicator of mastitis (low SCS is associated with low incidence of mastitis) and as a selection tool to reduce incidence of the disease (Schutz, 1994). Rodriguez-Zas et al. (2002) identified a QTL for SCS at approximately 60 cM on chromosome 21 in one of the families studied and another QTL for SCS at 84 cM. In addition, Heyen et al. (1999) identified a marker linked to a QTL affecting SCS at 33 cM, even though this marker was not highly significant. In this study, haplotypes A and E were associated with a significant increase in SCS vs. haplotype D, with other haplotypes having similar SCS as haplotype D (Table 5). Two copies of haplotype E are expected to increase SCS by 0.08, 0.18, 0.24, 0.16, and 0.12 points vs. haplotypes A, B, C, D, and F, respectively. Haplotype E, with a frequency of 26.5% in the Holstein population, was associated with a significant increase in SCS over haplotype D and less significant increase in milk yield vs. haplotypes A, B, and C. Thus, haplotype E increases both SCS and milk yield in the Holstein population.

In view of the observation that PI haplotypes show significant associations with SCS and that QTL for SCS were found in the PI region (Heyen et al., 1999; Rodriguez-Zas et al., 2002), we assume that the PI gene is the actual QTG or in close linkage disequilibrium with the QTG. However, additional fine mapping and molecular work is required to refine the PI region. In addition, the human PI gene plays a major role in the physiologic and biochemical responses in tissue repair of infection

or inflammation, either by inactivating proteases at the inflammation sites or other mechanisms (Blank and Brantly, 1994). The human and bovine genes demonstrate high sequence homology (79%); bovine PI might play a similar role to that of the human gene in the immune system, thereby affecting response to mastitis infection.

CONCLUSIONS

In summary, a combination of positional comparative candidate gene analysis (Rothschild and Soller, 1997) and previous QTL linkage mapping results were used to search for candidate genes affecting milk production and reproduction traits in dairy cattle. Given that haplotype D was associated with a decrease in SCS and an increase in milk yield, milk fat yield, milk protein yield, and PL, it would be desirable to make selection decisions favoring this haplotype. Although the causal mutation responsible for the quantitative effects has not been identified, we propose that the PI gene may be the actual QTG or in close linkage disequilibrium with the QTG. In both scenarios, PI haplotype information can be used in genetic improvement programs.

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