Assessment of milk quality using novel mutations of B2M gene in bovine DNA from milk

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1. Introduction

Increasing concerns about food safety generally and the impact of dairy products on human health specifically have resulted in tightening regulatory legislation and targeted supplier payment schemes based on the quality of milk composition (Friggens & Rasmussen, 2001). These concerns also provide a strong impetus to improve milk quality traits, such as milk yield (MY), milk fat percentage (MFP), milk protein percentage (MPP) and somatic cell count (SCC), each of which has been considered as an important determinant of product quality and resultant consumer satisfaction (VanRaden, 2006). Given this landscape, the development of increasingly sophisticated means for predicting and assessing milk composition would be expected to promote the growth of the dairy industry.

B2-microglobulin (B2M) is a 12-kD secreted protein which has crucial roles in a broad range of biological processes, notably immune modulation (Hofmann, Kiecker, Küchler, Kors, & Trefzer, 2011) and kidney function, and which contribute to the progression of cardiovascular diseases (Bash, Astor, & Coresh, 2010; Matsushita et al., 2010), peripheral arterial disease (Wilson et al., 2007), multiple myeloma (Rossi et al., 2010) and inflammatory diseases (Bianchi et al., 2001; Zissis et al., 2001). Genetic ablation in mice results in aberrant-immune cell composition, a phenotype which resembles that of humans who carry rare mutations of the B2M gene (Crump, Grusby, Glimcher, & Cantor, 1993; Liao, Bix, Zijlstra, Jaenisch, & Raulet, 1993; Sanchez et al., 2004; Worley et al., 2009). In beef cattle, 12 mutations in the regions of exon 2 and 4 of the B2M gene have previously been described and found to be associated with changes of milk protein and variable levels of serum immunoglobulin G (IgG) contributing to elevated SCC (Clawson et al., 2004; Sanchez et al., 2004; Worley et al., 2009).

This strong association to immune-regulatory function makes the B2M gene a particularly attractive candidate marker of milk quality. We have recently developed methods for efficient isolation of bovine genomic DNA directly from unprocessed milk samples, as an alternative to sampling...
blood which causes significant stress responses in cows (Liu, Gao, Yang, Ku, & Zan, 2014). In the current work, bovine genomic DNA extracted from milk was used to screen for potential mutations in the B2M gene of Holstein dairy cows by direct sequencing. Further, the relationship of these mutations to milk quality traits was examined. A total of five mutations were discovered and shown to be variably associated with key milk quality traits, including MY, MFP, MPP and SCC. As such, they may provide further tools for the assessment of measurable differences in milk quality traits in these herds.

2. Material and methods

2.1. Samples source and milk quality traits measurement

Raw milk samples (15 ml) were collected at the first milking of the day from 79 unrelated and randomly selected Chinese Holstein cows, all about 3 years old and in a single herd. Once collected, the samples were transported on ice packs and subsequently maintained at optimum low temperature prior to analysis. The milk quality traits MY, MFP, MPP and SCC were measured using standard protocols on a Foss Milkoscan FT120 instrument (Foss Electric, DK-3400 Hillerød, Denmark). Each milk quality trait was measured by a single investigator, in order to minimize measurement bias. Values were represented with mean ± standard error of the mean. Then, DNA was extracted from leukocytes isolated from the milk samples using a novel method that we have previously reported and based on long-fragment DNA amplification from small amounts of raw milk (Liu et al., 2014).

2.2. Detection of mutations and genotyping

Based on sequence of bovine B2M gene (GenBank accession No. NC_007308.5), one pair of primers (5′-GGC TTT CCC AGC ATC ACT AAC-3′ and 5′-TCA CAG CAC CAA ACT TAT CT-3′) was designed to amplify a 729-bp product of the B2M intron 3. Polymerase chain reaction (PCR) was conducted in 30 µl reaction mixtures containing 50 ng DNA templates, 10 pM of each primer, 0.20 mM dNTP, 2.5 mM MgCl2 and 0.5 U Taq DNA polymerase (TaKaRa, Dalian, PR China). The PCR protocol consisted of 95°C for 5 s followed by 30 cycles of 94°C for 30 s, 60°C annealing for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min. The end products were purified with a Wizard Prep PCR purification kit (Promega). The PCR products were sequenced using established protocols (Beijing Aolaibo Biotechnology, PR China; Applied Biosystems 3730xl DNA Sequencer, Foster City, CA, USA). Mutations in the B2M gene were identified across 79 different milk samples and compared with one another using SeqMan software (DNASTAR, Inc., Madison, WI, USA).

2.3. Statistic analysis

While a substantial measure of the variance in milk quality traits can be attributed to factors such as genotype, age, breed and herd status, and geographic location, the drivers of the remaining variability remain poorly defined. Since individual cow’s milk was collected, two factors (genotype and other random errors) were involved in the statistical model while the effects of cow factors and location were excluded during experimental design.

Following published approaches (Nei & Li, 1979; Nei & Roychoudhury, 1974), the established bioinformatic parameters for “proportions of genotypes”, allelic frequencies, gene heterozygosity and polymorphism information content (PIC) were statistically analyzed. Heterozygosity and PIC were used to indicate the extent of genetic variation at the B2M gene locus. The association between genotypes of mutation in B2M gene and the milk quality traits (MY, MFP, MPP and SCC) was analyzed by mathematical method and SAS software (SAS Institute Inc., Cary, NC, USA). A statistical linear model obtained was as follows:

\[ Y_i = \mu + G_i + \epsilon_i \]

where \( Y_i \) is the measured milk quality trait, \( \mu \) is the overall mean for each trait, \( G_i \) is the genotype effect and \( \epsilon_i \) is the random errors effect.

For the haplotype analyses, the model was subjected to the same covariates in a similar manner and the degree of linkage disequilibrium (LD), reflecting the nonrandom association of alleles at different loci, was investigated. Currently, the most commonly used parameters to determine LD are \( D' \) and \( r^2 \), where \( D' \) is a measure of LD relative to the maximum possible value (given the allelic frequency of the mutations) and was calculated from the frequencies of the haplotype of the mutation pairs. Meanwhile, \( r^2 \) is the square of correlation between pairs of mutations and can be used as a standardization measurement of LD between alleles existing at two loci. These two parameters of LD were measured using Haploview 2.05 software (Barrett, Fry, Maller, & Daly, 2005; Gabriel et al., 2002) after the haplotypes and their frequencies were inferred using a published algorithm (Stephens, Smith, & Donnelly, 2001). The phase probabilities of all the polymorphic sites for the haplotypes were calculated using PHASE software (http://depts.washington.edu/ventures/UW_Technology/Express_Licenses/PHASEv2.php).

3. Results

3.1. Determination of mutations in B2M gene

By direct DNA sequencing, five discrete mutations were identified in intron 3 of the B2M gene. Denoted as 7008G>T, 7026G>A, 7202A>G, 7432G>T and 7437G>C, they were all identified within a 729-bp PCR product, whose DNA sequencing maps are shown in Figure 1.

3.2. Analysis of bioinformatics parameters and association between mutations and milk quality traits

Within the population of 79 animals, we further analyzed the proportions of genotypes, allelic frequencies, gene heterozygosity and PIC of mutations, as summarized in Table 1. These data suggest that the identified mutations contribute to three separable genotypes representing the “common allele”, “heterozygotes” and “homozygotes” for the rare allele, respectively. The GT genotype on 7008G>T mutation contributed 0.6076 to this variability and representing more than half of the effect of three genotypes, and almost twice the proportion attributable to CC genotype. The GG genotype on the 7026G>A mutation, the AG genotype on the 7202A>G mutation and the GG genotype on the 7432G>T
respectively contributed 0.4684, 0.6456 and 0.5696 to the variability, which was almost twice the proportion attributable to the GA, GG and TT genotype, respectively. The GG genotype on the 7437G>C mutation contributed 0.6203 to the variability, which was almost three times the proportion attributable to the CC genotype. As to the allelic frequencies of the 5 mutations, the frequencies of the normal allele ranged from 0.5823 to 0.7089. Meanwhile, the ranges (from mutation sites 7437G>C to 7026G>A) of heterozygosis and PIC of the 5 mutations were from 0.4128 to 0.4865 and from 0.3276 to 0.3681, respectively, and there was no significant change in them.

Theoretically, there are 32 (2^5) possible haplotypes for the 5 mutations in the bovine B2M gene, although we only identified 6 genotyped mutations (GGAGG, TGAGG, GAGTC, TAGTC, GAAGG and GAGTG). Four common haplotypes (freq. > 0.1) were constructed and they also showed strong LDs (Figure 2(a,b)). According to LD analysis (Figure 2(b)), there was a tight LD ($r^2 > 0.33$) between each of the mutations, except for mutation 7008G>T which presented a weaker LD ($r^2 < 0.33$) with all the other mutations. Therefore, the five mutations were chosen as "tag mutations" for analysis of their associations with milk quality traits.

Meanwhile, the relationship between the genotypes of the 79 subject cows and the 4 milk quality traits was analyzed (phenotypic data not shown) and the associations summarized in Table 2. Notably, the mutation 7008G>T...
Table 2. Association of B2M mutation with milk quality traits on 79 milk samples.

| Traits                        | Mutation sites | Position | CC       | CR       | RR       | Genotypes (mean ± SE) | P value |
|-------------------------------|----------------|----------|----------|----------|----------|-----------------------|---------|
| Milk yield (MY) (kg)          | 7008G>T        | Intron 3 | 26.31 ± 1.97 | 26.35 ± 1.05 | 32.12 ± 2.35 | 0.1336               |
|                               | 7026G>A        | Intron 3 | 27.79 ± 1.37 | 25.10 ± 1.39 | 27.43 ± 1.73 | 0.4215               |
|                               | 7022A>G        | Intron 3 | 26.61 ± 1.06 | 29.48 ± 4.16 | 27.43 ± 1.73 | 0.6955               |
|                               | 7432G>T       | Intron 3 | 26.54 ± 1.11 | 28.65 ± 2.75 | 27.58 ± 1.44 | 0.7219               |
|                               | 7437G>C       | Intron 3 | 26.43 ± 1.02 | 28.20 ± 2.22 | 29.35 ± 2.20 | 0.5115               |
| Milk fat percentage (MFP) (%) | 7008G>T        | Intron 3 | 4.51 ± 0.13  | 4.52 ± 0.10  | 4.14 ± 0.33  | 0.3502               |
|                               | 7026G>A        | Intron 3 | 4.42 ± 0.12  | 4.67 ± 0.15  | 4.37 ± 0.14  | 0.3264               |
|                               | 7022A>G        | Intron 3 | 4.51 ± 0.09  | 4.45 ± 0.52  | 4.37 ± 0.14  | 0.7978               |
|                               | 7432G>T       | Intron 3 | 4.47 ± 0.10  | 4.58 ± 0.34  | 4.37 ± 0.14  | 0.786                |
|                               | 7437G>C       | Intron 3 | 4.45 ± 0.10  | 4.45 ± 0.28  | 4.60 ± 0.14  | 0.8577               |
| Milk protein percentage (MPP) (%) | 7008G>T      | Intron 3 | 3.10 ± 0.09a | 3.00 ± 0.05a | 2.67 ± 0.04b | 0.0237               |
|                               | 7026G>A        | Intron 3 | 3.01 ± 0.06  | 3.06 ± 0.08  | 2.80 ± 0.08  | 0.1323               |
|                               | 7022A>G        | Intron 3 | 3.06 ± 0.05b | 2.70 ± 0.10b | 2.80 ± 0.08b | 0.0138               |
|                               | 7432G>T       | Intron 3 | 3.06 ± 0.05b | 2.77 ± 0.11b | 2.88 ± 0.09b | 0.0446               |
|                               | 7437G>C       | Intron 3 | 3.07 ± 0.05b | 2.73 ± 0.09b | 2.83 ± 0.10b | 0.0106               |
| somatic cell count (SCC) (10⁶ cells/ml) | 7008G>T    | Intron 3 | 36.05 ± 0.93 | 34.11 ± 4.86 | 31.12 ± 1.60 | 0.1822               |
|                               | 7026G>A        | Intron 3 | 30.56 ± 5.00b | 44.99 ± 9.27b | 16.20 ± 3.39b | 0.0484               |
|                               | 7022A>G        | Intron 3 | 36.28 ± 4.92 | 28.23 ± 14.87 | 16.20 ± 3.39 | 0.1537               |
|                               | 7432G>T       | Intron 3 | 32.51 ± 4.63 | 32.30 ± 12.79 | 28.64 ± 8.90 | 0.9240               |
|                               | 7437G>C       | Intron 3 | 34.47 ± 4.65 | 26.50 ± 10.10 | 18.18 ± 5.03 | 0.3897               |

CC, CR and RR represent the common allele, heterozygotes and homozygotes for the rare allele, respectively. a,bMeans with different superscripts were significantly different (P < 0.05).

4. Discussion

We have identified five mutations in the B2M gene of dairy cows which display variable heterozygosity, frequency and PIC score, although the individual variances were relatively small (Table 1). In genetic linkage analysis, the PIC value was usually used to detect a specific mutation and a polymorphism within a population, and it depended on the number of available allele and frequency distribution (Wenzl et al., 2004). Originally defined as a codominant marker in a linkage study of a rare dominant disease, PIC has more recently been shown to be relevant regardless of the mode of phenotype inheritance (Shete, Tiwari, & Elston, 2000). Quantitatively, the degree of polymorphism reflects both the degree of heterozygosity and PIC value and classified as low (PIC < 0.25), median (0.25 < PIC < 0.5) or high (PIC > 0.5), with most populations being low or moderate (Mateescu et al., 2005). The PIC values in the present study ranged from 0.3276 to 0.3681, indicating largely moderate degrees of polymorphism in these particular populations.
B2M gene variants with immune function in animal models and human populations, there are still very little data relating such mutations to milk quality traits in dairy herd populations, in which the biology of milk composition and immune status are of paramount importance as determinants of food safety in a key worldwide human supply chain. Our study is a preliminary step in providing the essential tools for analyzing mutations and genetic effects at the bovine B2M gene locus.

5. Conclusion

In summary, we identified multiple mutations in intron 3 of the B2M immune-related gene using DNA isolated from milk samples and investigated its association with milk quality traits across a population of Holstein dairy cows. Analysis of the proportion of genotypes, allelic frequencies, heterozygosity and PIC was performed to explain the mutation distribution across the population. Association analysis revealed that mutations including 7008G>T, 7202A>G, 7432G>T and 7437G>C were all significantly associated with MPP (P = 0.0237, 0.0138, 0.0446 and 0.0106, respectively) and that 7026G>A was significantly associated with SCC (P = 0.0484). Thus, these mutations at the B2M locus should be useful for pre-evaluation and selection for milk quality by genotyping. The enhanced screening of raw milk for quality traits is required to meet increasing expectations of food safety and should translate to increased economic benefit for producers.

Disclosure statement

No potential conflict of interest was reported by the authors.

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