J. Dairy Sci. 93:1749–1753
doi:10.3168/jds.2009-2741

Short communication: Genetic variability in the predicted microRNA target sites of caprine casein genes

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ABSTRACT

The main goal of the current work was to identify single nucleotide polymorphisms (SNP) that might create or disrupt microRNA (miRNA) target sites in the caprine casein genes. The 3′ untranslated regions of the goat α_{S1}-, α_{S2}-, β-, and κ-casein genes (*CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3*, respectively) were resequenced in 25 individuals of the Murciano-Granadina, Cashmere, Canarian, Saanen, and Sahelian breeds. Five SNP were identified through this strategy: c.*175C > T at *CSN1S1*; c.*109T > C, c.*139G > C, and c.*160T > C at *CSN1S2*; and c.*216C > T at *CSN2*. Analysis with the Patrocles Finder tool predicted that all of these SNP are located within regions complementary to the seed of diverse miRNA sequences. These in silico results suggest that polymorphism at miRNA target sites might have some effect on casein expression. We explored this issue by genotyping the c.*175C > T SNP (*CSN1S1*) in 85 Murciano-Granadina goats with records for milk CSN1S1 concentrations. This substitution destroys a putative target site for miR-101, a miRNA known to be expressed in the bovine mammary gland. Although TT goats had higher levels (6.25 g/L) of CSN1S1 than their CT (6.05 g/L) and CC (6.04 g/L) counterparts, these differences were not significant. Experimental confirmation of the miRNA target sites predicted in the current work and performance of additional association analyses in other goat populations will be an essential step to find out if polymorphic miRNA target sites constitute an important source of variation in casein expression.

Key words: goat, casein, microRNA, polymorphism

MicroRNA sequences (**miRNA**) are small, single-stranded RNA that are able to downregulate gene expression by binding specific sequence motifs in the 3′ untranslated region (**UTR**) of target genes, an event that usually involves mRNA destabilization and repression of protein synthesis through a poorly understood mechanism (Baek et al., 2008; Selbach et al., 2008). In a typical mammalian genome, the expression of about one-third of the protein coding genes is modulated by miRNA, illustrating the pervasiveness and biological importance of this regulatory mechanism (Georges et al., 2007). Since the discovery of miRNA-based gene downregulation in *Caenorhabditis elegans* and *Drosophila melanogaster*, a considerable wealth of knowledge has been accumulated in domestic species by expanding the inventory of reported miRNA and their transcriptomic profiles in different tissues and developmental stages (McDaneld, 2009). In cattle, patterns of miRNA expression have been characterized in adipose tissue and mammary gland (Gu et al., 2007), immune and embryonic tissues (Coutinho et al., 2007), and even in specific cell types such as oocytes (Tesfaye et al., 2009) and alveolar macrophages (Xu et al., 2009). The demonstration that muscular hypertrophy in Texel sheep is associated with a G > A substitution creating an illegitimate target site for 2 skeletal muscle miRNA provided the first evidence that variation in miRNA target sites might have important phenotypic consequences in livestock (Clop et al., 2006).

Caseins are the most abundant milk proteins and they are encoded by a highly conserved cluster of genes, encompassing 250 to 350 kb (Rijnkels, 2002). Numbers of casein genes differ among species (Rijnkels, 2002). To date, 4 loci have been described in ruminants: α_{S1}-casein (*CSN1S1*), α_{S2}-casein (*CSN1S2*), β-casein (*CSN2*), and κ-casein (*CSN3*). Casein expression and secretion as micelles are highly regulated processes restricted to the lactation period and modulated by a complex network

Received September 16, 2009.
Accepted November 24, 2009.
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Table 1. List of SNP identified in the 3′ untranslated region of the goat casein genes

Gene[1]	Polymorphism	Frequency[2]
CSN1S1 (14 sequences)	c.*175C > T	C: 0.57 (8/14) T: 0.43 (6/14)
CSN1S2 (24 sequences)	c.*109T > C	T: 0.79 (19/24) C: 0.21 (5/24)
	c.*139G > C	G: 0.79 (19/24) C: 0.21 (5/24)
	c.*160T > C	T: 0.58 (14/24) C: 0.42 (10/24)
CSN2 (25 sequences)	c.*216C > T	C: 0.84 (21/25) T: 0.16 (4/25)

[1]*CSN1S1* = α_{S1}-casein, *CSN1S2* = α_{S2}-casein, *CSN2* = β-casein.

[2]Frequencies were calculated as the number of sequences that display each one of the 2 alternative alleles.

of environmental and genetic factors (Martin et al., 2002). Technological properties of milk are mainly determined by its casein content, highlighting the importance of understanding how these genes are regulated (Martin et al., 2002). In a recent study, Ogorevc et al. (2009) provided in silico evidence suggesting that several miRNA expressed in the bovine mammary gland might target casein genes, a feature that would affect their levels of expression. The main goal of the current work was to investigate the levels of variability in the 3′ UTR of goat casein genes and to evaluate the effect of this genetic variation in the creation or suppression of potential miRNA target sites.

The generation of a SNP data set for the 3′ UTR of the goat casein genes was achieved through a resequencing strategy. Genomic DNA samples from 25 Murciano-Granadina, Cashmere, Canarian, Saanen, and Sahelian goats were employed as templates in the amplification reactions. Because these breeds have very distinct origins, we assumed that we would have captured the most common variations existing at the goat casein loci. Five individuals per population were sequenced to characterize within-breed variability. Primer pairs employed in the amplification of the 3′ UTR regions are detailed in Supplementary Table 1 (available online at http://www.journalofdairyscience.org/). Polymerase chain reactions included 2.5 μL of PCR buffer, 0.75 μL of $MgCl_2$ (50 m*M*), 1 μL of dNTP (5 m*M*), 1.25 μL of each primer (10 μ*M*), 100 ng of genomic DNA, and 0.25 μL of *Taq* DNA polymerase (5 U/μL, Ecogen S.R.L., Barcelona, Spain) in a final volume of 25 μL. The thermal profile consisted of 35 cycles of 94°C for 1 min, annealing temperature (Supplementary Table 1; http://www.journalofdairyscience.org/) for 1 min and 72°C for 2 min. Amplified products were sequenced using the forward and reverse primers with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Sant Andreu de Llavaneres, Spain). Sequencing reactions were precipitated and purified by using Montage SEQ_{96} cleanup kit (Millipore Corporation, Billerica, MA) and analyzed in an ABI PRISM 3100 capillary electrophoresis device (Applied Biosystems). This approach allowed us to identify 5 putative SNP distributed as follows (Table 1): *CSN1S1* (1 SNP), *CSN1S2* (3 SNP), and *CSN2* (1 SNP). No polymorphic sites were found in the goat *CSN3* 3′ UTR region. Levels of variation in the 3′ UTR of caprine casein genes were similar to that reported in the human casein genes. In this way, screening of the Single Nucleotide Polymorphism Database (dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP) allowed us to retrieve 3, 3, and 1 putative polymorphisms located in the 3′ UTR of human *CSN1S1*, *CSN2*, and *CSN3* genes, respectively. In strong contrast, we did not find any polymorphic sites in cattle. This might be because of the poor annotation of the genetic variation existing in the bovine genome.

Prediction of polymorphic miRNA target sites in the goat *CSN1S1*, *CSN1S2*, and *CSN2* genes (*CSN3* was excluded from this analysis because its 3′ UTR was monomorphic) was performed with the Patrocles Finder tool (Hiard et al., 2010; http://www.patrocles.org/MyPatrocles.php). The Patrocles Finder tool can compare a pair of alleles differing at one or more positions and search for polymorphic miRNA target sites using 2 algorithms (Lewis et al., 2005; Xie et al., 2005).

Results generated with this online tool are shown in Table 2. Analysis of the *CSN1S1*-3′ UTR c.*175C > T SNP showed a polymorphic miRNA heptamer target site for miR-936, miR-101, and miR-144 (T allele). With regard to *CSN1S2*, Patrocles analysis predicted 7 (miR-220b, miR-500, miR-501-3p, miR-502, miR-502-3p, miR-767-5p, and miR-770-5p), 2 (miR-545, miR-873), and 1 (miR-665) polymorphic miRNA sites for c.*109T > C, c.*139G > C, and c.*160C > T SNP, respectively (Table 2). Finally, at polymorphism c.*216C > T of *CSN2*, the C allele present in the reference sequence generated a polymorphic potential target

Table 2. List of polymorphic microRNA target sites identified in the 3′ untranslated regions of goat casein genes with the Patrocles Finder tool (Clop et al., 2006; http://www.patrocles.org/MyPatrocles.php)

Target gene[1]	miRNA	Motif length	Variant holding the target	Species[2]	Reference[3]
CSN1S1	miR-101	Heptamer	c*175T	Hsa, Mmu, Cfa, Bta	3
	miR-144	Heptamer	c*175T	Hsa, Mmu, Cfa	1, 2
	miR-936	Heptamer	c*175T	Hsa	No reference
CSN1S2	miR-220b	Heptamer	c*109T	Hsa	1
	miR-500	Heptamer	c*109T	Mmu	1, 2
	miR-501–3p	Heptamer	c*109T	Hsa, Mmu	No reference
	miR-502	Heptamer	c*109T	Cfa	No reference
	miR-502–3p	Heptamer	c*109T	Hsa	No reference
	miR-545	Octamer	c*139G	Hsa	3, 4
	miR-665	Heptamer	c*160T	Hsa, Mmu	1
	miR-767–5p	Heptamer	c*109T	Hsa	No reference
	miR-770–5p	Octamer	c*109T	Mmu	No reference
	miR-873	Heptamer	c*139C	Hsa, Mmu	1
CSN2	miR-33a*	Heptamer	c*216C	Hsa, Mmu	1, 2
	miR-409–3p	Octamer	c*216C	Hsa, Mmu	No reference
	miR-653	Heptamer	c*216C	Hsa, Mmu	1, 2

[1]*CSN1S1* = α_{S1}-casein, *CSN1S2* = α_{S2}-casein, *CSN2* = β-casein.

[2]Species in which these microRNA have been described (according to Patrocles): Hsa = *Homo sapiens*, Mmu = *Mus musculus*, Cfa = *Canis familiaris*, Bta = *Bos taurus*.

[3]References in which these microRNA have been identified in cattle: 1 = Strozzi et al. (2009); 2 = Artzi et al. (2008); 3 = Coutinho et al. (2007); 4 = Tesfaye et al. (2009).

for human and mouse miR-409-3p (octamer motif) and miR-653 and miR-33a* (heptamer motifs). Although Patrocles suggested polymorphic targets for several miRNA, it included only one bovine miRNA. This is probably because of the poor characterization of the bovine miRNA, which is much less advanced than that of human and mouse. Moreover, Patrocles is based on the miRNA repertoire of the miRBase Sequence Database version 11.0 released on April 2008, which contained very few bovine miRNA compared with the current version. As predicted by interspecies sequence homology or found by short RNA library sequencing in bovine tissues (Coutinho et al., 2007; Artzi et al., 2008; Strozzi et al., 2009; Tesfaye et al., 2009), most of these miRNA are likely to be present in domestic ruminants (Table 2).

We performed an additional analysis with TargetScanHuman v. 5.1 (Lewis et al., 2003; Grimson et al., 2007) and miRanda (Enright et al., 2003), 2 online engines that detect miRNA target sites in species in which whole genome sequences are available. Bovine casein genes were used as templates in the bioinformatics analyses and information generated this way was extrapolated to goats. We were unable to make an in silico prediction for the *CSN1S2* gene with miRanda and TargetScan because there is no orthologous locus in the human genome. In silico predictions for miRNA target sites at *CSN1S1* and *CSN2* genes varied depending on the software employed (Supplementary Figure 1, supplementary files 1 to 4; http://www.journalofdairyscience.org/). TargetScan (bta-miR-101, bta-miR-204, and bta-miR-380-3p) and miRanda (bta-miR-21*, bta-miR-199a-3p, and bta-miR-204) identified 3 miRNA target sites at the bovine *CSN1S1* but only one was simultaneously identified in both analyses (Supplementary Figure 1, supplementary files 1 and 2; http://www.journalofdairyscience.org/). Similarly, the bovine *CSN2* gene had 1 (bta-miR-145) and 3 (bta-miR-23a, bta-miR-23b, and bta-miR-142) miRNA sites when analyzed with TargetScan and miRanda, respectively (Supplementary Figure 1, supplementary files 3 and 4; http://www.journalofdairyscience.org/). The lack of concordance between TargetScan and miRanda predictions might be because TargetScan allows the occurrence of mismatches as long as they are compensated for by conserved 3′ pairing sites, whereas miRanda demands strict complementarity at the seed region (Watanabe et al., 2007). Of note, miRanda predicted a large number of binding sites for human and murine miRNA in the bovine casein genes (supplementary files 1 and 3; http://www.journalofdairyscience.org/). Among these, miRanda coincided with Patrocles in the prediction of polymorphic miRNA target sites for hsa-miR-936 (*CSN1S1*) and hsa-miR-653 (*CSN2*).

Because sequence-based miRNA target predictions are prone to high false positive rates (about 20–30%), the validation of miRNA sites through an experimental approach is of outmost importance. We searched Tarbase v.5c (http://diana.cslab.ece.ntua.gr/tarbase) to look for miRNA targeting human and mouse casein genes but we did not find any positive matches. As an alternative strategy to further evaluate our in

Table 3. Frequencies and phenotypic effects of goat c.*175C > T polymorphism genotypes on milk α_{S1}-casein (CSN1S1) concentrations

	Genotype		
Item	CC	CT	TT
Absolute frequency (n = 85)	63	13	9
Milk CSN1S1 (g/L)	6.04 ± 0.76	6.05 ± 0.58	6.25 ± 0.45

silico predictions, we considered the expression of the predicted miRNA in the bovine mammary gland as evaluated by Gu et al. (2007). Interestingly, 7 of the miRNA we identified are expressed in the bovine udder (bta-miR-21*, bta-miR23a, bta-miR23b, bta-miR-101, bta-miR-142, bta-miR-145, and bta-miR-199a-3p) as shown by Gu et al. (2007). These findings are relevant because 30 to 50% of nonconserved miRNA target sites in the human 3′ UTR are functional when expressed in the same tissues as the cognate miRNA (Chen and Rajewsky, 2006).

With the aim of gaining new insights into the effect of polymorphic miRNA target sites on casein expression, we decided to genotype the c.*175C > T SNP (*CSN1S1*) in a population of 85 Murciano-Granadina goats with records of milk CSN1S1 concentration. There were several reasons for selecting this polymorphism to perform an association study: 1) the miR-101 target site was independently identified with Patrocles and TargetScan, 2) miRNA-101 is known to be expressed in the bovine mammary gland, and 3) there is evidence that miR-101 plays a fundamental role in the development of the mouse mammary gland and that it is able to suppress *CSN2* expression through an indirect mechanism (Tanaka et al., 2009). Our hypothesis was that the T allele should be associated with higher levels of CSN1S1 because this substitution disrupts the miR-101 target site. The polymorphic region was amplified with primers CSN1S1-SNP-Fw and CSN1S1-SNP-Rv (Supplementary Table 1; http://www.journalofdairyscience.org/) using the PCR conditions described above. Genotyping of the c.*175C > T polymorphism at *CSN1S1* was performed by primer-extension analysis using SnaPshot ddNTP Primer Extension Kit (Applied Biosystems), and the extension primer used was 5′-CTC TTT CTT CTT GAG TTC TC-3′. Levels of milk CSN1S1 were measured according to the protocols reported in Caravaca et al. (2008). Association analysis was done as indicated by Badaoui et al. (2007) using SAS software (SAS 9.2; SAS Inst. Inc., Cary, NC). Goat *CSN1S1* and *CSN3* genotypes had been previously determined as described in Caravaca et al. (2009) and introduced as fixed factors in the statistical model. This approach revealed that, although TT goats had more CSN1S1 in milk than their CC counterparts (a feature that is expected because TT goats lack the miRNA site), differences among C.*175C > T polymorphism genotypes were not statistically significant ($P = 0.86$; Table 3). In fact, the only 2 factors that had significant effects on milk CSN1S1 levels were month of lactation ($P < 0.0001$) and *CSN1S1* genotype (i.e., *CSN1S1* polymorphisms excluding c.*175C > T polymorphism; $P < 0.0001$). This lack of significance could be caused by 1) low experimental power linked to the reduced sample size, 2) environmental effects not corrected in our model, or 3) a false positive prediction of the miR101 target site in *CSN1S1*.

The main conclusion that can be derived from the current study is that the 3′ UTR of the goat *CSN1S1*, *CSN1S2*, and *CSN2* genes are polymorphic, with the level of variation being particularly high at the *CSN1S2* locus. Moreover, we have provided in silico evidence suggesting that the SNP we have identified create or abolish miRNA target sites at the 3′ UTR of these 3 casein genes. Further studies will be needed to confirm, at the experimental level, the existence of these miRNA sites and to determine if their variability has any effect on the translational rate of caseins.

ACKNOWLEDGMENTS

This work was funded with grants from the Spanish Ministry of Education and Science (AGL2002-04304-C03-02 GAN) and from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (RZ2007-00005-C02-01). A. Zidi received fellowships of the Spanish Agency of International Cooperation and Development and of the Centre for Agrigenomics Research.

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