Association of polymorphism within *LTF* gene promoter with lactoferrin concentration in milk of Holstein cows

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Abstract

This study analyzed the association between single nucleotide polymorphism (A/C) in position -28 located in the TATA box of *LTF* gene and the lactoferrin concentration in bovine milk secreted by healthy and infected udders. Out of 241, 69 cows were selected into the experimental group and were divided into 3 groups according to mean value of somatic cell count (SCC): I <180 000 cells/mL, II: 180 000 – 350 000 cells/mL and III > 350 000 cells/mL. In each SCC group, three *LTF* genotypes: AA, AC and CC were identified by PCR-SSCP method. A total of 604 milk samples were collected monthly and lactoferrin concentration was measured by ELISA. The 1-way ANOVA within SCC groups was performed to estimate association of -28 A/C genotypes with mean lactoferrin concentration per lactation. In the group of healthy cows (<180 000 cells/mL) LTF concentration in milk cows with the AA genotype (107.58 ± 17.92 μg/mL) was significantly higher than in homozygotes CC (52.09 ± 19.01 μg/mL). Unexpectedly, in cows with elevated SCC (>350 000 cells/mL) we observed a significant opposite relationship (207.21 ± 28.50 in CC *vs* 115.0 ± 28.6 μg/mL in AA). We hypothesized that a promoter with allele C, which cannot be recognized as a TATA sequence is becoming more accessible for other transcription factors, which may induce alternative *LTF* gene expression. We assume that our results demonstrate a very interesting effect of differential gene expression depending on polymorphism in a key regulatory motif (TATA box) and also on the health status of mammary tissues.

Key words: cattle, holstein, lactoferrin, gene, polymorphism, mastitis

Introduction

Lactoferrin (LTF) is an iron-binding 80-kDa glycoprotein, composed of 690 amino acids (Baker and Baker 2009) naturally occurring in milk. It participates in the innate response of the mammary gland against bacterial infections leading to mastitis (Rainard 1993, Chaneton et al. 2008). LTF plays multiple biological roles, ranging from direct activities against a large panel of microorganisms, including bacteria, viruses, fungi and parasites, to anti-inflammatory and anti-cancer activities (Wakabayashi et al. 2006, Berlutti et al. 2011, Garcia-Montoya et al. 2012). Granulocytes and mammary epithelial cells

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produce lactoferrin under hormonal regulation (Malewski 2002, Ward et al. 2005). Bovine LTF concentration in milk from healthy mammary glands is low: 100-400 µg/mL (Kutila et al. 2004) and significantly increases during immunological stress (Kawai et al. 1999, Hagiwara et al. 2003, Chaneton et al. 2008). A high positive correlation between somatic cell count (SCC) and LTF content in milk was reported by Hagiwara et al. (2003) and Cheng et al. (2008a). The highest LTF concentration is detected during dry-off and early mammary involution (Kutila et al. 2003, Newman et al. 2007, Piantoni et al. 2010). From 79 SNPs (Single Nucleotide Polymorphisms) identified in the bovine *LTF* gene (Seyfert et al. 1994, Li et al. 2004, Daly et al. 2006, O'Halloran et al. 2009, Huang et al. 2010, Bahar et al. 2011), substitution of A/C in position -28 within the TATA – box seems to have the most probable influence on *LTF* gene expression in the mammary gland. As reported by O'Halloran et al. (2010), Bahar et al. (2011) and our group (Zabolewicz et al. 2012) this SNP located in the key regulatory motif responsible for initiation of the gene transcription, affects the transcription complex and is associated with routine traits recorded in Holstein cattle such as SCC. So far, however, little is known about the effect of A/C point mutation within the TATA box on the LTF concentration, considering the health status of the udder. Therefore, the aim of the study was to assess association between SNP (A/C) in position -28 located in the TATA box of *LTF* gene and lactoferrin concentration in the milk secreted by healthy and infected udders.

Materials and Methods

Cows and milk sampling

Two hundred and forty-one cows from one Polish Holstein-Fresian herd were tested to identify the genotype of A/C polymorphism in position -28 of the *LTF* gene. Among them, 69 cows were selected for the experimental group. They had to fulfill two criteria: all cows were in the second lactation and belonging to one of three groups of somatic cell count. Since cows with CC genotypes occurred rarely, they limited the final number of cows in each experimental subgroup.

During routine milk recording, milk samples from selected cows were collected from June 2011 to December 2012 and frozen (-20°C) until used for assays. They were progeny of different sires. A total of 604 milk samples were collected monthly at least seven times during lactation (stage of lactation). The animals were held in free-stall barns. An animal feeding

system established over both the winter and summer season was based on TMR. The average milk yield was 8.882 ± 1.901 kg.

The relationship between stage of lactation, milk lactoferrin concentration, SCC was analyzed using 604 samples. SCC values were transformed into logarithmic form with the base of ten.

All experimental cows (n=69) were also split into 3 groups according to mean SCC value of the complete second lactation: group I: <180 000 cells/mL, II: 180 000 – 350 000 cells/mL and III > 350 000 cells/mL. In each SCC group three *LTF* genotypes were represented by 3-9 cows carrying AA, AC and CC *LTF* genotypes. The arithmetic mean of LTF concentration per lactation for each cow was used for statistical analysis.

SCC

The SCC in milk was determined with a Fossomatic Cell Counter and scored in an official milk recording system.

LTF content

Lactoferrin concentration (µg/mL) milk was measured using the Bovine Lactoferrin ELISA Quantification Kit (Bethyl Laboratories Inc.) according to the manufacturer's instructions. Absorbance was measured at 450 nm using the Multiscan®'s FC (Thermo Scientific). Milk samples were diluted 1:500, 1:1500, or 1:3,000 in sample buffer and each sample was duplicated within an assay. A standard curve was generated for each set of samples and the assay itself was then performed in duplicate. Mean values of LTF concentration (µg/mL) were positively skewed and were therefore transformed using the logarithm with the base ten.

Statistical Analysis

The obtained results were analyzed by ANOVA test of Statistica software version 10. (StatSoft, Inc. 2011). The distribution of milk LTF concentration (log10) was tested by a chi-square test for goodness of fit. The homogeneities of variance were analyzed by Levene's test in groups classified by the stage of lactation, SCC group and genotype of SNP (A/C). The analysis also included a 1-way ANOVA analysis using Bonferroni correction. A P value of less than 0.05 was considered significant and with <0.01 was highly significant.

Table 1. Genotype and allele frequency of *LTF* in analyzed Polish Hostein-Fresian herd.

Number of genotypes	Genotypes frequency (%)	Allele frequency
A/A – 132	54.8	A – 0.70
A/C – 72	29.9	
C/C – 37	15.3	C – 0.30
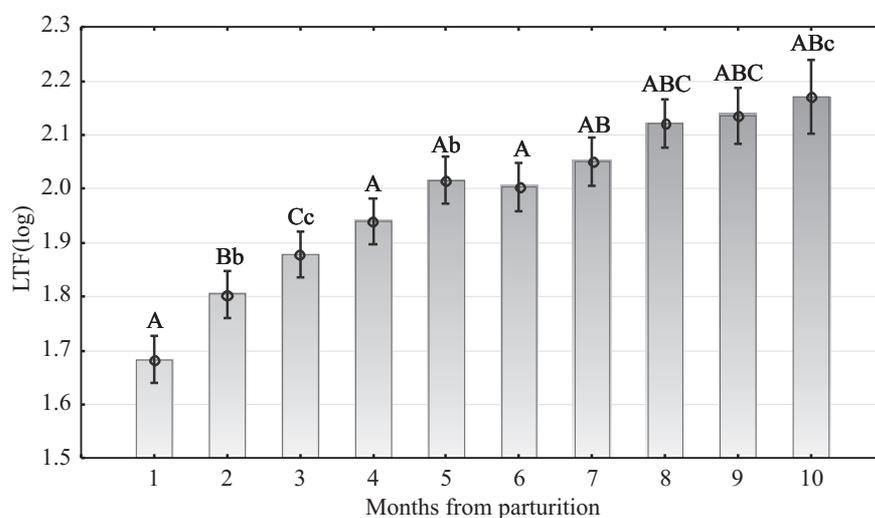
Fig. 1 Mean and ± SD of milk lactoferrin concentration (log transformed) during lactations; A,B,C The same letter indicate high significant (P<0.01) differences in the means; a,b,c The same letter indicate significant (P<0.05) differences in the means.
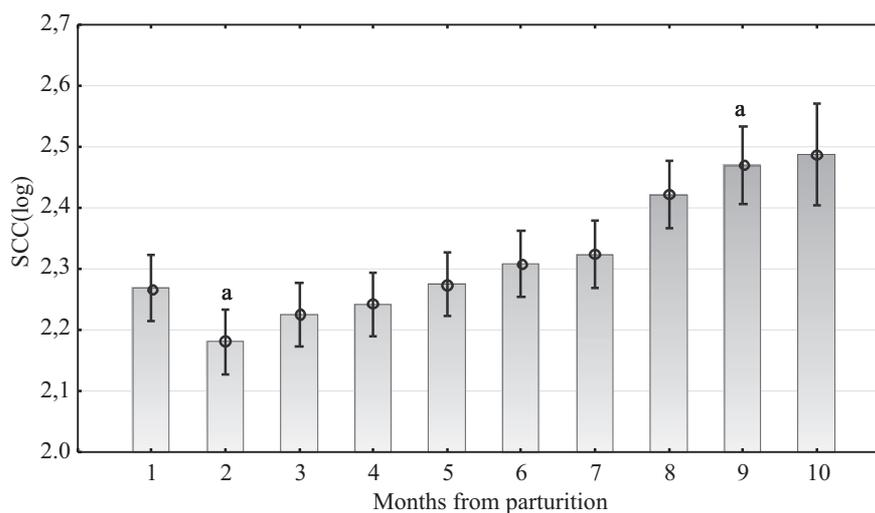
Fig. 2. Mean and ± SD of SCC (log transformed) during lactations; a Letter indicate significant (P<0.05) differences in the means.

SNP detection

The -28 A/C polymorphism within the TATA-box of the *LTF* promoter was identified by the PCR-SSCP method published by our group earlier (Zabolewicz et al. 2012).

In silico analysis of bovine LTF promoter

The relevant promoter region (-300 b to +1 relative to the transcription start side) of the NCBI bovine *LTF* gene sequence (GenBank Ref. AY319306, NC_007320) was analyzed using MATINSPECTOR

Table 2. Mean and ± SD of lactoferrin concentration in the milk from cows with different somatic cells count (1-way ANOVA).

SCC group (cells/mL)	Number	Milk LTF concentration (log)		P – value
		mean	±SD	
<180 000	25	**1.88[A,a]**	0.23	
180 000 – 350 000	20	**2.08[a]**	0.31	0.0004
>350 000	24	**2.17[A]**	0.21	
Total	69	2.04	0.28	

[a] Superscrips indicate high significant (P<0.01) differences in the means.
[A] Superscrips indicate significant (P<0.05) differences in the means.

Table 3. Relationship between *LTF* genotype and average concentration of lactoferrin in milk per lactation within SCC groups of cows (1-way ANOVA).

SCC group (cells/mL)	Genotype	Number	Milk LTF concentration (log)		P – value
			mean	SD	
<180 000	AA	9	**1.97[a]**	0.22	
	AC	8	1.95	0.24	0.0216
	CC	8	**1.70[a]**	0.12	
180 000-350 000	AA	8	2.13	0.11	
	AC	9	2.10	0.11	0.427
	CC	3	1.86	0.18	
>350 000	AA	9	**2.04[b]**	0.15	
	AC	6	2.21	0.24	0.039
	CC	9	**2.28[b]**	0.19	
Total		**69**	**2.09**	**0.28**	

[a,b] Superscrips (the same letter) indicate significant (P<0.05) differences in the means.

8.0 (Genomatix software GmbH) with the default settings. Putative transcription factor (Tf) binding sites were scored, using matrix similarity values >75%, on both strands of the DNA template (Cartharius et al. 2005). The analysis was performed using both allele sequences A and C at -28 polymorphic side.

Results

Genotyping of LTF A/C polymorphism

Among 241 cows screened by the SSCP method: 37, 72 and 132 turned to have CC, AC and AA genotype, respectively (Table 1). No significant differences in milk yield between cows of different *LTF* genotypes were observed (P=0.956). They account for 8 928 ± 1 309 kg for AA genotype, 8 783 ± 2 297 kg AC and 8 935 ± 2 133 kg for CC genotype (data not shown).

Lactoferrin content during lactation

Mean LTF concentration in milk of a total of 604 collected samples were 134.8 ± 154.4 µg/mL.

Fig. 1 shows the mean and ± SD of LTF concentration (log transformed) in milk from each of 10 periods of lactation. The concentration of LTF increased 3-fold during lactation from 1.68 ± 4.67 in the first month after parturition to 2.17 ± 4.66 at the end of lactation. The LTF concentrations measured in milk in the 1st month of lactation were significantly lower than in milk taken between 4th and 10th month after parturition. The average LTF concentration observed on the 60th day of lactation was significantly lower than that measured in the 5th month as well as in last four months of lactation. The LTF concentrations observed during the three months before end of lactation were significantly higher than those measured in the 3rd month after parturition.

SCC during lactation

The mean SCC of a total of 604 milk samples was 367 000 ± 685 000 cells/mL and ranged from 10 000 to 9 353 000 cells/mL. Changes in milk SCC in logarithmic form during lactation is shown in Fig. 2. Only the difference between SCC in milk samples taken in the 2nd and 9th month after parturition was significantly different (P=0.021).

Table 4. Putative transcription factor binding sequence altered by SNP -28 A/C.

Transcription factor	Recognition sequence[1,2]	Matrix similary	Target strand
Estrogen response elements	tcgtgagGTCAccgagcactgga	0.891	+
Bipartite binding site of VDR/RXR heterodimers, DR1 sites	cgagcactGGATaaagggacgcaga	0.763	+
Se-Cys tRNA gene transcription activating	gctcgttctgcgtCCCTttatccagtgctcg	0.787	–
GATA-binding factor 1	actgGATAaaggg	0.897	+

[1] Core sequence in CAPITALS.
[2] SNP loci underlined.

SCC association with LTF concentration

The average concentration of LTF per lactation (log transformed) differed significantly between selected SCC group and tended to increase proportionally to increase of the SCC (Table 2). The mean of LTF concentration in healthy cows (SCC<180 000 cells/mL) was 87.77 ± 36.35 μg/mL and was significantly lower (P = 0.0297) than that in milk from the SCC group of 180 000-350 000 cells/mL (mean content 150.60 ± 104.94 μg/mL.) The mean LTF concentration in milk of infected cows (>350 000 cells/mL) was 166.97 ± 91.27 μg/mL and was significantly higher (P = 0.000) than that in non-infected cows.

Association of LTF promoter genotypes with lactoferrin content in milk

Since the LTF content was significantly affected by SCC, 1-way ANOVA within SCC groups was performed to estimate the association of -28 SNP genotypes with the average lactoferrin concentration. (Table 3). In the group of healthy cows (<180 000 cells/mL) the average LTF concentration in cows with an AA genotype was significantly higher (107.58 ± 17.92 μg/mL) than in opposite homozygotes CC (52.09 ± 19.01 μg/mL). However, within the group of cows with infected mammary glands (>350 000 cells/mL) we observed a significant (P=0.035) opposite relationship (207.21 ± 28.50 in CC *vs* 115.06 ± 28.6 μg/mL in AA).

In silico identification of Tf binding sites

Analysis of the 300-bp promoter region of the bovine *LTF* -28 A allele generated a total of 92 Tf binding sites. The SNP-28 A/C alters the putative binding sequence for four identified transcription factors: Estrogen response elements (EREF), Bipartite binding side of VDR/RXR heterodimers – DR1 (VDR/RXR), Se-Cys tRNA gene transcription activating factor (STAF), GATA-binding factor 1 (GATA) (Tab.4). Analysis of the sequence with the substitution A>C at this position showed that two transcription factors: (GATA) and (STAF), lost putative binding sites (matrix similarity values <75%).

Discussion

In many countries, the effects of individual SNPs evenly spaced along the genome are currently applied for the estimation of genomic breeding value in dairy cattle (Hayes et al. 2009, Calus 2010, Szyda et al. 2011). These SNPs, mostly located in non-coding sequences, can indicate regions or neighboring genes contributing to genetic variation, but do not uncover casual mutation. Point mutation located in the functional segments of a gene-like promoter can potentially influence gene expression. The bovine lactoferrin gene was mapped to bovine chromosome 22 (Schwerin et al. 1994) in a region recognized as very rich in putative QTL for milk yield, milk energy yield, fat yield, protein percent, calf size, milking speed, SCC and clinical mastitis (Heyen et al. 1999, Rupp and Boichard 2003, Harder et al. 2006, Lipkin et al. 2008, Lund et al. 2008, Thomasen et al. 2008).

The results of many studies confirmed that *LTF* gene may be considered as QTL for milk performance traits and natural resistance to mastitis (Seyfert et al. 1996, Li et al. 2004, Daly et al. 2006, Kamiński et al. 2006, Huang et al. 2010, Sender et al. 2010, Bahar et al. 2011, Zabolewicz et al. 2012, Wojdak-Maksymiec et al. 2013).

In our work, using a Bovine Lactoferrin ELISA Kit, we found that the average LTF content was 134.8 ± 154.4 SD μg/mL and was very similar to the level observed by Arnould et al. (2009) (137.8 ± 176.7 μg/mL) who measured it in the huge number of samples (N=11.310) using mid-infrared spectrometry. A slightly lower average LTF content was obtained by Cheng et al. (2008a) (115.4 ± 67.7 μg/mL) by using the same kit as we did. Higher values were observed by Hagiwara et al. (2003) (169.1 ± 2.5 μg/mL) who measured LTF concentration by a single radial immunodiffusion test in Chinese and Japanese Holstein cattle. A lower lactoferrin content

(79.07-84.72 μg/mL) in the Polish Holstein Black-White population (N=559) was observed by Litwińczuk et al. (2011) by using reverse--phase HPLC.

The low LTF concentration during the first 2 months of lactation and the highest during the last 3 months could be explained by the dilution effect observed by Arnould et al. (2009) and Soyeurt et al. (2007) who obtained a negative genetic correlation between daily milk production and LTF content of -0.25 and -0.36, respectively. A significant increase in LTF concentration in the following stages of lactation was in agreement with the results obtained by other authors (Hagiwara et al. 2003, Cheng et al. 2008a). We also found a significantly higher LTF level in milk with higher SCC. This observation confirms the previous conclusions of many reports showing the increase in lactoferrin content in milk from infected udders compared with healthy cows (Kawai et al. 1999, Hagiwara et al. 2003, Cheng et al. 2008b, Arnould et al. 2009). LTF level in milk is believed to be a good indicator of mastitis because of a high positive correlation between LTF and SCC (Lindmark-Mansson 2006, Soyeurt et al. 2007, Arnould et al. 2009) and is also useful in detecting the false-positive results of screening tests for antibiotic residues in milk (Malinowski et al. 2008). Cheng et al. (2008b) observed a significant positive correlation of lactoferrin concentrations between serum and milk in early phase of the immune response. Therefore, it is thought that polymorphonuclear leukocytes are the source of lactoferrin in milk, especially during stimulation of the immune system. The elevation of LTF mRNA expressed in udder tissue during mastitis (Schmitz et al. 2004, Griesbeck-Zilch et al. 2008) proved the that LTF in the udder also originates from the secretory epithelium (Molenaar et al. 1992, Pfaffl et al. 2003, Zheng et al. 2005).

Among many candidate genes involved in natural resistance to mastitis, *LTF* seems to be good candidate. Intense research on the gene structure and its variation led to discovery of twenty-nine SNPs identified in the 2.2 -kb bovine lactoferrin promoter (Li et al. 2004, Daly et al. 2006, O'Halloran et al. 2009). Sixteen of them alter putative binding sequences of Tfs (Pawlik et al. 2009), suggesting high complexity of *LTF* gene expression. Moreover, two sites for TATA binding proteins (TBP) which play a major role in the transcription processes were identified in bovine *LFT* promoter: a non-canonical TATA-box (ATAAA) affected by SNP at -28 (Zheng et al. 2005) and a vertebrate TATA binding protein site affected by the SNP at -1702 (Bahar et al. 2011). The SNP-28 A/C alters the putative binding sequence for four transcription factors (Table 4). In analyzing milk LTF concentra-

tion in normal lactating cows (SCC<180 000 cells/mL), we found a significantly higher average lactoferrin content in milk of AA cows than cows carrying the CC genotype. Similar results were obtained by Bahar et al. (2011) in infection-free milk. Lactoferrin is an example of the gene which reacts to the environmental stimulus coming from the mammary gland. This hypothesis is supported by its parity-dependent effect (Wojdak-Maksymiec et al. 2013) and by our data (Table 2, 3) which show its dependence on SCC. Like many other milk components, fluctuations of LTF in the following stages of lactation are natural and are reflected by dynamic changes in mammary gland, especially in highly productive cows. Since lactoferrin is involved in response to bacterial infection (Chaneton et al. 2008), it is more interesting to show the overall effect of *LTF* polymorphism faced by health status of mammary gland than to trace its changes in the following stages of lactation. As we proved earlier, using the EMSA technique (Zabolewicz et al. 2012), a promoter sequence with the A allele was more efficiently bound by the nuclear proteins isolated from healthy mammary tissues than a promoter containing the C allele. This observation is in accordance with results published by Bahar et al. (2011) who demonstrated that promoter constructs, including allele A, exhibited 1.44 – fold higher basal transcriptional activity. Taken together, the transversion A to C disrupts a putative Tf binding site for TBP, GATA and STAF, resulting in a lower level of basal *LTF* gene expression in a healthy mammary gland.

Unexpectedly, an opposite relation between LTF concentration and *LTF* genotype was observed in cows showing the highest SCC (>350 000 cells/mL). Milk from cows carrying the CC genotype have a significantly higher LTF content than AA cows. This interesting phenomena might be explained by differential induction of Tfs depending on *LTF* genotype. We hypothesized that a promoter with allele C, which cannot be recognized as a TATA sequence and also as binding side for GATA or STAF becomes more accessible for EREF and VDR/RXR (Table 4), which may induce *LTF* gene expression. The EREF Tf functions under the influence of the reproductive hormone estrogen (Katzenellenbogen et al. 2000, Teng 2002). VDR/RXR is a multi-domain protein and nuclear receptor (NR) of vitamin D and functions as an obligate heterodimer with the retinoid X receptor (RXR) (Zhang et al. 2011, Gocek et al. 2012). This receptor belongs to the family of trans-acting transcriptional regulatory factors. The active form of vitamin D (1.25(OH)2D3) is a primary regulator of calcium and skeletal homeostasis (Adams and Hewison 2008) and is also required for optimal immune

response (Adams and Hewison 2010, Lippolis et al. 2011, Nelson et al. 2012). Bovine monocytes/macrophages produces 1.25(OH)2D3 from 25(OH)D3 both in vitro (Nelson et al. 2010b) and in vivo (Nelson et al. 2010a) following bacterial activation. Nelson et al. (2012) reports that increased 1.25(OH)2D3 synthesis is associated with activation of the VDR/RXR Tf in infected mammary glands. If we assume that *LTF* gene expression might be up-regulated by a higher level of 1.25(OH)2D3 during mammary infection as an effect of preferential binding of VDR/RXR to promoter with C allele, it might explain the higher concentration of LTF in milk of CC cows (having the highest SCC).

It was observed that lactoferrin content in milk is affected by A/C point mutation within TATA box of *LTF* gene in the way which is dependent on somatic cell counts. In the group of healthy cows LTF concentration in cows with the AA genotype was significantly higher than in homozygotes CC. Within groups of cows, however, with infected mammary gland opposite relationship was observed. We hypothesized that a promoter with allele C, which cannot be recognized as a TATA sequence is becoming more accessible for other transcription factors, which may induct alternative *LTF* gene expression.

Acknowledgments

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