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Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

Expression and polymorphism of ADAM32 gene and its association with somatic cell count in Holstein-Friesian cows*

Tadeusz Zabolewicz, Paweł Brym, Katarzyna Sitarz, Kamil Oleński, Barbara Bojarojć-Nosowicz, Stanisław Kamiński**

Department of Animal Genetics, University of Warmia and Mazury in Olsztyn, Oczapowskiego 5/114, 10-719 Olsztyn, Poland

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The aims of this study were to confirm ADAM32 gene expression in selected bovine tissues and identify potentially functional polymorphisms within the 5'flanking region. The associations between the polymorphism within *ADAM32* and somatic cell count in the Holstein-Friesian cow's milk were examined. Expression studies of *ADAM32* in different tissues performed by RT-PCR revealed the presence of the ADAM32 transcript in the liver, mammary and pituitary gland and leukocytes, except for monocytes. Comparative sequencing analysis of 870 bp PCR amplicons obtained from four different *Bos taurus* breeds and the *Bison bonasus* fragment of the *ADAM32* 5' flanking region showed a 21 bp insertion/deletion at position -541 nt to -520 nt and the occurrence of seven SNPs. *In silico* analysis of the 1180 bp 5' flanking region of *ADAM32* indicated that the 21 bp deletion abolished putative binding sites for six transcription factors associated with the immune system.
A population of one hundred and two Polish Holstein-Friesian bulls and 241 cows - progeny of 15 heterozygous (*InsDel*) sires were screened. The frequency of genotypes (for sires) were observed at 0.765, 0.235, 0.0 for *DelDel, InsDel* and *InsIns*, respectively, whereas for cows the frequencies are as follows: 0.365, 0.510 and 0.125. The association study performed within the group of daughters of heterozygous sires revealed that cows with the *DelDel* genotype showed significantly higher somatic cell counts than those with the *InsIns* genotype (*P*=0.013). No significant association was found between cows' genotypes and milk production traits.

KEY WORDS: cattle/ ADAM32 / polymorphism / *mastitis*

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**Corresponding author. tadeo@uwm.edu.pl

Susceptibility to udder infections referred to as *mastitis* causes great economical losses among high producing cows. Therefore, the aim of extensive research is to recognize the hereditary specificity of the mammary gland defense system [Walawski 1999]. ADAM32 - transmembrane protein belongs to the A Disintegrin and Metolloproteinase (ADAMs) superfamily. ADAMs mediate cell ectodomain shedding and non-proteolyic ligand binding, which are very important in a number of biological processes such as the interaction of the sperm and the egg [Cho 2012], cell fate determination, cell migration, wound healing, neurite and axon guidance [Edwards *et al*. 2008], heart development [Weber and Saftig 2012], immunity [Reiss and Saftig 2009, Tang *et al*. 2013], cell proliferation and angiogenesis [Brocker *et al*. 2009]. The contribution of other members of the ADAMs family (mainly ADAM17, ADAM10, ADAM9 and ADAM8) in immune response is commonly acknowledged [Sternlicht *et al*. 2005; Edwards *et al*. 2008, Breshears *et al*. 2012, Weber and Saftig 2012, Johnson and Johnson 2013].

Scanning of the Polish Holstein-Friesian genome using the Illumina Bovine SNP50 BeadChip showed a subregion on BTA 27 significantly associated with somatic cell count (SCC) in milk [Szyda *et al*. 2011]. This subregion contains six genes including *ADAM32*. The aims of the present study were to confirm ADAM32 gene expression in selected bovine tissues and identify potentially functional polymorphisms within the 5'gene region. The associations between the polymorphism within *ADAM32* and somatic cell count in the Holstein-Friesian cow's milk were examined.

Material and methods

ADAM32 gene expression

Blood and tissue samples from 2 Holstein-Friesian, 2 Limousine and 2 Hereford cows were collected. Blood samples were processed immediately after collection, while liver, pituitary and mammary tissues were stored in liquid nitrogen until RNA isolation. Peripheral blood mononuclear cells (PBMCs) and granulocytes were isolated by density gradient centrifugation on Histopaque 1077 based on the manufacturer's recommendations (Sigma, USA). Monocytes were separated by magnetic sorting using the MACS Technology (Miltenyi Biotec GmbH) according to the manufacturer's protocol with modifications described by Szczotka *et al*. [2012]. The quality of cell separation was confirmed by Giemsa stained smear microscopy analysis [Barcia 2007]. RNA isolation was performed with TRI-Pure (Roche) and the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's protocol. Only high quality samples characterized by RIN (RNA Integrity Number) >8.0, 28s/18s >1.6 and A_{260}/A_{280} >2.0 were processed. cDNA was synthesised with 1 µg total RNA, oligo-dT20 and the Transcriptor First Strand cDNA Synthesis Kit (Roche) in accordance with the manufacturer's instructions. RT-PCR primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3_www.cgi) based on NCBI record NC_007328.4: forward primer 5'-GCTACGCACCTCCAAATTGT 3' and

reverse primer 5' TTGGGATTCCTCTTTCTTGC 3' to generate a 206 bp product with the cDNA sequence and a 1356 bp product with genomic DNA as a target. PCR was performed for 5 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 60 s, followed by five cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 60 s and 30 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 60 s. Forward primer: 5' CCGAATATCATCCCTGCTT 3'and reverse primer: 5' GCAGGTCAGATCCACAACAGA 3' were used to amplify a 134 bp cDNA fragment of glyceraldehyde phosphate dehydrogenase (GAPDH) as a reference control [Brym *et al*. 2013]. The expected sizes of PCR products were controlled by 1.5% agarose gel/ethidium electrophoresis with the Low Molecular Weight DNA Ladder for *GAPDH* and with the 100 bp DNA Ladder (New England) for *ADAM32*.

Detection of gene polymorphism within ADAM32 promotor

In order to increase the possibility of identification of DNA polymorphism within the Adam32 gene, a group of diverse cattle breeds (Hereford, Holstein-Friesian, Jersey, Limusine) and an individual of a closely related species (*Bison bonasus*) were used for polymorphism screening purposes. Standard genomic DNA isolation from blood of eight animals was performed using the MasterPure for Blood Kit (Epicentre). For polymorphism screening within the 5' flanking sequence of ADAM32, 870 bp fragments were amplified by PCR, cleaned up using the Gel-Out Kit (A&A Biotechnology) and sequenced. Two primers were used: forward 5'-GGGGATGAGAGGAAAAGAGG 3'and revers e5'GTTAGCAAGCCACGAAGACC 3' designed using NCBI record NC_007328.4. Results were analysed with BLAST [Altschul *et al*. 1990] and CHROMAS Lite 2.1.1 (http://technelysium.com) software.

Population analysis

A fragment of the ADAM32 gene promotor -328 bp flanked by forward 5' GGTCTGTGAA CCACATGGAA 3' and a reverse primer 5' TCCCACAAACCTTTTGCTTC 3' was selected for population screening. PCR was performed for 30 cycles of 95°C for 30 s, 61°C for 30 s and 72°C for 60 s. Genotypes of the 21 bp insdel mutation were identified using two methods: 1) PCR-SSCP according to the standard procedure with modifications described by Kamiński *el al*. [2006], and 2) 4% polyacrylamide gel electrophoresis performed in the LI-COR 4200 Analyzer.

One hundred and two H-F bulls born between 1991 and 2003 were tested to identify the genotype of the 21 bp insdel mutation. Subsequently 241 cows - progeny of 15 sires heterozygous at the 21 bp insdel were included in the study. All cows were in the first lactation and their SCC was measured 10 times during lactation and scored in an official milk recording system. Cows were kept in 165 herds under similar rearing conditions: in tie stall barns and the Partly Mixed Ration feeding system. Cows were healthy except for udder diseases. Mean SCC values of the complete first lactation of each cow were calculated.

Statistical analysis

Distribution of milk production traits and SCC means (log transformed) of cows' first lactations were checked. Homogeneity of variances was estimated by Levene's test.

The following model of ANOVA was used to estimate an association of cows' 21 bp insdel genotype with milk performance traits and SCC.

$$y_{ijklm} = \mu + G_i + + A_j + P_k + S_l + e_{ijklm}$$

where:

y_{ijk} – SCC mean value (log transformed) of cow's first lactation;
μ – general mean;
G_i – fixed effect of genotype (i= 1, ., 3),);
A_j – fixed effect of age at calving (k= 1,…, 5);
P_k – fixed effect of productive season (l= 1, 2);
S_l – random effect of sire (j= 1, …, 15);
e_{ijkl} – random error.

Pearson's nonparametric chi-square test was performed to confirm the hypothesis that the studied population is at Hardy-Weinberg equilibrium for *ADAM32* 21bp insdel mutation. These computations were performed using the Statistica package programs (Statistica ver. 10, StatSoft, Inc., 2011)

***In silico* analysis of bovine ADAM32 promoter**

The relevant promoter region (-1180 b to +1 relative to the transcription start side) of the NCBI bovine ADAM32 gene sequence (NCBI record NC_007328.4) was analysed using MATINSPECTOR 8.2 (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 21bp *Insertion* and *Deletion* at -541nt to -520 nt.

Results and discussion

ADAM32 gene expression

Expression patterns of *ADAM32* in different tissues performed by RT-PCR revealed the presence of *ADAM32* transcripts in the liver, mammary and pituitary gland, PBMC, granulocytes and whole blood. A distinct signal of the putative 206 bp amplicon obtained on cDNA as a template was observed (Fig. 1). However, no significant signal was observed for monocytes. No signal of the 1356 bp PCR amplicon was produced in samples obtained from the udder, pituitary, monocytes, PBMC, granulocytes and whole blood predigested by DNaze. In contrast, in the liver sample deliberately not digested by DNaze a PCR product specific to genomic DNA as a template was found.

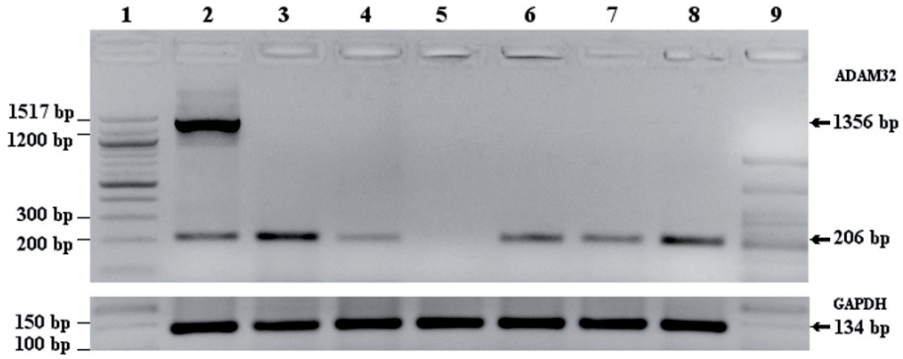

Fig. 1. ADAM32 gene expression in different bovine tissues. RT-PCR assay on samples prepared from: line 2 – liver (sample delibrately not DNaze digested), line 3 – mammary gland, line 4 – pituitary, line 5 – monocytes, line 6 – PBMC, line 7 – granulocytes and 8 – whole blood. Line 1 – 100 bp DNA Ladder and line 9 – Low Molecular Weight DNA Ladder (New England Biolabs, Inc). As a control GAPDH was amplified from cDNA.

Detection of polymorphism within promotor of ADAM32 gene

PCR performed with DNA isolated from blood of four different *Bos taurus* breeds and *Bison bonasus* generated a clear product of the expected size of 870 bp. After cleaning up and sequencing a comparative analysis showed a 21 bp insertion/deletion at position -541 nt to -520 nt and seven SNPs: at position -499 nt A/C, at position -573 nt A/G, at position -737 nt A/G, at position -766 nt A/G, at position -778 nt C/T, at position -779 nt C/G and A/C at position -784 nt.

***In silico* identification of Tf binding sites**

Analysis of the 1180 bp promoter region of the bovine *ADAM32 Ins* allele generated a total of 391 Tf binding sites. The 21 insdel at -541 to -520 nt alters the putative binding sequence for nine identified transcription factors (Tab. 1). Analysis of the sequence with the deletion 21 bp fragment showed that eight transcription factors lost putative binding sites. Six of them, i.e. the Positive Regulatory Domain I element binding factor, Tendon-specific bHLH transcription factor scleraxis, Transcription factor E2a (E12/E47), Tal-1alpha/E47 heterodimer, Neurogenin and NeuroD binding sites, Regulatory factor X 3 were determined as associated with immune system tissues. Moreover, mutation (*ADAM32 Del)* created new putative binding sites for Krueppel-like transcription factor 12 (AP-2rep), previously not recognised in the *ADAM32 Ins* allele sequence.

Association of 21 bp insdel polymorphism with production traits in Holstein-Friesian cows

Using two genotyping methods (Fig. 2), one hundred two Polish Holstein-Friesian bulls born between 1991 and 2003 were screened along with 241 cows.

The distribution of genotypes and allele frequency is presented in Table 2. Similarly to bulls (0.12), in cows (0.38) the *Ins* allele was rarer than the *Del* allele, (0.88 in bulls

Table 1. Putative transcription factor binding sequence altered by *Adam32* 21insdel mutation

Allele sequence	Transcription factor (presence of putative binding side)	Recognition sequence[1]	Matrix similary	Target strand
Ins	Positive Regulatory Domain I element binding factor 1	ggatataGAAAgtgtcatg	0.869	+
	Meis1b and Hoxa9 form heterodimeric binding complexes on target DNA	TGACactttctatat	0.780	-
	Pax-3 paired domain protein	agtgtCATGgcagatgctt	0.975	+
	Tendon-specific bHLH transcription factor scleraxis	cacaaagcatcTGCCatgaca	0.912	-
	Transcription factor E2a (E12/E47) (secondary DNA binding preference)	tcatggcaGATGctttg	0.946	+
	Tal-1alpha/E47 heterodimer	gtcatggCAGAtgctttgtga	0.888	+
	Neurogenin and NeuroD binding sites	aaagCATCtgcca	0.988	-
	Regulatory factor X, 3 (secondary DNA binding preference)	gcagatgctttGTGAcgat	0.861	+
Del	Krueppel-like factor 12 (AP-2rep)	gggcaGTGGatgctttg	0.922	+

[1]Core sequence in CAPITALS.

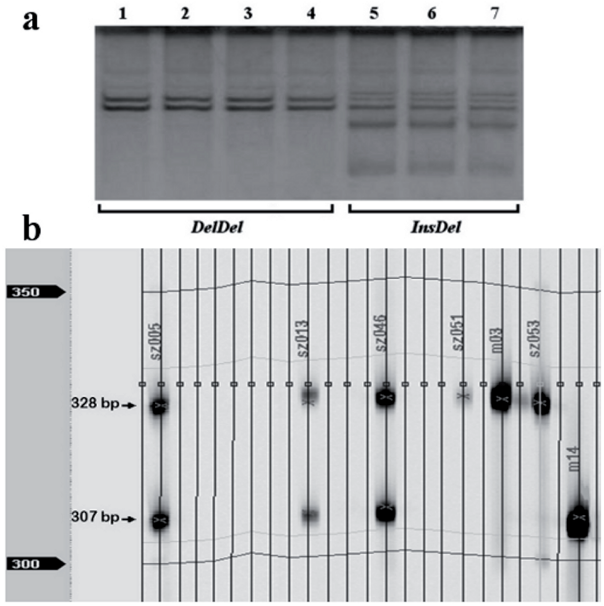

Fig. 2. Examples of genotyping of 21 bp insdel polymorphism: (a) – Bulls genotyped by SSCP. (b) – Cows genotyped by 4% polyacrylamide gel electrophoresis performed in the LI-COR system. Lines sz005, sz013 and sz046 – *InsDel*, lines sz051, m03 and sz053 – *InsIns*, line m14 – *DelDel.*

and 0.62 in cows). In cows, heterozygotes were found to be the most frequent (0.510). *DelDel* and *InsIns* homozygotes were found with a frequency of 0.365 and 0.125, respectively. In bulls the *DelDel* genotype was most frequent (0.765). No bull of the *InsIns* genotype was identified.

Table 2. Genotype and allele frequency of *ADAM32* 21 bp insdel polymorphism in goups of Holstein-Friesian bulls and cows

Group of animals (number)	Genotype frequency			Allele frequency	
	DelDel	*InsDel*	*InsIns*	*Del*	*Ins*
Bulls (102)	0.765	0.235	0.0	0.88	0.12
Cows (241)	0.365	0.510	0.125	0.62	0.38

Association study performed within a group of 241 daughters of heterozygous sires (Tab. 3) revealed that cows with the *DelDel* genotype showed significantly higher somatic cell counts than those with the *InsIns* genotype (*P*=0.013). No significant associations were found between cows' genotypes and milk production traits.

Preliminary Illumina Bovine SNP50 BeadChip analysis performed in the Polish Holstein-Friesian population showed a subregion on BTA 27, which affected SCC in milk [Szyda *et al*. 2011]. Within the subregion flanked by genome-wide significance SNP markers, ADAM32 is mapped (NC_ 007328.4) [Elsik *et al*. 2009]. This region was previously recognized as rich in putative QTLs for milk yield, milk protein yield and content [Viitala *et al*. 2003; Bagnato *et al*. 2008], for fat content in milk [Wang *et al*. 2012, Zhang *et al*. 1998], and for calving ease [McClure *et al*. 2010]. Other studies showed that this region of BTA27 may also be considered as a QTL for somatic cell score [Schrooten *et al*.2004; Tal-Stein *et al*. 2010] and clinical mastitis [Klungland *et al*. 2001, Rupp and Boichard 2003].

We intended to verify the potential involvement of *ADAM32* with mammary gland immune defense. Seven single nucleotide substitutions and one 21 bp insdel were identified within a sequence encompassing the promoter of bovine *ADAM32.* For population screening we selected the 21 bp insdel mutation and we developed two tests for individual genotyping. The first test based on PCR-SSCP was used in screening of 102 Polish Holstein-Friesian bulls. In this animal group we observed a very low frequency of the *Ins* allele and found no *InsIns* genotype. That may have been caused by the very intensive selection in sires, mainly focused on milk yield, and an unfavourable genetic correlation between production traits and udder health status [Koivula *et al*. 2004, Negussie *et al*. 2008]. The second test based on PCR and polyacrylamide gel electrophoresis was used to genotype daughters of 15 heterozygous *InsDel* bulls.

Table 3. Means and standard errors (SE) of milk performance traits in cows with different *ADAM32* genotypes

Genotype	N	Milk yield (kg)	Fat		Protein		log SCC
			yield (kg)	content (%)	yield (kg)	content (%)	
DelDel	88	6186.43 (155.80)	250.28 (6.45)	4.07 (0.05)	198.14 (5.53)	3.19 (0.03)	2.40[a] (0.05)
InsDel	123	6441.85 (133.17)	271.03 (6.16)	4.22 (0.05)	206.55 (4.66)	3.20 (0.02)	2.27 (0.04)
InsIns	30	5993.83 (262.30)	255.17 (11.74)	4.27 (0.10)	190.00 (8.55)	3.18 (0.04)	2.14[b] (0.07)

[ab]Witin columns means bearing different superscripts differ significanty at P<0.05.

Statistical analysis showed no association between any routinely evaluated production traits and the *ADAM32* genotype, except for SCC in the milk. Cows of the *ADAM32 DelDel* genotype have significantly higher SCCs than *InsIns*.

According to the MATINSPECTOR analysis the *ADAM32 Del* allelic sequence, eight transcription factors lost putative binding sides, six of them were found as involved in the immune system: the Positive Regulatory Domain I element binding factor, the Tendon-specific bHLH transcription factor scleraxis, Transcription factor E2a (E12/E47), Tal-1alpha/E47 heterodimer, Neurogenin and NeuroD binding sites, Regulatory factor X 3.

ADAMs are unique transmembrane proteins, as they are capable of mediating cell adhesion via their disintegrin and cysteine rich domains as well as the proteolytic release of cell surface molecules. Therefore, these proteases have been implicated in diverse (patho)physiologic processes including fertilization, neurogenesis, inflammatory diseases or cancer [Edwards *et al*. 2008]. Among the 21 human and 37 mouse identified ADAMs genes [Schwarz *et al*. 2013], *ADAM32* is poorly characterised in terms of its expression and function. To date in mammals the expression in human and mouse testes has been confirmed and an important role of this metalloproteinase in male reproduction and fertilisation has been confirmed. Choi *et al*. [2003] found predominant expression of mouse *ADAM32* during the meiotic prophase in pachytene spermatocytes and suggested a potential role of this protein in sperm development or fertilisation. Supporting results was obtained by Kim *et al*. [2006], who observed a large ADAM32 precursor (98 kDa) on the mouse spermatocyte surface, which processed to 44 kDa protein between the stage of testicular sperm and cauda epididymal sperm. Except for testes, Choi *et al*. [2003] detected ADAM32 gene expression at a low level in the murine epididymis, brain and heart. In our investigation we find expression of *ADAM32* in the bovine liver, mammary and pituitary gland, PBMC, granulocytes and whole blood, but not in monocytes. The presence of the ADAM32 gene expression in immune cells and mammary glands suggests a potential involvement of this protein in processes of natural resistance to udder inflammation. Previously, ADAM32 gene expression in domestic duck liver and its engagement in viral hepatitis pathogenesis was reported by Tang *et al*. [2013].

Mutation located in the functional segments of a gene promoter can potentially influence gene expression [Schild *et al*. 1994, Wagner *et al*. 1994, Kamiński 1996] and then moderate the health status of mammary tissues [Zabolewicz *et al*. 2012; 2014]. This relationship and the additionally recognised gene expression in mammary tissue and immune cells confirm our hypothesis that ADAM32 participates in bovine udder natural immune response.

The expression profile of the ADAM32 gene confirms that its product is involved in immune response. Moreover, it was shown that the 21 insdel polymorphism located in the 5' flanking region alters binding of 6 transcription factors known to be associated with tissues of the immune system. Potential importance of ADAM32 was confirmed on the population of cows – daughters of heterozygous sires for 21 bp

polymorphism. Cows with the *DelDel* genotype have significantly higher SCCs than those with *InsIns*. Taken together we conclude that the ADAM32 gene itself and its 21 bp insdel polymorphism are involved in immune response in the mammary gland manifested by changes in somatic cell counts.

A challenge for future research is to find out how insdel polymorphism of *ADAM32* affects expression of the gene itself in different tissues, especially those involved in natural immune response to mastitis and whether this polymorphism influences the content of ADAM32 protein in milk.

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