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Original article

Application of disease-associated differentially expressed genes – Mining for functional candidate genes for mastitis resistance in cattle

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Abstract – In this study the mRNA differential display method was applied to identify mastitis-associated expressed DNA sequences based on different expression patterns in mammary gland samples of non-infected and infected udder quarters of a cow. In total, 704 different cDNA bands were displayed in both udder samples. Five hundred-and-thirty two bands, (75.6%) were differentially displayed. Ninety prominent cDNA bands were isolated, re-amplified, cloned and sequenced resulting in 87 different sequences. Amongst the 19 expressed sequence tags showing a similarity with previously described genes, the majority of these sequences exhibited homology to protein kinase encoding genes (26.3%), to genes involved in the regulation of gene expression (26.3%), to growth and differentiation factor encoding genes (21.0%) and to immune response or inflammation marker encoding genes (21.0%). These sequences were shown to have mastitis-associated expression in the udder samples of animals with and without clinical mastitis by quantitative RT-PCR. They were mapped physically using a bovine-hamster somatic cell hybrid panel and a 5000 rad bovine whole genome radiation hybrid panel. According to their localization in QTL regions based on an established integrated marker/gene-map and their disease-associated expression, four genes (*AHCY*, *PRKDC*, *HNRPU*, *OSTF1*) were suggested as potentially involved in mastitis defense.

mastitis / expressed sequence tag / gene expression / cattle / RH mapping

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1. INTRODUCTION

Mastitis is a complex disease that is defined as an inflammation of the mammary gland and which results from the introduction and multiplication of pathogenic microorganisms as *Staphylococcus aureus* and *Streptococcus agalactiae* in the mammary gland [15]. In dairy cattle, mastitis is the most costly disease. Costs due to clinical mastitis include veterinary and treatment costs, reduced milk production in the remaining lactation, milk disposal due to antibiotic treatment, early culling, extra labor and decreased milk quality.

During the past 5 years, many experiments have identified a high number of different QTL regions in cattle affecting milk performance, growth, meat quality, exterior, but also functional traits such as mastitis resistance [2–4, 6, 19, 27, 34]. QTL positions and highly significant QTL effects repeatedly confirmed in independent studies emphasize the potential value of mapped QTL in selection for mastitis resistance using marker-assisted selection programs. Beyond fine mapping, however, the ultimate target of QTL analysis is the identification of the gene itself. One of the major efforts in this sense is the identification of coding sequences or transcript units [7], especially those that are localized in the QTL region of interest and that are trait-associated expressed. Identification of expressed sequence tags (EST) in tissues in different developmental stages or in phenotypically different individuals contributes not only to positional cloning by the establishment of high density transcript maps but also to a better understanding of the complex physiological process at the cellular level.

The changes in gene expression that are associated with mastitis are not well understood. In one of the first studies it was shown that the infection of multiparous Holstein cows with *Streptococcus agalactiae* significantly increased levels of mRNA coding for heat shock proteins, several growth factors, and the apoptosis marker testosterone-repressed prostate mucine-2 [30].

The technique of differential display of messenger RNA species originally described by [21], has been applied as a powerful tool for cloning genes that are differentially expressed in various tissues or under altered conditions in the same tissue [1, 10, 20, 22, 24, 28]. In this study, the mRNA differential display method was applied to identify mastitis-associated expressed DNA sequences based on different expression patterns in the mammary gland by a comparative analysis of non-infected and infected udder quarters of a cow. In a combined approach, these sequences were shown to have mastitis-associated expression in a representative number of udder samples of animals with and without clinical mastitis by quantitative RT-PCR using the LightCycler®. Their localisation was found to be in the vicinity of mapped QTL underlying the somatic cell score.

2. MATERIALS AND METHODS

2.1. Differential display RT-PCR (DDRT-PCR)

DDRT-PCR was performed essentially as described by [10]. Total RNA was extracted from infected and non-infected quarters of a lactating cow using the RNeasy Total RNA Kit (Qiagen, Hilden, Germany). The cDNA was generated by reverse transcription using the ExpandTM Reverse Transkriptase (Boehringer, Mannheim, Germany). For each cDNA sample a total of 6 primer combinations were used. The three downstream primers DP1 (5'-(T)₁₀G-3'), DP2 (5'-(T)₁₀C-3'), and DP3 (5'-(T)₁₀A-3') were combined with the upstream primers UP1 (5'-GTGAGCTCC-3') and UP2 (5'-AAGCTTCATTCCG-3'). PCR products were resolved on a 4% native polyacrylamide sequencing gel and the bands were visualized by conventional silver staining.

2.2. Cloning, cycle sequencing, sequence analysis, and primer design

The differentially displayed cDNA bands were cloned into Epicurian[®] Coli XL1-Blue competent cells (Stratagene, Heidelberg, Germany) using the Sure-Clone Ligation Kit (Pharmacia Biotech, Freiburg, Germany).

Five to ten clones of each partial library were randomly selected and sequenced by Taq cycle sequencing with a model 310C sequencer (Perkin Elmer/Applied Biosystems, Weiterstadt, Germany) using the universal M13 primers. Insert sequences were compared with sequences present in the EMBL/Genbank database using FASTA software [25]. As a criterion for homologous genes, a sequence identity of > 70% for at least 100 base pairs was defined. Primer pairs were designed using the OLIGO software (v.4, Natl. Biosciences Inc., Plymouth, MN, USA).

2.3. Study of gene expression

Transcript levels of identified differentially expressed genes were studied in unrelated lactating Holstein Friesian cows with (n = 10) and without (n = 10) clinical mastitis. Prior to slaughtering, the udders were examined for clinical mastitis. The results were verified histologically. Total RNA was extracted from udder samples using the RNeasy Total RNA Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. Synthesis of first strand cDNA was performed with MMLV-RT (Promega, Madison, USA) and random hexamer primers using 2 µg total RNA.

2.3.1. Semi-quantitative RT-PCR

The cDNA was diluted with 80 µL H₂O. Five-micro-litres of the dilution were used as the template for PCR analysis. Subsequent PCR was performed

Table I. List of mammary gland expressed sequence tags (EST) with similarity to known genes: homology, PCR conditions.
(continued on the next page)

Name of EST	GenBank Acc. No.	Length of EST [bp]	Sequences showing similarities with the bovine EST			Similarity		PCR-Amplification			RT-PCR
			Name of sequence	Species	Acc.-No.	[%]	overlap [bp]	Primer sequences	Annealing T	Length of product	- Semiquantitative RT-PCR (A)
								Forward (5'-3')	[°C]	[bp]	- LightCycler-PCR (B; fluorescence acquisition T)
fbn-eg001	BI347262	595	Vaccinia related kinase 2 (<i>VRK2</i>)	H. sapiens	AB000450	77.7	368	ATGGAGAGCTTGACTTGTTG GAGGAGGAGAAGCTGACTGG	55	282	-
fbn-eg002	BI347263	572	serine/threonine kinase 9 (<i>STK9</i>)	H. sapiens	NM_003159	86.4	322	TAGCATGCATGATTCTCTTC AGCCTTCATTGACCTTTTA	55	258	-
fbn-eg003	BI347264	471	phosphoinositide-specific phospholipase C epsilon (<i>PLCE</i>)	H. sapiens	AF190642	87.0	409	AGCGGGAAGTCTTCTCATAC ACAGAGCTTTCAGAACACC	55	279	-
fbn-eg009	BI347270	369	<i>LY75</i>	H. sapiens	AF064827	83.0	226	AGACATTGTTTGTGCTTTTT CCATACCATGTTACCTTTTT	50	316	-
fbn-eg010	BI347271	444	STE20 like kinase (<i>JIK</i>)	H. sapiens	AF181985	84.0	328	TTGGCCATCTCTCTATTCA GCAGCAGTAGTTAAGGTGT	55	251	A
fbn-eg018	BI347317	540	DNA-dependent protein kinase catalytic subunit (DNA-PKcs)	H. sapiens	U47077	83.9	471	ACCCAGATGACATCGACAGT GTGCGCCATCACAAAGAAC	45	367	-
fbn-eg022	BI347321	469	heterogeneous nuclear ribonucleoprotein U (<i>HNRPU</i>)	H. sapiens	AF068846	92.6	445	TTGAGATTGCTGCCCGTAAG GAACTGCATGTTCTGGTAGG	60	200	A
fbn-eg023	BI347322	186	serum amyloid A (<i>SAA3</i>)	H. sapiens	X13895	76.2	185	CTGACCCACCAGCTCTAAAG GAGCTCCGACAATGTCTAT	55	126	B (78°C)
fbn-eg028	BI347327	280	cytosolic ovarian carcinoma antigen 1 (<i>COVA1</i>)	H. sapiens	AF207881	94.5	271	CTGCCTGTGATACTGATTCT CAAAGCCAGCGTAAAAAC	55	174	B (78°C)
fbn-eg029	no entry	336	osteoclast stimulating factor I (<i>OSTFI</i>)	H. sapiens	AL133548	84.4	292	CAATCAAACCTAAGAACAAG TCAACATAAAGAACAGCACT	55	115	-
fbn-eg030	no entry	259	nuclear receptor subfamily 1 (<i>NR1D2</i>)	H. sapiens	D16815	88.8	250	no primer designed	-	-	-

Table I. Continued.

Name of EST	GenBank Acc. No.	Length of EST [bp]	Sequences showing similarities with the bovine EST		Similarity [%] overlap [bp]	PCR-Amplification		RT-PCR			
			Name of sequence	Species		Acc.-No.	Primer sequences		Annealing T	Length of product [bp]	
							Forward (5'-3') Reverse (5'-3')	[°C]			
fbn-ek005	B1347281	323	Neuroblast differentiation associated protein (AHNAK)	H. sapiens	M80899	80.0	308	CACCGGTAGCTCCTCTCAG GCCAGCTTTTGTGTTTATTA	55	209	B (80 °C)
fbn-ek012	B1347288	184	adenosylhomocysteinase	H. sapiens	AI312392	84.3	108	AGACAAGAGGAATGACAGCC TCTCGCTTTCTTTTAAACATT	50	127	-
fbn-ek018	B1347294	326	RAR-related orphan receptor A	H. sapiens	NM_001260	93.5	117	CTCTTTTCTCCAGTGTTC CCAAGTCCTAGGCTGTGTAA	50	243	-
fbn-ek020	B1347296	561	tumorprotein p53 (Tp53)	H. sapiens	X01405	97.4	533	CGTACTCCCTGCCCTCAAC GGCCCCAGACCATCGCTATC	50	196	-
fbn-ek021	B1347297	264	cell division protein kinase 8 (CDK8)	H. sapiens	NM_001260	93.5	117	GCCAAAACCTTGGCTACATA GTGCTCGTTCTTCTTGATA	50	188	-
fbn-ek030	B1347306	220	regulatory solute carrier protein, family 1, member 1 (RSC1A1)	H. sapiens	NM_006511	93.0	167	CTTGTCTGATGAATTGTCT GATAATAAAAAGCTTCTGTG	50	92	B (74 °C)
fbn-ek033	B1347310	336	signal sequence receptor, alpha (SSR1)	H. sapiens	AF156965	87.4	334	AACCAGCAGCCCGAGACCAG TCGTGCCGCCCCAGAGACAG	55	230	A
fbn-ek037	B1347314	471	aldoketoreductase (AKR1B1)	H. sapiens	NM_001628	70.0	127	GGGTGCGATATGTAGAGATT CTGCAAAAAGACCTAAGAGC	50	238	-

with *Taq* DNA polymerase (Promega, Mannheim, Germany) in 50 μ L of incubation buffer, and the primers of the corresponding EST (Tab. I) and of the internal β -actin standard were used (sense: AACTGGGACGACATG-GAGAAGAT, anti-sense: GCCAAGTCCAGACGCAGGAT) for 28 reaction cycles. Electrophoretic PCR patterns were measured densitometrically with the CAM imaging system (Cybertech, Berlin, Germany) and are represented as relative densitometric units (RDU). PCR of the respective samples with β -actin served as the reference to correct for different loadings of RNA.

2.3.2. Quantitative RT-PCR

Quantitative analysis of PCR products was carried out in the LightCycler[®] (Roche, Mannheim, Germany) according to optimized PCR protocols essentially as described by [29] using the specific primers of the corresponding EST (Tab. I) and LightCycler DNA Master SYBR Green I[®] (Roche, Mannheim, Germany). Based on the analysis of melting curves of the PCR products, a high temperature fluorescence acquisition point was estimated and included in the amplification cycle program (Tab. I). For all assays, an external standard curve was used based on a single stranded DNA molecule calculation. External DNA standard dilutions of each recombinant plasmid from single stranded DNA (10^x copies, $x = 1$ to 6) were generated from the cloned RT-PCR products into pUC18 vector (Pharmacia, Freiburg, Germany) linearized by a unique restriction digest.

2.4. Regional assignment by somatic cell genetics

The generated primer sets were used to identify the loci of the EST in the bovine syntenic groups essentially as described by [33] as well as in the recently published first generation radiation hybrid framework map of the cattle genome [5]. The PCR primer composition and PCR conditions are given in Table I. The EST were assigned to the genome by a two-point linkage which was computed using the RHMAPPER software [32]. The retention frequency was set to the genome average value of 0.22 calculated for the published framework map. For each EST the RH-PCR amplification was performed twice in complete independent experiments, and only concordant data were used.

2.5. Statistical analysis

All data were expressed as means \pm standard deviation (SD), and significance was accepted at $P < 0.05$. For all analyses, the SAS[®]/STAT package [26] was used. The means of the quantitative RT-PCR values for all EST of the two groups were compared using a t-test.

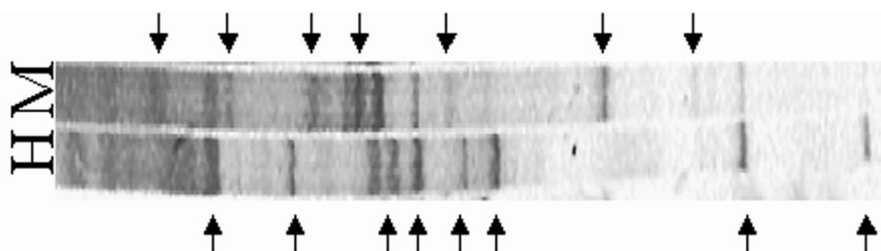


Figure 1. An example of an mRNA differential display of isolates of mastitis-infected (M) and non-mastitis infected (H) udder tissue from quarters of the same cow. Reverse transcription (RT) was done using downstream primer 2 (DP2: 5'-(T)₁₀C-3') and subsequent RT-PCR was done using DP 2 and upstream primers 1 (UP1: 5'-GTGAGCTCC-3'). Products were displayed on a 4% native DNA sequencing gel, and were visualized by conventional silver staining. The arrows indicate differentially displayed cDNA bands.

3. RESULTS

3.1. Identification of expressed mastitis-associated sequences displayed in DDRT-PCR

In total 704 different cDNA bands were displayed in both udder samples (see Fig. 1). Five-hundred-and-thirty-two bands (75.6%) were differentially displayed, of which 232 cDNA bands (43.6%) were more intensively displayed in the non-infected and 300 cDNA bands (56.4%) in the infected quarters of the udder. Amongst the differentially displayed cDNA bands, the most distinguished 90 were isolated, re-amplified, cloned and 5 to 10 clones per cDNA band were sequenced. In most cases, each band consisted of several cDNA. Altogether, 78 different mammary gland EST were identified using this approach. The nucleotide sequence data reported in this paper were submitted to GenBank and have been assigned the accession numbers BI347262 – BI347314. A genome database search showed that 25 EST were similar to previously described or hypothetical genes. Additionally, 11 EST showed similarity with EST of the database and 20 EST did not match any database entries. The rest were similar to repetitive, rRNA or cloned genomic sequences. The name and accession number of the sequences showing similarity to known genes, the length of overlapping fragments, the percentage of homology, the names of the homologous genes, and RT-PCR conditions are reported in Table I. The physiological function of the 19 EST was identified on the basis of their sequence similarity with known genes. The majority of these sequences exhibited homology to the protein kinase encoding genes (5), genes involved in the regulation of gene expression (5), growth and differentiation factors encoding genes (4) and immune response or inflammation marker encoding genes (4).

3.2. Mastitis significantly modifies expression profiles in the udder

The protein kinases encoding genes included the serine/threonine kinase 9 (*STK9*), the STE20-like kinase (*JIK*), the cell division protein kinase 8 (*CDK8*), the DNA-dependent protein kinase (*PRKDC*) and the vaccinia related kinase 2 (*VRK2*). The genes related to the regulation of gene expression included the S-adenosylhomocysteine hydrolase encoding gene (*AHCY*) acting as a competitive inhibitor of s-adenosyl-I-methionine-dependent methyl transferase reactions, the scaffold attachment factor A (*HNRPU*) that binds to pre-mRNA and is a component of ribonucleosomes, the signal sequence receptor α (*SSRI*) that regulates the retention of endoplasmatic proteins, and two genes coding for nuclear receptors (orphan nuclear receptor nr1d1, *NRD1*; nuclear receptor ror-alpha, *RORA*) binding DNA as a monomer to hormone response elements. The growth and differentiation factor encoding genes included three genes involved two regulatory factor encoding genes (osteoclast stimulating factor 1, *OSTF1*; regulatory solute carrier protein family 1, *RSC1A1*), a gene involved in cell differentiation (neuroblast differentiation associated protein ahnak, *AHNAK*) and a gene acting as a trans-activator negatively regulating cell division and cyclin-dependent kinases (cellular tumor antigen p53, *TP53*). The four genes involved in the immune response and inflammation processes were, respectively, the cystolic ovarian carcinoma antigen 1 (*COVA1*), the lymphocyte antigen 75 (*LY75*), the phospholipase C, epsilon (*PLCE*) and the serum amyloid A-3 (*SSA3*). To study the effect of mastitis infection on the gene expression pattern in the mammary gland, mRNA abundance of selected differentially displayed sequences characterizing the different pathways (*JIK*, *HNRPU*, *RSC1A1*, *AHNAK*, *SSRI*, *COVA1*, *SSA3*) was analyzed. The mRNA copy number (in 10 ng total RNA by Real-time PCR) varied between the different genes, from several hundreds to more than one million molecules, probably due to the varying physiological roles of the sequences. The mRNA abundance of these genes in the udder was analyzed in three independent repeated experiments.

Figure 2 represents the relative mean, standard deviation, x_{\min} - and x_{\max} -values of mastitis-associated transcript levels of the genes related to the mean of the non-infected animals. In cows exhibiting clinical mastitis, the mean mammary gland transcription levels of *COVA1*, *SAA3*, *RSC1A1* and *SSRI* were 1.8–4 times higher than in cows with non-infected udders. In contrast, the mean mRNA abundance of *AHNAK*, *JIK* and *HNRPU* was not significantly different between both groups. However, failing statistical significance is probably due to the extreme inter-individual variability observed in the mastitis group. As shown for *AHNAK*, *SAA3* and *RSC1A1*, mRNA abundance of individual animals with clinical mastitis ranges from values comparable to that of non-infected animals to about 14-fold increased concentrations (Fig. 3). In general, transcript levels of all genes analyzed were characterized by an

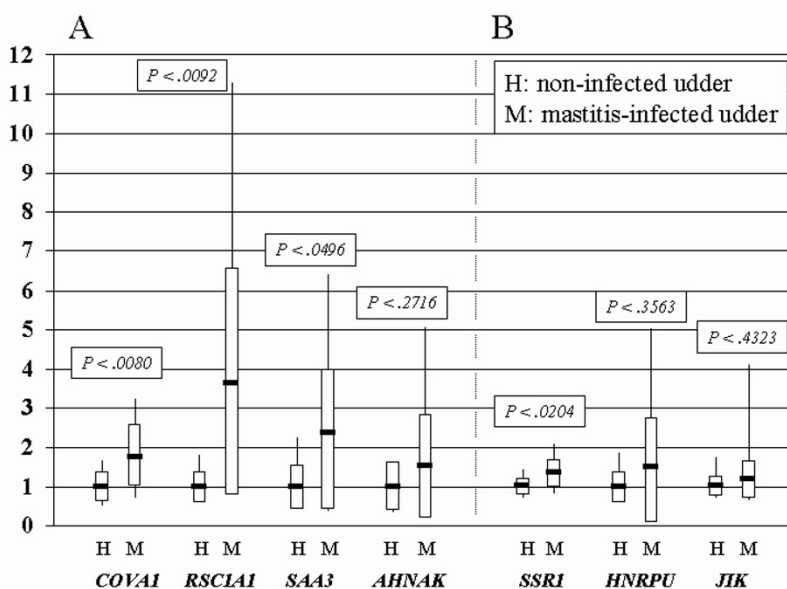


Figure 2. Mean transcript amounts in the mammary gland of ten cows with (M) and without (H) clinical mastitis, relative to the control group (animals without mastitis). The horizontal bold line represents the mean, the rectangle represents twice the SD, and the vertical bars correspond (upper bar) to the maximum and (lower bar) to the minimum. (A) Mean relative mRNA abundance (number of molecules per 10 ng total RNA) of the genes cystolic ovarian carcinoma antigen 1 (*COVA1*), regulatory solute carrier protein family 1 (*RSC1A1*), serum amyloid A-3 (*SSA3*), neuroblast differentiation associated protein ahnak (*AHNAK*). (B) Mean relative densitometric units of transcripts after semi-quantitative RT-PCR of the genes signal sequence receptor α (*SSRI*), scaffold attachment factor A (*HNRPU*), and STE20-like kinase (*JIK*) in both groups.

increased inter-individual variability in animals with clinical mastitis as shown by standard deviations significantly higher than that of the control group (Figs. 2 and 3).

3.3. Mastitis-associated expressed loci map within the vicinity of quantitative trait loci for somatic cell score in cattle

The four differentially expressed loci *OSTF1*, *AHCY*, *PRKDC* and *HNRPU* were mapped physically using a bovine-hamster somatic cell hybrid panel (SCP) and a 5000 rad bovine whole genome radiation hybrid panel (WGRH). These loci were assigned to the bovine syntenic groups and positioned in the recently established RH based ordered comparative map of the cattle genome [5]. The mapping results are summarized in Table II together with

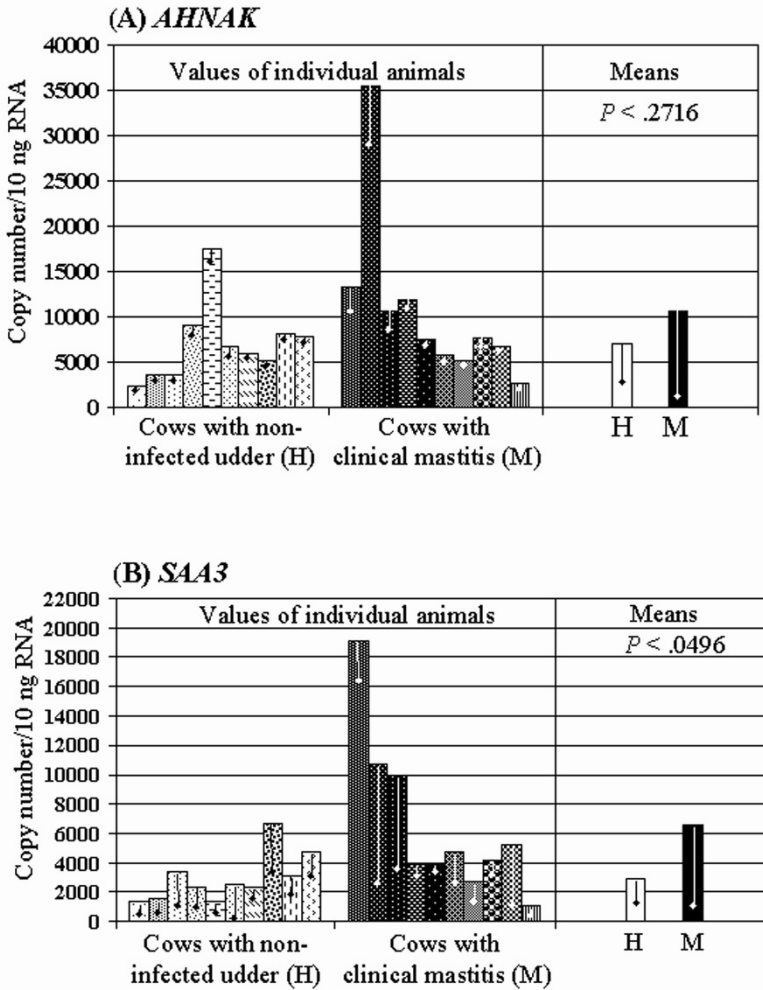


Figure 3. Individual and mean mammary gland mRNA abundance (number of molecules per 10 ng total cellular RNA) of the genes *AHNAK* (A) and *SAA3* (B) in 10 cows with and without clinical mastitis, respectively. Note that individual animals are characterized by specific column patterns. Bars represent SD.

the results from studies on the detection of quantitative trait loci (QTL) for the somatic cell score. In this table, the loci were ordered according to the bovine chromosome numbers. The SCP mapping data show the chromosome location of loci and the calculated concordance values. The WGRH mapping data show the loci location characterized by the nearest microsatellite markers in the RH framework map. In addition, their position in the bovine linkage MARC97 map [18] is given.

Table II. Results from regional assignment of mastitis-associated expressed loci by somatic cell genetics using a somatic cell panel (SCP) and whole-genome radiation hybrid panel (WGRH) and from studies on the detection of quantitative trait loci (QTL) for somatic cell score in cattle.

Locus name	Regional assignment			Position of nearest frame map micro-satellite marker within the MARC97 map [18] [cM]	QTL _{SCS} positions ¹	
	Chromosome BTA	SCP concordance value	WGRH5000-Mapping Nearest frame map [5] micro-satellite marker		[cM]	Reference
<i>OSTF1</i>	8	100	BM8129	70	44–84	[19]
<i>AHCY</i>	13	97	BMS995	84	91 104	[33] [6]
<i>PRKDC</i>	14	87	RM180	35	24–53	[33]
<i>HNRPU</i>	16	100	BMS1348	12	30	[6]

¹ Position on the marker map used in the study in which the QTL was detected.

4. DISCUSSION

This study compared mammary gland mRNA patterns of cows with and without clinical mastitis. DDRT-PCR was performed in infected and non-infected quarters of a lactating cow to identify mastitis-associated amplified sequences based on an identical genetic background. Between-cow variation was not considered using this qualitative approach. Differential expression, however, must be confirmed by classical Northern blot analysis, ribonuclease protection assay or quantitative PCR.

Based on 532 differentially displayed cDNA bands, we developed 78 molecular probes. Nineteen EST showing similarity to known genes were applied in transcription studies indicating that clinical mastitis significantly modifies gene expression in the mammary gland. Considering the physiological functions of these genes, significant changes in expression profile indicate a complex activation of gene expression associated with cell proliferation. Both up-regulated expressions of genes involved in cellular signal transduction (protein kinases: *STK9*, *JIK*, *CDK8*, *PRKDC*, *VRK2*, *TP53*; nuclear receptor encoding genes: *NRD1*, *RORA*; the cellular trans-activator TP53), and in the regulation of gene expression (*AHCY*, *SSR1*, *HNRPU*) and of genes involved in cell growth and differentiation (*OSTF1*, *RSC1A1*, *AHNAK*) characterize highly proliferating cells. Increased proliferation of cells is widely described as a pathophysiological effect of inflammation processes. However, in the present experimental approach of DDRT-PCR, the expression of genes related to increased cell proliferation dominated the mastitis-associated changes of the expression pattern in the mammary gland. Only four of the 19 genes, showing similarity to known genes, are involved in the immune response and inflammation processes. Two of these genes code for antigens (*COVA1*, *LY75*) whereas the two other genes code for the acute phase proteins phospholipase C, epsilon and serum amyloid A protein, which are early and sensitive inflammation markers [14,23,31]. On the contrary, no putative gene involved in mastitis defense could be detected using the DDRT-PCR approach.

However, the results of previous studies of mastitis-associated gene expression are in correspondence with the present findings. Inflammation of cows with *Streptococcus agalactiae* significantly increased the levels of mRNA coding for several growth factors [30]. Genes involved in cellular proliferation also represent putative candidate genes that might potentially affect both the resistance and etiology of disease. In humans, variants of genes coding for protein kinase that cause genetic disorders and diseases have been described. Defects in the myotonin protein kinase are the causes of myotonic dystrophy [11, 12], whereas defects in the pyruvate kinase genes are the most common cause of chronic hereditary nonspherocytic haemolytic anaemia [17]. Genetic variants

of DNA-dependent protein kinase that is involved in DNA double-stranded break repair and modulation of transcription are associated with severe combined immunodeficiency, type I [13]. Additionally, defects in *TP53* cause different malignomas as germ line cancers and Barrett adenocarcinomas [9, 16], and deficiency in *AHCY* is one cause of hypermethioninemia [8].

Based on the results of SHC/WGRH mapping and QTL mapping approaches [6, 19, 34], an established integrated marker/gene map allowed the identification of four genes potentially involved in mastitis resistance: *OSTF1* (BTA8), *AHCY* (BTA13), *PRKDC* (BTA14) and *HNRPU* (BTA16), showing that the combination of positional and functional candidate gene approaches represents a helpful prerequisite for cloning of candidate genes with underlying QTL effects. Trait-association of the corresponding gene variants is under investigation.

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