Expression of inflammation-mediated cluster of genes as a new marker of canine mammary malignancy

K. M. Pawlowski · A. Homa · M. Bulkowska · K. Majchrzak · T. Motyl · M. Król

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Abstract Because canine mammary tumours constitute a serious clinical problem and there are no good prognostic markers (only histopathological variables are used), the aim of the presented study was to find new malignancy markers as well as to identify intracellular pathways and biological processes characteristic for canine mammary malignancy. We compared gene expression of the most malignant mammary tumours (poorly differentiated cancers of the 3rd grade of malignancy) with less malignant tumours (well differentiated cancers of the 1st grade of malignancy). The results of our study indicated that in dogs the number of tumour-infiltrating myeloid cells or expression of myeloid-specific antigens by cancer cells is related to the cancer progression and may constitute a new marker of malignancy, however further studies in this field are required.

Keywords Canine mammary cancer · Malignancy markers · Microarrays · Real-time qPCR · Myeloid cells infiltration

Introduction

Spontaneous mammary tumours are the most prevalent type of malignant neoplasm in the bitch and woman with the three times over incidence in dog (MacEwen 1990). About 50% of all the mammary tumours are malignant (Misdorp 2002). The aetiology of mammary cancer is very complex and not clearly understood. The known mediators of tumourigenesis in both species are: genetic, hormonal, dietary, environmental and carcinogenic factors (Russo and Russo 1998). Moreover, both species live in the same conditions, thus the dog is a good model for breast cancer studies.

The role of oestrogens, progestins and growth hormone in canine mammary cancer development has been documented (Pawłowski et al. 2012). That is why mainly affected are not spayed female dogs in the middle age. The early ovariectomy reduces risk of mammary cancer development (Misdorp 1991). However, the high morbidity and mortality rate, which is caused by poor diagnostics and ineffective treatment strategies makes this problem still actual in both humans and dogs.

The conventional approach to cancer therapy provide treatment according to the organ in which the cancer originates. However, different intracellular signalling pathways are perturbed in the various cancers even if they represent the same type. Thus, the patients with the same type of cancer often have dissimilar genetic defects in their tumours and respond in a heterogeneous manner to anticancer agents (Veer van’t and Bernards 2008). Moreover, the diagnostic methodologies available in veterinary oncology may still be considered to be in progress. So far, only histopathological variables (tumour size, lymph node status, vascular invasion and tumour grade of differentiation) are used as prognostic parameters (Manuali et al. 2012).

Thus, the aim of the presented study was to find intracellular pathways and biological processes characteristic for canine mammary malignancy. We compared gene expression of the most malignant mammary tumours (poorly differentiated
cancers of the 3rd grade of malignancy) with less malignant tumours (well differentiated cancers of the 1st grade of malignancy) in order to find new diagnostic and prognostic markers.

Materials and methods

Tissue samples

Tumour samples of canine mammary cancers were obtained from patients subjected to surgery. The tumours then, were divided into two equal halves, one of them was fixed in 10 % neutral buffered formalin and routinely embedded in paraffin to perform histological assay. The other was snap frozen in liquid nitrogen and stored in −80 °C. Four μm samples from paraffin blocks were fixed on glass slides, stained with haematoxylin—eosin (HE) and examined by certified pathologists (prof. Dr. Elżbieta Malicka and Dr. Izabella Dolka, both from the Warsaw University of Life Sciences, Poland). The immunohistochemical examination of cytokeratin, vimentin, smooth muscle actin, s100 protein and p63

Table 1

| Gene symbol | Forward primer | Reverse primer | Optimum annealing temp. (°C) | Optimum annealing time (sec) |
|-------------|----------------|----------------|-------------------------------|-----------------------------|
| iil15       | CAGACTCACCAGAGGGAAA | CTGCTGTGAAGTCTGGGAGT | 60                          | 6                           |
| ergic2      | TGCCATCGTCTGCTACATT | CAGTCGCTTCCTCAGTCTCAT | 61                          | 9                           |
| elsph1      | CTTTCACATCACGTGACTCG | GTGTGGTGGGAGGTAGTTC | 60                          | 6                           |
| extl3       | AGCTTGGCTGGAAGGACG | TTATAGTCAAGGGCATATCC | 60                          | 6                           |
| rps19       | CCTTCCCTCTGAGGTCAGG | GTTCTCATCCTAGGGAGCAG | 61                          | 10                          |

Fig. 1 Gene Spring (Agilent, USA) diagrams of: a. boxplots showing median relative signal measured for each microarray; b. quality control gene expression in both microarray experiments (in dye-swaps) shows highly repeatable results; c. all genes expression in both microarray experiments (in dye-swaps), genes that differed significantly at p value <0.01 with fold cut-off = 1.5 (unpaired t-test and Benjamin-Hochberg FDR<5 % correction) are showed as blue points (they were taken to the further analyses)
Table 2: The list of up-regulated genes (↑) in canine mammary cancers of the 3rd grade of malignancy compared with the canine mammary cancers of the 1st grade of malignancy. Data was analyzed using Gene Spring software (Agilent, USA), \( p < 0.005, \text{Fold change} > 3 \)

| Fold Change | Gene symbol | Description |
|-------------|-------------|-------------|
| ↑5.0125217 | IL8         | Canis lupus familiaris interleukin 8 (IL8), mRNA [NM_001003200] |
| ↑4.714284  | FABP1       | Fatty acid binding protein Fragment [Source:UniProtKB/TrEMBL;Acc:Q95K85] [ENSCAFT0000011880] |
| ↑4.2913084 | MMP1        | Matrix metalloproteinase 1 |
| ↑4.2044907 | EXTL3       | Exostosin-like 3;EXTL3;ortholog |
| ↑3.957821  | CNGA1       | Canis lupus familiaris cyclic nucleotide gated channel alpha 1 (CNGA1), mRNA [NM_001003222] |
| ↑3.925793  | NELL2       | PREDICTED: Canis familiaris similar to Protein kinase C-binding protein NELL2 precursor (NEL-like protein 2) (NEL-related protein 2), transcript variant 2 (LOC477636), mRNA [XM_846523] |
| ↑3.9208682 | CNGA1       | Canis lupus familiaris cyclic nucleotide gated channel alpha 1 (CNGA1), mRNA [NM_001003222] |
| ↑3.416843  | MMP3        | Canis lupus familiaris matrix metallopeptidase 3 (stromelysin 1, progelatinase) (MMP3), mRNA [NM_001002967] |
| ↑3.2882302 | NELL2       | NELL-like 2 (chicken) [Source:HGNC Symbol;Acc:7751] [ENSCAFT0000015264] |
| ↑3.0424755 | MTMR10      | PREDICTED: Canis familiaris similar to phosphatidylinositol-3-phosphatase associated protein, transcript variant 4 (LOC479016), mRNA [XM_851476] |
| ↑2.972987 | ADCY8       | adenylate cyclase 8 (brain) [Source:HGNC Symbol;Acc:239] [ENSCAFT0000001672] |
| ↑2.596691 | MARCO       | macrophage receptor with collagenous structure [Source:HGNC Symbol;Acc:6895] [ENSCAFT0000007902] |
| ↑2.486832 | EMR3        | Canis lupus familiaris egf-like module containing, mucin-like, hormone receptor-like 3 (EMR3), mRNA [NM_001038666] |
| ↑2.31884  | IL6         | Canis lupus familiaris interleukin 6 (interferon, beta 2) (IL6), mRNA [NM_001003301] |
| ↑2.2737932 | SRGN        | PREDICTED: Canis familiaris similar to Secretory granule proteoglycan core protein precursor (Platelet proteoglycan core protein) (PG) (Hematopoetic proteoglycan core protein) (Serglycin) (LOC609421), mRNA [XM_846674] |
| ↑2.1735125 | ELSPBP1     | Epididymal sperm binding protein 1;ELSPBP1;ortholog |
| ↑2.1602907 | LEF1        | PREDICTED: Canis familiaris similar to lymphoid enhancer binding factor-1, transcript variant 7 (LOC478507), mRNA [XM_858241] |
| ↑2.1465235 | GAD1        | Canis lupus familiaris glutamate decarboxylase 1 (brain, 67 kDa) (GAD1), mRNA [NM_001097543] |
| ↑2.0999904 | CTRB1       | PREDICTED: Canis familiaris similar to chymotrypsinogen B1, transcript variant 1 (LOC479650), mRNA [XM_536782] |
| ↑2.0861115 | IL15        | Interleukin-15;IL15;ortholog |
| ↑2.001695  | DDIT3       | DNA-damage-inducible transcript 3 [Source:HGNC Symbol;Acc:2726] [ENSCAFT0000000367] |
| ↑1.975823 | PCSK2       | proprotein convertase subtilisin/kexin type 2 [Source:HGNC Symbol;Acc:8744] [ENSCAFT0000008876] |
| ↑1.9757178 | GPM6A       | PREDICTED: Canis familiaris similar to glycoprotein M6A isoform 1, transcript variant 6 (LOC475641) |
| ↑1.9687057 | HLA-DQB1    | Canis lupus familiaris major histocompatibility complex, class II, DQ beta 1 (HLA-DQB1), mRNA [NM_001014381] |
| ↑1.9438797 | SLC30A8     | solute carrier family 30 (zinc transporter), member 8 [Source:HGNC Symbol;Acc:20303] [ENSCAFT0000001287] |
| ↑1.9432139 | LYZL6       | Q6UW30_HUMAN (Q6UW30) TKAL754, partial (63 %) [TC51642] |
| ↑1.8990102 | CDA         | cytidine deaminase [Source:HGNC Symbol;Acc:1712] [ENSCAFT00000023893] |
| ↑1.8365646 | NELL1       | PREDICTED: Canis familiaris similar to nel-like 1 precursor (LOC476888), mRNA [XM_534090] |
| ↑1.8206882 | CELA1       | Canis lupus familiaris chymotrypsin-like elastase family, member 1 (CELA1), mRNA [NM_001038666] |
| ↑1.8029478 | CAMP        | Canis lupus familiaris cathelicidin antimicrobial peptide (CAMP), mRNA [NM_001003359] |
| ↑1.7610306 | ERGIC2      | Endoplasmic reticulum-Golgi intermediate compartment protein 2;ERGIC2;ortholog |
| ↑1.7566903 | PRKCQ       | protein kinase C, theta [Source:HGNC Symbol;Acc:9410] [ENSCAFT0000008336] |
protein expression was performed (data not shown). The
tumour types of specimens were classified based on
the World Health Organization (WHO) Histological
Classification and Mammary Tumours of the Dog and
Cat classification (Misdorp et al. 1999). Histological
tumour grading was conducted on HE-stained sections
using a Misdorp classification (2002). The mammary
carcinoma grading was assessed in respect to tubule
formation, degree of differentiation and mitotic index as. All
the tumours examined were classified as the 1st grade of
malignancy or the 3rd grade of malignancy (6 tumours in
each group). Unfortunately survival data of these dogs is
unavailable.

Microarray analyses

The total RNA from the samples was isolated using a
Total RNA kit (A&A Biotechnology, Poland) according
to the manufacturer’s protocol. Isolated RNA samples
were dissolved in RNase-free water. The quantity of
RNA was measured using NanoDrop (NanoDrop
Technologies USA). The samples with adequate amounts
of RNA were treated with DNaseI to eliminate a possi-
bility of DNA contamination. The samples were subse-
sequently purified using RNeasy MiniElute Cleanup Kit
(Qiagen, Germany). Finally RNA samples were analyzed
using BioAnalyzer (Agilent, USA) to measure the final
RNA quality and integrity.

The Quick Amp Labeling Kit (Agilent, USA) was
used to amplify and label target RNA to generate com-
plementary RNA (cRNA) for oligo microarrays used in
gene expression profiling and other downstream analy-
"Table 2 (continued)

| Fold Change | Gene symbol | Description |
|-------------|-------------|-------------|
| 33↑1.7566395 | TFP2        | tissue factor pathway inhibitor 2 [Source:HGNC Symbol;Acc:11761] [ENSCAFT0000023103] |
| 34↑1.7506666 | LAMP3       | lysosomal-associated membrane protein 3 [Source:HGNC Symbol;Acc:14582] [ENSCAFT0000018703] |
| 35↑1.725342  | S100P       | S100 calcium binding protein P [Source:HGNC Symbol;Acc:10504] [ENSCAFT00000022770] |
| 36↑1.7065927 | TREM1       | triggering receptor expressed on myeloid cells 1 [Source:HGNC Symbol;Acc:17760] [ENSCAFT0000002493] |
| 37↑1.6830823 | BCL2A1      | BCL2-related protein A1 [Source:HGNC Symbol;Acc:991] [ENSCAFT00000022179] |
| 38↑1.6449332 | IL33        | Canis lupus familiaris interleukin 33 (IL33), mRNA [NM_001003180] |
| 39↑1.597691  | AREGB       | amphiregulin B |

average of all labelled arrays, the dye effect on any
particular gene was cancelled. The hybridization was
performed with canine-specific AMADID Release GE
4x44K microarrays (Agilent, USA) using Gene Expression
Hybridization Kit (Agilent, USA) according to the manufac-
turer’s protocol.

Signal detection, quantification and analysis

Acquisition and analysis of hybridization intensities
were performed using DNA microarray scanner
(Agilent, USA). Then, the results were extracted using
Agilent’s Feature Extraction Software with normaliza-
tion and robust statistical analyses. Results were ana-
alyzed for statistical purposes using Future Extraction
and Gene Spring software (Agilent, USA). The unpaired
t-test with Benjamin-Hochberg FDR<5 % (false discov-
ery rate) correction was applied (with p value cut-off<0.01).
For further analysis we chose only these genes with values
within upper and lower cut-off (100.00 and 20.00, respective-
ly) in each of the slide, whose expression changed at least 1.5-
fold in each of examined slide. The area of the analyses
covered in this publication has been deposited in NCBI’s
Gene Expression Omnibus and is accessible via GEO Series
accession number GSE 44033.

Gene function was identified using the PANTHER
pathway analysis software (Mi et al. 2005) and
Pathway Studio software (Agilent, USA). PANTHER on-
line platform allowed for wide analysis of the Canis
familiaris regulated genes and also for statistical analy-
"Real-time qPCR

The mRNA sequences of the key genes were obtained from
NCBI database. Primers were designed using PRIMER3
software (free on-line access) and checked using Oligo
| Fold Change | Gene Symbol | Description |
|------------|-------------|-------------|
| 1.5897567 | SMOC1 | SPARC related modular calcium binding 1 [Source:HGNC Symbol;Acc:20318] [ENSCAFT00000026288] |
| 1.6253631 | SERPINE1 | Canis lupus familiaris serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (SERPINE1), mRNA [NM_001197095] |
| 1.6317778 | GDPD2 | glycerophosphodiester phosphodiesterase domain containing 2 [Source:HGNC Symbol;Acc:25974] [ENSCAFT00000026677] |
| 1.6928551 | TTC17 | tetratricopeptide repeat domain 17 [Source:HGNC Symbol;Acc:25596] [ENSCAFT00000010834] |
| 1.7700043 | PIP | prolactin-induced protein [Source:HGNC Symbol;Acc:8993] [ENSCAFT00000005869] |
| 1.9344714 | PPP2R2B | PREDICTED: Canis familiaris similar to protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform isoform 1, transcript variant 1 (LOC478053), mRNA [XM_535231] |
| 1.9801016 | ACAN | Canis lupus familiaris aggrecan (ACAN), mRNA [NM_001113455] |
| 2.0213842 | BMP7 | Bone morphogenetic protein 7 Fragment (BMP-7)(Osteogenic protein 1)(OP-1) [Source:UniProtKB/Swiss-Prot;Acc:P34819] [ENSCAFT00000019076] |
| 2.0953069 | PPP6R3 | PREDICTED: Canis familiaris similar to sporulation-induced transcript 4-associated protein, transcript variant 7 (LOC483688), mRNA [XM_858601] |
| 2.2811608 | NOTUM | notum pectinacetylesterase homolog (Drosophila) [Source:HGNC Symbol;Acc:27106] [ENSCAFT00000009543] |
| 2.3086379 | PRSS16 | protease, serine, 16 (thymus) [Source:HGNC Symbol;Acc:9480] [ENSCAFT00000017667] |
| 2.3557005 | LRP2 | low density lipoprotein receptor-related protein 2 [Source:HGNC Symbol;Acc:6694] [ENSCAFT00000019396] |
| 2.3742533 | FMOD | fibromodulin [Source:HGNC Symbol;Acc:3774] [ENSCAFT00000015038] |
| 2.3969395 | FABP3 | fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor) [Source:HGNC Symbol;Acc:3557] [ENSCAFT00000017685] |
| 2.5347118 | COL2A1 | Canis lupus familiaris collagen, type II, alpha 1 (COL2A1), mRNA [NM_001006951] |
| 2.630961 | ACSM4 | acyl-CoA synthetase medium-chain family member 4 [Source:HGNC Symbol;Acc:32016] [ENSCAFT00000028528] |
| 2.7111955 | PAQR8 | progestin and adipoQ receptor family member VIII [Source:HGNC Symbol;Acc:15708] [ENSCAFT0000003464] |
| 2.7223504 | SLC22A10 | solute carrier family 22, member 22, member 10 [Source:HGNC Symbol;Acc:18057] [ENSCAFT00000024230] |
| 2.7353601 | COL2A1 | Canis lupus familiaris collagen, type II, alpha 1 (COL2A1), mRNA [NM_001006951] |
| 2.74712 | TAF7 | acyl-CoA synthetase medium-chain family member 4 [Source:HGNC Symbol;Acc:32016] [ENSCAFT00000028528] |
| 2.8774078 | PAQR8 | progestin and adipoQ receptor family member VIII [Source:HGNC Symbol;Acc:15708] [ENSCAFT0000003464] |
| 3.2135046 | SCG2 | PREDICTED: Canis familiaris similar to Secretogranin-2 precursor (Secretogranin II) (SgiII) (Chromogranin C), transcript variant 1 (LOC488550), mRNA [XM_545669] |
| 3.331683 | EPYC | epiphycan [Source:HGNC Symbol;Acc:3053] [ENSCAFT0000009916] |
| 3.443157 | SERPINA9 | serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 9 [Source:HGNC Symbol;Acc:15995] [ENSCAFT00000028000] |
| 3.625558 | FXYD2 | FXYD domain containing ion transport regulator 2 [Source:HGNC Symbol;Acc:4026] [ENSCAFT00000020395] |
| 4.101486 | SCG2 | PREDICTED: Canis familiaris similar to Secretogranin-2 precursor (Secretogranin II) (SgiII) (Chromogranin C), transcript variant 1 (LOC488550), mRNA [XM_545669] |
| 4.2103615 | RIPPLY1 | PREDICTED: Canis familiaris similar to Down syndrome critical region homolog 6 (LOC610288), mRNA [XM_847751] |
| 4.6913886 | TAF7L | TAF7-like RNA polymerase II, TATA box binding protein (TBP)-associated factor, 50 kDa [Source:HGNC Symbol;Acc:11548] [ENSCAFT00000027954] |
| 5.1920776 | MYH2 | Canis lupus familiaris myosin, heavy chain 2, skeletal muscle, adult (MYH2), mRNA [NM_001076795] |
| 5.9604554 | MYH1 | Canis lupus familiaris myosin, heavy chain 1, skeletal muscle, adult (MYH1), mRNA [NM_001113717] |
| 7.343837 | POU1F1 | Canis lupus familiaris POU class 1 homeobox 1 (POU1F1), mRNA [NM_001006949] |
Calculator (free on-line access) and Primer-Blast (NCBI database). Primers’ sequences are listed in Table 1. Rps19 gene was used as a non-regulated, reference gene for normalization of target gene expression (Brinkhof et al. 2006; Etschmann et al. 2006). Quantitative RT-PCR was performed using fluorogenic Lightcycler Fast Strand DNA Sybr Green (Roche) and the Light Cycler (Roche). The results were analyzed using comparative Ct method (Schmittgen and Livak 2008). Relative transcript abundance of the gene equals ΔCt values (ΔCt = Ct reference – Ct target). Relative gene expression is expressed as ΔΔCt value (ΔΔCt=2^-ΔCt). The experiment was conducted in triplicates.

Then, to visualize the PCR product it was dedicated for electrophoresis in 2 % agarose gel (Sigma Aldrich), stained with ethidium bromide (Sigma Aldrich) and run for 60 min at 90 mV in 1× tris-borate-EDTA buffer. Then, the gel was visualized under UV light.

Results

Gene expression in canine mammary malignancy

The microarray-based transcriptional profile of the canine mammary cancers of the 3rd grade of malignancy was
compared to the canine mammary cancers of the 1st grade of malignancy used as a reference. For each comparison 2 separate dye-swap experiments were performed. This study showed 70 statistically significant \((p<0.005; \text{Fold change } = 1.5)\) regulated genes (Fig. 1). Further analysis showed 39 up-regulated genes (Table 2) and 31 down-regulated genes (Table 3) in canine mammary cancer of the 3rd grade of malignancy.

Function of identified genes

PANTHER analysis of identified up-regulated genes showed that they were mainly involved in biological processes such as: cellular process (NELL2, NELL1, CNGA1, PRKCQ, S100P, EMR3, PCSK2, IL15, IL8, MARCO, IL6, CAMP, GPM6A, BCL2A1), metabolic process (MMP3, LEF1, ADCY8, CELA1, PRKCQ, LYZL6, TFPI2, S100P, MMP1, PCSK2, MTMR10, CAMP, AMP3, EXTL3) and developmental process (NELL2, NELL1, PRKCQ, EMR3, PCSK2, IL8, GPM6A, BCL2A1) (Fig. 2a). The most significant pathway in which up-regulated genes \((n=12)\) were involved was the inflammation mediated by chemokine and cytokine signaling pathway (HLA-DQB1, NELL1, LYZL6, S100P, TFPI2, TREM1, EMR3, IL6, IL8, IL15, MARCO, CAMP). Analysis of the down-regulated genes showed that they were involved mainly in metabolic process, cellular process, cell communication and developmental process (Fig. 2b). Pathway analysis showed that these genes were mainly involved in cytoskeletal regulation by Rho GTPase, GnRH receptor pathway, inflammation mediated by chemokine and cytokine, nicotinic acetylcholine receptor signaling pathway and Wnt signaling pathway.

Real-time qPCR gene expression

For the purpose of microarray data validation, we have randomly selected 4 genes: il15, ergic2, elspb1 and extl3. Real-time qPCR results showed similar trends in gene expression changes as were observed in microarray studies (Fig. 3). The expression of examined genes was higher in the most malignant canine mammary cancers than in the tumours of the 1st grade of malignancy.

Discussion

Canine mammary cancer constitutes a serious clinical problem. That is a reason why its molecular biology has been systematically examined during the last few years (Rao et al. 2009; Pawlowski et al. 2011, Klopfleish et al. 2010; Pawlowski et al. 2013).

The very interesting study was conducted by Klopfleish et al. (2010) who identified a gene expression profile in canine mammary tumours that was associated with early metastatic spread to the lymph nodes. Based on the gene expression pattern of these tumours the authors were able to discriminate carcinomas with divergent metastatic potential despite similar histological features. Moreover, a partial overlap was found between the canine mammary “metastatic” gene expression profile and similar metastasis-associated gene expression “signature” of breast cancer (Veer et al. 2002).

Our previous study of gene expression in canine mammary tumours of various grade of malignancy showed that histological diagnosis was distinct from molecular diagnosis (Pawlowski et al. 2013). We have also identified cellular pathways and biological processes in which the most
significant up-regulated genes were involved. In the tumours of the 3rd grade of malignancy we identified interesting up-regulated cluster of genes related to immunological system. Their higher expression found in the most malignant cancers might be related with increased recruitment of hematopoietic cells into the tumour mass. Although the tumour is composed of various cells depending on the tumour type, myeloid cells seem to form a major component (Bingle et al. 2002). Clinical studies have shown a correlation between the number of myeloid cells (mainly macrophages) and a poor prognosis in many human cancers (e.g. breast, prostate, ovarian, etc.) (Judus et al. 1996). Our own studies conducted on canine mammary cancers have not shown any correlation between number of macrophages in tumour mass and a grade of tumour malignancy (Król et al. 2011). However, interestingly we observed expression of myeloid cell antigens in cancer cell lines and tissues (Król et al. 2011, 2012) which increased upon the co-culture of these both types of cells (Król et al. 2012).

We have shown that expression of typical macrophage antigens (CD14, CSF-1R) in canine mammary cancer tissues correlated with the tumour grade of malignancy (Król et al. 2011). Similarly, Dr. Pollard (2008) described that a gene expression signature characteristic for macrophages was an independent predictor of poor outcome in follicular lymphoma. Thus, these genes were typed as new malignancy markers.

Based on these results, the aim of the presented study was two-fold: 1) to compare gene expression in canine mammary tumours of the 1st and the 3rd grade malignancy in order to find new possible prognostic markers and 2) to validate whether genes characteristic for immunological system can constitute new markers of malignancy.

Similarly to our previous study (Pawłowski et al. 2013) we showed significant over-manifestation of genes related with chemokine and cytokine mediated signalling pathway (HLA-DQB1, NELL1, LYZL6, S100P, TFP12, TREM1, EMR3, IL6, IL8, IL15, MARCO, CAMP) (Fig. 2, Tables 2 and 3). A few of these genes seemed to be particularly interesting. For example, S100P calcium binding protein (which expression is regulated by androgens and IL6 – another up-regulated gene in the most malignant canine mammary tumours) is though as a new prognostic factor (Parkkila et al. 2008). A correlation was found between its increased expression and poor survival, cancer proliferation and increased resistance to chemotherapy (Maciejczyk et al. 2013). Our results are in accordance with clinical data as the cancers of high grade of malignancy (which express higher levels of S100P) are associated with an increased risk of death within 2 years after mastectomy (Karayannopoulou et al. 2005).

In the most malignant canine mammary cancers an increased expression of two metalloproteinases (MMPs): 1 and 3 was observed (Table 2). MMPs comprise a structurally and functionally related family degrading extracellular matrix and basement membrane barriers. That is why they are thought to play a key role in angiogenesis, inflammatory processes, cancer development and metastasis, as well as in proliferation and apoptosis (Sauter et al. 2008). Because of their role in the degradation of the extracellular matrix leading to tumor invasion and metastasis, they may also serve as prognostic markers (Pardo and Selman 2005, Brickerhoff and Matrisian 2002). In this context, MMPs have been focused on as targets for therapeutic strategies.

The metalloproteinases are also linked to specific aspects of an inflammatory or immune response, such as the generation of chemokine gradients or immune cell influx (Hojilla et al. 2008). In addition to the metalloproteinase-mediated generation of inflammation triggers, metalloproteinases are, in turn, utilized by immune cells to further propagate the inflammatory reaction. In breast cancer samples, MMPs are found in neutrophils, macrophages, and T lymphocytes as well as in cancer cells (Benaud et al. 1998).

Cancer development is a complex process. In addition to the cancer cell intrinsic factors, the cancer microenvironment composed of various cells influences the behavior of cancer cells. The results of our study indicate that in dogs the number of tumour-infiltrating myeloid cells or expression of myeloid-specific antigens by cancer cells is related to the cancer progression and may constitute a new marker of malignancy, however further studies in this field are required.

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