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Original article

Mismatch between transcriptomic and histopathologic picture of canine lymphomas

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Abstract

Lymphoma is one of the most common malignant tumours occurring in dogs. Since there is a constant need for new, more comprehensive laboratory diagnostic tools which permit the precise determination of many tumour-related factors we decided to verify whether the use of microarray analysis could be helpful in classifying lymphomas. The study was performed on samples collected from 7 dogs in which multicentric lymphoma was recognized. Among this group we were able to identify one sub-cluster of transcriptionally similar tumours, which completely differed in terms of the histopathological examination. Among them there were one diffuse large B cell lymphoma, one diffuse macronucleolated medium-sized cell lymphoma and one pleomorphic mixed small and large T-cell lymphoma. The lymphomas belonging to the sub-cluster differed from other analysed tumours in the expression of more than 100 genes of which only 18 were described earlier in regard to lymphomas.

Key words: dogs, lymphoma, DNA microarrays, histopathology, transcriptome

Introduction

Lymphoma (malignant lymphoma, lymphosarcoma) is one of the most common malignant tumours occurring in dogs. According to estimates, from 13 to 33 of 100 000 dogs may be affected each year. Breeds that are commonly affected include Boxers, Scottish Terriers, Airedale Terriers, Basset Hounds, Bulldogs,

German Shepherds and Bernese Mountain Dogs (Foulner-Fleury 2002, Jagielski 2002, Pastor 2009, Sapieryński 2010). The major clinical manifestation of canine lymphoma is a single, regional or systemic lymphadenopathy (Pastor 2009, Sapieryński 2009, 2010). The first step in the diagnostic procedure in these cases should be a cytological examination of the enlarged lymph node (Teske 1996, Foulner-Fleury 1997, Guija

de Arespacochaga 2007, Marconato 2010, Sapieryński 2010). In some cases establishing final diagnosis of lymphoma requires histopathological examination of the entire lymph node, or its sample collected during surgery, followed by additional laboratory tests – immunocytochemistry, immunohistochemistry, molecular diagnostic (Guija de Arespacochaga 2007, Sapieryński 2010) So far, microscopic evaluation of the tissue samples obtained using fine-needle biopsy (cytopathology) or surgical biopsy (histopathology), and stained with different histochemical techniques (for determination of tumour type and subtype, and malignancy grading) or immunohistochemical methods (immunophenotyping of tumour cells), are the most commonly used in malignancy grading (staging). Classification of tumours allows the prognosis, choice of treatment and prediction for recovery to be established. For example, determination of the type or subtype of lymphoma according to the updated Kiel Classification (low-grade lymphoma or high-grade lymphomas) based on cytopathological analysis allows the response to the treatment, life expectancy and time to first remission to be predicted (Teske 1994, Dobson 2001, Ponce 2004) A more specific histopathological assessment of lymphomas in dogs has been offered by Working Formulation classification, which enables a broadened categorisation of tumours, classified into three grades of histopathological malignancy, thus having an advantage over the Kiel classification. In some cases determination of immunophenotype of lymphoma is required, on the basis of the presence or absence of CD3 and CD79 alpha antigens on the surface of lymphoma cells. The lymphoma immunophenotype is one of the most important prognostic factors in dogs with recognized lymphoma.

However, there is a constant need for new and more comprehensive laboratory diagnostic tools which would allow the many indicators useful in giving the prognosis, choice of treatment and prediction of recovery to be precisely determined. Since the individual approach to cancer patients is becoming more and more justified, it seems that DNA microarray technology can be very useful in cancer diagnosis, providing a transcriptomic picture of a tumour in individual patients. Therefore we decided to verify whether the use of microarray analysis could be helpful in classifying lymphomas and identifying genes significantly regulated in sub-clusters determined in unsupervised classification of lymphoma transcriptomic profiles.

Materials and Methods

The idea of performed experiment, analyzes and calculations is presented schematically in Fig. 1.

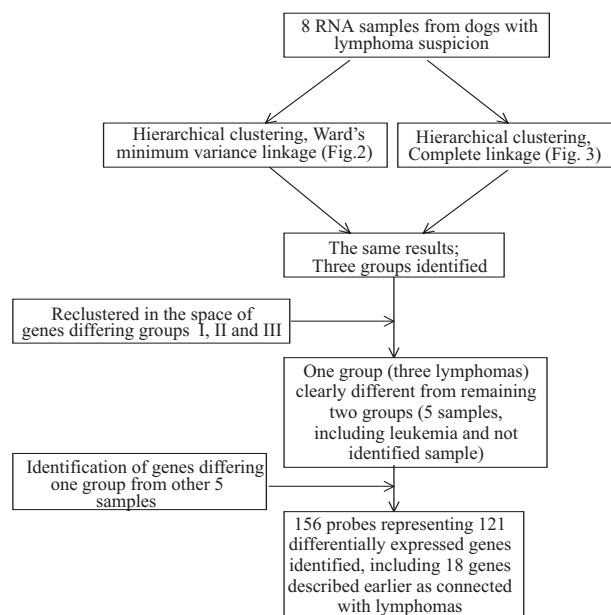


Fig. 1. The unsupervised classification of 8 RNA samples from dogs with suspicion of lymphomas. The diagram depicts the idea of performed analyses and calculations.

Patients' characteristics

The study was performed on samples collected from 7 dogs presented to the Small Animal Clinic, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, in which multicentric lymphoma was recognized based on clinical data, cytology and histology, and follow-up information. None of the dogs had received chemotherapy previously. In some of the examined animals antibiotic or anti-inflammatory therapy was conducted; however, in any case, reduction of lymph node size was observed. The patients' characteristics and results of histopathology are presented in Table 1.

Histopathology

Samples for histopathology and immunocytochemistry were collected during surgery. One of the affected lymph nodes obtained surgically, was fixed in 10% neutral buffered formalin, embedded in paraffin wax, cut in sections (3 μ m) and stained with hematoxylin and eosin. For immunohistochemical staining, tissue samples were processed in the same way. The expression of the CD3 and CD79 alpha antigens was stained using commercially available antibodies to the pan T-lymphocyte marker CD3 (antibody dilution 1:100) (Polyclonal Rabbit Anti-Human, Dako, Denmark) and B cell antigen receptor complex CD79 alpha (antibody dilution 1:50) (Monoclonal Mouse Anti-Human, Dako,

Table 1. Patient characteristics, diagnosis and Id number used in unsupervised classification analyses.

No	Patient characteristics	Diagnosis	Patient Id
1	Mixed-breed, 10-year-old, male	MMC cells	_36
2	Labrador retriever, 7-year-old, female	Myeloid leukaemia	_03
3	Miniature Schnauzer, male, 8-year-old	Large B cells	_91
4	Mixed-breed, 4-year-old, male	Large B cells	_95
5	Bernese mountain dog, 4-year-old, male	Large and small T cells	_07
6	Poodle standard, 11-year old, male	Pleomorphic T cells	_37
7	Yorkshire terrier, 12-year-old, male	Small B cells	_08

Denmark). Briefly, 3- μ m-thick sections on 2% silane coated slides were deparaffinized in xylenes and hydrated through alcohol gradients. Antigen unmasking was performed by microwave heating at 600W for 15 min. in 10 mM sodium citrate buffer, pH 6.0 and Tris/EDTA buffer. Sections were allowed to cool in the buffer at room temperature for 20 min and were rinsed in deionized H₂O twice, 2 min each. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min. Sections were incubated with antibodies for 1 h at room temperature in a humid chamber, and after extensive washing in Tris-buffered saline (TBS) (0.1 M Tris base, 0.9% NaCl, pH 7.4) were further incubated with a biotinylated secondary antibody. The following procedures were then carried out according to standard protocols using the EnVision™ System (Dako®, Denmark). The reactions were developed with 3-3'-diaminobenzidine (Dako®, Denmark), under microscopic control. Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene and mounted. Positive and negative immunohistochemical controls were performed. Tissue sections of formalin-fixed, paraffin-embedded normal canine lymph nodes were used as positive controls in every assay. Corresponding negative control sections were prepared by replacing only the primary antibody with TBS.

Based on histopathology and immunohistochemistry results, subtypes of lymphoma, including immunophenotypes, were established according to Working Formulation classification.

Ethics Statement

During the study all the regulations of the Polish Act concerning Animal Protection and the Act concerning Experiments on Animals were followed.

RNA isolation, validation, labelling and hybridization

Total RNA from tissues samples obtained by fine-needle aspiration (obtained during surgery) were isolated using a commercial kit for total RNA isolation (Total RNA; A&A BIOTECHNOLOGY, Poland) according to the manufacture's protocol. Isolated material was stored at -80°C for further analysis.

Isolated RNA samples were dissolved in RNase-free water and the RNA quantity was measured with the use of NanoDrop (NanoDrop Technologies, USA). Samples with an adequate amount of RNA were treated with DNase I to eliminate DNA contamination and then purified using an RNeasy MiniElute Cleanup Kit (Qiagen, Germany). Analysis of final RNA quality and integrity was performed using a BioAnalyzer (Agilent, USA).

The microarray experiment was performed using common reference design. On each two-colour microarray two RNA samples were hybridized – one from the tumour sample and one from reference RNA obtained by pooling RNA isolated from four healthy lymph nodes obtained from dogs during routine surgeries performed in the Small Animal Clinic of Faculty of Veterinary Medicine in Warsaw. The RNA from healthy dogs was not further investigated but was necessary for unsupervised analysis of transcriptomic profiles. All total RNA samples (10 μ g) were reverse-transcribed using a SuperScript Plus Indirect cDNA Labeling kit (Invitrogen, USA) according to the manufacturer's protocol. Single-strand cDNA was labelled with Alexa 555 (experimental samples) or Alexa 647 (reference pool) dyes (Invitrogen, USA). The efficiency of dye incorporation was measured using NanoDrop. We ran eight microarrays, one for every sample. For experimental purposes, we used *Canis familiaris* (dog) V1.0.1 microarrays, containing 25,383

Table 2. Primers, MgCl₂ amount and product size of genes used for Real-time PCR validation of experimental results.

Gene id	Primers sequence	MgCl ₂ (mM)	Product size (bp)
IFNA5	F: 5' – CGTGATGACCCAGAAGGTCT -3' R: 5' – AGGTCCTCAGGGTGGAGTCT -3'	3	188
STAT3	F: 5'- CAACCCCAAGAACGTGAACT 3' R: 5'-AGCCCACGTAATCTGACACC 3'	3	187
TIAM1	F: 5' – CCTCGTGCACAAGAAGAACA -3' R: 5' – CTGAAAGAGGAAGGCGTCAC -3'	3	250
HPRT	F:5'-AGCTTGCTGGTGAAAAGGAC -3' R: 5' – TTATAGTCAAGGGCATATCC -3'	3	100

oligonucleotide probes representing 27 039 transcripts (Operon, Germany). Hybridization was performed using an automatic hybridization station, HybArray12 (PerkinElmer, USA).

Hybridization, signal detection, quantification and analysis

Acquisition and analysis of hybridization intensities were performed using a ScanArray Express HT microarray scanner and ScanArray Express software (PerkinElmer, USA). Mean spot intensity values were automatically normalized (LOWESS method) by ScanArray Express software and used for further analyses.

Microarray data analysis

Purpose

The purpose of this analysis was to (a) identify groups of samples with similar patterns of gene expression, and (b) identify genes with most differential expression (DE) between the groups discovered in task (a). Task (a) was realized through unsupervised analysis (clustering) of samples, while task (b) was done using linear models for microarrays (limma) (Smyth 2004).

Data pre-processing

1. Probes with a very small level of expression were removed (the arbitrary threshold assumed for the average expression in all hybridizations was 100). Also probes with an expression value below zero in any of the hybridizations were removed. This reduced the number of probes to 24591.

2. The log₂ ratio of the tumour vs control channels was calculated (the M value), and the signal was median-centred.

3. Prior to unsupervised analysis, genes with similar expression patterns in the tumour and control groups were removed. The following criterion was adopted here: genes with a log ratio above 1.3 in at least 50% of samples were considered differentially expressed and were taken into account in clustering.

Unsupervised analysis

Unsupervised analysis (clustering) was performed for rows and columns of the gene expression matrix. Rows and columns correspond to genes and to samples, respectively. The measure of distance between vectors used by the clustering algorithm was defined as $1 - \text{cor}(v_1, v_2)$, where cor denotes the Spearman's correlation coefficient between vectors v_1, v_2 . Hierarchical clustering was used with two linkage functions: complete linkage and Ward's minimum variance.

Analysis of differential expression

Analysis was done using the limma package, with genes ranked by p-value of the t-test. Expression data were then re-clustered in the space of DE genes.

Real-time RT-PCR

All Real-time PCR analyses were performed on individual RNA samples isolated for microarray experiment. RNA was transcribed into cDNA using an Enhanced Avian HS RT-PCR-100Kit (Sigma-Aldrich, Germany) according to the manufacturer's protocol. The amount of cDNA obtained were measured spectrophotometrically in $\lambda = 230$ nm using NanoDrop 1000 spectrophotometer (NanoDrop® Technologies, USA) and then stored at -80°C.

The cDNA was reconstituted to a concentration of 50 ng/μl. PCR reaction was then run using a commercially available kit for Real-time PCR (LightCycler®

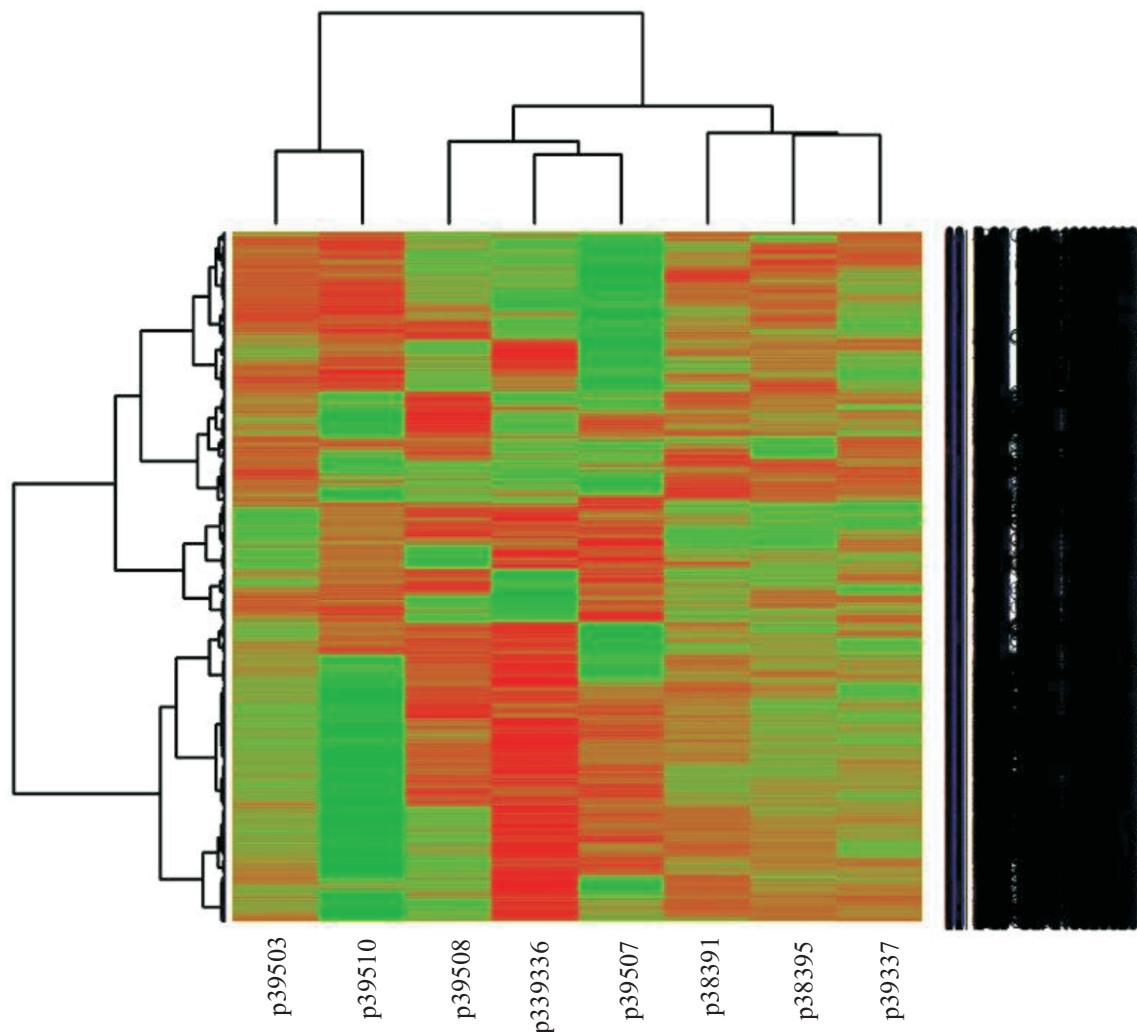


Fig. 2. Results of hierarchical clustering of 8 canine lymphoma cases using Ward's minimum variance linkage.

FastStart DNA Master SYBR Green I; Roche Diagnostic, Poland) using primers listed in Table 2.

Control and experimental samples, after placement into specific capillaries, were placed in a thermocycler (LightCycler, Roche Diagnostic, Poland). PCRs were run using LightCycler3 Front Screen software. The reaction conditions were as follows: preincubation – 95°C; denaturation after start of each cycle – 95°C; annealing – for INFA5, STAT3 and TIAM1: 58°C; for HPRT – 59°C; elongation – 72°C. The value of the threshold cycle was calculated using software integral to the LightCycler thermocycler. HPRT was used as internal control (reference gene). The value of the threshold cycle for each Real-time PCR reaction was measured in four repetitions. The value of the threshold cycle (CT) is the number of cycles in which the clear intensity of amplified RT-PCR product for target gene appears, and is inversely proportional to the amount of target gene mRNA in the sample. For each

sample the Δ CT value was calculated by subtraction of the CT value of the reference gene (HPRT) from the CT value of the gene investigated. In order to decrease the variability of measurements all the samples were normalized to the Δ CT value of the control sample in order to obtain $\Delta\Delta$ CT values. The relative expression of investigated genes was calculated using $2^{-\Delta\Delta CT}$ equation and was shown in arbitrary units. Such a method of data analysis is considered as standard in Real-time PCR analyses and gives similar results to measurements using a standard curve (Livak 2001). The specificity of PCR amplification was verified each time through melting curve analysis and the CT values for HPRT did not differ between groups. The differences in relative expression between genes were calculated using GraphPad Prism4 software (GraphPad Software, USA) with one-way ANOVA and Tukey test. Differences between average values with $p < 0.05$ were considered significant.

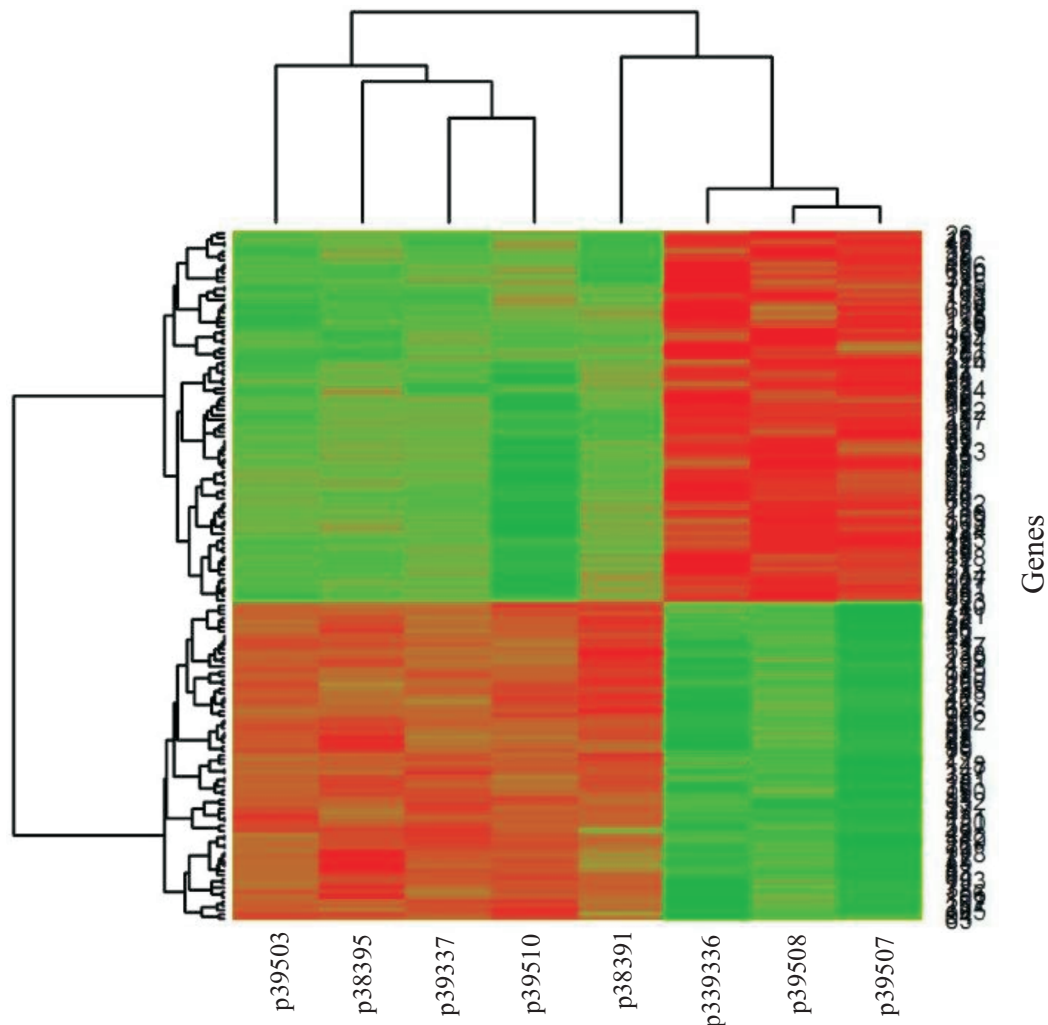


Fig. 3. Results of hierarchical clustering of 8 canine lymphoma cases using complete linkage.

Results

Results of histopathological and immunohistochemical examination

Histopathological analysis revealed that among eight investigated samples there were three cases of large B cell lymphoma, one MMC cell lymphoma, one large and small T cell lymphoma and one pleomorphic T cell lymphoma. In one case diagnosis was myeloid leukaemia (Table 1). RNA from this case was used in unsupervised classification as a kind of internal negative control.

Results of unsupervised analysis

Results of the complete linkage and Ward's minimum variance methods of clustering revealed three sub-cluster) of samples (2 last digits of sample id used):

I: _91, _95, _37; II: _07, _08, _36 and III: _03, _10 (Fig. 2). Based on these results we decided to consider samples _07, _08, and _36 as one sub-cluster and identify the genes which differentiate this sub-cluster from the remaining five samples.

Identification of differentially expressed genes

In this study, the following two sub-clusters were compared: I: _91, _95, _37, _03, _10; II: _07, _08, _36. One hundred and fifty six probes representing 121 genes, which had a BH-adjusted p-value of < 0.1, were declared differentially expressed (data not shown). The re-clustering of expression data in the space of DE genes indicated a similar pattern of expression in the sub-clusters of three samples (_07, _08, _36) which were clearly different from the remaining five samples (heatmaps and dendrograms in Fig. 3). It should be noted that the 156 probes declared as differentially

expressed showed the BH-adjusted p-values below 0.1, which is much lower than expected from the experiment design, under the assumption of no difference between the groups. A simulated experiment repeated for randomly generated data (24591 rows, 8 columns, data from $N(0,1)$), showed that the median of the smallest BH-adjusted p-value equals 0.5, with the probability of the smallest BH-adjusted p-value < 0.1 equal 7%, and the probability that the 5th smallest p-value is < 0.1 is below 1% (data not shown).

Ontology analysis of differentially expressed genes

The aim of the ontology analysis of differentially expressed genes was to identify the genes involved in lymphoma development. Analysis performed using Pathway Studio software identified 18 (out of 121) genes which were earlier described as involved in lymphoma development. From these we selected three genes (TIAM1, STAT3 and INFA5) for real-time PCR validation of microarray results.

Real-time PCR

Real-time PCR analysis of the three selected genes (TIAM3, STAT3 and INFA5) clearly showed a similar pattern of expression in samples _36, _08 and _07 (the sub-cluster identified based on unsupervised classification) and variable expression in the remaining five samples.

Discussion

Although in recent years considerable progress has been made in research investigating the biology and classification of dog lymphomas, the subject is still far from final elucidation. The establishment of a Veterinary Lymphoma Study Group was aimed especially at determining whether human classification systems (WHO classification) can be applied in canine lymphomas. At present various systems of lymphomas classification in dogs exist (WHO classification, Working Formulation, the updated Kiel classification) and they are based on numerous microscopic tumour features, including: cell morphology, immunophenotype, proliferative activity and affected tissue (lymph node, splenic) architecture. These systems allow specific lymphomas to be classified based on their malignancy level, which is of great importance during prognosis, choice of treatment and prediction of the reaction to the treatment (Teske 1996, Foulner-Fleury 1997, Foul-

ner-Fleury 2002, Guija de Arespacochaga 2007, Sapi-erzyński 2010) There are, however, a few factors which make histopathology- and cytopathology-based lymphoma classification imperfect. Firstly, studies concerning the concordance of the sub-classification of canine lymphoma show that there are some differences in the interpretation of canine lymphomas morphology between different, even well experienced, veterinary pathologists (Valli 2011). For example, in a recently published study the overall accuracy of canine lymphoma classification conducted by 17 experienced pathologists on 300 cases of canine lymphoma, which were classified according to the WHO system of classification, was 83 % (Valli 2011). Secondly, even in the same specific subtype of lymphoma some differences in responses to therapy and survival times exist, and it seems that classification criteria based solely on morphology may not be sufficient for the detailed subtyping of canine lymphomas (Marconato 2011). Additionally, many lymphomas in dogs are not as well described and characterised as human ones, and making precise therapeutic decisions based on their type is therefore not so easy. For this reason new method of classification of lymphoma, which could improve this unsatisfactory situation should be introduced into veterinary medicine. One of such potentially useful method, which can lead to precise diagnosis of lymphoma in dogs, is DNA microarray. Since the development of lymphomas is initiated by genetic alterations it seems that the type of these changes will determine the biological properties of the tumour. Moreover, genetic alterations leading to clinical lymphoma must result in pathologically changed gene expression in neoplastic cells. DNA microarray therefore seems to be a tool of great value in investigating the molecular background of lymphomas. The unique gene expression profile of the tumour could not only permit its classification but also indicate the potential target genes for treatment.

DNA microarrays were previously used in the classification of human lymphomas and enabled the identification of biologically and clinically distinctive subgroups of B-cell non-Hodgkin lymphomas (Ledieu 2005), as well as the peripheral T-cell lymphoma (Iqbal 2010). Results of above study were also used for the creation of a classifier permitting prognosis of angioimmunoblastic T-cell lymphoma. Such studies have not been performed in veterinary lymphomas. The use of DNA microarrays in canine lymphomas was described by Ledieu et al. (2005), but the authors investigated only two patients and were using human microarrays for the analyses. The results of their study are therefore difficult to interpret. Starkey and Murphy (2009) performed an investigation showing that fine-needle biopsies of lymph nodes could yield high quality material for gene expression profiling in dogs with lymphomas.

Table 3. Genes involved in lymphoma identified and included in the first sub-cluster of patients, based on unsupervised classification.

No	Gene symbol	Gene name	logFC	t	P.Value	adj.P.Val
1	ADCYAP1	Pituitary adenylate cyclase-activating polypeptide precursor (PACAP) [Contains: PACAP-related peptide (PRP-48); Pituitary adenylate cyclase-activating polypeptide 27 (PACAP-27) (PACAP27); Pituitary adenylate cyclase-activating polypeptide 38 (PACAP-38)]	1.596589	5.151343	0.000334	0.089049
2	CCL22	C-C motif chemokine 22 precursor (Small-inducible cytokine A22) (Macrophage-derived chemokine) (MDC(1-69)) (Stimulated T-cell chemotactic protein 1) (CC chemokine STCP-1)	-1.214320	-5.285102	0.000272	0.082592
3	E2F8	E2F transcription factor 8	1.782945	5.222778	0.000299	0.085957
4	EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	-1.237525	-4.932091	0.000469	0.095208
5	FBXL2	F-box and leucine-rich repeat protein 2	-1.588874	-5.457939	0.000210	0.079508
6	FGFR4	fibroblast growth factor receptor 4	-1.586812	-5.997000	0.000096	0.061865
7	FSCN1	Fascin (Singed-like protein) (55 kDa actin-bundling protein) (p55)	1.079527	5.165866	0.000326	0.088205
8	IFNA5	interferon-alpha	-1.475760	-5.134124	0.000343	0.089049
9	JMJD1C	jumonji domain containing 1C	1.537663	6.526064	0.000046	0.060733
10	NF2	neurofibromin 2 (merlin)	-1.479903	-6.074391	0.000086	0.061865
11	NME2	non-metastatic cells 2, protein (NM23B) expressed in	1.162605	5.219549	0.000301	0.085957
12	PADI2	peptidyl arginine deiminase, type II	1.889625	4.839142	0.000543	0.095208
13	PDCD1LG2	Programmed cell death 1 ligand 2 precursor (Programmed death ligand 2) (PD-L2) (PD-1-ligand 2) (PDCD1 ligand 2) (Butyrophilin B7-DC) (B7-DC) (CD273 antigen)	-1.795839	-7.568936	0.000012	0.056106
14	RAD51L3	RAD51-like 3 (<i>S. cerevisiae</i>)	-1.283854	-5.094146	0.000365	0.091196
15	RARG	retinoic acid receptor, gamma	1.262417	5.329666	0.000254	0.079882
16	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	1.497913	6.275587	0.000065	0.060733
17	TBK1	TANK-binding kinase 1	1.359606	4.964745	0.000446	0.095208
18	TIAM1	T-cell lymphoma invasion and metastasis 1	1.452279	4.815067	0.000564	0.096544

They also performed microarrays, but did not analyse their results beyond the clustering.

As far as we know this is the first study using DNA microarrays for the purpose of canine lymphoma classification. Thanks to the DNA microarrays we were able to create gene expression profile characteristic for each diagnosed lymphoma. Having obtained these profiles we decided to start our analysis from unsupervised classification (with two different methods), which divided the group of patients into three sub-clusters (Fig. 2). After comparing the gene expression of lymphomas between these sub-clusters it was noted that it is possible to find lymphomas with similar expression patterns. However, direct comparison of gene expression profiles revealed only two sub-clusters of similar expression (one comprising three cases, the second

comprising five cases) (Fig. 3). The smaller sub-cluster differed from the larger one in the expression of more than 100 genes of which 18 were described earlier as involved in lymphoma (Table 3). The other five cases (Fig. 3) included one myeloid leukemia and one unclassified lymphoma and therefore we did not interpret this group as one entity (although the results of re-clustering of genes and samples (Fig. 3) suggest that this is possible).

The most surprising result is that three lymphomas of common transcriptomic profile showed a completely different picture in the histopathological examination. Among them there was one diffuse large B cell lymphoma, one diffuse macronucleolated medium-sized cell lymphoma, and one pleomorphic mixed small and large T-cell lymphoma. This could indicate that the

phenotype of the tumour is not related to its genotype and that classification of the canine lymphomas based on their histology does not reflect their molecular background. However, the same conclusion could be drawn from the results of the Starkey and Murphy (2010) who also had 5 lymphomas (3 T-cell and 2 B-cell) and observed, based on gene expression profiles, that although two B-cell lymphomas created one sub-cluster and another two T-cell lymphomas created a second sub-cluster the third T-cell lymphoma had a distinct transcriptomic profile and was the least similar to any other lymphomas (both T-cell and B-cell). The authors suggested that such a result “may reflect the heterogeneity between the T-cell lymphomas that is not resolvable by the CD3-based immunophenotype classification” (Starkey 2009). Our results suggest that such heterogeneity could exist among many different types of lymphomas, not only the T-cell ones.

Based on generally accepted pathomorphological and immunophenotypical criteria the lymphomas included by us to one sub-cluster have some similarities and differences (two cases of B cell intermediate-grade tumours vs. one T cell high-grade tumour). The causes of these observed discrepancies can be various. Interestingly, the group of genes differentiating our two sub-clusters consisted both of genes related to lymphoma and genes not previously described as related to lymphoma. We cannot rule out that these two sub-groups of genes represent two completely different subpopulations of cells, since the material sampled for RNA isolation constituted part of the lymph node without further segregation of cells within a sample. The presence of some non-neoplastic lymphocytes within the lymph node could result from the immunological response directed towards neoplastic cells, and therefore these cells *per se* are not related to the transcriptomic pattern of the lymphoma but rather with the transcriptomic pattern of the lymph node during lymphoma. However, even if the non-lymphoma-related genes identified in our study were of non-neoplastic lymphocyte origin, this could suggest that the immunological response to neoplastic lymphocytes in the sub-cluster of three-lymphomas consist of cells transcriptomically different from the cells responsible for the immunological response to neoplastic lymphocytes in the five-tumours sub-cluster. Thus, when looking for transcriptomic markers of specific lymphoma type we cannot rule out that these markers could be of non-neoplastic origin.

The other explanation of mismatch between transcriptomic and histopathologic profile of lymphomas in our sub-clusters could be that CD3+ immunophenotyping is based mainly on changes at the protein level, and not the gene expression level. Al-

though DNA microarray allows us to create a quite precise transcriptomic portrait of a tumour, we cannot be sure how this portrait will be translated into a proteomic portrait of the lymphoma. Many transcriptomic differences could therefore be covered by the post-transcriptomic and translational processing.

We do not know whether such a mismatch between transcriptomic profile and histology could be of clinical relevance. To answer this question we need to analyse many more than 8 lymphomas, not only at the transcriptomic, but also at the proteomic level. Our results clearly show that the classification of canine lymphomas is a process that has only just started.

Acknowledgements

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