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Genomic and proteomic profiling for cancer diagnosis in dogs

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Abstract

Global gene expression, whereby tumours are classified according to similar gene expression patterns or ‘signatures’ regardless of cell morphology or tissue characteristics, is being increasingly used in both human and veterinary fields to assist in cancer diagnosis and prognosis. Many studies on canine tumours have focused on RNA expression using techniques such as microarrays or next generation sequencing, however, proteomic studies combining two-dimensional polyacrylamide gel electrophoresis or two-dimensional differential gel electrophoresis with mass spectrometry have also provided a wealth of data on gene expression in tumour tissues. In addition, proteomics has been instrumental in the search for tumour biomarkers in blood and other body fluids.

Keywords: Biomarkers; Gene expression signatures; Mass spectrometry; Microarray; Next generation sequencing
Introduction

A diagnosis of ‘cancer’ can be challenging in both dogs and man. Historically, it has relied on histopathological interpretation of biopsy samples with an emphasis on cell morphology and tissue architecture, using immunohistochemical (IHC) stains to confirm the precise cell of origin or specify subtype, to help with treatment decisions and estimates of prognosis.

Examination of single gene product expression by IHC can be useful diagnostically and practical to assess in the clinical laboratory setting, but more recently global gene expression is being used in both human and veterinary fields. This classifies tumours according to similar gene expression patterns or ‘signatures’ consisting of several tens or hundreds of genes, regardless of cell morphology or tissue characteristics. Ultimately, gene expression patterns from individual tumours may direct personal patient treatment plans, using therapy targeted to specific pathways of gene expression (Chantrill et al., 2015). The feasibility of prospective molecular profiling of canine cancers for personalised medicine (PM ed) has already been investigated successfully, making it a practical option for clinical use (Monks et al., 2013; Paoloni et al., 2014). In the shorter term, genomic profiling can usefully identify a few key genes for which IHC or RT-PCR assays can be developed for clinical diagnostic use.

Global gene expression is most easily measured using cellular RNA (Nambiar et al., 2005), however protein expression gives a more dynamic view, providing information on protein interactions, alternative splicing and post-translational modifications, as well as protein abundance (Maes et al., 2015). Global protein expression may therefore be of more use clinically than genomics, since it is proteins that perform most biological processes.
Most genomic and proteomic profiling studies focus on tumour tissue, however, since biopsy samples can be time consuming, stressful and expensive to obtain, the use of tumour biomarkers in blood or other body fluids (urine, cerebrospinal fluid) for diagnosis and monitoring of treatment response is an attractive tool for clinicians (McCaw et al., 2007). In addition, circulating tumour DNA released from tumour cells in the blood can reflect the genomic changes in the tumour itself and is an exciting prospect for determining tumour gene expression from the blood (Schaefer et al., 2007; Forshew et al., 2012; Bidard et al., 2013).

This review will summarise recent developments in genomic and proteomic profiling of canine tumour tissue and blood, which could be of value in diagnosis and therapeutic decisions. It includes the most relevant publications, but is not an exhaustive list of all veterinary studies using genomic and proteomic profiling.

**Genomic Profiling - techniques**

Sequencing of the canine genome (Lindblad-Toh et al., 2005) and more recent improvements in genome detail (Hoeppner et al., 2014) have facilitated high throughput genomic or gene expression profiling (GEP) of canine tumour tissues.

Initial global gene expression studies in dogs used microarray techniques whereby extracted RNA was converted to a fluorescently labelled cDNA and hybridised to DNA coding sequences attached to a solid surface platform (Nambiar et al., 2005); either cDNA fragments (Buishand et al., 2013) or short synthesised oligonucleotide segments (25-60bp) representing the whole canine genome (Klopflieisch et al., 2010b; Scott et al., 2011). The
location and intensity of the cDNA identified which genes were expressed and their level of
eexpression (Nambiar et al., 2005).

Since microarray techniques rely on hybridisation between nucleic acids, cross-
hybridisation makes analysis of highly related sequences difficult, prior knowledge of gene
sequences being investigated is necessary to attach these to the array, and detection of low
abundance genes is difficult (Shendure, 2008). More recently, massively parallel sequencing
of nucleic acids (next generation sequencing, NGS) has proved more popular because it does
not rely on hybridisation and provides extra information on splice variants, polymorphisms
and possible mutations. Interpreting the significance of mutations can be challenging,
however, since many are bystander mutations which do not lead to cell transformation and in
addition, redundancy in cancer pathways means that the biological impact of gene
modifications is not always immediately apparent as an oncogenic effect. For transcriptomic
NGS (RNA-Seq), RNA is converted to cDNA fragments, adaptors are attached to one
(single-end sequencing) or both ends (pair-end sequencing) and fragments are sequenced to
give reads of 30-400 base pairs depending on the technology used. Resulting reads are either
compared to a reference genome or assembled without the genomic sequence to give detail of
gene expression and transcript structure. With decreasing costs, NGS is now preferred over
microarray methods (Wang et al., 2009), although both techniques require enormous
bioinformatic input for manipulation and interpretation of the data.

Genomic profiling - common veterinary tumour types

GEP has been conducted in a variety of canine tumour types (Table 1), although the
data obtained are not yet in diagnostic use in most cases.
Conventional diagnosis of canine lymphoma is based on either cytological or histological interpretation of cell morphology and architecture, often using human classification systems (Teske et al., 1994; Fournel-Fleury et al., 1997; Vezzali et al., 2010; Valli et al., 2011). Application of the revised WHO classification scheme has identified the most common canine subtypes as being diffuse large B cell (DLBCL; 48%), peripheral T cell lymphoma not otherwise specified (PTCL-NOS; 14%), T-zone lymphoma (13%; TZL), T-cell lymphoblastic lymphoma (T-LBL; 4%) and marginal zone lymphoma (4%; MZL) (Valli et al., 2011) although additional criteria such as phenotype, cytogenetic and molecular changes are rarely available for dogs as part of their routine diagnostic investigations.

Molecular profiling of canine lymphoma is still in its infancy, however, a microarray analysis of 35 lymphomas of the six most common subtypes (Frantz et al., 2013) was able to split the tumours into B (DLBCL, MZL, Burkitt and Burkitt-like lymphoma [BL]) and T cell phenotypes (T-LBL, TZL, PTCL-NOS) according to gene expression. Furthermore a clear difference in gene signature split the T cells into low and high grade tumours but the B cell tumours were less easily separated. Mindful of the need for their data to be diagnostically relevant, the researchers developed a benchtop diagnostic test based on qRT-PCR of four genes that could reliably classify an independent cohort of 17 canine lymphomas into the three main subgroups. To distinguish B or T phenotype, the ratio of CD28 to ABCA5 expression was calculated with a value of >1 indicating T cell, and <1 indicating B cell. To separate each T cell tumour according to grade, the expression ratio for CCDC3 to SMOC2 was calculated with a value of >1 indicating low grade T cell tumour and <1 indicating high grade. Although technically possible to carry out qRT-PCR within the scope of a diagnostic laboratory, it remains to be seen whether this gene expression test is adopted commercially.
Other research groups have focussed on molecular subtyping of DLBCL, the most common subtype in both dogs and man. Human DLBCL is further divided into activated B cell (ABC) or germinal centre B cell (GCB) on the basis of gene expression, an important prognostic classification with only 16% of ABC patients alive at 5 years compared to 76% of GCB patients (Richards et al., 2013; Richards and Suter, 2015). The ABC subtype is characterised by B cell receptor pathway signalling and constitutive canonical NF-κB activity and a similar subtype with NF-κB activity has been identified in dogs (Gaurnier-Hausser et al., 2011; Mudaliar et al., 2013; Richards et al., 2013). Although the gene signatures that typically separate human ABC and GCB DLBCL do not separate dog DLBCL as reliably, the pathways and biologic processes that distinguish GC and post GC groups are shared between species (Richards et al., 2013). A canine specific set of differentially expressed genes separates two distinct groups with significantly different survival as do IgH somatic hypermutations (ongoing or static). Immunohistochemical algorithms based on antigens expressed by GC or post GC cells (CD10, BCL6 and MUM1) are used to identify the ABC/GCB subtypes in man as a diagnostically more useful surrogate for GEP, however, in dogs IHC seems less useful since only CD10 stained a moderate number of DLBCL samples, with BCL6 and MUM1/IRF4 rarely expressed (Richards et al., 2013). Unfortunately this means diagnostic application of DLBCL subtyping by IHC will be more complicated in the dog until new markers can be identified and tested.

The molecular classification of canine lymphoma holds promise for future therapeutic developments. Intra-nodal injection of an NF-κB essential modulator-binding domain peptide in four dogs with relapsed DLBCL expressing NF-κB, produced a marked reduction in tumour mass in three cases by inhibiting NF-κB expression (Gaurnier-Hausser et
al., 2011), and oral administration of the bruton tyrosine kinase inhibitor PCI-32765 (ibrutinib) to block B cell receptor signalling in eight treatment naïve or relapsed lymphoma dogs produced a partial response (3/8) or stable disease (3/8) (Honigberg et al., 2010). Thus with more accurate classification of canine tumours based on gene expression, more tailored and targeted drug therapy should be possible for individual patients.

Mammary tumours

GEP of canine mammary tumours is still in its infancy but has much potential when compared to molecular classification of human breast cancer. Although the IHC detection of oestrogen receptor (ER), progesterone receptor (PR), and Human epidermal growth factor receptor 2 (HER2) in human breast cancer has been routine diagnostic practice for decades to assist with prognosis and therapeutic intervention, genomic profiling has greatly refined their usage. GEP of human breast tumours using microarray analysis first identified four major groups with different gene signatures: luminal, HER2 enriched, basal-like and normal (Perou et al., 1999; Perou et al., 2000). Subsequent studies split the luminal group into luminal A (high ER expression) and luminal B (low to moderate ER expression), and showed gene expression patterns could be linked to overall survival, disease relapse, metastasis and chemotherapy response (Toss and Cristofanilli, 2015). Since then, these molecular subtypes have been adopted in routine clinical practice, but for ease of clinical diagnostics, they are identified using the three basic IHC markers, with treatment tailored accordingly e.g. endocrine therapy for luminal A and B, and HER2 therapy for HER2-enriched. The basal-like subtype, recognised as carrying a poor prognosis was originally defined as being triple (ER, PR, HER2)-negative (TN), but more recently it has been emphasised that the two terms are not synonymous, with basal-like tumours forming only a proportion of triple negative tumours (Foulkes et al., 2010). Molecular classification of TN tumours has identified six gene
expression subtypes: two basal-like, an immunomodulatory, a mesenchymal, a mesenchymal stem-like and a luminal androgen receptor (AR) subtype (Lehmann et al., 2011; Turner and Reis-Filho, 2013). Since the AR luminal subtype has a better overall survival, it is important to distinguish these from basal-like tumours especially since recent clinical trials with androgen receptor antagonists have shown therapeutic benefit.

Since malignant canine mammary tumours share many clinical properties with their human counterparts, several IHC studies have examined whether the same molecular subtypes can be identified in dogs. One study (Gama et al., 2008) used an IHC panel based on five markers (ER, HER2, cytokeratin 5, p63 and P-cadherin) to evaluate 96 malignant carcinomas (simple or complex) and carcinomas and identified the four main human subtypes: luminal A (44.8%), luminal B (13.5%), HER2 (8.3%) and basal (29.2%). In contrast, a smaller IHC study on 44 mammary carcinomas (simple or complex) and one squamous cell carcinoma identified luminal A and B and basal subtypes, but no HER2 enriched tumours (Sassi et al., 2010). The basal subtype was associated with a poor prognosis (shorter overall and disease-free survival) in the first, but not the second study. A more recent study focussed on canine mammary tumours which were phenotypically TN in an attempt to distinguish these from the basal-like breast cancer molecular subtype (Kim et al., 2013). Of the 45 TN tumours (33 simple or mixed carcinomas and 12 special types) examined, 43 expressed at least one basal marker (CK14, 5/6, p63 or EGFR), and 34 expressed more than two markers and were therefore considered to have a basal-like phenotype with a worse prognosis (Kim et al 2013). Although these three studies show that there may be some overlap of the molecular subtypes between dog and man, it has yet to be shown that the full molecular signatures are similar between the two species. More studies, as well as standardisation of methodologies in terms of antibody clone and dilution, and antigen
retrieval treatment are needed before diagnostic laboratories are likely to adopt IHC with these markers as routine procedure. While subtyping of human breast tumours determines treatment selection, the benefit of anti-estrogens and antiHER2 therapy has not been established in dogs, meaning that subtyping may have less therapeutic value in this species.

GEP studies on small numbers of canine mammary tumours have been conducted; however, data are limited and have not yet translated to routine diagnosis. Using a dog specific cDNA microarray, one study examined gene expression in 21 mammary tumours compared to normal tissue and progesterone-induced mammary hyperplasia (Rao et al., 2009). The mammary tumours differentially expressed genes involved in cell motility, cytoskeleton organisation and extracellular matrix production, although the expression signatures of benign \( n = 4 \) and malignant \( n = 17 \) tumours, which might be of more use diagnostically, were not compared (Rao et al., 2009). A microarray study on 18 mammary carcinomas compared gene expression to histological grade (Pawlowski et al., 2013b). Five key genes were identified as being either upregulated (sehrl, mipep, relaxin) or downregulated (magi3, zfp37) in high grade tumours, and protein expression was verified by IHC to demonstrate that this gene set has potential to distinguish high grade malignancy (Pawlowski et al., 2013c). Using different microarrays, the same researchers also compared six low and six high grade tumours and found that poorly differentiated tumours often contain upregulated chemokine and cytokine mediated signalling pathways (Pawlowski et al., 2013a), consistent with conventional histological grading of tumours which often takes into account presence of inflammatory response as one criterion of malignancy (Ehrhart et al., 2013).

Although canine metastatic carcinomas can be differentiated from both normal mammary gland and non-metastatic carcinomas by global gene expression (Klopfleisch et al., 2010b, 2011), a small number of key genes that could be detected by IHC in a diagnostic setting to
predict metastatic potential, has not been finalised as yet. Further microarray studies on isolated populations of cancer cells separated from background stroma are needed to clarify the genetic contribution of each cell type to breast cancer progression.

Canine mammary tumours may be complex (epithelial and myoepithelial components) or mixed (osseocartilaginous) as well as forming simple carcinomas as in human breast cancer and the underlying molecular mechanisms for each still need to be established. Using paired-end whole genome sequencing, whole-exome sequencing and RNA-seq, Liu et al (2014) showed that while mammary carcinomas have extensive genomic aberrations, complex tumours contain mostly epigenomic aberrations (Liu et al., 2014). A further study using microarray analysis separated gene expression of canine mammary carcinomas from sarcomas and identified homeobox genes as overexpressed in the latter (Wensman et al., 2009). Such studies may have diagnostic potential in particularly undifferentiated tumours and further work may relate these gene signatures to prognosis or to key pathways for targeted chemotherapy.

In human medicine, five commercial multi-gene profiling tests have been developed to analyse individual patient breast tumour samples and provide prognostic information based on more reliable, reproducible and less subjective techniques than IHC (Toss and Cristofanilli, 2015). Some just predict low or high risk e.g. MammaPrint (70 genes), but the Prediction Analysis of Microarray (PAM50–50 genes) and BluePrint (80 genes) determine the four major intrinsic subtypes (Luminal A/B, HER2-enriched or basal-like) which is helpful for treatment decisions and prognosis. The comparative predictive value of these tests is still being evaluated in independent clinical trials. Ultimately, similar tests could be developed for dogs, if GEP of mammary tumours reveals defined subtypes.
Peripheral nerve sheath tumours (PNST) and fibrosarcomas (FSA) can be difficult to distinguish by morphological characteristics alone or using IHC markers. Klopfleisch et al. (2013) therefore looked at gene expression in five PNST and five FSA using microarrays and identified 45 gene products that were differentially expressed (Klopfleisch et al., 2013). Seven genes known to be specifically expressed in neuroectodermal tissues were upregulated in PNSTs compared to eight genes associated with carcinogenesis which were more highly expressed in FSA. Interestingly, when the same group tried to validate RT-PCR assays for these genes to differentiate PNST from FSA which had been classified using conventional IHC markers (S100, laminin and PGP9.5), the potential PNST markers GLI1 and CLEC3B were able to differentiate the two tumour types with a sensitivity of 89% and specificity of 87%, while the potential FSA markers FHL2-Ex4 and FHL2-Ex9 were unable to separate the tumour types reliably (sensitivity 50%, specificity 88%) (Meyer and Klopfleisch, 2014).

As well as distinguishing between different types of soft tissue sarcoma, genomic profiling can assist subclassification within sarcoma types. Microarray analysis of 15 Histiocytic sarcomas in the Flatcoated retriever identified nine genes that separated tumour from normal spleen (five downregulated; four upregulated) (Boerkamp et al., 2013) but also identified further genes that were differentially expressed between the two clinical presentations of the disease: the soft tissue form affecting limb muscles and joints and the visceral form affecting internal organs with widespread dissemination (Boerkamp et al., 2014). qRT-PCR confirmed the differential expression of three genes: C6 upregulated and VLEC12A and CCL5 downregulated in the visceral compared to the soft tissue form. Whilst this could be adopted as a useful assay in a diagnostic laboratory, it is arguable that the two...
forms of the disease are generally distinguished quite easily by clinicians, without the need for such a test.

GEP of canine haemangiosarcomas has also helped identify tumour subtypes. After candidate gene investigations had failed to show abnormalities in the PTEN/Akt pathway, VHL or Ras genes, microarray analysis was used to examine global gene expression of ten haemangiosarcoma samples cultured to enrich for endothelial cells prior to analysis and compared to four splenic haematomas (normal endothelium) (Tamburini et al., 2010). Gene expression data indicated that the two main processes central to the pathogenesis of canine haemangiosarcoma were inflammation and angiogenesis and it was hypothesised that, rather than the inflammatory component being recruited to the tumour microenvironment, it may be derived from a shared haematopoietic/endothelial progenitor i.e. a single lineage gives rise to both endothelial and haematopoietic progenitors. Subsequent genome-wide expression profiling of 24 canine haemangiosarcoma tissue samples using NGS supported the idea of a multipotent progenitor differentiating into three distinct subtypes: angiogenesis, inflammation and adipogenesis (Gorden et al., 2014). The three gene signatures were then validated in 47 other tumour samples using RNA-seq analysis. It remains to be seen whether these subtypes have different clinical outcomes and whether diagnostic assays to differentiate them would be of value.

Osteosarcoma

Gene expression studies of canine osteosarcoma (OSA) have focussed on the comparative nature of the disease with its human counterpart. Microarray analysis of 15 dog and 15 paediatric osteosarcomas was not able to distinguish the human and canine diseases by their gene expression signatures and two genes consistently expressed in dog tissues (IL-8
and SLC1A3) were associated with a poor outcome in an independent group of children with OSA (Paoloni et al., 2009). The heterogeneous nature of the tumours has hampered genomic analysis, so another study of 26 OSA used low-passage cells lines to filter out stromal contributions (Scott et al., 2011). This identified two groups of tumours with robustly different gene signatures and which had significantly different survival times. The gene signatures were used to segregate reliably, further data sets from dogs and humans, suggesting there is potential for this to be applied in a diagnostic setting, if the key genes in the signature could be narrowed down. A previous microarray study on 32 OSA frozen tissue samples was also able to distinguish two groups based on long and short survival times with upregulated genes in the short survivors involved in proliferation, drug resistance or metastasis (Selvarajah et al., 2009).

**Mast cell tumour**

Differentiation of low grade from high grade mast cell tumours (MCT) is difficult based on histological appearance alone despite the used of different grading systems (Kiupel et al., 2011; Patnaik et al., 1984). A recent microarray study of 51 canine MCT was unable to separate the samples clearly into well-defined groups on the basis of unsupervised gene expression clustering because of too much sample variability, however comparison of differentiated and undifferentiated tumours based on histological criteria, identified clear differences in gene expression relating to cell cycle, DNA replication, p53 signalling, nucleotide excision repair and pyrimidine metabolism (Giantin et al., 2014). The two groups had significantly different survival times, and four genes in particular, FOXM1, GSN, FEN1 and KPNA2 were linked to MCT-related mortality. For practical diagnostic purposes, qRT-PCR assays were developed for 13 transcripts which most reliably separated differentiated
and undifferentiated tumours, and the authors suggest these could be developed as a useful
and cheap benchtop diagnostic test to predict MCT outcome regardless of histological grade.

Proteomic profiling - techniques

Protein expression profiling in clinical practice has lagged behind genomic
techniques in both human and veterinary fields (Kycko and Reichert, 2014; Maes et al., 2015)
but has great potential, particularly in identifying cancer biomarkers to assist with diagnosis,
treatment selection and monitoring of patients.

To detect proteins of both high and low abundance, protein extracts must be
fractionated using techniques such as liquid chromatography (LC) or gel electrophoresis (GE)
(Maes et al., 2015; Matharoo-Ball et al., 2008). Two dimensional polyacrylamide gel
electrophoresis (2D-PAGE) which separates according to charge (isoelectric focusing) before
molecular weight achieves better resolution than 1D-PAGE. To identify the separated
proteins, bands or spots visualised by staining are excised, digested with proteases to small
fragments which are then ionized and analysed by mass spectrometry (MS). Electrospray
ionization (ESI) is usually combined with LC in tandem (LC-MS/MS) whereas matrix-
assisted laser desorption/ionization (MALDI) or surface enhanced laser desorption/ionization
(SELDI) are often combined with time-of flight (TOF) MS. The peptide sequences obtained
by MS are matched to known protein databases to identify the relevant proteins in a ‘bottom-
up’ approach. The alternative ‘top-down’ approach in which intact proteins are identified
without prior enzymatic digestion (Gregorich and Ge, 2014) results in reduced sample
complexity but suffers from molecular weight limitations in protein solubility, separation and
MS analysis (Matharoo-Ball et al., 2008; Gregorich and Ge, 2014).
To overcome the low throughput limitations of 2D-PAGE (one sample on one gel), two dimensional differential gel electrophoresis (2D-DIGE) has been developed, in which two samples and an internal control are labelled with different fluorescent cyanine dyes, then run together on a single gel to allow the ratio of the sample proteins to be determined at each separated spot on the gel (Matharoo-Ball et al., 2008; Maes et al., 2015).

**Proteomic Profiling – common veterinary tumour types**

A limited number of veterinary studies have used proteomics (Table 2), most comparing gene expression in tumour to normal tissue. Formalin fixed paraffin embedded sections are less reliable than fresh-frozen tissue, particularly for the high molecular weight subproteome (Tanca et al., 2012; Tanca et al., 2013). Blood and other body fluids including dog tears have also been examined in the search for biomarkers to diagnose and monitor cancer patients (de Freitas Campos et al., 2008).

**Lymphoma**

Only one study has examined canine lymphoid tissue using proteomics, comparing 11 lymph nodes from dogs with B cell lymphoma to 13 from normal dogs using 2D-PAGE. Ninety-three differentially expressed spots analysed by MALDI-TOF MS revealed three down regulated (prolidase, triosephosphate isomerase and glutathione S transferase) and one upregulated protein (macrophage capping protein) in lymphoma (McCaw et al., 2007). Further studies are needed to confirm whether these proteins could represent potentially useful biomarkers for diagnosis or classification.

**Blood**
In contrast, several proteomic studies have examined serum from dogs with lymphoma. One, comparing serum samples from 29 dogs with B cell lymphoma and 87 control dogs (healthy, with non-cancer diseases, or non-lymphoma cancers) using ion exchange chromatography and SELDI-TOF MS identified three protein peaks with biomarker potential (Gaines et al., 2007). These separated the lymphoma cases from the controls in a classification tree with positive and negative predictive values of 78% and 99% (97% sensitivity, 91% specificity, 92% accuracy), however the identity of the proteins was not given.

A second larger study using the same proteomic technique compared serum samples from 87 lymphoma patients and 92 non-lymphoma samples (diseased or healthy). Although 19 serum peaks were differentially expressed, two biomarkers in particular were able to differentiate control and lymphoma patients and when tested on a separate cohort of 96 patients gave positive and negative predictive values of 80% and 88% (sensitivity 75%, specificity 91%) (Ratcliffe et al., 2009). These were later revealed as the acute phase proteins, haptoglobin and C-reactive protein (CRP) which have been combined with a unique algorithm to produce the canine lymphoma blood test (cLBT) (Alexandrakis et al., 2014). Although these proteins are not specific to lymphoma, the cLBT score appears useful in predicting prognosis and monitoring remission status whilst on treatment and is marketed as a commercial diagnostic test (Avacta) for lymphoma.

Using a different 2D gel-based approach combining agarose and PAGE techniques with MALDI-TOF MS, another study (Atherton et al., 2013) examined the sera of three lymphoma cases compared to two normal healthy dogs. Ten proteins were identified in at least one lymphoma case but not in control dogs, with haptoglobin detected in all three
lymphoma cases. Other acute phase proteins, ±2 macroglobulin, inter-±-trypsin inhibitor and
±-chymotrypsin were identified in lymphoma patients, consistent with an inflammatory
component being present. Kininogen was present in control sera but absent from all three
lymphoma cases. Although interesting, the data need to be verified in a larger cohort of dogs
to see whether these proteins have diagnostic potential in the clinical setting.

A another small study on three dogs with lymphoma, two with transitional cell
carcinomas of the bladder and seven control dogs, looked specifically at serum glycoproteins
using lectin affinity capture prior to global internal standard technology (GIST) stable isotope
labelling, LC and MALDI-TOF MS (Wilson et al., 2008). Eleven fucosylated proteins were
common to all three dogs with lymphoma and upregulated over 50% compared to controls.
In one dog, the fucosylated protein levels fluctuated in response to chemotherapy treatment,
suggesting the potential for disease monitoring. Further studies are needed, however, before
these proteins can be used diagnostically.

Mammary tumours

To search for protein expression differences between long and short survivors with
mammary tumours, Klopfeisch et al (2010) compared the proteome of six mammary
carcinomas with lymph node metastasis at presentation (survival time < six months) to six
mammary carcinomas without metastasis at presentation (metastasis-free survival time > two
years). Using 2D-DIGE and MALDI-TOF MS, they identified 11 upregulated and ten
downregulated proteins in metastatic tumours, 19 of which were reported previously in
metastatic human tumours including breast (Klopfeisch et al., 2010a). Most proteins were
involved in cellular functions related to metastatic spread such as proliferation, cell adhesion,
extracellular matrix remodelling, and hypoxia resistance. In a similar study comparing six normal, six benign, six non-metastatic and six metastatic carcinomas, step wise changes in protein expression were identified, with the biggest change in protein expression detected by the switch to metastasis (Klose et al., 2011). Eleven of 48 proteins increased in metastatic tumours were involved in proliferation (PCNA, RAN binding protein 1, phosphoglycerate mutase 1, siderophilin and Rho-GTPase activating protein) and cell motility (tropomyosin 1 and 3, myosin light chain 2, gelsolin and calumenin) and have been identified in the previous proteomic or genomic studies (Klopfleisch et al., 2010a; Rao et al., 2009), suggesting they are promising candidates for prognostic markers or therapeutic targets.

Blood

In a slightly different approach, serum from 15 dogs with mammary tumours and 15 healthy controls (Zamani-Ahmadmahmudi et al., 2014) was hybridised by western blot to a membrane transferred from a 2D-PAGE gel on which proteins extracted from a mammary tumour cell line had been separated. Four immunoreactive autoantigens (manganese-superoxide dismutase, triose phosphate isomerase, alpha-enolase, phosphoglycerate mutase1) were identified by cutting out spots from the original gel and subsequent MALDI-TOF MS, and all four showed higher expression in tumour tissue compared to control samples using IHC and western blotting and stimulated autoantibody responses in human breast cancer. Further work is needed to determine whether these proteins are useful in diagnostic classification or as prognostic biomarkers in dogs.

Prostatic tumours

Tissue
A 2D-DIGE and MALDI-TOF MS study of three canine prostatic tumours compared them to normal prostate or bladder and identified nine proteins differentially upregulated over 2.5 fold; three being significantly overexpressed: keratin 7, glucose-regulated protein GRP78 and endoplasmin (GRP74) (LeRoy et al., 2007). Keratin 7 assists classification of anaplastic human prostatic neoplasms that are of transitional origin, so has diagnostic potential for the dog. A previous IHC study in dogs, however, did not find it helpful to discriminate prostatic neoplasms from transitional cell carcinoma (LeRoy et al., 2004) although others found it correlated with castration status in younger dogs (Sorenmo et al., 2003).

Mast cell tumours

In an attempt to find better diagnostic methods to subclassify canine MCT, a proteomic study using 2D-DIGE and MALDI-TOF MS identified 13 proteins which were differentially expressed, with four stress response proteins upregulated in high grade tumours, and proteins associated with cell motility and metastasis either increased or decreased (Schlieben et al., 2012). Only five low and five high grade tumours were examined but their grade was confirmed using both Kiupel and Patnaik grading systems and reduced expression of tryptase on proteomic analysis of the high grade tumours was consistent with their dedifferentiation. This proteomic study was performed prior to the genomic study discussed above (Giantin et al., 2014), however, there was no overlap in the genes identified by the two different methodologies. Further studies are needed to find proteins that reliably differentiate the biological grade of mast cell tumours and can be used in clinical diagnosis.

Conclusion
Although case numbers are small, the existing genomic and proteomic canine studies have already identified key genes and gene signatures which have potential for diagnostic use. Further validation of these marker genes on more clinical samples is now needed before applying them to diagnostic use in clinical pathology laboratories. Although progress is slow, the possibility of individualised patient care based on molecular or proteomic profiling of clinical samples may become reality in the not-too-distant future.

Conflict of interest statement

The author has no financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the review.

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### Table 1

Veterinary studies using genomic profiling.

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<td>17 T cell</td>
<td>Microarray</td>
<td>GEP separates B and T cell tumours and low and high grade T cell tumours. Ratio CD28:ABCAS &gt;1 predicts T cell</td>
<td>Frantz et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>17 B cell</td>
<td>Canine genome 2.0 GeneChip</td>
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<tr>
<td></td>
<td>1 nonT-nonB cell</td>
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<tr>
<td>LSA</td>
<td>58 B cell</td>
<td>Microarray</td>
<td>Two separate groups of DLBCL with different progression free and overall survival times, similar to ABC and GCB subtypes in human DLBCL</td>
<td>Richards et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Canine genome 2.0 GeneChip</td>
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<tr>
<td>LSA</td>
<td>23 DLBCL 10 normal LNs</td>
<td>Microarray</td>
<td>NF-kBp65 canonical pathway activated as in human DLBCL</td>
<td>Mudaliar et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Canine genome 2.0 GeneChip</td>
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<tr>
<td>MT</td>
<td>21 tumours (4 benign, 17 malignant) 8 normal mammary tissue 8 progesterone-induced hyperplasia</td>
<td>Microarray</td>
<td>Tumours had expression of ECM, cell motility and invasion genes (FN1, SPARC, CTHRC1, FHL2) and expression of cytoskeletal organisation genes (DES, ACTN1, NEB, DAG1)</td>
<td>Rao et al. (2009)</td>
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<tr>
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<td>Dog specific cDNA</td>
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<tr>
<td>MT</td>
<td>18 CA (6 well, 6 moderately, 6 poorly differentiated)</td>
<td>Microarray</td>
<td>High grade CA have expression of Sehrl, mipep, relaxin and expression of Magi3, zfP37</td>
<td>Pawlowski et al. (2013b, 2013c)</td>
</tr>
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<td>Dog specific cDNA (as in Rao et al, 2009)</td>
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<tr>
<td>MT</td>
<td>12 CA (6 well, 6 poorly differentiated)</td>
<td>Microarray</td>
<td>High grade CA express myeloid specific antigens and have expression of chemokine and cytokine mediated pathways (e.g. S100P), and ECM/inflammatory response genes (MMP1 and 3)</td>
<td>Pawlowski et al. (2013a)</td>
</tr>
<tr>
<td></td>
<td>Oligo-microarray</td>
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<tr>
<td>MT</td>
<td>13 LN +ve simple CA 14 LN -ve simple CA</td>
<td>Microarray</td>
<td>Metastatic CA have expression of cell cycle, matrix modulation, protein folding and proteasomal degradation genes and expression of cell differentiation, growth factor pathway and actin organization genes</td>
<td>Klopfleisch et al. (2010)</td>
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<tr>
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<td>Canine genome 2.0 GeneChip</td>
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<tr>
<td>MT</td>
<td>13 LN +ve simple CA 12 corresponding normal glands</td>
<td>Microarray</td>
<td>Metastatic CA have expression of cell division, invasion genes (MMP, SERPINE1, TIMP3) and expression of chemokine and cytokine mediated pathways (e.g. S100P), and ECM/inflammatory response genes</td>
<td>Klopfleisch et al. (2011)</td>
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<tr>
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<td>Canine genome 2.0 GeneChip</td>
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<tr>
<td>MT</td>
<td>7 simple CA 4 complex CA</td>
<td>NGS</td>
<td>Extensive genomic aberrations in simple CA. Epigenomic changes in complex CA.</td>
<td>Liu et al. (2014)</td>
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<tr>
<td>MT</td>
<td>7 simple CA 6 FSA 5 OSA 4 normal tissues</td>
<td>Microarray</td>
<td>CA expressed epithelial markers and cell adhesion genes. SA expressed mesenchymal differentiation genes and homeobox genes previously linked to craniofacial bone formation</td>
<td>Wensman et al. (2009)</td>
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<td>Canine genome 2.0 GeneChip</td>
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<tr>
<td>STS</td>
<td>5 FSA 5 PNST</td>
<td>Microarray</td>
<td>Neuroectodermal genes (FMN2, KIF1B, GLI1, ROBO1, NMUR2, DOK4 and HMG20B) ‘in PNSTs. Carcinogenesis genes (FHL2, PLAGL1, FNP1L1, BAG2, HK1, CSK and Cox5A) ‘in FSAs’</td>
<td>Klopfleisch et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Canine genome 2.0 GeneChip</td>
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<tr>
<td>HisSA (FCR)</td>
<td>7 visceral tumours 6 soft tissue (limb)</td>
<td>Microarray</td>
<td>PPBP, SpiC, VCAM1, ENPEP, ITGAD ‘and GTSF1, Col3a1, CD90 and LUM ‘in both</td>
<td>Boerkamp et al. (2013)</td>
</tr>
</tbody>
</table>

Note: "Microarray" and "Canine genome 2.0 GeneChip" are different types of microarrays used in these studies, with Canine genome 2.0 GeneChip likely providing more detailed genetic information due to its ability to measure a larger number of genes.

[a]: Some studies used the Affymetrix Canine Genome 2.0 GeneChip array to perform the microarray analysis.

[b]: One study used oligo-microarrays, a type of microarray that can provide specific information about the expression of certain genes.

[c]: NGS refers to Next-Generation Sequencing, a technique used to sequence the DNA of a sample.
<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>Sample Description</th>
<th>Expression Platform</th>
<th>Gene Set</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HisSA (FCR)</td>
<td>8 visceral tumours, 7 soft tissue (limb), 8 pooled normal organs</td>
<td>Microarray, Canine Gene Expression V1</td>
<td>C6 was ‘+’ and CLEC12A and CCL5 were ‘−’ in the visceral compared to the soft tissue form</td>
<td>Boerkamp et al. (2013)</td>
</tr>
<tr>
<td>HSA</td>
<td>10 tumour samples, 4 non tumour (low passage cell culture)</td>
<td>Microarray, Canine genome 2.0 GeneChip</td>
<td>Three distinct tumor subtypes associated with angiogenesis (group 1), inflammation (group 2), and adipogenesis (group 3). Possibly a common progenitor.</td>
<td>Tamburini et al. (2010)</td>
</tr>
<tr>
<td>HSA</td>
<td>12 tissue samples &amp; 18 cell lines (microarray)</td>
<td>Microarray, Canine Gene Expression V1b and NGSc</td>
<td>Expression signatures could not distinguish the canine and human diseases</td>
<td>Gorden et al. (2014)</td>
</tr>
<tr>
<td>OSA</td>
<td>15 dog tumours, 15 human tumours</td>
<td>Microarray, Canine genome v1.0 Human Genome U133Aa</td>
<td>Gene expression identifies two prognostic groups based on survival time &lt; or &gt;6 months.</td>
<td>Paoloni et al. (2009)</td>
</tr>
<tr>
<td>OSA</td>
<td>32 tumours</td>
<td>Microarray, Dog specific cDNA</td>
<td>Differential gene expression segregates dog samples into two groups with differential survival probabilities and applies to human datasets</td>
<td>Scott et al. (2011)</td>
</tr>
<tr>
<td>OSA</td>
<td>26 low passage cell lines, 1 osteomyelitis, 6 tumours</td>
<td>Microarray, Canine genome 2.0 GeneChipa</td>
<td>13 genes involved in cell cycle, DNA replication, p53 signaling pathway, nucleotide excision repair and pyrimidine metabolism can separate undifferentiated and differentiated MCT.</td>
<td>Giantin et al. (2014)</td>
</tr>
<tr>
<td>MCT</td>
<td>51 tumours (5 undifferentiated, 13 differentiated reference samples)</td>
<td>Microarray, Canine Gene Expression V2</td>
<td>FOXM1, GSN, FEN1 and KPN2A expression related to increased mortality</td>
<td></td>
</tr>
</tbody>
</table>

736 ABC – activated B cell, CA – carcinomas, DLBCL -Diffuse large B cell, ECM – extracellular matrix, FCR – flatcoated retriever, FSA-fibrosarcoma, GCB – germinal centre B cell, GEP- Gene expression profiling, LSA – lymphoma, MT – mammary tumour, HSA – haemangiosarcoma, HisSA – histiocytic sarcoma, MCT – mast cell tumour, SA – sarcomas, STS – soft tissue sarcoma, NGS-next generation sequencing, OSA- osteosarcoma, PNST – peripheral nerve sheath tumour, +ve -positive, -ve – negative, ABCA5- ATP-Binding Cassette, Sub-Family A (ABC1), Member 5; CCD3-cytoplasmic cluster of differentiation 3, SM02-small organ 2, NF-kB – Nuclear factor kappa B; FN1-fibronectin1; SPARC – secreted protein acidic cysteine rich, CTHRC1- Collagen, Triple Helix Repeat Containing 1, FHL2 - Four And A Half Lim Domains 2, DES-desmin, ACTN1-actinin alpha 1, NEB -nebulin, DAG1-dystroglycan 1, MMP - matrix metalloproteinase, SERPINE1- Serpin Peptidase Inhibitor Clade E, TIMP – tissue inhibitor of metalloproteinase, EGF - epidermal growth factor, EGFR – epidermal growth factor receptor, MAP2K6- Mitogen-Activated Protein Kinase Kinase 6, STAT- signal transducer and activator of transcription, CLDN5-claudin 5, CTNNAL1- Catenin Alpha-Like 1, MUC1-mucin 1, PECAM1- Platelet/Endothelial Cell Adhesion Molecule 1, ANGPT 2- Angiopoietin 2, ANGPTL1-4- Angiopoietin-like 1-4, FGFI- C-Fos Induced Growth Factor, TIE1- Tyrosine Kinase With Immunoglobulin-Like And EGF-Like Domains 1, FGFR1 -fibroblast growth factor receptor 1, GHR, PDGFR – platelet derived growth factor receptor, TGFB – transforming growth factor beta receptor, FNM2-formin 2, KIF1B- Kinesin Family Member 1B, GLI1- GLI Family Zinc Finger 1, ROBO1- Roundabout 1, NMUR2- Neuromedin U
Receptor 2, DOK4- Docking Protein 4, HMG20B- High Mobility Group 20B, PLAGL1- Pleomorphic Adenoma Gene-Like 1, FNBP1L- Fructose-1,6-Bisphosphatase 1-like, BAG2- BCL2-Associated Athanogene 2, HK1- Hexokinase 1, CSK- c-src tyrosine kinase, Cox5A- Cytochrome C Oxidase Subunit Va, PPBP- Pro-Platelet Basic Protein, SpiC- Spi-C Transcription Factor, VCAM1- Vascular Cell Adhesion Molecule 1, ENPEP- Glutamyl Aminopeptidase, ITGAD- Integrin, Alpha D, GTSF1- Gametocyte Specific Factor 1, Col3a1-Collagen, Type III, Alpha 1, CD90-cluster of differentiation 90, LUM-lumican, CLEC12A- C-type lectin-like domain family 12, member A, CCL5- Chemokine (C-C Motif) Ligand 5, FOXM1- Forkhead Box M1, GSN-gelsolin, FEN1- Flap Structure-Specific Endonuclease 1, KPNA2- Karyopherin Alpha 2.

a Affymetrix.
b Agilent Technologies.
c Illumina.
<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Cases</th>
<th>Technique</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSA</td>
<td>Lymph nodes from 11 B cell 13 normal</td>
<td>2D-PAGE and MALDI-TOF MS</td>
<td>Prolidase (proline dipeptidase), triosephosphate isomerase, and glutathione S-transferase down-regulated and macrophage capping protein up-regulated in lymphoma samples</td>
<td>McCaw et al. (2007)</td>
</tr>
<tr>
<td>LSA</td>
<td>Serum from 29 dogs - B cell, 87 dogs - healthy or nonLSA cancer</td>
<td>IEC and SELDI-TOF MS</td>
<td>Three biomarker protein peaks identified which could separate control from B cell lymphoma dogs</td>
<td>Gaines et al. (2007)</td>
</tr>
<tr>
<td>LSA</td>
<td>Serum from 87 dogs - LSA, 92 dogs - healthy or diseased</td>
<td>A agarose and PAGE electrophoresis and MALDI-TOF MS</td>
<td>Two biomarker protein peaks identified which could separate lymphoma and non-lymphoma patients.</td>
<td>Ratcliffe et al. (2009)</td>
</tr>
<tr>
<td>LSA</td>
<td>Serum from 3 dogs - LSA, 2 dogs - healthy</td>
<td>LA capture GIST stable isotope labelling, LC and MALDI-TOF MS</td>
<td>Haptoglobin identified in all three lymphoma dogs Kininogen absent in all three lymphoma dogs but present in the sera of healthy dogs</td>
<td>Atherton et al. (2013)</td>
</tr>
<tr>
<td>LSA</td>
<td>Serum from 3 dogs - LSA, 2 dogs - TCC bladder, 7 dogs - control</td>
<td>2D-DIGE and MALDI-TOF MS</td>
<td>11 fucosylated peptides ' by &gt;50% in all 3 lymphoma cases compared to normal. In one lymphoma case, 46 upregulated fucosylated peptides “ post-chemotherapy, and then subsequently ‘ upon recurrence and 9 ‘ post-chemotherapy and “ upon recurrence</td>
<td>Wilson et al. (2008)</td>
</tr>
<tr>
<td>M</td>
<td>6 LN +ve carcinomas (&lt;6 months survival), 6 LN –ve carcinomas (&gt;2 years survival)</td>
<td>2D-DIGE and MALDI-TOF MS</td>
<td>Proteins ‘ in LN +ve carcinomas: proliferating cell nuclear antigen, ferritin light chain, bomapin, tropomyosin 3, thioredoxin-containing domain C5, adenosin, ornithine aminotransferase, coronin 1A, RAN-binding protein 1,3-phosphoglycerate dehydrogenase, &amp; eukaryotic translation elongation factor 1. Proteins “ in LN +ve carcinomas: calretinin, myosin, light chain 2, peroxiredoxin 6, maspin, ibrinogen beta chain, vinculin, isocitrate dehydrogenase 1, tropomyosin 1, annexin A5, and Rho GTPase activating protein 1</td>
<td>Klopfleisch et al. (2010)</td>
</tr>
<tr>
<td>M</td>
<td>6 normal tissues, 6 adenomas, 6 LN –ve carcinomas, 6 LN +ve carcinomas</td>
<td>2D-DIGE and MALDI-TOF MS</td>
<td>Differences in malignancy are associated with a stepwise but not linear change in protein expression levels Acquisition of metastatic potential is associated with the strongest changes in protein expression levels</td>
<td>Klose et al. (2011)</td>
</tr>
<tr>
<td>M</td>
<td>Serum from 15 dogs with tumours, 15 healthy controls</td>
<td>Hybridisation to cell line sep by 2D-PAGE, and MALDI-TOF MS</td>
<td>Four autoantigens: manganese-superoxide dismutase, triose phosphate isomerase, alpha-enolase, and phosphoglyceraldehyde mutase 1 with ‘ immunoreactivity in tumour samples identified as biomarker candidates</td>
<td>Zamani-Ahmadmah mudi et al. (2014)</td>
</tr>
<tr>
<td>PT</td>
<td>3 prostate carcinomas, 6 normal prostate, 6 normal bladder</td>
<td>2D-DIGE and MALDI-TOF MS</td>
<td>Three proteins (keratin 7, GRP78, and endoplasmin) were ‘ in the carcinomas compared with normal prostate or bladder</td>
<td>LeRoy et al. (2007)</td>
</tr>
<tr>
<td>MCT</td>
<td>5 Low grade, 5 High grade</td>
<td>2D-DIGE and MALDI-TOF MS</td>
<td>Four stress response proteins (HSPA9, PDIA3, TCP1A, TCP1E) were ‘ in high-grade tumours. Proteins associated with cell motility and metastasis were either ‘ (WDR1, ACTR3, ANXA6) or “ (ANXA2, ACTB)</td>
<td>Schlieben et al. (2012)</td>
</tr>
</tbody>
</table>
+ve - positive, -ve - negative, 2D-DIGE - 2 dimensional differential gel electrophoresis, 2D - PAGE -
2dimensional polyacrylamide gel electrophoresis, GIST - global internal standard technology, IEC-Ion
exchange chromatography, LA - lectin affinity, LC - liquid chromatography, LN - lymph node, LSA-
lymphoma, MCT - mast cell tumour, MT - mammary tumour, PT - prostate tumour, MALDI-TOF MS - matrix-
assisted laser desorption/ionisation with time of flight mass spectrometry, SELDI-TOF MS - surface-enhanced
laser desorption/ionisation with time of flight mass spectrometry, sep-separated, TCC - transitional cell
carcinoma, GRP78-glucose regulated protein 78, HSPA9- Heat Shock 70kDa Protein 9 (Mortalin), PDIA3-
Protein Disulfide Isomerase Family A, Member 3, TCP1A- T-complex polypeptide 1 alpha, TCP1E- T-complex
polypeptide 1 epsilon, WDR1- WD Repeat Domain 1, ACTR3- Actin-Related Protein 3 Homolog, ANXA6-
annexin A6, ANXA2- annexin A6, ACTB- actin beta, RAN - RAS-related Nuclear protein.