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Update on Genomics in Veterinary Oncology

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Abstract

The release of an annotated human genome sequence assembly and the emergence of genomics technologies has led to significant advances in our understanding of many human diseases including cancers. As DNA sequencing technology has become less costly, the field of comparative genomics has progressed rapidly and attention has turned now to generating whole genome assemblies and dedicated genomics resources for veterinary species. Such progress brings a whole new series of opportunities to advance veterinary medicine. Many human and animal diseases share a pathogenetic basis and so while veterinary species need advances in biomedical research in their own right, the consideration of companion animals also as good comparative models for human disease saw the emergence of the 'One Medicine' concept. The future of many areas of human and veterinary biomedical research are very much interdependent, with one of the closest associations being in oncology. It is inevitable that veterinary oncology will benefit enormously from data derived from genomics and that this era will see a huge shift in the ways in which companion animal cancer patients are evaluated and subsequently treated. Here we will review some of advancements of genomics as they relate to veterinary oncology.

Keywords

Veterinary; oncology; genomics; cancer; technology

Introduction

Advances in the use of genomics tools in medicine have been Cancer in humans represents a plethora of diseases that have resulted in pain, suffering and death for many generations. Even now, as we are firmly established in the first decade of the 21st Century, cancer remains a leading cause of death in the USA, second only to cardiac disease [1]. During the last 50 years of the 20th century, improvements in diet combined with advances in biomedical research have resulted in a >60% reduction in the mortality rate of cardiac disease. During the same time, however, the mortality rate associated with cancer has been reduced by < 5% (<http://www.cancer.org>). A component of this apparent minimal reduction in cancer mortality may be associated with improved healthcare and so an increase in longevity; the longer we live the more likely we are to develop a cancer. Treatments for a variety of cancers are certainly prolonging life of patients, with the trend in 5-year survival continuing to increase each decade.

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According to the American Cancer Society, between 1975–2004 the 5-year survival rate for all cancers increased from 50% to 66% (<http://www.cancer.org>). As with humans, our primary companion animals, dogs and cats, also now benefit from advances in health care and are thus tending to live longer lives, with a similar elevation in the number of our pets that become diagnosed with a cancer.

According to recent figures released by the American Pet Products Manufacturers' Association (APPMA), almost two thirds of US households provide a home for a pet and in 2008 the estimated number of dogs in the USA is ~73,000,000 while the number of cats is in the region of 90,000,000. The APPMA estimated that in 2008 Americans will spend almost \$10.5 billion on the overall health care for their pets, with the average lifetime cost of care for either a medium-size dog or a cat being ~\$10,500. Globally, the growth of the pharmaceutical market for companion animals is now at a rate comparable to that of human healthcare (<http://www.bioportfolio.com>) and while the actual cost of providing healthcare to companion animal cancer patients is unknown, with the millions of animals treated each year the cost is anticipated to be several billion dollars per annum in the USA alone.

Statistics on incidence of cancer in pet animals are not widely available. The most widely cited study remains one that was published 40 years ago [2] which looked at overall incidence rates (IR) of cancer in dogs and cats based on a three year survey of veterinary practices in California between 1963 and 1966. While this study cannot have considered the impact of lifespan of healthcare improvements made over the previous four decades, it provided data indicating that annual IR for all cancers in dogs was 381/100,000/year and an IR for cats was 155.8/100,000/year. Among the major cancers in dogs, mammary cancer was by far the highest at 198.8/100,000 with non-melanoma skin cancer at 90.4/100,000 and then several other cancers all being in the region of 20–35/100,000. In cats, the major cancer was reported as of lymphoid origin (48.1/100,000), non-melanoma skin cancers (34.7/100,000) and mammary cancers (25.4/100,000).

In a recent study in Europe looking at a 17 year period (1985–2002), the estimated annual IR of cancer in the dog was reported to be 99.3/100,000 for male dogs and 272.1/100,000 in female dogs [3]. This study revealed the three fold higher overall rate in females was attributed to the high risk of mammary cancer in females, which accounts for approx 70% of all cancer cases. Mammary cancer (IR= 191.8/100,000) aside, the highest incident rates in females was for canine lymphoma (22.9/100,000). In males the highest incident rates were for canine lymphoma and skin cancers (both at 19.1/100,000).

While we know now that many cancers in humans and companion animals are highly comparable diseases, the approach to treating these cancers has been historically far from parallel. In human medicine it is accepted that the driving aim of cancer treatment is to 'cure' the patient. There are three approaches to treating cancer in humans - surgery, radiation therapy and chemotherapy. In treating human patients a combination of these three approaches is often used as part of a highly aggressively regime to keep patients alive as long as possible. In veterinary medicine there is a general acceptance that euthanasia is one possible outcome. As such many animals are still not treated for their cancer and many of those that are treated may receive treatments that are less aggressive and more palliative. However, with all three of the treatment approaches used in human oncology now available to veterinary oncology, the field is presented with new opportunities to embrace new options other than euthanasia. Though we may be a long way from curing cancers, there is optimism that we will be able to treat cancers in pets with improved therapies that not only extend life but also maintain a high quality of life. While it has been only in the past 10–15 years that the important fields of veterinary oncology and radiation oncology were accepted by the American College of Veterinary Internal Medicine/Radiology as being recognized board-certified disciplines, these areas have served

to increase awareness of the role that studies of animal/dog cancers can play in furthering our understating of cancer [4,5].

One cancer – different outcomes

Cancer refers to a myriad of diseases and for decades it has been through the eyes of the experienced cancer pathologist that the ‘type’ of cancer has been determined. Subclassification of malignancies provides more opportunity to correlate the tumors of individual patients with their clinical and biologic behavior. Conventional histological approaches advanced the field for many years and the application of immunohistochemistry to determine the presence of specific cell surface markers is still used widely to sub-divide tumors.

Since the discovery that DNA provides the code to life, there has been a global effort to understand how the genome sequences of numerous organisms are related to their health. Leading the genomics revolution has been the study of the human genome that, since the release of the first draft genome sequence just a few years ago [6], has had an enormous impact on human cancer research. In veterinary medicine, the lack of advanced molecular genomic tools specific to non-human species has hampered our abilities to take full advantage of this exciting and promising new field. The vision of leaders in genomics recognized the power of using comparative analysis of other animals to benefit our understanding of the human genome. This led to a series of non-human genomes entering the pipeline for full sequencing. In July 2005, the release of a publicly accessible annotated genome assembly of the domestic dog [7] and subsequently a draft sequence of the domestic cat [8,9] changed the landscape for canine and feline cancer research. With the release of the horse genome imminent, the future of companion animal veterinary oncology is now presented with a series of exciting new opportunities that were previously considered well beyond its reach.

Application of genome technologies to veterinary oncology

Molecular Cytogenetics – pre and post genome assembly—Just as the pathologist gains more detailed visual clues about the cells comprising a tissue section by rotating the objective turret of his/her microscope to a higher powered lens, so the molecular biologist seeks to use higher resolution molecular tools and approaches to hone in on the genome, from the chromosome to the DNA sequence of the gene. Prior to the availability of a genome sequence for any species, investigations of genome organization in cancers relied primarily on the use of molecular cytogenetics, while determination of specific mutations at the DNA level relied on targeted use of the polymerase chain reaction (PCR). With complete genome sequences now available for dog, cat and horse, we are presented with the tools needed to ask very specific questions about genomes and cancer.

It is widely accepted that malignant transformation requires the accumulation of genetic alterations, or lesions. At the sub-cellular level, many of these changes are evident as alterations to chromosome number and/or structure. The development of molecular cytogenetics, using fluorescence *in situ* hybridization (FISH) technology, has played a significant role in our understanding of cancer biology by providing a means for ‘interrogating’ tumor cells for such gross karyotypic changes. The field of molecular cytogenetics provides a highly visual approach to identify chromosome aberrations that are recurrent and thus associated with initiation/progression of malignancy, ‘vs’ those that are random and thus a result of the chaotic genome organization associated with tumor cells. Many forms of human cancer are so closely associated with specific chromosome aberrations that the aberrations are regarded now as diagnostic for the cancer. Some chromosome aberrations result in the gain or loss of chromosomal material (numerical changes), whilst others result in a reorganization of chromosomal material (structural changes) with either a net change in DNA copy number (imbalanced rearrangements) or no net change in DNA copy number (balanced aberrations)

(Figure 1). While numerical changes alter the copy number of genes in the genome, structural changes frequently bring together genes that have been spatially separated in the genome for millions of years. The interaction between these new ‘neighbors’ in the cancer genome often leads to the altered regulation of genes and/or the generation of new gene products that may act to drive the cell to form a cancer. Knowledge of such gene products provides an opportunity to develop new therapies for treatment of cancers, using the hypothesis that if we are able to identify the biological drivers of a cancer we may be able to block their effects and so inhibit cancer progression. In addition, for many human cancers there is a correlation between the presence of certain genomic aberrations and the clinical outcome of the tumor and/or the tumor’s response to therapy. For this reason many chromosome aberrations are of prognostic value and this information may be used by clinicians to determine the most appropriate therapy and likely survival times [10].

Evolutionarily conserved genomic changes in cancers

Perhaps the most widely investigated chromosome aberration associated with cancers in people is the Philadelphia chromosome, first described almost half a century ago in patients with chronic myelogenous leukemia (CML) [11]. This aberrant human chromosome (HSA) is the result of a translocation event that brings together the *c-abl* oncogene [located at HSA 9q34 (*ABL* locus)] and the breakpoint cluster region (BCR) [located at HSA 22q11] to form a derivative human chromosome 22, technically described as t(9;22)(q34;q11) and referred to as the Philadelphia (Ph) chromosome [12]. The juxtaposition of BCR and ABL is considered a hallmark feature of CML, reported in over 95% of CML patients [13]. The biological consequence of the generation of this fusion is elevation of tyrosine kinase activity, with the consequential proliferation of white blood cells. The identification that a compound, STI571 (imatinib mesylate) could act as an antagonist to this fusion protein (bcr-abl tyrosine kinase) and prevent blast crisis [14,15] led to clinical trials and the development of Gleevec® [13] that (with some exceptions) is now generally considered standard of care for patients shown to present with the Philadelphia chromosome. In May 2001, the FDA approved Gleevec for first line treatment of CML and over the following two years almost 90% of patients were free of disease worsening, with an estimated overall survival rate of 91% and a cytogenetic response in up to 60% of patients [16,17]. Cytogenetic response remains an important surrogate marker of survival in human CML patients [18,19]

While very rare in veterinary species CML has been reported in dogs, all of which had a poor prognosis [20–23]. A recent study of canine CML showed that dogs diagnosed with CML also presented with a functional active BCR-ABL translocation [23] (Figure 2). These data suggest that, cost aside, treatment with Gleevec (using careful monitoring for liver toxicity) could be an option for therapy of canine CML. This study resulted in the first molecular cytogenetic test for the presence of a clinically significant genomic alteration in a veterinary cancer and has since been used to identify the Raleigh chromosome in a further 10 dogs presenting with CML (Breen, unpublished). In the same study [23], the presence of RB1 deletions in canine patients presenting with chronic lymphocytic leukemias and MYC-IgH translocations in canine patients diagnosed with Burkitt lymphoma were also reported. These findings reinforce the concept that as mammals, humans and dogs may be considered temporally separated, differential organizations of the same collection of ancestrally related genes. Since we have shown that genetic ‘lesions’ associated with human cancers may be similarly associated in cancers of veterinary species, therapies developed for malignancies with specific cytogenetic signatures in human cancers may become applicable to provide improved treatments for cancers in our pet dogs and cats. This of course assumes that we are able to define the evolutionarily conserved signatures in our pets and that the pharmacologic effects are considered efficacious. There is little doubt that with the new genomics resource now available to the veterinary biomedical researches (see below), similar associations will be discovered for a variety of animal cancers

and that cytogenetic screening of cancers in our pets could become common practice in veterinary oncology.

Microarrays in cancer – CHIP before you CHOP?

While conventional and molecular cytogenetics are able to reveal and characterize gross genomic alterations in cancers, the process is limited by the resolution of fluorescence microscopy. The emergence of genome sequences for a variety of veterinary species has allowed for the development of new microarray based technologies, that facilitate whole genome, or gene targeted, profiling to be performed at a considerably higher resolution and throughput. The major microarray platforms available for veterinary oncology were initially genomic and cDNA microarrays generated by depositing DNA fragments onto a glass or silicon surface and then binding the sequences to the surface. These arrays generally comprise several hundred to several thousand spots, or features, according to their intended use. More recently high-density oligonucleotide arrays, where short DNA sequences are synthesized directly on the surface of the ‘chip’ have allowed tens to hundreds of thousand of features to be represented on the array and thus increase resolution substantially. Depending on the design, either of these types of microarray may be used to establish DNA copy number variation and gene expression levels, while the latter may be designed specifically to define single base pair changes (single nucleotide polymorphisms, SNPs) at many thousand of points throughout the genome.

Comparative genomic hybridization (CGH)— allows for genome wide evaluation of DNA copy number aberrations at a much higher throughput and resolution than is possible with conventional approaches, thus allowing a more accurate and faster rate of data accumulation. The emergence of complete genome assemblies enables the generation of genome integrated molecular cytogenetic resources. For example, a recent study by Thomas et al [24] reported on the generation of over 2,000 canine bacterial artificial chromosome (BAC) clones, each selected from the canine genome assembly at approx 1Mb intervals and each cytogenetically verified by FISH analysis. Using this genome assembly integrated collection of clones, Thomas et al [24] generated a genomic microarray that was validated for use in array based comparative genomic hybridization analysis (aCGH) of canine tumor DNA samples. This approach has already been used to analyze several types of canine cancer [24–27]. aCGH analysis of human cancers has been refined to determine DNA copy number changes at progressively higher resolutions and the veterinary field is following close behind. High density oligonucleotide arrays that allow scanning of DNA copy number variation in intervals of just a few kilobases of genome sequence are now being used [28]. An illustration of the rapid change in resolution of aCGH in just the past five years is shown in Figure 3. The use of this approach to define recurrent region of genomes that are subject to aberration is a powerful means to hone in on cancer-associated genes.

Gene expression – the impact of genetic changes in cancer—Another contribution to veterinary cancer research is from the analysis of levels of gene expression. The genetic machinery of the cell tightly regulates the expression of genes, with different cell types having characteristic patterns of gene expression. Using microarrays with highly specific probes, the level of expression of thousands of genes may be assessed simultaneously, a process referred to as gene expression profiling. While there are a variety of fabrication processes for such microarrays, the end result is to generate data that highlights the genes that have become deregulated in the tissue of interest. The study of gene expression profiles in large numbers of human cancers has revealed characteristic patterns of gene expression associated with key clinical features such as specific subtype of a malignancy, response to a particular therapy, duration of remission and anticipated survival. In the dog, gene expression profiling has been used to reveal genes associated with intracranial malignancies [29] and to identify that high

expression of the membrane-cytoskeleton linker ezrin in dog tumors was associated with early development of metastases [30].

Figure 4 illustrates how data generated from gene expression profiling are obtained and subsequently compiled to generate clusters of tumors that share commonality determined by the relative expression levels of numerous genes. These gene expression signatures are being investigated for their association with biological behavior of tumors.

MicroRNA profiling: While expression profiling is able to determine the level of mRNA transcripts in cell populations, this does not necessarily correspond to the level of protein product that the cells will ultimately generate. MicroRNAs, or miRNAs, are a class of small noncoding RNA species that are known to have critical functions across various biological processes, serving as key regulatory molecules. From a cancer perspective some miRNAs are known to regulate cell proliferation and apoptosis while others have been shown play crucial roles in cancer cell growth. Disturbance of miRNA expression may thus play a role in the initiation and progression of cancers. For example, over expressed miRNAs in cancers, such as mir-17–92, may function as oncogenes and promote cancer development by negatively regulating tumor suppressor genes and/or genes that control cell differentiation or apoptosis [31–34]. This cluster of miRNAs has been reported also in canine tissues [35]. The development of miRNA ‘chips’ [36–38] thus provides another means to evaluate simultaneously the role of these key regulators in determining prognosis in cancer patients [39]. In addition, miRNAs may have the potential for use as therapeutic agents that could be a powerful tool in cancer prevention and treatment.

Genome wide association studies (GWAS) to define predisposition to cancer—

Early cancer detection is affected by the fact that animals do not communicate their ill health until such a time that their physiological stress provides key indicators; loss of weight, loss of appetite, lameness, lack of interest, coughing etc. While we strive to promote routine health screens and work towards developing means to detect cancers earlier, it is also important to highlight that cancer is a genetic disease and as with other genetic diseases it should be possible to predict which individuals have a genome indicating a cancer predisposition.

It is widely accepted that mapping disease genes in veterinary species is made possible through the development of genomic resources. Cancer is a complex disease process that is associated with numerous genes and so teasing out the major effectors in human populations that present with a high level of locus and phenotypic variation is a challenge. The demographic history of purebred dog breeds has resulted in a genetic structure within dog populations that allows association studies to be performed on considerably smaller cohorts and thus for diseases genes to be identified more readily. There are now numerous reports of mapping canine disease genes, both simple and complex, via genome wide genotyping studies [40,41], illustrating the broader importance of the dog as a comparative model system.

One of the mapping tools for association studies is to consider the haplotypes shared between individuals presenting with the same disease phenotype. Such haplotypes may be scored by a variety of means, with the most informative method being one that assesses variation at the highest density. The densest form of polymorphism in the genome is that which relies on variation at the level of a single base pair. Such changes are called single nucleotide polymorphisms (SNPs). In the domestic dog, the average SNP frequency has been estimated to be 1 every 1,000 bp [7] and researchers are currently identifying the frequency and distribution of SNPs as part of the development of high quality genome assemblies for other veterinary species, including cat and horse. Of great significance to veterinary biomedical research is that association studies is the fact that such a high SNP frequency in the domestic dog means that while association studies in human population may require assessment of

500,000 SNPs, similar studies in purebred dogs require as few as 10,000 SNPs [7]. Genome wide association studies using populations of dogs will likely proceed much faster and at a greatly reduced cost than would be the case using human populations. For diseases with a shared pathogenetic basis in human and dog, gene discovery may thus originate from studies of the dog and then translate to human. Analysis of haplotype in different breeds suggests that a common set of SNPs would be informative for most breeds of dog, but that selection of breeds to be studied is of great importance. Several SNP genome wide association studies to define genes associated with specific phenotypes have already been reported [42–44] and several studies are ongoing to identify genomic regions and ultimately genes associated with a variety of canine cancers. The SNP frequency in the emerging genomes of the cat and horse suggest that both species have a rate the same as the dog (Lindblad-Toh, pers. Comm.). With almost one million SNPs reported in the horse genome (<http://www.broad.mit.edu/mammals/horse/snp>) and SNP discovery underway for the cat, genome wide association mapping studies will play a key role in investigations of both feline and equine cancers.

Conclusion

Regardless of the approach used to identify cancer associated genes, once identified such genes will be evaluated specifically for their role in the initiation and/or progression of the associated cancers and for their association with response to current and emerging therapies. This work will encourage the development of new therapies for our animal cancer patients, ultimately prolonging the length and quality of life for our pets. It is inevitable that the field of veterinary oncology will very soon be presented with new opportunities to become involved in this rapidly progressing genomics pipeline. The companion animal population in the USA provides a source of naturally occurring cancers that may be suitable for inclusion into research projects as we endeavor to define the biological process underling the cancer process, and subsequently to become involved in clinical trials of new therapies. Owners of animal cancer patients along with their primary veterinarians and oncology specialists will thus play a key role in determining the pace at which this research progresses. Public and professional awareness and acceptance of progress should help to motivate people to submit biospecimens from their companions to provide material for ongoing research initiatives. One such initiative from the Canine Comparative Oncology and Genomics Consortium is the Pfizer-CCOGC Biospecimen Repository (www.CCOGC.net). The CCOGC determined that an essential resource needed to advance veterinary and comparative cancer research was a well-described repository of tissues (tumor and normal) from tumor bearing dogs. Established in 2007, the CCOGC aim to collect biospecimens from 3,000 dogs with seven specific cancer types. Using standard operating procedures, samples from suitable cases are submitted by participating collection sites to the Pfizer-CCOGC's repository in Frederick Maryland. A caBIG compliant web-based relational database has been implemented to track sample shipment and to describe sample characteristics and clinical annotation of patients. Once populated, samples will be made available for inclusion in research projects, following appropriate review by an independent scientific advisory board. Progress in advancing veterinary genomics will likely not be limited by technology or lack of suitable sample materials. Key discoveries towards easing incidence and the pain and suffering resulting from cancers in our pets will be expedited by increasing communication and awareness between researchers, practicing veterinarians and their clients. Veterinarians will soon be able to present to their clients a whole new era of genetic testing, counseling and tailored therapy for numerous disorders, including cancer.

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Figure 1. Schematic demonstration of examples of structural and numerical chromosome aberrations

A) Single homologues of two example chromosomes; on the left the larger submetacentric chromosome has been divided in regions A and B on the p-arm and regions C-H on the q-arm; on the right the smaller metacentric chromosome has regions 1 and 2 comprising the p-arm and regions 3 and 4 comprising the q-arm. B and C shows balanced chromosome aberrations in which the organization of the segments has altered but their copy number has not changed; in B a reciprocal translocation between the two chromosomes has resulted in two new derivative chromosomes, in which the q arms have been exchanged; in C a paracentric inversion in the q arm of the larger chromosome has resulted in a change of segment order. These changes would go undetected in comparative hybridization analysis and would require cytogenetic evaluation to identify these changes. Panels D, E and F shows structural changes that are associated with gain or loss of chromosomal segments of the larger chromosome; D shows deletion of region E, E shows a pericentric inversion with loss of region A, F shows a duplication of region E.

All three of these aberrations change the DNA content of the cell and would be readily detected using CGH analysis. All three would also be visualized using molecular cytogenetic evaluation with suitable probes.

Figure 2. Identification of the 'Raleigh' chromosomes in dogs diagnosed with chronic myelogenous leukemia

A. Metaphase preparation and interphase nucleus from a clinically healthy dog to which canine bacterial artificial chromosome (BAC) clones representing the genes *ABL* (yellow signal) and *BCR* (green signal) showing the localization of these loci to CFA 9 and 26, respectively (see inset). B) Hybridization of the same *BCR* and *ABL* BAC clones to a metaphase preparation and interphase nuclei derived from a CML patient. In the metaphase spread, while one copy each of CFA 9 and 26 appear normal (lower right) the presence of a derivative chromosome showing co-localization of yellow and green signal (inset) indicates the presence of the 'Raleigh' chromosome. Similarly, juxtaposition of one green and one yellow signal in each of the two interphase nuclei confirm this translocation event

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Figure 3. Advances in resolution of DNA copy number variation using array based comparative genomic hybridization (aCGH)

A) aCGH data of a canine tumor sample using a 1Mb BAC array [24] indicates genome wide DNA copy number variation of 2,200 canine BAC clones. There is indication of copy number increase for regions of dog chromosomes (CFA) 6,9, 21 and 26 and evident of copy number decrease for segments of CFA 8, 19, 26 and 28. There is evidence of a homozygous deletion of a single BAC clone in the distal half of CFA 8 (circled). B) genome wide aCGH analysis of the same case on a high resolution oligonucleotide array, assessing copy number at 385,000 points in the canine genome and with an effective resolution of 50Kb. It is evident that the overall copy number aberrations visible in panel B are grossly comparable to those in panel A

although additional copy number changes are evident that lay between the loci assessed on the BAC array. C) aCGH data specifically from CFA 8 of panel B); when the copy number data are evaluated at this resolution it is evident that the copy number aberration at the distal end of the chromosome may now be further refine to a region that spans fewer than 1.5 Mb between 45 Mb and 46.5 Mb (red lines). Interestingly, this higher resolution platform also identified a smaller deletion of less than 1 Mb at the proximal end of CFA8 within the region ~5.5 Mb-6.0 Mb. This deletion lay between BAC clones on the 1 Mb array and so was not detected in panel A.

Figure 4. Gene expression analysis in canine cancers

A) Scan data resulting from the use of a small 3,840 feature cDNA spotted microarray. RNA from two samples were differentially labeled (reference = red; tumor = green) and c-hybridized to the array. Following hybridization the microarray was scanned at 5 μ m resolution, acquiring the two color planes. The strength of the hybridization signal on each spot relates the abundance of mRNA population of the corresponding gene. On the right a merged image is shown to visually compare gene expression levels of both samples. B) Hierarchical cluster analysis 100 genes identified as showing differential regulation of 60 canine cancer specimens. Each column represents a different patient and each row represents a different gene. Blue represents increased expression and yellow represents reduced expression, relative to non-neoplastic

cells. Despite being of the same tumor type, it is evident that these 60 cases of the same histological type have quite distinct gene expression profiles and associating these with response to therapy and survival will lead to more sophisticated cancer patient management.