DISSERTATION

UTILIZATION OF A CANINE CANCER CELL LINE (FACC) PANEL IN COMPARATIVE AND TRANSLATIONAL STUDIES OF GENE EXPRESSION AND DRUG SENSITIVITY

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ABSTRACT

UTILIZATION OF A CANINE CANCER CELL LINE (FACC) PANEL IN COMPARATIVE AND TRANSLATIONAL STUDIES OF GENE EXPRESSION AND DRUG SENSITIVITY

Canine cancer is the leading cause of death in adult dogs. The use of the canine cancer model in translational research is growing in popularity due to the many biologic and genetic similarities it shares with human cancers. Cancer cell tissue culture has long been an established tool for expanding our understanding of cancer processes and for development of novel cancer treatments. With the high rate of genomic advancements in cancer research over the last decade human cancer cell line panels that combine pharmacologic and genomic information have proven very helpful in elucidating the complex relationships between gene expression and drug response in cancer. We have assembled a panel of canine cancer cell lines at the Flint Animal Cancer Center (FACC) at Colorado State University to be utilized in a similar fashion as a tool to advance canine cancer research. The purpose of these studies is to describe the characteristics of the FACC panel with the available genomic and drug sensitivity data we have generated, and to show its utility in comparative and translational oncology by focusing specifically on canine melanoma and osteosarcoma.

We were able to confirm our panel of cell lines as being of canine origin and determined their genetic fingerprint through PCR and microsatellite analyses, creating a point of reference for validation in future studies and collaborations. Gene expression microarray analysis allowed for further molecular characterization of the panel, showing that similar tumor types tended to cluster together based on general as well as cancer specific gene expression patterns. In vitro
studies that measure phenotypic differences in the panel can be coupled with genomic data, resulting in the identification of potential gene targets worthy of further exploration. We also showed that human and canine cancer cells are similarly sensitive to common chemotherapy.

Next we utilized the FACC panel in a comparative analysis to determine if signaling pathways important in human melanoma were also activated and sensitive to targeted inhibition in canine melanoma. We were able to show that despite apparent differences in the mechanism of pathway activation, human and canine melanoma tumors and cell lines shared constitutive signaling of the MAPK and PI3K/AKT pathways, and responded similarly to targeted inhibition. These data suggest that studies involving pathway-targeted inhibition in either canine or human melanoma could potentially be directly translatable to each other.

Evidence of genetic similarities between human and canine cancers led us to ask whether or not non-pathway focused gene expression models for predicting drug sensitivity could be developed in an interspecies manner. We were able to show that models built on canine datasets using human derived gene signatures successfully predicted response to chemotherapy in canine osteosarcoma patients. When compared to a large historical cohort, dogs that received the treatment our models predicted them to be sensitive to lived significantly longer disease-free. Taken together, these studies show that human and canine cancers share strong molecular similarities that can be used advantageously to develop better treatment strategies in both species.
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CHAPTER 1

Literature Review

CANINE CANCER AS A MODEL

Canine cancer statistics

Cancer is the second leading cause of human death in the United States, with a 2014 study estimating 1,665,540 new diagnosed cases and 585,720 deaths each year (American Cancer Society, 2014). Advancements in health care for companion animals in recent years including better vaccines, diet, the implementation of leash laws, and better diagnostic tools have resulted in longer living pets, leading to increases in age-related disease such as cancer (Paoloni and Khanna, 2007). Of the estimated 65 million dogs in the United States, cancer is the leading cause of death in adult dogs, reaching 45% in of dogs 10 years or older. An estimated 6 million new cancer diagnoses are made in the population each year (Mazcko, 2012; O'Donoghue et al., 2010; Ranieri, 2013). Put more simply, it is estimated that 1 in 2 dogs will get cancer, and 1 in 4 dogs will die from cancer, showing that similar to humans the pet dog population is deeply impacted by this disease and is in need of continued support in developing new and better ways of combating cancer (Paoloni and Khanna, 2007).

Dogs get many of the same cancers as humans, including osteosarcoma, melanoma, non-Hodgkin’s lymphoma, leukemia, soft tissue sarcoma, as well as cancers of the prostate, lung, bladder, head and neck, and breast (Bergman, 2007; Caserto, 2013; Fenger et al., 2014; Ito et al., 2014; Knapp et al., 2014; Paoloni and Khanna, 2007; Porrello et al., 2006; Richards and Suter,
According to a UK study, malignant tumors with the highest incidence are mast cell tumors followed by soft tissue sarcoma, lymphoma, osteosarcoma, and mammary tumors (Dobson, 2013). Certain breeds have been associated with a higher risk of developing specific cancers. For example, Rottweiler, Irish Wolfhound, and Great Dane breeds are at higher risk to develop osteosarcoma (Dobson, 2013). Bull Terrier, Boxer, Golden and Labrador Retriever, and Bulldog breeds are at a higher risk to develop mast cell tumors (Dobson, 2013). Golden Retrievers are also at high risk for developing other cancers such as lymphoma, oral melanoma, fibrosarcoma, and histiocytic tumors, resulting in the reported cancer death rate of 60% for this more susceptible breed (Dobson, 2013; Hovan, 2006).

**History of veterinary oncology**

The relatively new field of veterinary oncology has advanced steadily since its beginnings more than 50 years ago. In 1958 a veterinarian named Gordon Theilen wrote a report describing a herd of cattle where multiple leukemias were observed (Theilen, 2013). This was the start of his very influential path as a pioneer for this emerging field. A few years later in 1962 the New York Academy of Science sponsored a meeting entitled “Tumors in Animals” where Dr. Theilen and others presented papers describing animal cancers. From that point on a small group of research and clinical veterinarians began to meet to discuss their interests in animal cancers, eventually leading to the creation of the Veterinary Cancer Society (VCS) in 1977 (Paoloni and Khanna, 2007; Theilen, 2013). The decade before another group called the International Association for Comparative Research on Leukemia and Related Diseases (CRLRD) was created whose primary interest was in studying the basic mechanisms and relations between viruses and cancer in different species including birds, rodents, and mammals (Theilen, 2013). In 1972 at UC Davis Dr. Theilen helped develop the first veterinary cancer
medicine specialty program (Theilen, 2013). In 1988 Medical Oncology was officially added to the American College of Veterinary Internal Medicine (ACVIM). Alice Villalobos, a former veterinary student under Dr. Theilen at UC Davis, was responsible for establishing the first veterinary practice that was strictly devoted to cancer treatment in southern California called the “Animal Oncology Consultation Service and Animal Cancer Center” (Theilen, 2013). Now there are similar animal cancer centers established all over the world.

One animal cancer center in particular at Colorado State University (CSU) got its start thanks to the efforts of two more pioneers: Dr. Ed Gillette, a veterinarian and radiation biologist, and Dr. Steve Withrow, a veterinary surgeon (Flint Animal Cancer Center, 2014; Theilen, 2013). Starting in the late seventies they hypothesized that they could treat animals with cancer using strategies similar to those used in people. They also hypothesized that the spontaneous cancers arising in dogs would make an effective translational model for human cancer based on the many similarities of tumors between species. After years of hard work the CSU Flint Animal Cancer Center (FACC) was finally established in 2002 (Flint Animal Cancer Center, 2014). Today, the FACC is now recognized all over the world as a leader in cancer research and clinical veterinary oncology, with a strong research program in multiple areas such as radiation oncology, molecular genetics, pathology, immunology, pharmacology, musculoskeletal oncology, and experimental therapeutics (Flint Animal Cancer Center, 2014). The FACC also has a large clinical team with expertise in medicine, radiation, and surgical oncology that offers high quality care for animals with cancer (Flint Animal Cancer Center, 2014).

Another advantageous development in veterinary oncology was the launching of the Comparative Oncology Program (COP) by the National Cancer Institute’s Center for Cancer Research (CCR) in 2003 (Mazcko, 2012). Its focus is to increase understanding of cancer
biology and to improve the development of novel human cancer treatments by incorporating pet animals into the process. One way that the COP accomplishes this is through the management of the Comparative Oncology Trials Consortium (COTC), a network of 20 comparative oncology centers that design and carry out clinical trials in canine cancer patients. The resulting data from these canine clinical trials are utilized in the design of human phase I and II clinical trials (Mazcko, 2014).

In anticipation of the completion of the Canine Genome Project in 2005 where 99% of the canine genome consisting of 2.5 billion base pairs was sequenced, a group of veterinary and medical oncologists, geneticists, biologists, and pathologists gathered in 2004 in Boston to share their interest in comparative research of human and canine genomics as it relates to cancer. The group was established in 2006 and was named the Canine Comparative Oncology and Genomics Consortium (CCOGC) (Canine Comparative Oncology & Genomics Consortium, 2015; Lindblad-Toh et al., 2005). One of its priorities was to develop a biospecimen repository which would contain tumor, normal tissue, blood, and urine samples. Currently the Pfizer CCOGC Biospecimen Repository has limited the collection to certain cancers with a high comparative value with human cancers, namely lymphoma, osteosarcoma, melanoma, hemangioma, lung cancer, soft tissue sarcoma, and mast cell tumors (Canine Comparative Oncology & Genomics Consortium, 2015). Currently there are ten veterinary institutions in the United States participating as collection sites for this repository, which provides samples for investigators all over the world (Lana, 2014).

Interestingly, the FACC, which is included in this group of collection sites used by the CCOGC, established its tissue archiving program 3 years prior in 2003. Today more than 21,000 samples of tissue, blood and urine for both canine and feline cancers have been collected in the
archive which is an impressive resource for researchers from around the world (Lana, 2014). Genomic studies in veterinary and comparative oncology are now emerging with force thanks to genomic advances and the recent availability of tumor samples.

Before this new field of veterinary oncology had begun to gain traction, the only treatment option for animals with cancer was surgical excision of the tumor, and if that failed to cure, euthanasia (Theilen, 2013). Thankfully, today along with surgical oncology there are many more options available to canine cancer patients including chemotherapy, radiation therapy, immunotherapy, and molecular targeted therapy (Withrow, 2013). Veterinary oncology has come a long way in a short time, spurred on by ever growing interest in advancing health care for animals with cancer, and by the increasing number of studies showing that cancers in companion animals (specifically pet dogs) share many of the same characteristics as human cancers. The more we learn of companion animal models of cancer the more potential is apparent for translational applications for human research.

**Advantages of the canine cancer model**

Murine models have been and continue to be invaluable in studying the biology behind cancer initiation, promotion, and progression (Ranieri, 2013). However, other aspects of human cancers are better represented by a canine cancer model. One of the most notable advantages is the fact that the tumors in pet dogs arise spontaneously which represents the human condition much more closely than many murine models where tumors are artificially introduced through transplantation or genetic manipulation (Withrow, 2013). Dogs also are exposed to similar environmental risk factors by living in the same conditions as their owners and may reflect any epidemiological changes observed in human cancer development (Dobson, 2013; Withrow, 2013). Canine tumors have similar histology to human tumors, they grow within the context of
an intact immune system, and they display tumor heterogeneity between individuals and within the tumor itself. This heterogeneity leads to issues of cancer resistance, recurrence, and metastasis in the dog as is seen in human cancer (Ranieri, 2013; Withrow, 2013).

The larger body size of dogs compared to rodents holds many advantages as well. Repeated sample collection over time from the same animal is possible in the dog, whereas in most murine models the sacrifice of multiple animals is needed to form a composite collection of samples representing different time points. Larger tumor size allows for more tissue and/or blood to be available for molecular analysis. Larger body size is also important in achieving a drug dose in dogs that is more comparable to doses given to people (Withrow, 2013).

Canine cancer shares high genetic similarity with the human disease. Genetic lineages between dogs and humans are more similar than rodents in terms of nucleotide divergence as well as genetic rearrangements (Paoloni and Khanna, 2007). Many of the cancer-driving genes in humans are also found to play roles in canine cancers (Withrow, 2013). For example, the same TP53 alterations that have been documented in human breast cancers, sarcomas and lymphomas have also been reported in the same cancers in the dog (Haga et al., 2001; Hershey et al., 2005; Setoguchi et al., 2001). Another example is found in v-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog (c-Kit), a tyrosine kinase growth factor receptor which is known to have activating mutations in human gastrointestinal stromal tumors as well as canine mast cell tumors. Despite the differences in tumor type, the common mechanism of mutant c-Kit activation allows studies targeting this known cancer pathway to be translatable between species (Da Ros et al., 2014; London et al., 1999; Pryer et al., 2003; Yamamoto and Oda, 2015). A cluster analysis of gene expression profiles of canine and human osteosarcoma and corresponding normal tissues resulted in the separation of normal tissues by species, but
interspersion of all osteosarcoma samples regardless of species, showing the genetic similarities between the cancers of human and dog are more similar to each other than their normal tissue counterparts (Paoloni et al., 2009). Genetic similarities between human and dog cancers have also been reported for lymphoma, breast, soft tissue sarcoma, and glioma (Mudaliar et al., 2013; Paoloni and Khanna, 2008; Uva et al., 2009).

Another advantage of the pet dog model of cancer is the availability of veterinary clinical trials. Since there is a lack of “standard of care” for the treatment of canine cancer patients, novel therapies can be administered in a trial setting before other known treatments have failed (Vail and MacEwen, 2000; Withrow, 2013). Veterinary clinical trials can occur in a pre-Investigational New Drug (IND) setting, requiring less paperwork and time for approval. The cost of clinical trials in companion animals is significantly less than for human trials, and high levels of owner compliance is advantageous for the quick populating of trials (Vail and MacEwen, 2000). The shorter time frame associated with the life span of dogs and cancer progression allows researchers to generate and analyze data as early as 6-18 months in dog trials, hastening results needed to increase cancer knowledge for dogs with potentially translational conclusions (Paoloni and Khanna, 2007).

**Disadvantages of the canine cancer model**

Although the larger body size is amenable to repeated sample collection and achieving doses nearer to what’s possible in humans, it also means higher quantities of drug are needed compared to rodent models and thus higher costs (Gordon and Khanna, 2010). The life span of dogs allows data to be generated much faster than in humans, but studies in dogs are also much slower to complete than rodent models (Paoloni and Khanna, 2008). Studies with pet dogs in a clinical trial setting are by nature “uncontrolled” compared to rodent studies making it more
difficult to confidently associate toxicity with the drug under investigation. Also, attempts to bring more control into clinical trials by increasing study entry requirements to reduce the number of clinical variables can make the study model less similar to the human population it is meant to reflect (Paoloni and Khanna, 2008).

Another potential issue is that the most common canine cancers are not the most common human cancers. Dogs generally get a lot of sarcomas and lymphomas but have much lower incidences of the common human tumors such as breast, prostate, colon and lung. The disadvantage lies in the difficulty in populating a clinical trial in dogs with a rare cancer because of its translational significance for humans (Paoloni and Khanna, 2008). On a molecular level, the similarities between many human and canine cancers do not necessarily mean that a molecular target of interest in humans will be seen in the dog. Often times the mechanisms in a canine cancer are understudied or they are simply different than their human counterparts (Gordon and Khanna, 2010).

The general lack of available data from canine cancer studies makes it difficult to acquire funding to perform large studies. Dog owners usually are not responsible for costs when their dog is enrolled in a clinical trial, so the difficulty in obtaining funding from trial sponsors can also be a challenge for veterinary oncology (Paoloni and Khanna, 2008).

**CANCER AS A MOLECULAR DISEASE**

*Role of genomic instability and mutation in cancer*

In 1914 a German biologist, Theodor Boveri, famously hypothesized in a manuscript that abnormal chromosome arrangements may be a driving factor in tumorigenesis (Boveri, 2008).
Boveri confirmed what the German researcher David Hansemann had observed years earlier that cancer cells tended to have more chromosomes than normal cells, and that multipolar mitoses was a likely cause (Bignold et al., 2006). Interestingly, genomic instability is now documented in the majority of solid tumors and adult-onset leukemias (Lord and Ashworth, 2012). Indeed, 100 years after Boveri’s death, cancer is now known to be a molecular disease that arises from the slow accumulation of genetic alterations that transform normal cells into a malignant state that is no longer bound by the majority of tight regulations over growth, proliferation, invasion, and survival imposed by the organism. These genetic alterations can vary in size and severity from large abnormal changes in the number and structure of chromosomes all the way down to small single nucleotide changes in genes that play roles in malignant transformation (Curtin, 2012; Rajagopalan and Lengauer, 2004).

Whereas normal human cells contain 46 chromosomes, cancer cells often contain between 60 and 90 (Rajagopalan and Lengauer, 2004). The chromosomes of cancer cells also contain structural abnormalities including inversions, deletions, duplications, and translocations. This phenomenon of numerical and structural changes seen in chromosomes is called aneuploidy. Aneuploid or chromosomally unstable cancers generally have a worse prognosis than diploid cancers, and studies have correlated the degree of aneuploidy with disease severity (Watanabe et al., 2001; Zhou et al., 2002). There is a debate to whether aneuploidy is essential for tumorigenesis, or whether it is just a by-product of de-regulated growth. The mechanisms of developing aneuploidy are also not well understood. Defects in mitotic machinery, tetraploidization events through whole genome duplication, and abnormal numbers of centrosomes have been suggested as leading to aneuploidy (Dutrillaux et al., 1991; Gisselsson et al., 2002; Lingle et al., 2002; Saunders et al., 2000). 15% of colon cancers exhibit microsatellite
instability (MIN), which is seen when simple repeat sequences in the genome suffer a high rate of mutation due to loss of mismatch repair function (Marra and Boland, 1995; Peltomaki, 2001; Yamamoto et al., 2002a). MIN-cells are generally not aneuploid in nature. A study that compared the rate of chromosome gain or loss between MIN cells and non-MIN cells reported a much higher rate in non-MIN cells, a phenomenon that was termed chromosomal instability (CIN) (Lengauer et al., 1997). It is possible CIN can lead to aneuploidy and be a driving factor in tumorigenesis through amplifying oncogenes or creating loss of heterozygosity in tumor suppressor genes. Studies are providing evidence that defects in mitotic spindle checkpoints may be a primary player in CIN (Cahill et al., 1998).

Alterations in the DNA sequence of cells are relatively common, either due to mistakes during DNA replication or exposure to DNA damaging agents such as ultraviolet light and ionizing radiation, environmental factors like cigarette smoke and industrial chemicals, as well as many chemotherapeutic drugs (Lord and Ashworth, 2012). Astonishingly, ultraviolet light alone is capable of causing 10,000 DNA lesions in a single cell per day (Hoeijmakers, 2009). Unrepaired lesions can result in permanent changes such as nucleotide substitutions, duplications, and deletions in the genetic code. If these small alterations in genes result in a mutant genotype that confers a survival advantage, then clonal expansion of the altered cells can occur. Mathematical models have suggested that six to ten of these driver mutations with their resulting clonal expansions are required for most cancers to fully mature (Knudson, 2001; Nowak et al., 2002).

Thankfully, the cell has highly sensitive mechanisms dedicated to identifying and repairing problems in the DNA, which collectively are known as the DNA damage response (DDR) (Lord and Ashworth, 2012). The different mechanisms of DDR include direct repair,
base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), non-homologous end-joining (NHEJ), homologous recombination repair (HHR) (Curtin, 2012). Direct repair generally involves repairing O\textsuperscript{6}-methylguanine lesions which can cause G:C to A:T substitutions during replication. O\textsuperscript{6}-methylguanine DNA methyltransferase (MGMT) is responsible for removing the incorrect methylation before replication (Curtin, 2012). BER is used to repair damaged bases and single-strand breaks, the most common endogenous lesions. Damaged bases are removed by glycosylases followed by endonucleases causing the single-strand break which signals the binding of poly (ADP-ribose) polymerase 1 (PARP1) and PARP2 which recruit the remaining players namely DNA polymerases and DNA ligases (Curtin, 2012). NER repairs bulkier lesions that cause distortions in the structure of the DNA helix but operates in the same fashion as BER (Cleaver et al., 2009). MMR is very important in correcting DNA replication mistakes that cause base mismatches. The newly synthesized DNA surrounding the mismatch is excised and replaced with resynthesized DNA (Jiricny, 2006). Important proteins in this process are Mut S protein homolog 2 (MSH2) and MutL Homolog 1 (MLH1) which aid in detecting the lesions and recruiting DNA polymerases. Double-strand breaks in DNA are usually dealt with through either NHEJ or HHR. HHR acts primarily in the S and G2 phases of the cell cycle. The damaged section of DNA is removed and the original DNA sequence is resynthesized with the help of a homologous sister chromatid (Moynahan and Jasin, 2010). NHEJ can occur at any time, and is simpler in that it directly ligates the two ends of the double strand break. However, the rate of mutation is much higher in NHEJ than in HHR (Lieber, 2010).

For cancer to develop, multiple gene mutation events must occur over time in a single cell. Interestingly, cancer cells have been observed to increase their rates of mutation, which
presumably serves to speed up the accumulation of mutations needed for full malignant transformation. This is often achieved through defects in components of the DDR, or by increased sensitivity to mutagenic agents. TP53 is mutated in over half of all human cancers, making it the most commonly mutated gene. Interestingly, TP53 encodes the protein p53, which as a result of its numerous duties in maintaining genomic integrity has become known as the “guardian of the genome” (Lane, 1992). DNA damage of several types can activate p53, which is capable of signaling cell cycle arrest, activating DDR, or even initiate programmed cell death (Salk et al., 2010). It is also interesting that in many early-onset hereditary cancers there are germline mutations found in genes involved in DNA repair and maintenance, as is the case with xeroderma pigmentosum patients and those with Li-Fraumeni, Bloom, and Werner syndromes (Cleaver, 2004; Ellis et al., 1995; Kamath-Loeb et al., 2007; Levine, 1997). Additionally, one of the hallmarks of cancer is to enable replicative immortality, which eliminates another preventative mechanism for genetic mutation. With each cell division, there are chances replication mistakes will be missed and incorporated into the genes that are potential drivers for cancer, commonly known as oncogenes and tumor suppressors (Salk et al., 2010).

**Oncogenes and tumor suppressors**

The work of Peyton Rous in the early 1900’s and his discovery of the tumor-causing Rous sarcoma virus in chickens led to the eventual discovery of genes capable of transforming normal cells into tumor cells. These genes were named oncogenes, and were originally thought to originate from viruses. Later research indicated that viruses incorporated normal cellular genes responsible for controlling growth and altered them for the purpose of transforming other cells after viral infection (Weinberg, 2007g). These novel discoveries led to the popular theory in the 1970’s that most cancers were caused by viral mechanisms. However, efforts to identify
viruses responsible for every known cancer were largely unsuccessful (Weinberg, 2007a). Today it is known that only 10-15% of cancers are caused by viruses worldwide. Oncogenic viruses can transform normal cell by various mechanisms, the two more prominent themes being the promotion of genomic instability or the integration of the viral genome and elevating viral oncogene expression (Chen et al., 2014b). After a new DNA transfection technique was developed in 1972 an alternate theory that cancers were caused by virus-unassociated alterations in the genome of normal cells was explored. The theory was supported by early DNA transfection experiments where DNA from 3-MC-transformed mouse cell lines was extracted and transfected into NIH 3T3 cells, causing the growth of tumorigenic foci (Weinberg, 2007a).

As more cellular oncogenes were identified, many were observed to resemble those originally found in viruses. There is a large collection of precursor oncogenes (proto-oncogenes) conserved across mammals that can be activated into oncogenes through either viral mechanisms or somatic mutation. Harvey rat sarcoma viral oncogene homolog (HRAS) was the first oncogene where the mutation of a single base was all that was different than the normal proto-oncogene. This change resulted in a frameshift in the reading frame of the gene resulting in an altered protein with abnormal function (Weinberg, 2007a). This discovery was quite significant for cancer research, and revealed one of the major mechanisms of oncogene activation. Other mechanisms include proviral insertion, gene amplification, and chromosomal translocation. Both proviral insertion and chromosomal translocation can result in the proto-oncogene being put in control of a foreign promoter which can lead to constitutive activation. Gene amplification can result by preferential replication of specific segments of chromosomal DNA, leading to increased expression of an oncogene (Weinberg, 2007a).
Oncogenes originate from genes responsible for the critical functions of cell growth, proliferation, and/or survival. Under normal circumstances, the actions of these genes are under tight regulation. The transformation of a proto-oncogene to an oncogene results in gained ability to send growth-promoting signals regardless whether the conditions are appropriate for such growth or not. Uncontrolled cell growth and division is a result of both constitutive signaling from oncogenes as well as alterations in regulatory genes responsible for keeping cell growth and division in check. The other side of the coin in this scenario is the impairment, silencing, or deletion of tumor suppressor genes. Tumor suppressor genes have various functions, but they all share a common trait: the risk of cancer development increases if their expression is lost due to various mechanisms. A frequent characteristic of tumor suppressor genes is that they have experienced a loss of heterozygosity (LOH) event in their alleles. This can happen through genetic mutation, mitotic recombination resulting in homologous chromosome with identical mutant alleles, or gene silencing through methylation of its promoter. If the remaining allele has been inactivated through mutation or methylation, then the tumor suppressor gene is lost (Weinberg, 2007f). The rate of tumor suppressor gene loss in cancer development is much higher than the rate of activation of oncogenes.

The two arguably most important tumor suppressor genes in cancer are retinoblastoma (RB1) and TP53. RB was identified in the rare childhood cancer from which it was named and helped to illuminate the mystery behind familial cancer risk. People who have inherited a germline mutation in the RB allele have a much higher risk of developing the disease, which was evident in the emergence of bilateral tumors. The most characterized role of pRb is in regulation of the cell cycle. The decision of the cell to commit to enter S-phase from G1 is dependent on the phosphorylation status of pRb. Understandably, pRb function is reported to be lost or
diminished in many cancers (Indovina et al., 2013). This can be achieved through genetic mutation, hyperphosphorylation, or interactions with viral or cellular oncogenes (Weinberg, 2007d). TP53 encodes p53, a master regulator of cell proliferation and survival. In response to cellular stress or DNA damage, p53 can promote senescence, cell cycle arrest, and even apoptosis. The p53 pathway is disrupted in the majority of human tumors, half of these due to mutations in TP53 (Weinberg, 2007c).

The combination of activated oncogenes in conjunction with the loss of tumor suppressor genes not only leads to transformation, but can also contribute to a specific phenomenon called “oncogene addiction”. The growing number of inactivated genes can make a cancer cell less adaptable and increasingly more dependent on one or very few genes for survival (Weinstein and Joe, 2008). Researchers and drug developers have taken advantage of this phenomenon by targeting those oncogenes specifically, resulting in dramatic results. The first time this was seen was with trastuzumab, an antibody that targets human epidermal growth factor receptor 2 (HER2), a receptor tyrosine kinase in breast cancer patients. Other examples include imatinib which has shown to be very effective in chronic myeloid leukemia patients with the BCR-ABL oncogene and also gastrointestinal stromal tumors that express the oncogene KIT (Weinstein and Joe, 2008). More recently some exciting advancements in melanoma have come from the development of specific inhibitors of oncogenic V-Raf murine sarcoma viral oncogene homolog B (BRAF), which is reported to be present in 50-60% of human melanomas. Although most successful responses to these types of drugs usually end eventually in tumor relapse, it is a great example of how our increased knowledge of the molecular underpinnings of cancer has resulted in progress in the form of improved survival times. Oncogenes and tumor suppressor genes both participate in cell signaling pathways in order to exert their tumor-driving or tumor-preventing
effects. The study of molecular pathways that are essential for cancer development is critical for our growing understanding of the disease which will hopefully translate into improved therapeutic strategies.

**Signaling pathways important for cancer**

Cancer development involves many cellular processes. For a cell to transform, it must acquire several genetic mutations to deregulate growth and proliferation. It must also promote survival by inhibiting the natural defense systems of cell cycle checkpoints, the induction of senescence, and programmed cell death. Also, as cancer further develops towards malignancy, it must gain ability to migrate and invade into surrounding tissues, induce angiogenesis to facilitate increased nutrients for the growing tumor as well as provide routes for further expansion via metastasis to distant sites. All of these processes are regulated by signaling pathways in the cell, and many components of these pathways are proteins found to be activated or inactivated in cancers. Commonly activated proteins involve kinases of different kinds including receptor tyrosine kinases, cytoplasmic tyrosine kinases, lipid kinsases, small GTPases, nuclear receptors, transcription factors, chromatin remodelers, cell cycle effectors, and components of developmental pathways (Sever and Brugge, 2015). Negative regulators in signaling pathways such as tumor suppressor genes are commonly inactivated or lost.

There are several important signaling pathways in cancer. Developmental pathways such as Wnt, Notch, and Hedgehog signaling are important for regulating cell fate and differentiation, as well as proliferation and migration (Muller *et al.*, 2007). Transforming growth factor beta (TGF-β) signaling is important for pathogenesis of most carcinomas, and can increase invasiveness of more advanced tumors (Weinberg, 2007b). Janus kinase-Signal Transducer and Activator or Transcription (Jak-STAT) signaling has important roles in cell growth, proliferation,
and survival. Nuclear factor kappa B (NFκB) signaling is important for inhibiting apoptosis and promoting survival. Rhodopsin (Rho) signaling is instrumental in cytoskeleton remodeling and cell attachments, allowing cells to migrate (Weinberg, 2007b).

Two pathways that are very important in many cancers due to their effects in most of the processes described above are the Phosphoinositide 3-kinase/v-AKT murine Thymoma Viral Oncogene (PI3K/AKT) and RAF/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (RAF/MEK/ERK, or MAPK) pathways. They influence several processes including promoting genomic instability, cell proliferation, survival, and angiogenesis (Sever and Brugge, 2015). The PI3K/AKT pathway can inhibit the DNA damage response and promote survival of cells with damaged DNA. AKT inhibits DNA repair via homologous recombination through phosphorylation of checkpoint proteins Checkpoint, S. pombe, homolog of, 1 (CHK1) and DNA topoisomerase 2-binding protein 1 (TOPBP1). AKT can also prevent Breast Cancer 1, Early Onset (BRCA1) from associating with sites of DNA damage. This contributes to genomic instability (XU 2012a). The MAPK pathway also inhibits apoptosis which can lead to survival of DNA damaged cells, and a study has shown that hyperactivation of MAPK signaling leads to genomic instability (Saavedra et al., 1999).

Proliferation can be regulated by the PI3K/AKT pathway in multiple ways. AKT can inhibit the Tuberous Sclerosis 1 (TSC1-TSC2) complex, allowing GTP-bound Ras homolog enriched in brain (Rheb) to activate Mammalian target of rapamycin complex 1 (mTORC1) which promotes protein synthesis needed during cell cycle progression (Richardson et al., 2004). AKT also directly inhibits cell cycle inhibitors p27 and p21, and indirectly blocks transcription of cell cycle inhibitors p27 and Retinoblastoma-like protein 2 (RBL2) (Burgering and Medema, 2003; Rossig et al., 2001). Additionally, it can cause p53 degradation through phosphorylation
of the ubiquitin ligase MDM2 oncogene, E3 ubiquitin protein ligase (MDM2), removing an important cell cycle regulator (Xu et al., 2012). A downstream target of MAPK signaling is the stabilization of V-Myc avian myelocytomatosis viral oncogene homolog (Myc) through ERK phosphorylation. The oncogene Myc is a transcription factor that can induce several genes that promote cell proliferation including G1/S cyclins, cyclin-dependent kinases, and E2F-family transcription factors (Duronio and Xiong, 2013). ERK phosphorylation of ELK1, member of ETS oncogene family (ELK1) leads to the induction of the oncogene FBJ murine osteosarcoma viral oncogene homolog (FOS), which leads to production of the transcription factor Activating protein 1 (AP1), involved in the regulation of several pro-proliferation genes (Murphy et al., 2002).

Evading the cell’s mechanisms for programmed death is an essential characteristic of cancer. The PI3K/AKT pathway regulates apoptosis by blocking the induction of death ligands Fas Ligand (FasL) and Tumor necrosis factor (ligand) superfamily, member 10 (TRAIL) through phosphorylation of Forkhead box protein O3 A (FoxO3A). AKT activates X-linked inhibitor of apoptosis (XIAP), an apoptosis inhibitor, as wells as NF-κB which regulates antiapoptotic proteins such as B-Cell CLL/Lymphoma 2 (BCL2), BCLxl, and MC1L (Cagnol and Chambard, 2010; Shen and Tergaonkar, 2009; Zhang et al., 2011). Both AKT and ribosomal s6 kinase (RSK), a kinase regulated by ERK, inhibit the proapoptotic Bcl2-family member BCL2 antagonist of cell death (Bad). ERK has also been shown to target BCL2-like 11 (apoptosis facilitator) (Bim), another proapoptotic protein, and Nuclear factor of kappa light polypeptide gene enhancer (IκBa), an NF-κB inhibitor, for degradation, showing the MAPK pathway plays a role in survival as well (Ghoda et al., 1997).
Angiogenesis, the formation of new blood vessels, is needed as tumors grow to the point that existing vessels can no longer provide the tumor core with oxygen and nutrients. Signalling pathways usually used for wound healing are used by cancer to facilitate this angiogenesis, which involve the proteins vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), interleukin 8, and angiopoietin (Sever and Brugge, 2015). Hypoxia-inducible factor 1 (HIF1) production in cancer cells is increased through PI3K/AKT signaling, which leads to the synthesis of VEGF. PI3K/AKT signaling is also involved in the production of nitric oxide, important factors for angiogenesis (Ward and Thompson, 2012). Thrombospondin 1 (Tsp1) has an inhibitory role in angiogenesis, which cancers must overcome. MAPK signaling through Ras and Myc is known to repress the expression of Tsp1, leading to deregulation of the angiogenesis process (Green, 2014). The PI3K/AKT and MAPK pathways have also been shown to regulate other cancer processes including cell metabolism, cell polarity and migration, and cell differentiation. Due to the several cancer processes that these 2 pathways regulate, it is not surprising that many researchers have focused here on identifying molecular targets for drug development.

*Molecularly targeted agents for cancer therapy*

Signaling pathways represent an attractive source of potential therapeutic targets, and as our knowledge of molecular biology of cancer has increased in recent years much focus has been on developing and optimizing targeted therapy as an alternative to traditional cytotoxic agents. In contrast to blindly screening compounds for anti-cancer activity and determining their mechanism of action afterwards, researchers are identifying targets that make sense biologically based on what is known about oncogenes and over-expressed pathways and then screening specifically for compounds with activity against those targets. Good targets have molecular
properties that are amenable for agents such as monoclonal antibodies or low-molecular weight drugs (Weinberg, 2007e). For example, monoclonal antibodies work well with proteins that are either on the surface of the cell or in the extracellular space. Low-molecular weight drugs work well with enzymes that have accessible catalytic clefts. Additionally, a target is attractive if its inhibition leads to a block in proliferation, an induction of apoptosis, or a sensitization to another therapy (Weinberg, 2007e).

Success has been found most often with targeting kinases, early examples being the monoclonal antibody trastuzumab for HER2-driven breast cancers, and the small molecule inhibitors gefitinib and erlotinib which both target EGFR-driven tumors such as non-small lung cancer and prostate cancer (Rask-Andersen et al., 2014). The most famous example is probably the 2003 FDA-approved of the small molecule kinase inhibitor imatinib for the Bcr-Abl-driven chronic myeloid leukemia (Fausel, 2007). A long term study has recently reported that 82% of patients treated with imatinib are still alive without disease progression after 10 years (Kalmanti et al., 2015). This unprecedented success led to a massive effort that continues today of identifying other kinase targets that are drivers for other cancers. The 2011 FDA approval of the mutant BRAFV600E kinase inhibitor vemurafenib has revolutionized treatment for 50-60% of melanoma patients (Kim et al., 2014a). Presently, 39 kinase inhibitors are FDA-approved for various diseases, the majority being cancers (Rask-Andersen et al., 2014).

Other pathways where non-kinase targets have been investigated involve DNA repair, apoptosis, angiogenesis, as well as others. PARP inhibitors were first identified in synthetic lethal screens of BRCA1/2 mutant ovarian and breast cancer cells (Bertwistle and Ashworth, 1999). PARP is recruited for the repair of damaged DNA, but is also involved in cell proliferation, differentiation, and transformation (de Murcia et al., 1991). P53 which regulates
cell cycle arrest and apoptosis is targeted for degradation by the ubiquitin ligase activities of MDM2/X expression is elevated in most cancers, making them an attractive target (Momand et al., 1992; Wade et al., 2013). MDM2/X inhibitors serve to reactivate p53, which have the potential to be combined with non-targeted DNA damaging agents as well as targeted therapy that promotes apoptosis (Wade and Wahl, 2009; Zhang et al., 2010). Growth factors can be potential targets as is the case with the monoclonal antibody bevacizumab that binds to VEGF-A, important in angiogenesis signaling (Cook and Figg, 2010). Many oncogenes and tumor suppressor genes encode transcription factors, making them historically difficult to target. In particular, members of the NFκB, AP-1, and STAT transcription factor families are involved in cancer processes and are potential targets (Libermann and Zerbini, 2006). Myc, a major oncogene whose role as a transcription factor influences several cancer pathways, is a target that has been investigated for drug development, but there are challenges. Myc activation is predominantly caused by over-expression or de-regulation, not by activating mutations, and it has no intrinsic enzymatic activity (Fletcher and Prochownik, 2014). Strategies for Myc inhibition have involved attempts to target protein-protein interactions, or synthetic lethal inhibitors, which have not produced effective results (Fletcher and Prochownik, 2014).

Challenges that face targeted therapy including kinase inhibitors involve acquired or intrinsic resistance. As cancers advance they become increasingly complex in their repertoire of genetic alterations, making resistance more likely. Even in the most successful targeted therapies, relapse is common or inevitable. Mechanisms of resistance typically fall into 3 main categories: on-target mutations, bypass signaling, or phenotypic transformation to a different histology or morphology (Pazarentzos and Bivona, 2015). Research in overcoming resistance to targeted agents has increased recently. Resistance to BRAFV600E-specific inhibitors in human
melanoma is being combated by investigating drug combination strategies that address MAPK pathway reactivation via multiple mechanisms (Chapman, 2013). As we increase our molecular knowledge in the context of therapy resistance it will hopefully result in improved strategies to treat cancer with molecularly targeted therapies. Translational models to study such pathway interactions with targeted agents would be invaluable for this work to continue to move forward.

COMPARATIVE ONCOLOGY OF MELANOMA

Epidemiology of human and canine melanoma

Melanoma is an aggressive cancer that is known to be resistant to therapy in both human and dogs. In the United States there were approximately 76,000 new cases and 9700 expected deaths in people in 2014 (Siegel et al., 2014). Melanoma accounts for 4-7% of all canine cancer, 9-20% of skin cancers and is the most common oral tumor in dogs (Aronsohn MG, 1990; Marino et al., 1995; Moulton, 1990; Theon et al., 1997). Malignant melanoma is responsible for 75% of all skin cancer deaths in humans.

Certain factors increase human melanoma risk. Family history, fair skin, having multiple moles, immunosupression, and UVR exposure have been associated with developing the disease. The strongest risk factors for human melanoma include intense intermittent ultraviolet radiation (UVR) exposure and having severe sunburns as a child (Whiteman et al., 2001). UVR exposure in the skin stimulates melanin production, generates reactive oxygen species (ROS), and can form thymine dimers which if not repaired can be mutagenic. The effects of ROS in the melanocyte include DNA strand breaks, chromosomal damage, and enzyme deactivation which lead to cell death and/or transformation (Breimer, 1990). Melanocytes that have sustained ROS-
mediated damage can attempt to repair the damaged DNA or can undergo apoptosis through the assistance of p53. If the melanocytes have genetic alterations that prevent them from repairing the DNA damage, then cell division occurs in the place of apoptosis, which can lead to increases in genetic mutation (Tornaletti and Pfeifer, 1994).

In dogs, risk factors are not well established, but it is unlikely that UVR plays a causative role due to the fact that most breeds are protected by their coat of hair (Bergman, 2013). It is generally seen more in older dogs, and there are certain breeds that appear to be associated with higher risks of developing oral melanoma, namely Scottish Terriers, Golden Retrievers, Poodles, and Dachshunds (Bergman, 2013; Goldschmidt, 1985). The biologic behavior of canine oral melanomas is determined generally by a combination of factors such as the size and stage of the tumor, the location on the body, and other histological parameters (Bergman, 2007).

**Comparative biology of melanoma**

In both humans and dogs melanomas arise from melanocytes, pigment-producing cells in the body that are responsible for pigmentation as well as protection from UVR. Melanocytes are found in multiple sites of the body including skin, the eye, mucosal and acral sites, and the meninges (Bergman, 2007; Lo and Fisher, 2014). Human malignant melanoma is found most commonly on the skin, although the less common mucosal and acral sites are known to be more aggressive and prone to metastasis. In contrast, more than 80% of canine melanocytic neoplasms of the skin are benign, presumably because of the role of the haired skin in UVR protection (Goldschmidt, 2002; Green et al., 2006). Malignant melanoma is more commonly found in the oral cavity and digits of the dog (Aronsohn, 1990; Marino et al., 1995; Moulton, 1990; Theon et al., 1997), and like the human cancer is also locally invasive and highly metastatic.
The World Health Organization (WHO) has determined the staging system for canine oral melanoma is based on tumor size and metastasis. A tumor with a diameter less than or equal to 2 cm with no nodal involvement or metastasis is considered stage I. Tumors with diameters between 2 and 4 cm and no nodal involvement or metastasis are considered stage II, and tumors greater than 4 cm with no nodal involvement or metastasis are considered stage III. Tumors between 2 and 4 cm but with nodal involvement are also considered stage III. If metastasis is present than the tumor is classified as stage IV (Bergman, 2013). In a 2014 retrospective study involving 70 cases the median survival time for canine oral melanoma with stage I is 28 months. Stage II patients have median survival times of 26 months, and stage III patients only 6 months (Tuohy et al., 2014). Since tumor size is not scaled based on body size and other histological factors are ignored in this system many veterinary oncologists are actively searching for better prognostic factors to form an alternate staging system. Some negative factors discovered so far include incomplete surgical margins, a tumor mitotic index higher than 3, and the presence of bone invasion and lysis (Bergman, 2007).

In contrast, the American Joint Committee on Cancer (AJCC) TNM system for staging human melanoma is more complicated and dependent on additional factors such as tumor thickness, mitotic rate, whether the tumor is ulcerated or not and whether the tumor has spread to lymph nodes and other parts of the body (National Cancer Institute, 2015). The 5 year survival rate of people with stage I and II melanoma ranges from 53-97%. Stage III melanoma patients have 5 year survival rates from 40-78%. Unfortunately, only 15-20% of stage IV melanoma patients will survive 5 years (American Cancer Society, 2015). It is clear that prognosis is dismal for advanced stages of the disease for both species.
Comparative genetics and molecular biology of melanoma

In this new genomics era much research has been dedicated to the discovery of novel cancer-driving mutations to increase knowledge and to identify molecular targets for therapy. The RAS family of genes which are involved in signal transduction of mitogenic signals from the plasma membrane to the cytoplasm and nucleus are commonly mutated in many cancers including human melanoma. The most common mutated RAS gene in human melanoma is neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) at codon 61 (Bos, 1988). 15-20% of human melanomas are reported to have mutations in NRAS (Albino et al., 1984; Davies et al., 2002). Other early discoveries came from genome-wide association studies of familial melanoma that identified high risk genes cyclin-dependent kinase inhibitor 2A (CDKN2A), melanocortin 1 receptor (MC1R) and tyrosinase (Bishop et al., 2009; Hussussian et al., 1994). CDKN2A encodes p14ARF and p164a, tumor suppressors that are involved in regulating pathways driven by p53 and pRb (Lo and Fisher, 2014).

The roles of CDKN1A and CDKN2A, p16, Phosphatase and tensin homolog (PTEN), pRb and p53 have also been investigated in canine melanoma. In a study of 7 canine melanoma cell lines and 27 tumors, a large reduction in expression of p16 and/or PTEN was observed (Koenig et al., 2002). Although TP53 mutations were not found in canine melanoma, p53 along with pRb was observed to be excluded from the nuclear compartment (Koenig et al., 2002). These reports suggest that similar to human melanoma an inactivation of the p16/pRb pathway is commonly seen in canine melanoma.

A major breakthrough in melanoma genomics occurred in 2002, when a genome-wide screen resulted in a report that BRAF mutations are commonly found in human cancers, with the highest frequency observed in melanoma (Davies et al., 2002). BRAF is a serine/threonine
kinase that lies immediately downstream of NRAS in the MAPK signaling pathway. Other studies have documented BRAF mutations in approximately 50-60% of melanomas, most of them a somatic point mutation which substitutes a glutamic acid for a valine at the 600 amino acid position (V600E) (Ascierto et al., 2012). This discovery has revealed a major target for cancer therapy and has led to the development of specific inhibitors to BRAF V600E which are now FDA-approved for melanoma treatment (Ballantyne and Garnock-Jones, 2013; Kim et al., 2014a). Despite the significant improvement in patient response to these new agents, relapse is inevitable in almost all of these patients, and 20% of the mutant BRAF melanoma patients have an intrinsic resistance to these new inhibitors (Girotti et al., 2015). An emerging area of research is dedicated to understanding and combating the development of resistance. Multiple methods of drug resistance have been identified, many of them leading to reactivation of the MAPK pathway. Some of these include acquiring secondary NRAS mutations, amplification of receptor tyrosine kinase signaling, amplification of mutant BRAF, upregulation of the MEK kinase COT, or the development of MEK mutations (Chapman, 2013; Girotti et al., 2015; Johannessen et al., 2010; Nazarian et al., 2010; Straussman et al., 2012; Vergani et al., 2011; Villanueva et al., 2010; Wilson et al., 2012).

Currently a small number of studies have shown that the common NRAS and BRAF mutations seen in human melanoma are rare or absent in canine melanomas. In particular, in a study of 16 dogs with melanoma 2 were found to have the common NRAS Q61R mutation (Mayr et al., 2003). In another study of 17 dogs no BRAF V600E mutations were found. However, in those same dogs lacking BRAF mutations constitutive activation of ERK1/2, a kinase downstream of BRAF in the MAPK pathway, was observed (Shelly et al., 2005). Expression of phosphorylated ERK1/2 and AKT was observed in 77 and 52% of canine oral
melanoma rumor samples in another study, as well as weak or absent PTEN expression, which is often seen in human melanoma (Bogenrieder, 2010; Davies et al., 2009; Simpson et al., 2014; Zhou et al., 2000). These reports suggest that although the specific common mutations may not be common between human and canine melanoma, the same signaling pathways important to melanoma appear similarly altered.

*Treatment of human and canine melanoma*

In recent years many advances in human melanoma research have resulted in multiple new therapies receiving FDA approval. However, surgical tumor resection still remains the only curative treatment strategy if the disease is caught early (Lee et al., 2013). For the majority of the last 200 years advancements in melanoma was primarily based on developing better surgical techniques and tumor staging protocols. Starting in the 1950’s and 60’s with the observations of high rates of spontaneous tumor regressions in melanoma, researchers began investigating immunolotherapeutic strategies to treat the disease (Baker, 1964). During the 1970’s new developments in melanoma therapy included a better tumor classification system and the beginnings of lymphoscintigraphy, a technique of locating lymph nodes for resection. Chemotherapy was introduced at this time but was found to not be very effective (Lee et al., 2013). However, in 1975 the FDA approved dacarbazine as the first drug for systemic therapy of metastatic melanoma, an alkylating agent that binds to DNA and causes cell death. Sadly, it has been shown to produce responses in only 30% of patients, and is currently the only chemotherapeutic to ever receive approval for melanoma treatment, despite multiple attempts to improve outcome with newer chemotherapeutics and combination strategies (Lee et al., 2013).

Advancements in immunotherapy led to the FDA approval in 1996 of interferon alpha 2 beta (IFNα2b), an antiviral drug that later has been recognized to play a role in activating the
immune system, and in 1998, high-dose interleukin 2 (IL-2), cytokines that stimulate the expansion of T cells. In a randomized controlled study IFNα2b showed significant benefit in relapse-free and overall survival in metastatic melanoma patients (Brassard et al., 2002; Kirkwood et al., 1996).

Before 2011, the only approved agents for human melanoma therapy were dacarbazine, IFNα2b, and IL-2. Within the last 4 years advancements in melanoma targeted therapy and immunotherapy have exploded leading to the approval of 8 new therapies. The 3 new drugs in immunotherapy are involved in immune checkpoint inhibition. Ipilimumab is a humanized monoclonal antibody that blocks cytotoxic T lymphocyte antigen-4 (CTLA-4), a T cell receptor which inhibits T cell activation. It was shown to prolong survival in advanced melanoma and was approved in 2011 (Hodi et al., 2010). Pembrolizumab and nivolumab are monoclonal antibodies that target programmed cell death-1 (PD-1), another inhibitory T cell receptor, and have been associated with higher response rates and less toxicities than ipilimumab in clinical studies (Hamid et al., 2013; Topalian et al., 2012). A pegylated form of IFNα2b that is able to stay in the bloodstream longer and can be given at a lower dose was also approved in 2011.

The remaining 4 recently approved therapies for melanoma, vemurafenib, dabrafenib, trametinib, and dabrafenib/trametinib combination, have been developed in response to the discovery of MAPK pathway addiction via the mutant BRAFV600E observed in over half of melanoma tumors. Vemurafenib is a selective ATP-competitive inhibitor of the V600E mutant form of BRAF. In a phase I trial it was shown to be well tolerated and 81% of patients with the V600E mutation responded (Flaherty et al., 2010). Vemurafenib performed well in a Phase II trial of 132 BRAF- mutant melanoma patients, of which 53% responded, with a median duration of response of 6.7 months (Sosman et al., 2012). These encouraging results were repeated in a
Phase III trial where 675 patients were randomized into 2 arms to receive either vemurafenib or dacarbazine. 84% of patients receiving vemurafenib survived 6 months compared to 64% of patients receiving dacarbazine. The vemurafenib cohort had response rates of 48% compared to the 5% response seen in the dacarbazine cohort. The overall survival time of patients receiving vemurafenib was 13.6 months compared to 9.7 months with dacarbazine (Chapman et al., 2011). These impressive results led to the FDA approval of vemurafenib in 2011 (fda.gov).

Dabrafenib is also a selective BRAF V600E inhibitor similar to vemurafenib, but it is thought to have higher selectivity, lower toxicity, and has been shown to have antitumor effects intracranially, an important characteristic for the potential treatment of melanoma metastases in the brain (Long et al., 2012; Martin-Liberal and Larkin, 2014). In a Phase I trial at the determined recommended dose of 150 mg twice daily 50% of patients responded (Falchook et al., 2012). In a Phase II trial with V600E and V600K BRAF mutated melanoma patients, 59% of BRAF V600E patients responded to dabrafenib, 7% of them a complete response (Ascierto et al., 2013). The Phase III trial comparing dabrafenib to dacarbazine showed similar results as with vemurafenib. Specifically, 50% of the dabrafenib cohort responded, compared to the 7% response seen in the dacarbazine cohort. The median progression free survival time in the dabrafenib cohort was significantly better at 5.1 months compared to 2.7 months for dacarbazine (Hauschild et al., 2012).

Trametinib is an inhibitor of MEK1 and MEK2, kinases downstream of the RAF kinases in the MAPK pathway. In the past MEK inhibitors have been studied for melanoma therapy, but they failed to impress in clinical trials. A possible problem with these earlier studies was that the mutational status of BRAF or NRAS was not taken into account (Bennouna et al., 2011; Bodoky et al., 2012; Hainsworth et al., 2010; Hayes et al., 2012; Kirkwood et al., 2012; O'Neil et al.,
Constructing trials for MEK inhibitors by selecting patients with RAS or RAF mutations has improved results and has ultimately led to the first MEK inhibitor trametinib to be approved by the FDA for advanced BRAF-mutant melanoma (Martin-Liberal and Larkin, 2014; Wright and McCormack, 2013). In a Phase III trial 22% of patients responded to trametinib compared to the 8% that responded to dacarbazine, and the median progression free survival time was 4.8 months vs. 1.5 months, respectively (Flaherty et al., 2012b).

The latest targeted therapy to receive FDA approval is the combination of the BRAF-mutant inhibitor dabrafenib with the MEK inhibitor trametinib (Bodoky et al., 2012; Hainsworth et al., 2010; Hayes et al., 2012; Kirkwood et al., 2012; O'Neil et al., 2011; U.S. Food and Drug Administration, 2014). A Phase I/II trial determined that both drugs could be used at full dose and be well tolerated. It was shown that dabrafenib plus trametinib treatment achieved improved responses compared to dabrafenib alone, with response rates of 76% and 54%, respectively. The median progression free survival time of the 2 groups was 9.4 and 5.8 months (Flaherty et al., 2012a).

Although these advances in targeted therapy for human melanoma are exciting, it is important to note that relapse is inevitable in virtually all of these patients who responded to RAF and MEK inhibition. Investigating mechanisms behind this acquired drug resistance has been a hot area of research, and the general consensus is that the majority of these mechanisms involve reactivation of the MAPK pathway (Chapman, 2013). Combination strategies that address problems with secondary mutations, pathway redundancy and up-regulation of upstream signals are needed in the future as the new wave of advancements for melanoma.

Unfortunately for canine melanoma patients, advancements in therapy have not been as fruitful in recent years. Typical treatment for canine melanoma involves local tumor control
through surgery and/or radiation therapy followed by systemic therapy (Gonzalez-Garcia *et al.*, 2005; Liptak, 2012; Liptak, 2013; Tanaka *et al.*, 2004). Cutaneous melanomas generally require only a lumpectomy, whereas for melanomas in other sites wider excisions are needed (Bergman, 2007). The mean survival time for dogs treated solely with surgical resection range from 150 to 318 days with only 35% surviving longer than a year (Bergman, 2013). However, a recent study of canine oral melanoma reported a median survival time of 874 days for patients treated with surgery alone, compared to 396 days for surgery plus adjuvant chemotherapy (Tuohy *et al.*, 2014).

Radiation therapy can be implemented when the melanoma is not resectable by surgery, the surgery results in incomplete margins, or when local metastasis to neighboring lymph nodes has occurred (Bergman, 2007). The overall response rates to radiation therapy have been reported between 82 and 94% with a mean survival time of 5.3 to 11.9 months (Bateman *et al.*, 1994; Freeman *et al.*, 2003; Murphy *et al.*, 2005; Proulx *et al.*, 2003). The reported rates of cancer recurrence after radiation are variable, but one study reported 26% of dogs with microscopic disease had local recurrence (Proulx *et al.*, 2003).

Adjuvant therapy after local tumor control with surgery and/or radiation has usually involved chemotherapy such as carboplatin or cisplatin (Murphy *et al.*, 2005; Proulx *et al.*, 2003; Tanaka *et al.*, 2004). Generally this strategy has yielded unsatisfactory results, although a recent 2014 study reported that 17 canine oral melanoma patients administered carboplatin in the post-surgical adjuvant setting had an overall progression free survival time of 259 days and overall survival time of 440 days (Dank *et al.*, 2014). In another recent retrospective study of 63 dogs between 2001 and 2011, however, it was reported that the addition of carboplatin did not significantly increase survival times compared to surgery alone (Brockley *et al.*, 2013).
Immunotherapy in the adjuvant setting has been an area of much research in canine melanoma. Multiple strategies have been investigated including the use of autologous and allogeneic tumor cell vaccines, liposomal-encapsulated nonspecific immunostimulators, intralesional FAS ligand DNA, bacterial superantigens, and canine dendritic cell vaccines (Alexander et al., 2006; Bianco et al., 2003; Dow et al., 1998; Gyorffy et al., 2005; Helfand et al., 1994; Hogge et al., 1999; MacEwen et al., 1999; MacEwen et al., 1986). Some of these strategies have shown some antitumor responses, but they are associated with many disadvantages such as lack of reproducibility, high expense, and dependency on establishing cell lines from tumor samples.

A new approach to tumor vaccines in recent years has been to use DNA vaccines with plasmids containing clones of human tyrosinase antigen, a melanomal glycoprotein found to be overexpressed in melanomas across species, which is homologous to the canine protein. This xenogeneic DNA vaccine (huTyr) can stimulate the immune system in dogs against tyrosinase antigen, which should simultaneously attack the tumor because of the antigen homology between species (Bergman et al., 2006; Guevara-Patino et al., 2003). In a study of 9 dogs with stage II, III, and IV melanoma, 1 dog had a complete response to the vaccine, and 4 other dogs survived between 421 and 588+ days (Bergman et al., 2003). In a larger multi-center clinical trial comparing 58 dogs receiving the huTyr vaccine to 53 historical controls they observed the vaccinated dogs survived significantly longer and tolerated the treatment well (Grosenbaugh et al., 2011). These results led to the USDA licensing the huTyr vaccine (Oncept) for the treatment of canine melanoma in 2010 ("USDA licenses DNA", 2010). However, a recent restrospective analysis of medical records of 45 dogs with melanoma revealed that patients receiving the Oncept melanoma vaccine did not have improved progression free survival, disease-free
survival, or median survival time compared to dogs that did not receive the vaccine (Ottnod et al., 2013). Furthermore, in another recent study of 151 dogs it was concluded that systemic adjuvant therapy of any kind (chemotherapy, radiation therapy, or vaccine therapy) did not confer any significant survival advantage compared to surgery alone (Boston et al., 2014). Taken together, despite the advances in immunotherapy that have been achieved in canine melanoma, surgery remains the cornerstone for treatment, which is not curative for patients with metastatic disease. New approaches and advancements are sorely needed for this deadly cancer.

Molecular targeted agents which have had recent success in human melanoma may be the next chapter in the evolution of therapy for melanoma in dogs. Studies have already begun for search of potential molecular targets for drug development, but many more are needed. The handful of published studies to date have identified possible targets in receptor tyrosine kinases such as insulin-like growth factor 1 receptor (IGF1-R), c-Kit, PDGFR, and FGFR, and the proteosome (Ito et al., 2013; Marech et al., 2014; Thamm et al., 2010; Thamm et al., 2012). The miRNA miR-203 has been identified to be a tumor-suppressive miRNA that has inhibited growth of both human and canine melanoma cells (Noguchi et al., 2014). As discussed earlier, although activating mutations in the molecular targets NRAS and BRAF that are common in human melanoma have been shown to be rare in dogs, evidence of PI3K/AKT and MAPK pathway activation in canine melanoma suggests that inhibitors to these specific pathways may induce a response in canine melanoma and further investigation is warranted.
COMPARATIVE ONCOLOGY OF OSTEOSARCOMA

Epidemiology of human and canine osteosarcoma

Osteosarcoma (OSA) is the most common primary bone tumor in humans, although its incidence is rare at 600-1000 cases per year, or 1-3 cases per million (Fenger et al., 2014; Morello et al., 2011; World Health Organization, 2013). It occurs primarily in children and adolescents with the peak onset from 10-14 years, but there is a second peak later on after 50 years of age (Fenger et al., 2014; World Health Organization, 2013). Human OSA occurs mainly in the long bones of the appendicular skeleton near the metaphyseal growth plate. The most common sites are the femur, tibia, and humerus, but OSA can also occur less frequently in the skull, jaw, and pelvis (World Health Organization, 2013). Over the last 15 years no improvements in survival have been made towards this aggressive and highly metastatic cancer, which causes death in 30-40% of affected children (Fenger et al., 2014).

Like in humans, OSA is the most common primary tumor of the bone in dogs, comprising 85-98% of all canine bone tumors (Dernell, 2007; Liptak, 2004). There is an early incident peak at 18-24 months, but unlike human OSA the majority of dogs with this disease are between 7 and 9 years of age (Dernell, 2007). Canine OSA also occurs mainly in the long bones near the metaphyseal regions, with incidences in the fore limbs twice that of the hind limbs. The distal radius and proximal humerus are the most common sites in dogs, followed by distal femur, proximal and distal tibia (Dernell, 2007; Straw et al., 1990). Similar to humans, the lack of recent success in improving outcome means that over 90% of dogs with OSA eventually succumb to disease (Fenger et al., 2014).
There have been multiple risk factors identified for human OSA. Rapid growth and turnover of bone during puberty is associated with risk of OSA development, as the incidence peak occurs a little earlier for girls than boys, coinciding with the different time of puberty onset between girls and boys (Pritchard-Jones et al., 2006). OSA has also been associated with height as children with taller statures appear to have an elevated risk (Cotterill et al., 2004). Environmental factor such as exposure to ionizing radiation has been shown to cause OSA in 3% of people, sometimes taking as long as 30 years to become evident (Logue and Cairnduff, 1991).

Certain bone diseases as well as heritable syndromes have been associated with risk of developing OSA. Paget’s disease, a bone disease that can affect people 50 years or older and cause bone remodeling with weakened tissue, has been reported to cause OSA in 1% of affected patients (Seton, 2013; Smith et al., 1984). 12% of patients with Li-Fraumeni syndrome, an autosomal dominant disorder caused by germline mutations in TP53, have been reported to develop OSA (Gokgoz et al., 2001; Srivastava et al., 1993). Similarly, germline mutations in the RB tumor suppressor gene has been associated with increasing risk of OSA development several hundred-fold (Hansen et al., 1985). There are several autosomal recessive syndromes related to mutations in the RecQ DNA helicase family that increase the risk of OSA incidence. OSA development occurs in 32, 10, 3, and 13% of Rothmund-Thomson, Werner’s, Bloms, and RAPADILINO syndrome patients have been reported to develop OSA, respectively (German, 1997; Rosen et al., 1970; Siitonen et al., 2003; Wang et al., 2003; Wang et al., 2001).

Similar risk factors have also been associated with increased risk of OSA in dogs. The disease is mostly seen in larger breeds, as only 5% of OSA occurs in dogs that weigh less than 15 kg (Dernell, 2007). Specific breeds with known predispositions for the disease include Greyhounds, Rottweiler, Great Danes, Saint Bernards, Doberman Pinschers, Irish Setters,
Golden Retrievers, and German Shepherds (Rosenberger et al., 2007; Ru et al., 1998). OSA incidence has been reported to be inherited in a familial pattern in Saint Bernards, Rottweilers, and Scottish Deerhounds (Misdorp, 1980; Phillips et al., 2007).

Similar to human OSA, exposure to ionizing radiation has been shown to cause OSA in dogs. Experimental exposure studies in Beagles caused the development of OSA in 3 separate studies (Lloyd et al., 1993; Lloyd et al., 1994; Muggenburg et al., 1996). Radiation therapy for the treatment of canine cancer has also been reported to result in the development of a secondary OSA in small group of dogs (Dickinson et al., 2001; Gillette et al., 1990; Powers et al., 1989).

Neutered dogs have a higher risk of developing OSA than intact dogs, suggesting the role of sex hormones in the disease. A large study of 683 Rottweilers who had undergone a gonadectomy before turning 1 years old concluded that a lifetime exposure to gonadal hormones reduced the risk of developing OSA (Cooley et al., 2002).

OSA development has been documented in cases where dogs had previously received metallic implants during orthopedic procedures, suggesting an association of risk (Boudrieau et al., 2005; Harasen and Simko, 2008; Murphy et al., 1997; Sinibaldi et al., 1982). It has been hypothesized that implant complications such as infection, instability and corrosion may be causative factors (Stevenson, 1991). However, the fact that these reports are few compared to the large number of implant procedures occurring in dogs and there is an absence of studies confirming the role of metallic implants in sarcoma development offers the counter viewpoint that OSA occurring at the same sites of implants may be merely coincidental (Murphy et al., 1997). Bone diseases such as bone infarcts and osteochondritis dissecans have also been associated with OSA in a few reports, suggesting a possible role (Dubielzig et al., 1981; Holmberg et al., 2004; Marcellin-Little et al., 1999).
Although the hereditary genetic disorders linked to human OSA have not been identified in dogs, there is evidence of inherited risk of OSA development associated with specific breeds. Scottish deerhounds, Rottweilers, greyhounds, Great Danes, Saint Bernards, and Irish wolfhounds are at higher risk for OSA than other breeds. The narrow genetic diversity found within breeds is advantageous for possibly identifying hereditary factors in disease. For example, Rottweilers have a much higher frequency of germline mutations in Met Proto-Oncogene (MET), a receptor tyrosine kinase known to be dysregulated in both human and canine OSA, at 70%, compared to less than 5% for other breeds (Liao et al., 2006; MacEwen et al., 2003; Patane et al., 2006). A recent genome wide association study (GWAS) in Greyhounds, Rottweilers, and Irish wolfhounds identified 33 inherited risk loci for OSA that explained between 55 and 85% of the phenotypic variance in each breed. The strongest associated loci in Greyhounds lies upstream of CDNK2A/B genes, which encode cyclin-dependent kinase inhibitors p16\(^{INK4a}\), p15\(^{INK4b}\), and p14ARF involved in cell cycle progression (Karlsson et al., 2013). Future studies have the potential of identifying even more hereditary factors related to OSA in dogs.

**Comparative biology of osteosarcoma**

The growth and remodeling of normal bone depends mainly on 2 types of cells: osteoblasts and osteoclasts. Osteoblasts play roles in bone formation, whereas osteoclasts play roles in bone resorption. The breaking down of bone and building new bone matrix is essential for bone growth and this process is extremely active during times of rapid body growth found with puberty (Kansara et al., 2014). Osteoblasts are mesenchymal cells located in the bone marrow that go through different phases of differentiation before reaching their terminal stage (World Health Organization, 2013). The mesenchymal cells first differentiate into pre-
osteoblasts, which are responsible for the expression of alkaline phosphatase, parathyroid hormone, and type I collagen (Wang et al., 1999). Mature osteoblasts are responsible for synthesizing and precursors to type I collagen which comprises 95% of the bone matrix. The terminal differentiation stage forms the osteocyte, which becomes embedded in the bone matrix (Mutsaers and Walkley, 2014).

OSA is commonly accepted to arise from malignant osteoblasts that share characteristics with immature osteoblasts and form immature bone and osteoid tissue (Mutxaers and Walkley, 2014; World Health Organization, 2013). However, a recent study reported for the first time that OSA can arise from osteocytes (Sottnik et al., 2014). Additionally, since the disease can have chondroblastic, fibroblastic, and osteoblastic components, it is possible that the cell of origin may be a more pluripotent precursor cell (Gorlick, 2009; Wilson et al., 2008). The disease in both human and dog can be divided into histological subtypes based on location, cell type and tumor grade: conventional, low-grade central, periosteal, parosteal, telangiectatic, chondroblastic and small cell (World Health Organization, 2013). Although the histological subtypes have not been observed to influence biological behavior of the tumor, high grade tumors are associated with a poor prognosis (Kirpensteijn et al., 2002; Klein and Siegal, 2006).

OSA is locally very aggressive in nature and can cause both lysis and new bone formation at the same time. Soft tissue swelling often occurs at the local site, and pathological fracture are not uncommon in both human and dogs (Ehrhart, 2013). Humans and dogs can present with lameness, swelling at the primary site, and pathological fracture at the time of diagnosis. It is interesting to note that human and canine OSA cannot be distinguished from each other using radiographs, as both present the classical “sunburst” pattern of combined bone lysis/formation (Ehrhart, 2013; Gorlick, 2010).
OSA in both species is highly metastatic in nature, and predominantly occurs at early stages via hematogenous spread to the lung, and to other sites such as bone and soft tissues at lower frequencies. Twenty and 10% of human and canine OSA patients have respectively detectable metastasis at time of diagnosis (Morello et al., 2011). In humans, improved therapy protocols have increased the 5 year survival rates to 60-70% in patients with no detectable metastases at presentation. However, for patients presenting with metastases the 5 year survival rates are only 10-30% (Allison et al., 2012; Bielack et al., 2002; Harris et al., 1998; Meyers et al., 2005). Outcomes for dogs are even bleaker, with 90% of them developing gross metastases even after good local control of the primary tumor, and long-term survival rates at only 10-15% (Ehrhart, 2013; Gorlick, 2010; Withrow et al., 1991). This suggests that canine OSA may behave more aggressively than human OSA, but that has been debated. It has been proposed that higher dose intensity of neoadjuvant chemotherapy in humans is associated with the longer survival rates (Delepine et al., 1996; Kawai et al., 1996). However, a recent study involving 470 dogs with appendicular osteosarcoma treated with doxorubicin and/or carboplatin showed no association with dose intensity of chemotherapy with development of metastases or survival (Selmic et al., 2014). It is clear that new therapies are needed for metastatic OSA patients of both species.

The staging system for OSA was devised for humans and is implemented in canine OSA as well. It is based on 3 types of information: histological grade (G), primary tumor location (T), and regional or distant metastasis (M) (Ehrhart, 2013; Zhou et al., 2000). Stage I includes low-grade (G1) tumors without metastasis. It is further subdivided based on whether the primary tumor is intracompartmental (T1) or extracompartmental (T2). Stage II includes high grade (G2) tumors without metastasis. Stage III tumors have evidence of regional or distant metastasis,
regardless of histological grade (Ehrhart, 2013). Stage III canine patients that present with metastasis have a poor prognosis. Studies have reported the median survival time at 76 days. Dogs with soft tissue metastases had the shortest survival times (19 days), followed by lung metastases (59 days) and bone metastases (132 days). The treatment strategy associated with the longest survival times (130 days) was palliative radiation therapy combined with chemotherapy (Boston et al., 2006; Hillers et al., 2005).

**Comparative genetics and molecular biology of osteosarcoma**

Human and canine OSA are characterized as genomically unstable tumors with abnormal karyotypes. Structural changes that have been documented include chromosomal translocations, rearrangements, deletions, and changes in DNA copy number (Bayani et al., 2007; Chen et al., 2014a; Kuijjer et al., 2013a; Maeda et al., 2012; Mayr et al., 1991; Selvarajah et al., 2008; Thomas et al., 2009). WGAS and whole genome sequencing studies have identified that 33% of human OSA experience chromothrypsis, a catastrophic event that causes hundreds of chromosomal changes at the same time, and over 50% exhibit kataegis, regions of localized hypermutations in the genome (Chen et al., 2014a; Stephens et al., 2011). This complex genomic instability has made it difficult to identify consistent chromosomal alterations as possible factors in tumorigenesis. However, it is suggestive that early stages in OSA development most likely involve changes in DNA repair and chromosomal segregation mechanisms (Fenger et al., 2014).

In 2013 the first large scale GWAS study for human OSA identified glutamate receptor, metabotropic 4 (GRM4) to be associated with susceptibility for the disease (Savage et al., 2013). Another recent GWAS study in a Chinese population confirmed GRM4 to be associated with both susceptibility and metastasis of OSA (Jiang et al., 2014). GRM4 is involved in intracellular
signaling and inhibiting the cyclic AMP (cAMP) signaling cascade (Griffin et al., 2004; Molyneux et al., 2010). Glutamate signaling is involved in regulating bone physiology, and is expressed in both osteoblast and osteoclast cells. Interestingly, GRM4 is associated with poor prognosis in multiple cancers including colorectal cancer, rhabdomyosarcoma, multiple myeloma, and pediatric CNS tumors (Brocke et al., 2010; Chang et al., 2005; Luksch et al., 2011; Stepulak et al., 2009). Glutamate signaling has also been suggested as playing a role in canine OSA as well, with the results of a GWAS study in large dog breeds identifying glutamate receptor ionotropic, kainite 4 (GRIK4) associate with the development of OSA (Karlsson et al., 2013).

Although etiology of this disease is still not elucidated, studies have suggested that tumorigenesis is associated with changes in an assortment of different genes. Alterations in the tumor suppressor proteins p53, Rb, and PTEN, overexpression of oncogenes erbB-2 and MET, as well as signaling disregulation in pathways involving STAT3, VEGF, PDGF, mTOR, c-Kit, metalloproteininases, and ezrin have all been implicated as playing a role in tumorigenesis (Mueller et al., 2007; O'Day and Gorlick, 2009; Ta et al., 2009).

Somatic mutations in TP53 and RB are the most common specific genetic alterations reported in OSA, which have been verified by whole genome sequencing (Chen et al., 2014a). In a study of 196 human tumors, 19.4% had mutations in TP53 (Wunder et al., 2005). In a more recent and smaller study of 34 human tumors, TP53 mutations were identified in 28 of them (82%) (Chen et al., 2014a). Similarly, over-expression of p53 has been reported in 60% of canine OSA cell lines and 41 and 67% of canine OSA tumors, and this over-expression correlates with missense point mutations within the DNA-binding domain (Kirpensteijn et al., 2008; Levine and Fleischli, 2000; Loukopoulos et al., 2003). Another study of canine OSA
observed 72% of samples over-expressed p53, with a higher prevalence occurring in the appendicular skeleton, suggesting a correlation of p53 expression and aggressiveness of the tumor (Sagartz et al., 1996). Importantly, overexpression of p53 has also been seen in canine osteosarcoma cell lines (Loukopoulos et al., 2004). RB1 alterations occur in 30 to 75% of human OSA (Ottaviani and Jaffe, 2009). In dogs, a recent study identified a loss in DNA copy number for RB1 in 30% of canine OSA tumors, which contributed to the observed reduction or absence of RB1 expression in 62% of the 38 samples (Thomas et al., 2009).

Deletions in PTEN, a tumor suppressor of the PI3K/AKT pathway, are commonly seen in both human and canine OSA. In one human study it was reported to be deleted in 48% of the samples (Freeman et al., 2008). Canine OSA studies report PTEN deletions both in cell lines and in 30 and 42% of tumors (Angstadt et al., 2011; Levine et al., 2002; Thomas et al., 2009).

Multiple molecular pathways are activated in human and canine OSA which may prove advantageous for identifying therapeutic targets. The receptor tyrosine kinase MET is expressed in both human and dog disease, and heterodimerization of MET with EGFR and Macrophage stimulating 1 receptor (C-Met-Related Tyrosine Kinase) (Ron), a receptor tyrosine kinase in the MET family, has been shown to promote resistance to targeted therapy in human cancers (Liao et al., 2005; MacEwen et al., 2003; McCleese et al., 2013). In canine OSA cell lines, a novel MET inhibitor PF2362376 was shown to inhibit proliferation and block migration, invasion, and colony formation (Liao et al., 2007). Another study treated canine OSA lines with a combination of the MET and EGFR inhibitors crizotinib and gefitinib and observed that they inhibited proliferation with an additive effect, supporting the idea of targeting MET, EGFR, and RON signaling in OSA (McCleese et al., 2013).
STAT3 is constitutively activated in human and canine OSA cell lines and canine OSA tumors. High expression of STAT3 has been associated with a worse prognosis in human OSA patients. Also, expression profiling of pediatric OSA showed that genes regulated by STAT3 that are involved in cell migration and remodeling are associated with poor prognosis (Mintz et al., 2005; Ryu et al., 2010; Wang et al., 2011). When human and canine OSA cell lines were subjected to STAT3 inhibition either indirectly through targeting Src kinases upstream or directly through inhibiting STAT3 DNA binding or its expression, a decrease in proliferation and an increase in apoptosis were observed (Fossey et al., 2009). Two novel inhibitors FLL32, a curcumin analog, and LLL12 a small molecule STAT3 inhibitor, have been investigated in both human and canine OSA. In dog cell lines FLL312 decreased STAT3 DNA binding, expression, and induced apoptosis (Fossey et al., 2011). LLL12 reduced STAT3 phosphorylation, decreased proliferation, induced apoptosis, decreased the expression of STAT3 transcriptional targets, and also had a synergistic effect with doxorubicin in dog cells (Couto et al., 2012). In human OSA cells and murine xenograft tumors FLL312 and LLL12 have been observed to inhibit STAT3 phosphorylation, induce apoptosis, decrease migration of cells, and inhibited tumor growth (Onimoe et al., 2012). These studies suggest that targeting STAT3 signaling is an attractive possibility for both human and canine OSA.

Ezrin is a protein that links the cell membrane to the cytoskeleton, and murine models have shown that it is necessary for metastasis in OSA (Khanna et al., 2004). High ezrin expression is associated with early metastasis in canine OSA tumors and with a lower 2 year survival rate in human OSA patients (Khanna et al., 2004; Wang et al., 2013). In a tissue microarray study of 73 canine OSA tumors with known clinical outcome data, high ezrin staining was significantly associated with a shorter median DFI, compared to low ezrin staining.
Small molecule library screenings have identified 2 inhibitors to ezrin, NSC305787 and NSC668394, which in early studies have been shown to inhibit ezrin phosphorylation, the interaction between ezrin and actin, and ezrin-mediated motility of OSA cells. In murine models, these inhibitors inhibited lung metastasis after tail vein injection (Bulut et al., 2012). Since metastasis is the leading cause of OSA death, further study into ezrin targeting in both the human and dog may prove beneficial.

Survivin is a member of the inhibitor of apoptosis (IAP) family and is involved in cell division as well as apoptosis. It is over-expressed in human and canine cancers. Expression of survivin was detected in 65 of 67 canine primary OSA samples and the intensity of expression correlated with DFI, the lower and higher expressing groups being 331 and 173 days, respectively (Shoeneman et al., 2012). In contrast, another study in canine OSA observed that in 17 samples moderate to high survivin expression correlated with longer overall survival, suggesting it to be a positive and not a negative prognostic factor (Bongiovanni et al., 2012). Similar conflicting results have been seen in human OSA. Human studies have correlated high survivin expression with presence of metastasis, suggesting it to be a negative prognostic factor, but another study correlated high survivin expression with longer overall survival, suggesting it to be a positive prognostic factor (Osaka et al., 2006; Osaka et al., 2007; Trieb et al., 2003). Cellular localization of survivin may play a role in the contradicting results, as in both cases where it was reported a positive prognostic factor survivin expression was localized in the nucleus. EZN-3042, a locked nucleic acid antisense oligonucleotide that down-regulates surviving mRNA expression, has been recently shown to inhibit growth, induce apoptosis and enhance chemosensitivity in canine OSA cell lines. Also, in canine OSA xenografts EZN-3042 was able to decrease survivin transcription and protein production (Shoeneman et al., 2014).
Advances in gene expression analysis have allowed comparative analysis between human and canine cancers, including OSA. On a more global level, studies have compared orthologous gene signatures between human and canine OSA samples in unsupervised hierarchical cluster analyses, and observed that samples did not segregate based on species (Paoloni et al., 2009). Also, an array comparative genomic hybridization (aCGH) study in canine OSA identified recurrent high-frequency DNA copy number changes in the syntenic regions where studies of human OSA report recurrent genome imbalances (Angstadt et al., 2011; Angstadt et al., 2012). Taken together, there are many genetic and molecular similarities between human and canine OSA, which presents an opportunity to perform more comparative research which could translate to improved clinical outcomes for both species.

_Treatment of human and canine osteosarcoma_

Surgery remains the standard for local tumor control in OSA for both humans and dogs, but because of the early presence of undetectable microscopic metastases, surgery alone is not effective. Only 15-17% of human patients survive when treated with only surgical resection of the tumor (Bernthal et al., 2012; Link et al., 1986). In dogs the median survival time with surgery alone is only 103-175 days, with 1- and 2- year survival rates at 11-20% and 2-4% (Berg et al., 1992; Brodey and Abt, 1976; Mauldin et al., 1988; Shapiro et al., 1988; Spodnick et al., 1992; Straw et al., 1991; Thompson, 1991). In the 1970’s chemotherapy was introduced in the adjuvant setting post surgery in humans, and survival rates tripled for patients with non-metastatic disease (Jaffe et al., 1974).

The two main options for surgery in OSA are amputation and limb-sparing surgery. There is no significant difference in survival or in rate of tumor recurrence between these two options in both humans and dogs, if adequate chemotherapy is administered (Federman et al.,
2009; Messerschmitt et al., 2009; Straw and Withrow, 1996). In humans, improved imaging techniques and the positive effects of neoadjuvant chemotherapy have caused a large shift away from amputation towards limb-sparing surgery (Bielack et al., 2009; Jaffe, 2009). More than 85% of appendicular OSA patients are eligible for limb-sparing surgery, but only if it is possible to get wide surgical margins (Federman et al., 2009; Messerschmitt et al., 2009). In a retrospective study of 200 human pediatric OSA patients who received limb-sparing surgery, 9% had local recurrence. Post-recurrence survival significantly correlated with the length of resection margins, with the longest survival times occurring with margins greater or equal to 1 cm (Loh et al., 2014).

In contrast, amputation continues to be the main standard of care for dogs with appendicular OSA. It is best for local tumor control, it avoids the risk of pathological fractures, and it eliminates pain and is well tolerated by the patient (Brodey and Abt, 1976; Spodnick et al., 1992). Limb-sparing surgery has had good results for OSA in the distal radius, but at other sites there is a greater chance of complications resulting in a poor functioning limb (Kuntz et al., 1998; Morello et al., 2001). In dogs it is not advisable in cases of extreme obesity, neurological disease, or concurrent debilitating orthopedic disease (Brodey and Abt, 1976; Spodnick et al., 1992). An interesting discovery that has been reported in both human and canine OSA is that an infection of the surgical site after limb-sparing surgery is associated with longer median survival times than non-infected sites (685 versus 289 days) (Jeys et al., 2007; Lascelles et al., 2005; Liptak et al., 2006). This may be suggestive of an immune-activating mechanism in these cases. The physical similarities between humans and dogs such as body size, tumor biology, and anatomy makes canine OSA a good model for the design of new and improved strategies and devices for limb-sparing procedures (Paoloni and Khanna, 2008; Withrow and Wilkins, 2010).
Limb-sparing techniques that have been developed in the dog include filling the bone defect with frozen cortical allograft, an endoprosthesis, or neoplastic bone that has been resected and either pasteurized, autoclaved, or irradiated (Boston et al., 2007; Buracco et al., 2002; LaRue et al., 1989; Liptak et al., 2004a; Liptak et al., 2004b; Massin et al., 1995; Morello et al., 2001; Morello et al., 2003; Yamamoto et al., 2002b).

The main therapy for OSA currently in human patients is neoadjuvant chemotherapy followed by surgery, and then adjuvant combination chemotherapy. Today the 5-year survival rate of patients without metastasis is 60-70% (Ta et al., 2009). When these survivors were assessed for long term follow up at 10, 15, and 20 years survival rates were 93.5%, 90.4%, and 88.6%, respectively (Nagarajan et al., 2011). This suggests the majority of these patients are essentially cured from the disease. For a patient with detectable metastases at time of presentation, however, the 5 year survival rate is only 20%, and that hasn’t improved in the last 30 years (Quinn et al., 2001). A 5-fold increase in survival has been observed with surgical excision of all detectable OSA tumors compared to just removing the primary tumor (Morello et al., 2011).

Canine OSA is thought to be more aggressive than its human counterpart, and that is reflected in the observation that despite surgical amputation and adjuvant chemotherapy, greater than 50% of patients will not live past 1 year, and 90% will not survive 2 years (Ehrhart, 2013). This is likely due to the presence of micrometastases that has been reported to occur in 90% of dogs (MacEwen and Kurzman, 1996; O'Brien et al., 1993; Selvarajah and Kirpensteijn, 2010). New therapies that target metastasis are desperately needed in dogs with OSA.

The introduction of chemotherapeutics into treatment strategies for OSA has done much to slow down metastatic growth and improve survival times in both the human and dog. By
definition chemotherapy drugs are designed to target rapidly dividing cells. Although there still is no confirmed optimal combination of chemotherapy yet in human OSA, the often used regimen in the United States and Europe consists of a combination of high dose methotrexate, doxorubicin, and cisplatin (Rainusso et al., 2013). Methotrexate, an antimitabolite that is a folate analog, inhibits purine and thymidylate biosynthesis necessary in DNA replication via the inhibition of the dihydrofolate reductase enzyme (Allegra et al., 1986). Doxorubicin, an antitumor antibiotic, is an anthracycline that has been shown to intercalate DNA, inhibit topoisomerase II, RNA and DNA polymerases, thioredoxin reductase, generate reactive oxygen species, and alter Ca\textsuperscript{2+} homeostasis (Bachur et al., 1978; Doroshow, 1985; Mau and Powis, 1992; Pessah et al., 1990; Tewey et al., 1984; Zunino, 1975). Platinum agents such as cisplatin and carboplatin exert antitumor effects through covalent binding of DNA by forming interstrand or intrastrand cross-links, which blocks strand separation during replication and transcription (Fichtinger-Schepman et al., 1985).

The use of chemotherapy in humans is done in the neo-adjuvant and adjuvant setting with surgery. Surgery usually occurs 3-4 weeks after the last dose of neo-adjuvant chemotherapy, and then chemotherapy begins again 2 weeks post surgery (Morello et al., 2011). In dogs with OSA, neoadjuvant chemotherapy is not commonly used. There is no survival advantage associated with employing early post-surgery administration of chemotherapy in dogs (Berg et al., 1997; Dernell, 2007; Fenger et al., 2014).

A strong prognostic factor in human OSA is the effect of chemotherapy in the neoadjuvant setting. Patients whose primary tumor experience > 90% necrosis (good responder) have a better prognosis than those that do not (poor responder) (Jawad et al., 2011). The 5-year event free survival rates for poor and good responders are 35-45% and 70-80%, respectively.
(Whelan et al., 2006). Good responders will usually be assigned the same regimen of chemotherapy in the post-surgery setting, whereas poor responders will be assigned to a salvage therapy regimen, which might include an increased dose of the same drugs or a different protocol (Messerschmitt et al., 2009).

For dogs with OSA, although no standard protocol exists for chemotherapy, it most often includes doxorubicin and/or a platinum agent such as cisplatin or carboplatin. Carboplatin is a second generation platinum agent that has shown comparable efficacy as cisplatin but with less nephrotoxicity, and has recently widely supplanted cisplatin use in dogs (Ehrhart, 2013). There is no clear evidence that combination chemotherapy is superior to single agent therapy, but it may have the advantage of reducing negative side effects (Szewczyk et al., 2015). In canine OSA the addition of chemotherapy to surgery and/or radiation therapy has increased mean survival time from 103-175 days to 262-450 days. The 1- and 2-year survival rates with chemotherapy is 31-48% and 10-26% (Dernell, 2007). Chemotherapy has been shown to be less effective in the presence of detectable macroscopic metastases (Ogilvie et al., 1993).

Used as a single agent, doxorubicin has slowed metastasis with 1-, 2-, and 3-year survival rates of 35, 17, and 9% (Moore et al., 2007). A clinical study of 102 OSA dogs that received combined doxorubicin and cisplatin treatment concurrently ran into severe toxicity issues, resulting in death in 10% of the test population (Berg et al., 1997). Combination protocols that alternate the drug doses have been more tolerable. The alternating combination of doxorubicin and carboplatin in a study of 32 OSA dogs had a median DFI of 227 days and survival time of 320 days (Kent et al., 2004).

Although the use of systemic chemotherapy for the management of metastasis in OSA remains the most effective treatment strategy, clinical trials attempting to further optimize
chemotherapy in OSA have not resulted in significantly improved outcomes (Fenger et al., 2014). It is not probable that new cytotoxic drugs or increasing dose of existing agents will translate to better clinical results.

The role of radiation therapy (RT) in the management of OSA is mainly for palliation of unresectable tumors in both humans and dogs, or for patients with incomplete margins after surgical excision (Federman et al., 2009). Osteolytic bone pain is a large contributor to morbidity in humans and dogs and can have a great negative impact on quality of life (Fenger et al., 2014). RT has been shown to be the most effective treatment for bone pain in OSA. The combination of chemotherapy and RT has resulted in long-term remissions in some human patients, and certain chemotherapies such as ifosfamide, cisplatin, and high dose methotrexate are reported to play a role in increasing the effectiveness of RT (Errani et al., 2011; Ozaki et al., 2002). In a 2012 study involving 50 dogs with OSA that received palliative RT alone, or combined with chemotherapy, pamidronate, or both, it was observed that dogs that receive RT with chemotherapy had a MST of 307 days, whereas combining RT with just pamidronate resulted in a MST of only 69 days (Oblak et al., 2012).

Newer radiation techniques such as intraoperative radiation therapy (IORT) and stereotactic radiotherapy (SRT) have been applied in both human and dog OSA patients (Liptak et al., 2004a; Oya et al., 2001). IORT involves surgically exposing affected bone in order to receive a direct dose of RT, or it can imply removal of affected bone followed by RT and re-engraftment. It has the advantage of preserving limb function in anatomic sites such as the proximal humerus that are not good sites for surgical limb-sparing techniques (Kuntz et al., 1998). IORT resulted in a MST of 298 days for dogs with appendicular sarcoma in (Ehrhart,
However, high complication rates with orthopedic implants and infection of treated bone has led to this strategy not being recommended.

SRT incorporates image guidance systems to deliver high dose RT to the tumor while sparing the surrounding normal tissues via a sharp drop off of dose intensity (Ehrhart, 2013). It is also a good option to preserve limb function in sites not amenable to surgical limb-sparing and it has the advantage over IORT in that no surgery is required. The primary complication with this technique is pathological fracture, and it appears that lesions that are small and more blastic than lytic are better suited for this procedure (Ehrhart, 2013). In a small study of 11 dogs with a protocol developed at the University of Florida SRT resulted in excellent limb function and a MST of 363 days (Farese et al., 2004). At Colorado State University a protocol has been developed that incorporates chemotherapy before and after receiving fractions of SRT followed by standard adjuvant chemotherapy protocols at 3 week intervals. For the first 50 dogs that have received treatment, MST is reported to be 275 days and the overall limb survival rate is 83% (Ehrhart, 2013).

Immunotherapy for effective treatment of OSA is still considered to be in its infancy, although ironically it actually started over 100 years ago with the work of the surgeon William Coley. He treated patients with bone and soft tissue sarcomas with heat-killed Streptococcus pyogenes and Serratia marcescens and observed tumor regressions, though not a durable response (Coley, 1910). Later on in the 1970’s a clinical study treated 17 OSA patients after tumor resection with bacillus Calmette-Guerin and an allogeneic tumor cell vaccine. They observed 18% of treated patients survived without disease recurrence, as opposed to 12 patients not treated with immunotherapy who all died after tumor relapse (Eilber et al., 1975).
Recently, a new field of osteoimmunology has emerged due to the growing knowledge of the crosstalk that occurs between bone cells and immune cells. Osteoblasts play a role in the development of hematopoietic stem cells (HSCs), and in turn bone homeostasis is regulated by the immune system especially in times of immune activation (Lorenzo and Choi, 2005). Certain signaling pathways such as TGFβ and receptor activator of nuclear factor-κB (RANK-RANKL) are involved in bone as well as the immune system (Kansara et al., 2014). These discoveries show the potential immunology could have as a treatment strategy in OSA, and the recent successes of new immunotherapies in other cancers may hopefully translate into OSA in the future.

Interestingly, studies in canine OSA have already played an important role in the development of immunotherapy in humans, specifically with the development of liposome-encapsulated muramyl tripeptide-phosphatidylethanolamine (L-MTP-PE). L-MTP-PE is a lipophilic derivative of muramyl dipeptide, a synthetic analog of a component of the cell wall in *Mycobacterium*. It’s been shown to cause cytotoxicity in canine OSA cell lines by enhancing the tumoricidal properties of alveolar macrophages (Kurzman et al., 1999). In a randomized double-blind trial L-MTP-PE was administered to 14 canine OSA patients immediately following amputation, while 13 dogs were treated with empty liposomes. The MST for dogs treated with L-MTP-PE was 222 days, compared to 77 days for non-treated dogs (MacEwen et al., 1989). This led to 2 additional clinical trials investigating the combined use of L-MTP-PE and cisplatin chemotherapy. In the first trial after amputation and 3 months of cisplatin treatment, dogs were randomized to receive L-MTP-PE or placebo control. In the second trial they investigated using L-MTP-PE concurrently with cisplatin after amputation. The results showed that although there was no survival advantage for using L-MTP-PE concurrently with cisplatin, using L-MTP-PE
after the administration of cisplatin resulted in a MST of 14.4 months, which was significantly longer than the placebo group (Kurzman et al., 1995). These canine OSA trials were used for the scientific rationale for a phase III evaluation of L-MTP-PE in human pediatric OSA. In a human clinical trial it was observed that overall survival at 6 years improved from 70% to 78% (Meyers et al., 2008). Compared to patients receiving chemotherapy alone, the addition of L-MTP-PE added an average 2.58 years of life (Chou et al., 2009; Johal et al., 2013). These results led to the approval of L-MTP-PE for treatment of metastatic OSA by the European Medicines Agency.

Other immunological strategies that have been investigated in OSA include type I interferon therapy, T cell check point inhibitors, and monoclonal antibody therapies. IFNα is reported to inhibit proliferation in OSA cell lines and sensitize them to chemotherapy such as doxorubicin (Beresford et al., 1990; Yuan et al., 2007). In a small clinical trial IFNα appeared to improve survival and cause partial tumor regressions, however in a large transatlantic collaborative clinical trial pegylated IFNα showed little effect when added to standard chemotherapeutic protocols (Bielack, 2014; Strander, 2007).

T cell check point inhibitors that have had recent success in other cancers target CTLA4, PD1, or PDL1. CTLA4 polymorphisms have been correlated with a higher risk of OSA development (He et al., 2014; Liu et al., 2011). Also, a study has associated high mutational load with overall response rates to anti-PD1 and PDL1 agents in several cancers (Champiat et al., 2014). Mouse models of metastatic osteosarcoma, targeting PD1-PDL1 interactions resulted in improved survival (Lussier, 2013). No OSA patients have been treated with T cell check point inhibitors currently, but they are definitely strategies worthy of pursuit.

Although it has proved difficult to identify antigens specific to OSA, a few have been identified such as disialyl anglioside (GD2), folate receptor-α (FOLR1), and CD146 (Navid et
A Phase I trial is open currently that will investigate the use of humanized GD2-specific monoclonal antibodies in children and adolescents with relapsed or refractory osteosarcoma, neuroblastoma, or melanoma (Gorlick et al., 2013). There is a lot of potential for improved immunotherapeutic strategies in OSA, and their development will require immunocompetent model systems. Thankfully, dogs develop OSA with fully intact immune systems and share such biologic and genetic similarities to human OSA, providing a model system that would be hard to surpass for investigating immunotherapy. Although the genetic complexity of OSA has made it difficult to identify molecular targets, researchers have investigated several different novel strategies for treating the disease in humans and dogs. Results in patients in general have been less than optimal. The main strategies explored have been overcoming drug resistance, altering the bone microenvironment, and inhibiting cell signaling pathways (Luetke et al., 2014). Many cancers acquire resistance to drugs as a result of expression of drug efflux transporters, such as P-glycoprotein, which is encoded by the \textit{MDRI}/\textit{ABCB1} gene. In dogs, silencing the \textit{MDRI} mRNA has been shown to decrease the expression of drug efflux pumps which resulted in increased cytotoxicity of chemotherapeutic drugs (Kimura et al., 2013; Sha et al., 2013). Early drugs designed in humans to inhibit ABC transporters were challenged with problems due to high toxicity of chemotherapeutic agents. Novel inhibitors are being designed and investigated currently. Curcumin, a phenolic compound that has been shown to inhibit the expression and function of \textit{ABCB1} and \textit{ABCG2}, is currently in Phase I/II trials to study its efficacy in high-grade relapsed or metastatic OSA (NCT00689195) (Anuchapreeda et al., 2006; Chearwae et al., 2006; Hattinger et al., 2010).
Another strategy that has been investigated is to target the changing microenvironment surrounding the bone tumor. Osteoclasts play an important role in causing lysis of normal bone, which in turn releases factors that are required for tumor growth. Osteoclast differentiation is dependent on RANK/RANKL signaling, and a study associated high RANKL expression with poor response to neoadjuvant chemotherapy and survival in OSA cells (Lee et al., 2011). Early studies of rodent OSA models that were treated with siRNAs targeting RANKL showed no effect on tumor growth, but it may provide benefit when combined with chemotherapy (Rousseau et al., 2011). Matrix metalloproteinases (MMPs) serve to aid in the processes of invasion and tumor metastasis, and MMP-2 and MMP-9 have been documented to be active in canine OSA cell lines (Lana et al., 2000; Loukopoulos et al., 2004). BAY 12-9566, a specific inhibitor for MMP-2 and MMP-9, was investigated in canine OSA in a prospective double blind, randomized, placebo-controlled clinical trial. Unfortunately, no significant improvement in DFI or survival was observed (Moore et al., 2007). Bisphosphonates have been used in human and canine OSA due to their ability to inhibit cell proliferation, induce apoptosis, decrease VEGF expression, and prevent bone loss via inhibition of osteoclast function (Kansara et al., 2014). In humans they found that the bisphosphonate pamidronate when combined with chemotherapy in a phase II trial resulted in more durable limb reconstructions (Meyers et al., 2011). Pamidronate has been studied in canine OSA as well, and has resulted in improved pain alleviation and limb function compared to palliative RT alone (Fan et al., 2009; Fan et al., 2007). Currently studies are investigating zoledronate, a more potent bisphosphonate that can be more safely administered, in human and dogs with OSA. The role of Hedgehog (HH), Notch and WNT pathways in bone development and their reported role in OSA development makes them an attractive target as well. Preclinical investigations of inhibitors to these three pathways have resulted in
encouraging antitumor effects (Cai et al., 2014; Kansara et al., 2009; Kolb et al., 2012; Lo et al., 2014; Mu et al., 2013; Rubin et al., 2010). Clinical trials have begun targeting these pathways in combination, but results are pending (NCT01154452) (Kansara et al., 2014).

Receptor tyrosine kinases play a major signaling role in many cancers, and several have been identified to be over-expressed in OSA, such as IGF1R, VEGFR, PDGFR, HER2, and MET. IGF-1 signaling is important for skeletal growth, and IGF-1 expression has been reported in human and canine OSA cell lines (Polednak, 1985; Savage and Mirabello, 2011). IGF-1 exposure causes proliferation and antiapoptotic responses (Bostedt et al., 2001; MacEwen et al., 2004). In humans, IGF-1 expression associates with surgical stage, distant metastasis, and poor survival (Wang et al., 2012). When IGF-1 signalling was inhibited by either a small molecule drug (OSI-906) or by RNA interference in human OSA cell lines, proliferation and invasion decreased while sensitivity to radiation increased (Kuijjer et al., 2013b; Wang et al., 2009). In dogs the efficacy of OncoLAR was investigated, an analog of somatostatin known to inhibit IGF-1 concentrations, in 44 canine OSA patients after amputation and chemotherapy. Compared to a vehicle control, although IGF-1 concentrations decreased in the patients, no clinical improvement was observed (Khanna et al., 2002).

Multi-targeted RTK inhibitors have been tested in human and canine OSA. In dogs toceranib has activity against VEGF receptor, PDGF receptor, and KIT and is used for canine mast cell tumors. In 23 dogs with pulmonary OSA metastases, toceranib treatment resulted in 43.5% of the patients achieving stable disease, and 4.3% a partial response (London et al., 2012). In humans, pazopanib targets KIT, VEGF receptor, FGFR, and PDGFR. A Phase II trial of pazopanib in humans is currently underway (NCT01759303) (Kansara et al., 2014). Other signaling pathways that have been investigated include SRC, mTOR, Aurora kinase, VEGF, p53,
and cyclin-dependent kinases (Cheok et al., 2007; Hingorani et al., 2009; Kaya et al., 2000; Maris et al., 2010; Tovar et al., 2006; Zhou et al., 2010). These new targeted therapies are not yet considered successful in OSA, but as our understanding increases of how these different pathways work together to promote tumorigenesis, growth and metastasis new therapies may one day improve clinical outcome for OSA patients. With the 70% success rate seen with combination chemotherapy in human patients, however, perhaps one of the first steps in improving therapy for canine OSA would be to learn better ways of utilizing and combining existing chemotherapeutics.

PREDICTING RESPONSE TO THERAPY IN INDIVIDUAL PATIENTS

Role of biomarkers in cancer

A measurable characteristic that can serve as an indicator of biological conditions such as normal or pathogenic processes, or pharmacologic responses to therapy can be considered a biological marker, or “biomarker” (Nowsheen et al., 2012). Traditional biomarkers in cancer have often been identified through pathological analysis such as histological grade of the tumor. The expansion of our knowledge of the molecular basis of cancer has led to the identification of many molecular biomarkers, many of them being oncogenes and tumor suppressors. Cancer biomarkers can be classified into 4 different subgroups: diagnostic, prognostic, predictive, and pharmacodynamic (Sawyers, 2008). Diagnostic biomarkers naturally can be used to help in diagnosis of the cancer type or subtype. Molecular diagnostic biomarkers are important in this area because often tumors that appear similar histologically arise from different driver mutations, resulting in differences in response to therapy (Majewski and Bernards, 2011). They can be
identified through the discovery of key mutations and pathways important in tumor development and proliferation (Kalia, 2015). Prognostic biomarkers are used heavily by clinicians in order to make correlations with clinical endpoints, and are not therapy-specific. These can involve somatic germline mutations, alterations in DNA methylation, circulating tumor cells in the blood or increases in microRNA expression (Kalia, 2015; Nowsheen et al., 2012). Predictive biomarkers are used in outcome prediction of specific therapies, which can be useful in correctly matching up patients with a therapy they could potentially benefit the most from. Pharmacodynamic biomarkers have the potential to predict response depending on drug dose levels. Both predictive and pharmacodynamic biomarkers could have a major impact on the cost of certain therapies, ensuring that newly designed and costly treatments are being used on the population subset of cancer patients that would have the maximum benefit (Fojo and Grady, 2009). Some biomarkers can fall into more than one of these subgroups.

Molecular alterations in cancer with potential biomarker characteristics can be detected by multiple methods, including expression profiling of both microRNAs and genes, multiplex polymerase chain reaction (PCR), genome hybridization, DNA microarrays and sequencing, mass-spectrometry, proteomic profiling, molecular imaging, and measuring tumor specific antibodies and circulating tumor cells (Sawyers, 2008).

In recent years molecular biomarkers have made a large impact in several cancers, including chromic myeloid leukemia (CML), colorectal cancer (CRC), breast cancer, non-small cell lung cancer (NSCLC), and melanoma. CML is caused by the fusion protein Bcr-Abl, generated by a specific chromosomal translocation on chromosome 22 (Daley et al., 1990; Lugo et al., 1990; McLaughlin et al., 1987). CML patients with this abnormality are recommended to be treated with a Bcr-Abl inhibitor as first line therapy, which has led to improvements in long-
term survival based on surrogate endpoints (Colburn, 2000). Currently 5 Bcr-Abl inhibitors have been approved by the FDA for treatment of CML patients positive for the Bcr-Abl biomarker.

There have been multiple molecular biomarkers identified for CRC. EGFR is over-expressed in 70% of CRC tumors, which has led to the utilization of EGFR inhibitors such as cetuximab and panitumumab for treatment. This has led to prolonged survival, especially when combined with chemotherapy (Kalia, 2015). However, 40% of CRC tumors have a G13D mutation in K-Ras, the only predictive biomarker for anti-EGFR treatment. Tumors with this particular K-Ras mutation are predicted to not respond to cetuximab. A diagnostic kit to determine the mutational status of K-Ras has recently been approved to determine whether CRC patients should receive cetuximab or not (Chung and Christianson, 2014). The presence of BRAFV600E mutations in 35-45% of CRC tumors is prognostic of poor response to 1st line therapies (Kalia, 2015). Additionally, fluorouracil, an often used chemotherapy in CRC could be highly toxic for 3-5% of patients that have deficiencies in dihydropyrimidine dehydrogenase (DPD), making it an important biomarker to be tested for before treatment decisions are made (Chung and Christianson, 2014).

Breast cancers that are positive for estrogen receptor (ER) and progesterone receptor (PR) respond well to hormonal therapies and are associated with better disease-free survival (Harvey et al., 1999). Hormonal therapy is recommended for all breast cancer patients who are ER+, regardless of levels (Chung and Christianson, 2014). HER2 expression is present in 15-25% of invasive breast cancers and is a predictive biomarker for favorable response to trastuzumab, a HER2-targeting antibody, as well as topoisomerase II inhibitors such as the chemotherapeutic agent doxorubicin (Chung and Christianson, 2014; Salomon et al., 1995). A genetic test called Oncotype Dx has been developed that measures the genetic signature of 21 genes that is used for
prognosis of non-negative, ER+ breast cancer patients, but is currently lacking enough evidence for routine use (Kalia, 2015).

In NSCLC patients, newly identified biomarkers include EGFR, ERRC, and RRM1 gene expression, as well as K-Ras mutations. Patients who express EGFR have been shown to respond well to gefitinib and erlotinib, anti-EGFR tyrosine kinase inhibitors. When compared with chemotherapy, EGFR inhibition has led to significantly longer disease-free survival, increased response, and a better quality of life with less toxicity (Duffy, 2013; Moran and Sequist, 2012; Rossi et al., 2013; Soria et al., 2012). The last few years have been exciting for melanoma research, with the identification of the strong biomarker of the mutant BRAFV600E that is found in over 50% of patients. Agents designed to specifically target this mutant kinase has led to tumor regressions in this large subset of patients (Rossi et al., 2013).

Biomarkers also play a huge role in diagnosis and prognosis of canine cancers. The identification of molecular biomarkers has also begun, although in many cases the needed validation through the use of large sample datasets has been lacking. This has led to inconsistent results with contradicting reports for several well known biomarkers in humans, such as ER, PR, and HER2 expression in canine mammary tumors (Pena et al., 2014). Studies in acute myeloid leukemia (AML) in dogs have discovered mutations in fms-related tyrosine kinase 3 (FLT3), C-kit, and Ras (Juopperi et al., 2011). FLT3 expression has also been documented in human AML as well. As more genetic studies are performed in canine cancers, the use of molecular biomarkers will undoubtedly increase like we are seeing in human oncology.

There are several challenges with the identification and use of biomarkers at present. Although much progress has been made in identifying novel molecular targets and translating that into novel therapies for cancer patients, less progress has been made in correctly identifying
the subset of patients who will best respond (Sawyers, 2008). Thousands of biomarkers are reported in journal articles each year, but the majority has not been sufficiently validated for widespread use in the clinic. The poor availability of tumor tissue, especially in the case of solid tumors, has impeded validation efforts, as large numbers are needed (Sawyers, 2008). Developing new non-invasive ways of detecting biomarkers such as finding serological markers circulating in the blood can hopefully lead to improvements in this area. Also, often biomarkers are only detected after their levels have elevated once the tumor has advanced to late stage, limiting their benefit. Lack of standardized methods for determining positivity of certain biomarkers is an issue that needs addressing as well (Nowsheen et al., 2012). Despite these challenges, the future is looking bright for the increased utilization of molecular biomarkers in oncology. Incorporating histological and molecular data for each individual’s tumor will strengthen our efforts of achieving improved personalized medicine for the treatment of cancer in humans as well as dogs.

**Multi-gene signatures of drug response**

As mentioned previously with the example of the 21-gene Oncotype DX test that is used in breast cancer patients, biomarkers need not exist solely as single entities but can also consist as a collection of multiple genes. Although single gene biomarkers have been moderately successful for general diagnostic, prognostic, and/or predictive purposes, a more detailed prediction of highly individualized cancer processes may benefit from more information than a single gene. Much work has been done attempting to identify multi-gene signatures describing different characteristics of cancer including aggressiveness, metastatic ability, responsiveness to therapy, etc. Using known cancer biology and pathway knowledge to identify candidate genes in the gene signature identification process can be considered a “biased” approach. The 21 genes of
the Oncotype DX test for breast cancer is an example of such a strategy, as they were identified from a selection of 250 candidate genes involved in proliferation and invasion, as well as genes related to HER2/neu, ER, and/or PR expression (Paik et al., 2004). There are advantages to this approach, but it leaves the possibility that a highly predictive undiscovered biomarker may go undetected. With the new genomic technologies available that allow for quick analysis of the molecular expression of thousands of genes, the chance of discovering novel biomarkers has increased greatly. An unbiased approach that relies solely on mathematical and statistical methods for selecting genes without the influence of current cancer biology knowledge may serve to further illuminate our understanding of cancer behavior.

Methods of identifying gene signatures can involve differential expression analysis between 2 different groups with an opposite characteristic (for example, metastatic versus non-metastatic, or drug sensitive versus drug resistant), using one of several statistical approaches including two-sample t-tests, empirical Bayesian methods, and generalized likelihood ratio test (Cheng et al., 2010). Correlation methods can also be used with the entire population of samples instead of comparing 2 distinct subgroups where the level of gene expression is correlated to a specific parameter like overall survival. Significant Analysis of Microrrays (SAM) is another popular method that makes use of permutation tests to estimate the false discovery rate (FDR) of differentially expressed genes (Tusher et al., 2001).

Multi-gene signatures have great potential to advance personalized medicine for cancer therapy, since the added number of components can increase the complexity of prediction that is needed to more fully “tailor” a therapeutic strategy to the individual. Much caution must be exercised, however, in this type of genomic strategy. Risks inherent to these types of studies include the risk of “overfitting” the model on the training dataset, as well as inadequate model
validation prior to use in a prospective setting (Taylor et al., 2008). An unfortunate example of this type of method being performed without proper validation has been seen by the suspension of clinical trials in human lung and breast cancer using multi-gene prediction models to determine drug sensitivity (Bonnefoi et al., 2011; Potti et al., 2006). Although the researchers responsible for generating the models used separate training and testing gene expression datasets, the model building on the training set was aided by the use of gene clusters that were derived from the combined training and testing datasets. This resulted in a testing set being used that was not completely independent from the model building process (Taylor et al., 2008). It is essential that proper validation of these signatures is performed before similar genomic methods can move forward in the clinic. What type of models could be used to provide the necessary validation? The use of advanced animal cancer models that are routinely treated with similar therapies in a clinical setting would serve this purpose very well, especially given the fact that much evidence points to the genetic similarities between cancer in humans and dogs.

One of the difficulties of developing these same genomic methods in a canine model is the lack of available drug sensitivity and genomic data. In human cancer research this type of data is ever growing and publicly accessible. Human cancer cell line panels from the National Cancer Institute (NCI60) as well as the Genomics of Drug Sensitivity in Cancer project (GDSC) are dedicated to studying the relationship between gene expression and drug sensitivity. Combined these panels represent hundreds of cell lines of varying tumor types and over 100,000 compounds used for drug screening (Shoemaker, 2006; Yang et al., 2013). The establishment of a similar canine cancer cell line panel is underway but in terms of panel size and available drug data it is far behind its human counterparts. In order to utilize the canine cancer model in the validation of multi-gene prediction models, the possibility of cross-species testing may be
required. How would this be done? A recent method of prediction model building that has begun to gain traction named the co-expression extrapolation (COXEN) method might be the answer.

In 2007 the COXEN method was described by Lee and Theodorescu (Lee et al., 2007). The original problem they were addressing was how to generate prediction models for drug sensitivity in human bladder tumors. They wanted to utilize the human NCI60 cancer cell line panel and its wealth of drug screening data, but no bladder cancer cell lines were represented. They devised a method where after a gene signature was identified through the utilization of drug and gene expression data in a reference set (NCI60 panel), genes that do not share strong co-expression patterns with a secondary target dataset of interest were filtered out (bladder cancer panel). Determining strong concordant expression between the two datasets involves a series of correlations of the genes of the signature against each other, both separately in each dataset and then between datasets (Lee et al., 2007). The filtering step results in a subset of the gene signature that is both predictive of drug sensitivity and compatible with the target dataset, and is used in prediction model building for datasets similar to the target dataset. This method has been successful in predicting drug sensitivity in cell lines and/or clinical outcome in cancer patients for bladder, ovarian, breast, and non-small cell lung cancer (Ferriss et al., 2012; Kim et al., 2014b; Lee et al., 2010; Lee et al., 2007; Nagji et al., 2010; Shen et al., 2012). COXEN has utility for in silico screens for potential anti-cancer compounds, and has been used to identify drugs with high-predicted activity against human bladder cancers (Smith et al., 2011). It has also been utilized to predict radiosensitivity in head and neck squamous cell carcinomas (Williams et al., 2011). One study used the COXEN method to predict the risk of atherosclerosis in different types of leukocytes by first identifying a risk signature from monocyte data of a high risk
population followed by 3 separate co-expression steps with different leukocyte datasets (macrophages, circulating T cells, and whole white blood cells), eventually leading to 3 tailored prediction models for risk in each cell type (Cheng et al., 2012). These examples illustrate the versatility and robustness of this method. A clinical trial is currently underway investigating the ability of COXEN models to predict complete pathological response between two highly used combinations of neo-adjuvant chemotherapy for bladder cancer (SWOG, S1314) (Dinney et al., 2014). Could the COXEN method be used in a cross-species manner to develop models of drug sensitivity from human reference sets that would be predictive in canine cancer? Could future testing of the COXEN method in a veterinary clinical trial setting serve as pre-clinical validation for human COXEN clinical trials?

**PROJECT RATIONALE**

Cancer is the second leading cause of death in humans and the leading cause of death in adult dogs. Significant advancements in cancer research through the years have most often begun with *in vitro* studies via the utilization of cancer cell tissue culture. Traditionally cancer cell lines have been utilized to study processes of cancer biology as well as drug screening for novel anti-cancer agents. They also play a major role in *in vivo* xenograft tumor model studies in rodents. Recent advancements in cancer genomics has resulted in increased understanding of genetic heterogeneity of tumors, a phenomenon that is also reflected in tumor-derived cell lines. Understanding these genetic changes and their effects on cancer biology and therapeutic response is a major area of cancer research, in which cancer cell lines continue to be invaluable tools. Additionally, these genetic advancements in both human and canine cancer have revealed
a high degree of genetic similarity between the species, bolstering the emerging strategy of using companion animal models of cancer for translational research.

There are several publicly available human cancer cell line panels used currently that strive to address the relationship between genetic alterations in cancers and drug sensitivity. They represent a wealth of information advantageous for researchers of human cancer. Unfortunately, similar cell line panels for canine cancer have not been established to such a degree. This led us to our hypothesis that canine cancer cell line panels can be useful in informatics approaches for comparative and translational oncology. We tested this hypothesis in Chapter 2 (The Flint Animal Cancer Center (FACC) canine tumor cell line panel: A resource for veterinary drug discovery, comparative oncology, and translational medicine).

This chapter describes the characterization of a panel of 29 validated canine cancer cell lines representing multiple tumor types that is currently being used at the FACC. Characteristics of the cell lines include tumor type information, cell line origins, and xenograft potential. In this chapter we also describe and present our cell validation procedures using microsatellite analysis to confirm the genetic fingerprint of each cell line. Initial drug screening data with common chemotherapeutics are presented, as well as some molecular characterization via principal component analysis and unsupervised hierarchical clustering. Also described is an example of potential application of the panel by looking at the identification of genes/gene signatures that are predictive of migration/invasion capacity in canine osteosarcoma cell lines.

After describing the genotypic and phenotypic characteristics of the FACC panel, we then endeavoured to utilize the panel to study the relationship between gene expression and drug sensitivity between human and canine melanoma. Interestingly, the majority of canine melanomas in the FACC originate from oral sites, whereas most of the recent advancements in
pathway targeting of human melanoma is based on somatic mutations in BRAF, which are predominantly observed in cutaneous melanoma of the skin. The few small studies in canine melanoma to date report no identified BRAF mutations, concluding that these alterations are rare events in dogs with this disease. However, studies have also given evidence that both the PI3K/AKT and MAPK pathways do appear to play some role in canine melanoma. This led us to hypothesize that although malignant melanoma appears predominantly at different anatomical sites in humans and dogs, on a molecular level they would share similar pathway activation and will respond similarly to pathway inhibitors. We tested this hypothesis in Chapter Three (Comparative analysis of MAPK and PI3K/AKT pathway activation and inhibition in human and canine melanoma). This chapter details a comparative pathway analysis we performed between melanoma tumor samples versus normal tissue of both human and dog via microarray gene expression analysis followed by pathway analysis of differentially expressed genes. We found through two separate analyses that the most differentially expressed pathways between tumor and normal samples were shared between species, and that specifically the MAPK and PI3K/AKT pathways showed similar patterns of deregulation in both human and canine samples. Additionally, we report that in an unsupervised cluster analysis of genes of the MAPK and PI3K/AKT pathways that were differentially expressed between tumor and normal samples in both human and dog, human and canine tumor samples interspersed. This chapter also details a comparative analysis of pathway activation and inhibition in human and canine cell lines. Mutational analysis of specific BRAF V600E and NRAS Q61R mutations performed in human melanoma cell lines and FACC canine melanoma cell lines confirmed that these mutations are rare in dogs, suggestive that alternate mechanisms of pathway activation must exist in canine melanoma. Through serum starvation assays, cell cycle analysis and pharmacological
targeting of the PI3K/AKT and MAPK pathways singly and in combination we were able to show that these pathways are constitutively active and respond similarly to pathway inhibition in both species.

The results of chapter 3 were encouraging for the application of molecularly targeted drugs in canine cancer. However, the use of cytotoxic chemotherapy remains the standard for many tumor types both in human and dogs. In human osteosarcoma, for example, strategies involving neoadjuvant chemotherapy followed by surgery and adjuvant chemotherapy that has led to near 70% of patients achieving disease free survival at 5 years, with the majority of these patients essentially “cured” after 20 years. In contrast, 50% of dogs with osteosarcoma receiving amputation and the adjuvant chemotherapy doxorubicin and/or carboplatin don’t survive 1 year. Non-pathway focused genomic approaches have been developed that use multi-gene prediction models to predict chemosensitivity in cancer cell lines and tumors. One of these methods, called COXEN, has the ability to extrapolate gene signatures identified in a reference set into a target set that does not match histologically. We wondered whether this method would be able to perform in a different species, or more impressively in a cross-species manner. This was exciting to us because of the possibility of developing multi-gene prediction models from our canine FACC panel. Additionally, the idea of testing this method in a cross-species manner between human and canine datasets could potentially allow canine cancer researchers access to the wealth of available human datasets.

These ideas led us to the hypothesis that the COXEN method can be utilized in an intra- and interspecies manner to predict doxorubicin and/or carboplatin sensitivity in canine osteosarcoma. This hypothesis was tested in Chapter 4 (Gene expression models for predicting drug response in canine osteosarcoma). In this chapter we selected and ran
microarray analyses on 33 canine osteosarcoma tumor samples from the FACC archives, as well as obtain publicly available microarray data on an additional 16 tumor samples with known clinical outcome after treatment with amputation followed by doxorubicin and/or platinum agents. We showed that gene signatures for doxorubicin sensitivity identified from either the human NCI60 cell line panel or our FACC panel were able to successfully sort poor and good responders in tumor samples after unsupervised clustering. We also detail our results of several iterations of prediction model building for doxorubicin and carboplatin sensitivity in FACC cell lines as well as canine osteosarcoma tumors. Our data show that predictive models built across species can effectively predict clinical outcome in dogs with bone tumors, suggesting that chemotherapy protocols can be improved in canine osteosarcoma by implementing genomic methods in order to predict drugs with the most benefit for the individual patient.

The main goal of this project was to show the utility of the FACC panel in comparative and translational studies in cancer. Hopefully this type of research can lead to a closer “marriage” of human and canine oncology, where studies in either species can be viewed as beneficial and translatable to the other. Canine oncology has much to benefit from the recent strides that are happening today in human cancer research. Alternatively, human studies could be further improved on by implementing canine cancer models for pre-clinical validation, a strategy that could potential save a lot of time and money in the long process of developing new therapeutic strategies.
REFERENCES


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CHAPTER 2

The Flint Animal Cancer Center (FACC) Canine Tumor Cell Line Panel: A Resource for Veterinary Drug Discovery, Comparative Oncology and Translational Medicine

SUMMARY

Mammalian cell tissue culture has been a critical tool leading to our current understanding of cancer including many aspects of cellular transformation, growth, and response to therapies. The current use of large panels of cell lines with associated phenotypic and genotypic information now allows for informatics approaches and \textit{in silico} screens to rapidly test hypotheses based on simple as well as complex relationships. Current cell line panels with large amounts of associated drug sensitivity and genomics data are comprised of human cancer cell lines (i.e. NCI60, GDSC). Canine cancer is becoming increasingly recognized as an important contributor to cancer research as a spontaneous large animal model with application in basic and translational studies. We have assembled a panel of canine cancer cell lines to facilitate studies in canine cancer and report here phenotypic and genotypic data associated with these cells.

INTRODUCTION

Mammalian cell tissue culture is invaluable for studying biological processes and is a fundamental tool used in many laboratories worldwide. The practice of maintaining mammalian cells in a culture system began in the early 20\textsuperscript{th} century (Harrison, 1910) with these techniques

\footnote{Thanks to Dan Gustafson, Dawn Duval, Doug Thamm, Deanna Dailey, and Laird Klippenstein for contributions.}
quickly being utilized to study the growth of cancers \textit{in vitro} including a breast adenocarcinoma from a dog (Carrel and Burrows, 1911). By 1950 the use of animal cell culture had become routine, and in 1951 the HeLa cell line was established, the first human cell line developed from a cancer patient (Scherer \textit{et al.}, 1953; Sharma \textit{et al.}, 2010). In vitro studies using cancer cell lines play a large role in cancer drug discovery and development, providing crucial data on drug effects and cancer biology in the early pre-clinical stages, many of which would be unethical to explore in patients. This information is key in the decision process for drugs moving forward into expensive and time consuming clinical trials (Weinstein, 2012).

The emergence of new genomic technologies in the last decade has revolutionized cancer research and revealed to researchers that genetic heterogeneity is inherent across the whole human tumor population as well as within histological tumor types. Importantly, this heterogeneity is highly similar between primary tumors and tumor-derived cell lines according to multiple studies including breast cancer, melanoma, and non-small cell lung cancer (Lin \textit{et al.}, 2008; Neve \textit{et al.}, 2006; Sos \textit{et al.}, 2009). This has led to renewed interest in creating cancer cell line panels as a model system to further explore genetic effects on cancer biology and therapeutic response (Sharma \textit{et al.}, 2010). The most well known human cancer cell line panel dedicated to this purpose was developed by the National Cancer Institute (NCI60 panel), consisting of 60 cell lines of various tumor types that has been used to screen over 100,000 compounds for anti-cancer activity (Shoemaker, 2006). The panel has also undergone expression profiling at the DNA, RNA, protein, and chromosomal levels (Weinstein \textit{et al.}, 1997).

More recently cell line panels from the Genomics and Drug Sensitivity in Cancer project (GDSC) and the Broad Institute\textquoteleft s Cancer Cell Line Encyclopedia (CCLE) have been established consisting each of around 1000 cell lines. These panels have been screened against 138 and 24
cancer drugs, respectively (Barretina et al., 2012; Yang et al., 2013b). Gene expression, chromosomal copy number and sequencing data are available for the CCLE, whereas generated genomic data for the GDSC panel includes data on gene expression, point mutations, gene amplifications and deletions, sites of microsatellite instability, and DNA rearrangements (Barretina et al., 2012; Yang et al., 2013b). Fortunately, unique genomic data from these large cell line panels can be shared for 496 cell lines that overlap CCLE and GDSC panels, and 55 cell lines of the NCI60 that are found on either the CCLE or the GDSC panels. In order to better translate discovered genetic relationships of drug response from cell lines to tumors, available genomic resources such as the NCI’s Cancer Genome Atlas (TCGA) have been established containing exon and whole genome sequencing as well as gene expression data for thousands of tumor samples representing 33 tumor types (Cancer Genome Atlas Research Network, 2011). These resources are invaluable for the development of more personalized therapeutic strategies for the treatment of cancer.

Similar cancer cell line panels for canine cancer at such a scale are currently non-existent. Small collections of canine cancer cell lines exist at various institutions but the range of data is often limited. The purpose of this paper is to describe the first diverse canine cancer cell line panel of its kind, comprised of 29 validated cell lines representing multiple tumor types. Herein we will report the characteristics of the Flint Animal Cancer Center (FACC) panel and the accompanying genomic profiling that have been performed as well as its potential applications for comparative and translational oncology.
MATERIALS AND METHODS

Cell culture

FACC cell lines were acquired from other institutions, purchased from the American Type Culture Collection (ATCC), or established from tumor samples from the FACC archive (see Table 2.1). During cell viability assays all cells were maintained in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100ug/mL) and incubated at 37°C in a humidified atmosphere of 5% CO₂:95% air.

Cell line validation

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen) from 1-5 x10⁶ cells. A multiplex PCR reaction was conducted on 50-400 ng of isolated genomic DNA to confirm the species of origin of each cell line as previously described (Cooper et al., 2007). Upon confirmation as a canine cell line, each line was subsequently analyzed by short tandem repeat profiling using the Canine Stockmarks Genotyping Kit (Life Technologies) per the manufacturer’s protocol and as previously described (O'Donoghue et al., 2011a). The PCR products were analyzed via capillary electrophoresis as follows: 1.5 μl of diluted PCR product (1:5 to 1:10 dilution) was mixed with 0.5 μl size standard and 10 μl of highly deionized formamide. Samples were run using POP7 polymer and the array length was 50 cm. Run conditions were identical to the default run module except injection time was increased from 15 to 24 sec, and scan number was shortened from 1,800 scans to 1,750. Fragment lengths for each locus were recorded and for ease of analysis rounded to the nearest common allelic size whole number. This information is maintained in an excel datasheet that can be sorted by allelic size to
ease comparisons between cell lines. Where possible cell line duplicates from multiple laboratories were analyzed to confirm identity.

**Microarray gene expression analysis**

RNA was extracted from cell lines using the RNeasy kit (Qiagen, Valencia, CA) according to manufacturer’s protocol. A DNA digestion step was included using the RNase-Free DNAs Set to ensure RNA purity (Qiagen, Valencia, CA). Yield and integrity of RNA was examined via a NanoDrop 1000 spectrophotometer (Thermo Scientific, Asheville, NC) and a Bioanalyzer (Agilent, Sanat Clara, CA). Samples were hybridized onto both Affymetrix GeneChip Canine Genome 2.0 arrays and the Canine Gene 1.0 ST arrays at the Genomics and Microarray Core at the University of Colorado Denver. Resulting CEL files were then imported into Bioconductor (Gentleman et al., 2004) and intensity values were preprocessed with the Robust Multi-Array Average (RMA) algorithm.

**Unsupervised clustering and principal component analysis**

Affymetrix Canine 2.0 Microarray Gene Expression data was processed using RMA and the log 2 transformed gene expression data was ranked based on the standard deviation of each gene across all sample sets. The top 100 most variant genes were selected for unsupervised clustering using the CIMminer website (http://discover.nci.nih.gov/cimminer/). Gene expression data was also sorted for the 522 cancer genes currently annotated in the Cosmic Cancer Gene Database (http://cancer.sanger.ac.uk/cosmic). These cancer genes were then ranked based on the standard deviation of each cancer gene across all samples to identify the most variant genes. The top 100 most variant cancer genes were used in unsupervised clustering to generate heatmaps using CIMminer. Euclidian distancing and average linkage were the
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1 ATCC, American Type Culture Collection; UWM, University of Wisconsin-Madison; CSU, Colorado State University; UCSF, University of California-San Francisco; Tokyo, University of Tokyo; WSU, Washington State University; AU, Auburn University; PU, Purdue University; Upenn, University of Pennsylvania; OSU, The Ohio State University; Aus, Veterinary University of Austria; UI, University of Illionois at Urbana-Champaign.
parameters chosen for the analysis. Principal components analysis was conducted on the whole gene expression data set and a graph of the first 2 components was generated using Bioconductor.

**Cell viability assays**

Drug sensitivity data was generated via a resazurin-based bioreductive fluorometric assay (Alamar Blue) for cisplatin (CIS), carboplatin (CARBO), doxorubicin (DOX), lomustine (LOM), paclitaxel (PTX) and vinblastine (VBL) in the FACC panel as follows: Cells were plated in 96 well plates at a density of 1500 – 5000 cells in 100 uL per well, depending on growth rate. 24 hours after initial plating, serial doses of the drugs in 100 uL of media were added to the plates, including vehicle control wells and blank wells with only media. Drug incubation lasted 48 hours after which old media containing drug was replaced with 200 uL fresh media, and 20 uL of Alamar Blue solution (200 ug Resazurin Salt/ml in PBS) was added to each well. Following 2-4 hours of incubation, fluorescence was measured on a 96 well plate reader with emission wavelength parameters of 530 and excitation of 590. Experiments were performed at least in triplicate, and medial dose (Dm) values were calculated.

**Cell transfections**

8 osteosarcoma cell lines (HMPOS, OSA8, Gracie, Abrams, Moresco, McKinley, Vogel, and D17) were stably transfected with the lentiviral-based vector CellPlayer™ NucLight Red (Lenti, EF-1 alpha, puro) (Cat # 4476, Essen BioScience, Ann Arbor, MI) according to manufacturer protocols. Transfected cells were selected in 2ug / mL of puromycin, followed by 1ug/mL for maintenance. Transfected cells were maintained with Corning’s DMEM/F12 50/50 medium (Mediatech, Manassas, VA) with L-glutamine and added penicillin/streptomycin (1%), sodium pyruvate (1%), and fetal bovine serum (10%).
**Migration and invasion assays**

Migration and invasion assays of 8 osteosarcoma cell lines transfected for red nuclear fluorescence were performed with the Incucyte Zoom® live cell imaging system and accessories according to manufacturer protocols (Essen Bioscience, Manasses, VA). Briefly, for the migration assay cells were plated in 6 replicates in a range of cell plating densities (20,000, 25,000, 30,000, and 35,000 cells/well). 18 hours after plating uniform “wounds” were made in each well using the WoundMaker™ tool followed by rinsing off the dislodged cells and replenishing with 100 uL of media in each well. Plates were then placed in the Incucyte Zoom machine inside the incubator, and images of each well were scanned every 2 hours for a total of 48 hours.

For invasion assays, 96 well plates were coated with a thin layer of Matrigel (ref # 356234, Corning) overnight, followed by plating of each cell line in 4 replicates at 35,000 cells/well. Twenty hours after plating, wounds were made followed by the addition of 50 uL of Matrigel into each well. After Matrigel solidified 100 uL of media was added to each well, and the plate was placed in the Incucyte Zoom for incubation. Scans were made every 3 hours for 24 hours. Plates and materials were kept cold during the addition of Matrigel. Analysis was performed using Incucyte software.

**Differential expression analysis and pathway analysis**

For the osteosarcoma study the Canine Gene 1.0 ST array CEL files for the 8 osteosarcoma cell lines from the FACC that have been transfected to display red nuclear fluorescence were uploaded into Bioconductor and preprocessed by RMA. Absent probeset IDs (PSIDs) were filtered out and then samples were grouped according to their migration and invasion capacity. The “fast” migration/invasion group was Gracie, McKinley, and Vogel cell
The “slow” migration/invasion group was Moresco, HMPOS, and D17. The intermediate group, OSA8 and Abrams, were left out of further analysis. Differential gene expression analysis was performed via the Linear Models for Microarray Data (Limma) package in R using p-values < 0.001 and an absolute log fold change > 1 as cutoffs. Resulting DEGs were then used for pathway analysis using Enrichr (http://amp.pharm.mssm.edu/Enrichr/).

RESULTS

Cell Line Validation

Tumor-derived canine cancer cell lines have been established or acquired from multiple sources over the years at the Flint Animal Cancer Center (FACC) at Colorado State University. Currently, our panel consists of 29 cell lines representing 11 different tumor types: 10 osteosarcomas, 5 melanomas, 2 mammary carcinomas, 1 hemangiosarcoma, 2 bladder carcinomas, 3 lymphomas, 1 leukemia, 1 mast cell tumor, 2 histiocytic sarcomas, 1 thyroid carcinoma and 1 soft tissue sarcoma. Cell line names, origins, and known xenograft potential of the “FACC panel” are described in Table 2.1. All of the cell lines were validated previously as being canine of origin (O'Donoghue et al., 2011a). To address the possibility of cross-contamination and uniqueness of the individual lines if the FACC panel, short tandem repeat (STR) analysis was used for further validation. The fragment size rounded to the nearest common whole number at each allele from 10 loci is listed in Table 2.2. When possible, cell lines obtained from multiple laboratories were assessed to confirm the identity of a given cell type, however source genomic material for these lines was not available. Based on this analysis, the genetic identity of 29 cell lines was confirmed. The Fitz and Den-HSA hemangiosarcoma
### Table 2.2 Allelic sizes of the commonly used cell lines as determined using the Canine Stockmarks Genotyping Kit.

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<th>FHC 2010 (210-260 bp)</th>
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<th>PEZ 3 (95-154 bp)</th>
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a Allele sizes are rounded to the nearest common allelic size whole number in order to ease comparisons between samples.

b Positive control represents a canine genomic DNA sample included in the Stockmarks kit.
cell lines were found to be genetically identical (O'Donoghue et al., 2011b), as were the MH and Nike histiocytic sarcoma cell lines. In addition, the CMT12 and CMT27 canine mammary cell lines exhibited conserved profiles at 8 of the 10 loci and 90% homology overall suggesting the potential for a common source for these cell lines (O'Donoghue et al., 2011b).

**Molecular characterization of cell lines**

In order to characterize the FACC panel on a molecular level and to facilitate future genomic studies, all 29 cell lines have undergone molecular profiling on the mRNA expression level. The Affymetrix GeneChip Canine Genome 2.0 array contains over 43,000 probesets mapping to over 20,000 genes. In contrast to the 3’-biased probesets of the 2.0 array, the FACC panel has also recently been profiled with the Canine Gene 1.0 ST arrays which performs a whole-transcriptome analysis with over 195,000 probesets spread out across each exon of the genes. Additionally, microRNA expression has been profiled in the panel using Affymetrix GeneChip miRNA 4.0 arrays which contain all microRNA in the miRBase Release 20 (www.affymetrix.com). To show that the two types of mRNA gene expression arrays were complementary with each other in the FACC panel, correlations were performed between both the Canine 2.0 and 1.0 ST arrays for a selection of known cancer genes in each cell line. We observed an average correlation coefficient of 0.6905 for all 29 cell lines (p < 1.0E-7), which was highly significant (Figure 2.1). It is important to note that the extracted RNA used for microarray analysis on both platforms was performed in different labs in the FACC at different times, suggestive of strong conservation of genotypic features in these cell lines.

Histological characteristics are routinely used by pathologists for distinguishing different tumor types from each other, and gene expression patterns can be used in similar ways for unsupervised hierarchal cluster analysis. Using the 2.0 expression data, the first 2 principal
Figure 2.1 Correlations of cancer genes between Canine 2.0 and 1.0 ST arrays. Gene expression from 319 selected cancer genes were correlated between to Affymetrix microarray platforms (Canine 1.0 ST array data on x-axis, Canine 2.0 array data on y-axis) in the FACC panel. Correlation coefficients for each cell line was averaged.

components from the PCA analysis of the gene expression data were plotted with PC1 on the x-axis and PC2 on the Y-axis (Figure 2.2). The distribution of the samples in this graph is in roughly 3 groups. 6 of the 7 hematopoietic cell lines, including the CLBL1, CLL1390, 1771, OSW, C2, and Nike cell lines, are distributed across the top of the graph. A group on the lower right side of the graph contains 8 of the 10 osteosarcoma cell lines and the Bliley transitional cell
carcinoma line. Above this group and to the left is a group containing the other 2 osteosarcoma cell lines that were derived from metastatic tumors, Abrams and D17; the melanoma cell lines, the mammary carcinoma cell lines, one histiocytic sarcoma cell line DH82, and 3 soft tissues sarcomas including the 2 hemangiosarcoma cell lines that were genetically identical based on STR analysis. To further assess the defining gene expression characteristics of each of these cell lines, a cluster analysis heat map was generated using the top 100 most variant genes.
This unbiased cluster analysis separated the cell lines into 2 primary groups: The first group located at the top of the heat map contains 6 hematopoietic cell lines: OSW, Nike, C2, CLL1390, 1771, and CLBL1. Interestingly, the DH82 histiocytic cell line was placed in the other large group, but separated out with the Gracie osteosarcoma cell line and the CML10C2 melanoma cell line. The remainder of the cell lines was clustered into 2 secondary groups. The first group comprised of the next 7 cell lines as listed on the left axis of the figure contains primarily carcinoma cell lines: K9TCC; CMT12, adjacent to CMT27; Bliley, and CTAC (thyroid carcinoma) In addition, the juxtaposed DEN and Fitz hemangiosarcoma cell lines are a subgroup within this branch. The final group of 13 cell lines is dominated by 9 osteosarcoma cell lines, the STSA-1 soft tissue sarcoma cell line, and 3 melanoma cell lines. The genes dictating these groups are shown along the bottom of the figure. Elevated expression of cell-type specific markers such as lymphocyte cytosolic protein 1 (LCP1), cytokeratins (KRT8 and KRT18), epithelial cell adhesion molecule (EPCAM), and collagen, type I, alpha 1 (COL1A1) contribute primarily to the separation of the various cancer cell histiotypes. In order to explore the alterations in gene expression that contribute to the unrestrained growth of these cell lines, the gene expression data was sorted for the 522 genes identified as contributing to the development and progression of various cancers in the COSMIC Cancer Gene Census. The genes were once again ranked according the standard deviation of each cancer gene across all samples to identify the most variant genes and cluster analysis of the 100 most variant cancer genes was generated (Figure 2.4). As previously, the unbiased cluster analysis separated the cell lines into 2 primary groups: the hematopoietic cell lines at the bottom of the figures and a large grouping containing a combination of the carcinomas and sarcomas at the top of the figure. As before, these cell lines are separated into 3 subgroups; the first containing the mammary carcinomas, hemangiosarcomas
Figure 2.3. Cluster analysis using the Top 100 most variant genes separates the samples into groups with similar histiotypes. Affymetrix Canine 2.0 gene array data was ranked based on the standard deviation of each gene across all samples and nonbiased cluster analysis of the 100 most variant genes was performed using CIMminer.
and the K9TCC and Bliley transitional cell carcinomas. The next subgroup is a combination of the sarcoma and melanoma lines, as well as the thyroid carcinoma cell line. One notable exception is the Gracie osteosarcoma cell line which was separated from all the other sarcoma and carcinoma cell lines. These separations are again largely due to the overexpression of cell-specific markers that have been shown to contribute to cancer development including \textit{COL1A1} and \textit{LCP1}. In addition, examination of the generated heat map does provide potential evidence of genetic drivers of cancer development. For example, elevated expression of \textit{KIT} is observed in the C2 and 1771 cell lines. In addition, the C2 cell line carries an activating mutation in the \textit{KIT} gene (Halsey \textit{et al.}, 2014). Decreased expression of PTEN is observed in the CMT12 and 27 mammary cell lines, the HMPOS, Abrams, and OSA8 osteosarcoma cell lines, and the CLL1390 and DH82 cell lines. A smaller decrease in expression is also observed in the OSW cell line. Previous studies using FISH analysis of the CLL1390 cell line indicated complete loss of the PTEN gene (Seiser \textit{et al.}, 2013). Similarly, deletions of the region in chromosome 26 containing the PTEN gene have been observed in 40.7% of canine histiocytic sarcomas (Hedan \textit{et al.}, 2011) and 30% of canine osteosarcomas (Angstadt \textit{et al.}, 2011). Interestingly, the canine mammary carcinomas both appear to over-express epidermal growth factor receptor (EGFR). The Gracie osteosarcoma cell line is clustered separately from the other osteosarcoma cell lines. Examination of the Gracie cell line gene expression profile reveals the elevated expression of ETV1, notable for its fusion to the EWS gene in human Ewing’s sarcoma and TMPRSS2 in prostate cancers (Oh \textit{et al.}, 2012). This cell line also exhibits reduced expression of several markers elevated in the majority of the osteosarcoma cell lines including: \textit{COL1A1}, \textit{FGFR2}, and \textit{CDH11}.
Figure 2.4. Cluster analysis using the Top 100 most variant cancer genes separates the samples into groups with similar histiotypes and may identify critical genetic drivers. Affymetrix Canine 2.0 gene array data was sorted for the 522 cancer genes currently annotated in the Cosmic Cancer Gene Database. These cancer genes were then ranked based on the standard deviation across all samples and nonbiased cluster analysis was performed using CIMminer.
Pharmacologic screening

Currently, there are 3 cancer drugs approved by the FDA in canine oncology. Palladia and the conditionally approved masitinib are indicated for the treatment of canine mast cell tumors (Food and Drug Administration, 2009; Heflin, 2011). Paccal Vet-CA1 is conditionally approved to treat canine head and neck squamous cell carcinoma and mammary carcinoma (Oasmia Pharmaceutical AB, 2014). Many drugs commonly used to treat dogs with cancer are considered “off-label”. One of the purposes of this panel is for drug screening in order to identify beneficial pairings of human-approved or novel therapeutics with a given tumor type in canine cancer. Cytotoxic chemotherapy is commonly used in the treatment of canine cancers, so we have begun to screen the FACC panel with these drugs. In Figure 2.5 the drug sensitivity data in the FACC panel is compared to the human NCI60 cancer panel to 6 chemotherapeutics: cisplatin (CIS), paclitaxel (PTX), lomustine (CCNU), vinblastine (VBL), carboplatin (CARBO), and doxorubicin (DOX). Statistical testing revealed that with the exception of CIS, the means of the drug sensitivity ranges between human and canine panels were significantly different from each other. Additionally, the variances in the data were also significantly different for CARBO and PTX. Overall, however, the patterns of drug sensitivity and variances observed for each agent in both human and canine panels shared general trends, and the mean Log GI50 values for each agent showed a significant cross-species correlation (r = 0.88, p = 0.0194, Pearson). These data suggest that human and canine cancer cell lines respond in similar ranges to cytotoxic agents.

Differential gene expression and pathway analysis for osteosarcoma cell lines with high migration/invasion capacity

Canine osteosarcoma is a highly aggressive tumor that is rarely curable even after limb amputation and adjuvant chemotherapy due to its propensity for early metastasis (Dernell, 2007).
Studies have identified genes that are associated with metastasis by profiling samples of primary tumor and tumor metastases. For example, high expression of ezrin, a protein that is important at the plasma membrane and cytoskeleton interface, has been associated with early development of metastasis in canine osteosarcoma tumors (Khanna et al., 2004). Interestingly, in a xenograft study determining the effect of transplantation site on tumor growth and metastasis of canine osteosarcoma cell lines, the intratibial injected tumor cells had greater metastatic potential and exhibited the highest expression of ezrin (Jaroensong et al., 2012). In order to identify a gene signature predictive of migration and invasion capacity, we performed scratch wound migration and invasion assays using the Incucyte Zoom® live cell imaging system in 8 osteosarcoma cell lines of the FACC panel followed by differential gene expression analysis. The cell lines were

Figure 2.5 Human and canine cancer cells are similarly sensitive to chemotherapy. GI50 ranges of the human NCI60 panel to 6 chemotherapeutics were compared to the ranges generated in the canine FACC panel via Alamar Blue assays.
stably transfected with a nuclear red fluorescence marker to allow for automated cell counting for analysis. We observed a stratification of cell lines in both migration and invasive capacity 24 hours post scratch, where the wounds were closed in certain some cell lines but not all (Figure 2.6). The percentage of cells in the wound was calculated over time, and area under the curve was calculated for all cell lines (Figure 2.6). We observed that the top 3 cell lines with the best ability to fill in the wound in both the migration and invasion assays were the Gracie, McKinley, and Vogel cell lines. Interestingly, although all 3 of these lines had nearly identical migration capacity, Gracie was superior in invading through matrigel (Figure 2.6). The 3 cell lines that were in the bottom 4 of both assays were D17, Moresco, and HMPOS cells. In both assays there was a cell line with intermediate capacity: OSA8 cells for migration, and Abrams for invasion.

From this data we selected 2 groups for differential gene expression analysis: the “fast” cell lines Gracie, McKinley, and Vogel versus the “slow” cell lines D17, Moresco, and HMPOS. Limma analysis was performed and a combination of p-value <0.001 and an absolute log fold change > 1 were used as cutoffs for significantly different genes. This resulted in the identification of 184 PSIDs mapping to 70 differentially expressed genes (DEGs) as shown in Table 2.3. Several of the DEGs encode proteins with known functions involved in cell adhesion, migration, and cellular signaling. Studies have associated some of these proteins with metastasis, Phosphatase, Receptor Type, K (PTPRK), and secreted fizzled-related protein 4 (SFRP4). EphA2, an ephrin receptor, has been shown to play a role in metastasis in human renal cell carcinoma, head and neck squamous cell carcinomas, thyroid, prostate, and gastric cancers (Chen et al., 2014a; Chen et al., 2014b; Li et al., 2014; Liu et al., 2012; O'Malley et al., 2012). EphA2 has also been suggested to play a role in metastasis of human osteosarcoma (Fritsche-Guenther et al., 2010). Targeting Flt1, a VEGF receptor, with an anti-Flt1 peptide has been shown to inhibit
Figure 2.6 Migration and invasion of osteosarcoma cell lines. Scratch wound assays with or without Matrigel (for invasion and migration, respectively) were performed with 8 osteosarcoma cell lines and live images were recorded periodically over 24 hours. A) images of migration assay at 0 and 24 hours for a “fast” and “slow” cell line. B) images of invasion assay at 0 and 24 hours for a “fast” and “slow” cell line. C & D) Area under the curve measurements are compared for each cell line for the migration (C) and invasion (D) assay.
Table 2.3 Differentially expressed genes between fast and slow migration/invasion osteosarcoma cell lines.

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<td>0.000570</td>
<td>APC</td>
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* multiple probesets identified for this gene in the analysis; FC=Fold Change
tumor growth and metastasis in VEGF-secreting cancer cell xenografts (Bae et al., 2005). Additionally, Flt1 has been identified as a biomarker for tumor cell spread of primary ovarian cancer to the bone marrow, and its increased expression is correlated with decreased disease-free survival (Wimberger et al., 2014). PTPRK, the top DEG from our analysis, has been reported to be involved in adhesion, invasion, and metastasis in breast cancer cells and rhabdoid tumors (Gadd et al., 2010; Sun et al., 2013). SFRP4 is a soluble modulator of Wnt signaling that is lost in more aggressive ovarian cancers and has been shown to inhibit metastasis in prostate cancer (Ford et al., 2013; Horvath et al., 2007). Upregulation of SFRP4 has also been associated with cell motility in canine mammary adenocarcinoma lung metastases (Krol et al., 2010).

To determine which pathways are most enriched by our identified list of DEGs, we performed pathway analysis using the web tool Enricher (http://amp.pharm.mssm.edu/Enrichr/). Enricher has the capability to compare our DEG list against a collection of gene sets obtained from multiple sources including KEGG, WikiPathway, and Reactome databases. Pathway analysis results are shown in Table 2.4. Selecting only those pathways that were significantly enriched with our DEG list, we saw the majority of results were involved in focal adhesion and extracellular matrix interactions. Additionally, pathways for ion channel transport and the activation of complement pathway proteins were highlighted. Interestingly, gene sets that arose from experimental kinase perturbations identified 6 kinases whose list of affected proteins significantly overlapped our list of DEGs: Kinase suppressor of Ras 1 (KSR1), fibroblast growth factor receptor 1 (FGFR1), bone morphogenetic protein receptor, type II and IA (BMPR2 and BMPR1A), and the V-Erb B2 avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2). FGFR1 has been implicated in promoting invasion of non-small cell lung cancer, Ewing’s sarcoma, and breast and prostate cancers.
## Table 2.4 Differentially expressed pathways between fast and slow migration/invasion osteosarcoma cell lines.

<table>
<thead>
<tr>
<th>Pathway/Gene set term</th>
<th>Adj. P-value</th>
<th>Z-score</th>
<th>Genes</th>
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<td>Small cell lung cancer</td>
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<td><strong>WikiPathways 2015</strong></td>
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<tr>
<td>Focal Adhesion (Homo sapiens)</td>
<td>0.027546168</td>
<td>-2.107929051</td>
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<td>Focal Adhesion (Mus musculus)</td>
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<td>-2.106479856</td>
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<td>Iron uptake and transport (Homo sapiens)</td>
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<td>-1.809175801</td>
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<td><strong>Reactome 2015</strong></td>
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<tr>
<td>Ion channel transport</td>
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<tr>
<td>Stimuli-sensing channels</td>
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<td>Laminin interactions</td>
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<td>-1.986561131</td>
<td>LAMA2; ITGA3; ITGA2</td>
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<tr>
<td>Activation of C3 and C5</td>
<td>0.02183961</td>
<td>-1.013182587</td>
<td>CFB; C2</td>
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<tr>
<td><strong>Kinase Perturbations from GEO</strong></td>
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<tr>
<td>KSR1</td>
<td>0.010257433</td>
<td>-1.85901876</td>
<td>COL4A2; ANXA3; ITGA3; OGN; CYTIP; CLCN3; EPHA2</td>
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<td>FGFR1</td>
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<td>-1.567057353</td>
<td>STEAP3; SLCO4A2; FAP; UBA7; LAMA2; OSBPL3; MS4A7; RGS10; TRIM14; CYTIP; PARP8</td>
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<tr>
<td>BMPR2</td>
<td>0.010257433</td>
<td>-1.163566117</td>
<td>BBS2; DOCK3; STEAP3; FLT1; SFMBY2; L1CAM; C2; SEC14L2; ARHGAP31; COL4A2; MEP1B; RGS10; CYTIP; SULT4A1; CFB; EPHA2</td>
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<tr>
<td>BMPR1A</td>
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<td>-1.017027569</td>
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<tr>
<td>ERBB2</td>
<td>0.017952662</td>
<td>-0.88633252</td>
<td>DOCK5; SLCO4A2; PFKRTK; FBLIM1; MS4A7; DYSF; ABRACL; CLCN3; ANXA3; ITGA3; VDR; ITGA2; NGDN; CRIM1; L1CAM; VAX2; SFRP4; ARHGAP31; FAP; COL4A2; PSA1; PXDN; RGS10; TRIM14; EPHA2; PTPN3</td>
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</tbody>
</table>
(Kamura et al., 2010; Wendt et al., 2014; Yang et al., 2013a; Zhao et al., 2015). BMPR2 and KSR1 have been associated with breast cancer metastasis (Owens et al., 2012; Salerno et al., 2005). ERBB2 has been associated with osteosarcoma prognosis, but there are conflicting reports (Gorlick et al., 2014; Liu et al., 2014). These data suggest potential targeting strategies some of which have not been considered before for canine osteosarcoma.

**DISCUSSION**

Since the early 1900’s, tissue culture cell lines from canine tumors were starting to become established. A recent Pub Med search using the terms “canine cancer cell line” retrieved almost 1200 articles. Despite the long history and the substantial quantity of research being performed with canine cancer cells, *in vitro* panels have not yet been developed to the scale that has occurred in human cancer. As a step towards that goal, we have introduced and described a validated panel of 29 canine cancer cell lines collected from multiple sources or established at the FACC at Colorado State University. Individual cell lines in the panel have already been used in several studies across the population of cancer researchers. A Pub Med search using the name of each cell line combined with the canine tumor type as keywords resulted in 94 articles involving one or more of 20 of the 29 cell lines in the FACC panel. In these studies the canine cancer cell lines are used to investigate the effects of genes or drugs or both on different cancer processes.

In this new genomics era for cancer research, combining genotypic, phenotypic, and pharmacologic data to reveal novel relationships has been essential for the many recent discoveries that have culminated in improved clinical outcomes. Our canine panel has
undergone 2 types of mRNA expression profiling, and has been used for screening of several established and novel anti-cancer agents. With these new tools, the possibilities for comparative and translational applications with human cancer research are becoming readily apparent. Dogs with cancer can potentially benefit from new discoveries made in human oncology, and conversely, human research can benefit through the integration of canine cancer models for pre-clinical validation studies.

Currently the size of the FACC panel is relatively small when compared to similar human panel such as the NCI60, the GDSC, or the CCLE. There are challenges inherent to that fact. Although there are 11 different tumor types within the panel, 5 of these types are represented by a single cell line, making it extremely difficult to form experimental conclusions with a large degree of confidence. Indeed, the panel should be considered a “jumping off” point towards further advanced experimentation in a similar way as initial microarray analyses are typically viewed as “hypothesis-generating” experiments. Efforts to increase the size of the panel through the establishment of new cell lines from tumor samples is difficult because of low take rate, the danger of contamination and outgrowth of competing fibroblasts, as well as the obstacle of overcoming anoikis-related cell death after loss of contact with their extracellular matrix (Cheung et al., 2014). A possible solution to this problem for the future would be for a call for a greater collaborative effort across several institutions. The sharing of canine cell line resources has helped the FACC panel grow to what it is today, and the fastest way for future growth would undoubtedly be from an increase of collaborations with fellow researchers that have additional established cell lines to contribute. The formation of the FACC panel is a great step in the right direction towards improving informatics approaches to canine cancer, but it is only one step of many.
Another future direction that we are currently considering is to expand upon our available gene expression profiling to include next generation genomic data such as exome and whole genome sequencing, and array comparative genome hybridization (Array CGH) data. Sequencing would be an invaluable tool in identifying potential mutations and/or deletions in oncogenes and tumor suppressors across the entire genome. Array CGH would allow us to investigate alterations in copy number of genes and their role in canine cancer progression.

In conclusion, we have introduced a new valuable resource for canine cancer studies in the FACC panel. With its potential for testing various cancer processes and pharmacological screening connected with genomic data, we hope it will serve to facilitate studies that can further shorten the gap between human and canine oncology, leading to novel discoveries and better designed treatments for dogs with cancer.
REFERENCES


model for human histiocytic cancer identifies deletion of tumor suppressor genes and highlights influence of genetic background on tumor behavior. BMC Cancer 11, 201.


CHAPTER 3

Comparative Analysis of MAPK and PI3K/AKT Pathway Activation and Inhibition in Human and Canine Melanoma

SUMMARY

The lack of advanced animal models of human cancers is considered a barrier to developing effective therapeutics. Canine and human melanomas are histologically disparate but show similar disease progression and response to therapies. The purpose of these studies was to compare human and canine melanoma tumors and cell lines regarding MAPK and PI3K/AKT signaling dysregulation, and response to select molecularly targeted agents. Pathway activation was investigated via microarray and mutational analysis. Growth inhibition and cell cycle effects were assessed for pathway inhibitors AZD6244 (MAPK) and rapamycin (PI3K/AKT) in human and canine melanoma cells. Human and canine melanoma share similar differential gene expression patterns within the MAPK and PI3K/AKT pathways. Constitutive pathway activation and similar sensitivity to AZD6244 and rapamycin was observed in human and canine cells. These results show that human and canine melanoma share activation and sensitivity to inhibition of cancer related signaling pathways despite differences in activating mutations.

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INTRODUCTION

Human malignant melanoma has a dismal prognosis due to rapid disease progression and resistance to standard therapies, with the last 40 years of research producing minimal improvement in overall survival. Recent advances in immunotherapy and molecular targeted agents are promising, however. In 2011, three new drugs were approved for the treatment of melanoma: PEG-interferon α2b, ipilimumab, and vemurafenib. PEG-interferon α2b is a cytokine shown in a recent phase III clinical trial to be as effective as standard high dose interferon, with easier delivery and less toxicity (Eggermont and Robert, 2011). Ipilimumab, an anti-CLA-4 monoclonal antibody that increases immune response through inhibiting T-cell suppression, was approved for first and second line treatment of advanced melanoma (Eggermont and Robert, 2011; Weber, 2007). Vemurafenib is a selective inhibitor of the V600E mutant of v-Raf murine sarcoma viral oncogene homolog B1 (BRAF), a commonly found mutation in melanoma. Patients treated with vemurafenib in a recent randomized phase III trial experienced improved overall response rate, progression-free survival, and 12 month overall survival rate when compared to patients treated with standard of care dacarbazine (Chapman et al., 2011; Sharma et al., 2012).

Oncogenic BRAF and neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) mutations, resulting in constitutive activation of the RAF/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (RAF/MEK/ERK or MAPK) and phosphoinositide 3 kinase/protein kinase B (PI3K/AKT) signaling pathways, have been documented in 50-60% and 15-20% of human melanomas, respectively (Ascierto et al., 2012; Kelleher and McArthur, 2012). NRAS lies upstream of BRAF and is able to activate both the MAPK and PI3K/AKT
pathways. The fact that mutations in these two genes have been found to be mutually exclusive, combined with evidence that the PI3K/AKT signaling pathway inhibitor PTEN is often deleted alongside mutations in BRAF, suggests that signaling through both the MAPK and PI3K/AKT pathways are highly important in human melanoma pathogenesis (Goel et al., 2006). Agents targeting these pathways have demonstrated promising antitumor activity both in vitro and in vivo, but efficacy seems to be limited by acquired resistance clinically (Livingstone et al., 2010). Although an overall response rate of 80% to vemurafenib in V600E mutant BRAF expressing melanoma patients was reported, almost all patients eventually progressed due to the appearance of refractory disease (Fisher and Larkin, 2012; Roukos, 2011). Multiple mechanisms of resistance have been identified, including isoform switching whereby the targeted protein is replaced in order to maintain signaling in the pathway (Dumaz, 2011). The targeted gene may also escape inhibition through mutation or gene amplification, and crosstalk with or activation of alternate signaling pathways with redundant downstream effects allowing circumvention of drug activity. (Corcoran et al., 2011; Dumaz, 2011). Although it is clear that MAPK and PI3K/AKT pathways remain attractive targets in cancer therapy, therapeutic strategies of inhibition require optimization and assessment of resistance mechanisms for more robust responses to occur.

Highly predictive translational disease models are invaluable tools in cancer drug development. The estimated time to bring a new cancer drug to market is 12-15 years with a cost of 800 million to 2 billion dollars (Gavura, 2011; Masia, 2008) thus necessitating comprehensive and effective pre-clinical screening. Genetically modified mice can be effective for identifying molecular mechanisms involved in melanomagenesis, but typically are not applicable for studying advanced disease and therapeutics (Becker et al., 2010). Xeno-transplantation models are generally better for studying advanced melanoma, but the necessity of
immunodeficient recipients negates a comprehensive assessment of efficacy still. Unfortunately, murine melanoma models used in drug development often generate highly efficacious preclinical results, but in human clinical trials the drugs fail to impress (Becker et al., 2010). Advanced animal models that closely resemble the human disease can provide preclinical data that can improve decisions made early in this lengthy and expensive process, and pet dogs with cancer are being increasingly recognized as an asset to this regard for numerous reasons, including: (I) tumors arise spontaneously and occur at an equivalent or higher rate than in humans (Paoloni and Khanna, 2008; Vail DM, 2004); (II) dogs are biologically and genetically more similar to humans than rodents, and they are exposed to the same environmental factors as their owners (Paoloni and Khanna, 2008; Vail DM, 2004); (III) larger body sizes allow for repeated sample collection from the same animal over the treatment period, including normal tissues and fluids for assessing drug activity as well as multiple tumor biopsies; (IV) the cost of veterinary clinical trials is significantly less expensive than their human counterparts, can occur before Investigational New Drug (IND) application submission and typically study a patient population that is not heavily pre-treated (Paoloni and Khanna, 2008; Vail DM, 2004); and (V) human and canine cancers have been reported to have comparable clinical outcomes to cancer therapy (Paoloni and Khanna, 2008).

Canine melanoma can occur on many anatomic sites with the most clinically significant malignant melanomas occurring in the oral cavity and digit nail beds (Bergman PJ, 2013). Like human melanoma it is a deadly disease with a high propensity for metastasis and poor prognosis when discovered in advanced stages with survival times of dogs with advanced disease being less than 5 months following local excision. Radiation treatment has been reported to modestly extend survival, but canine melanoma has historically been resistant to standard cytotoxic
chemotherapies (Bergman PJ, 2013). Similar to human melanoma, evidence suggests that immunotherapy may be efficacious for canine melanoma, most recently illustrated with the conditional licensing of a DNA-based vaccine, although conclusive results are lacking (Bergman, 2007; Dow et al., 1998; MacEwen et al., 1999). New strategies for combating this highly aggressive cancer in dogs are desperately needed and may provide an avenue for translational studies for therapy of human melanoma.

Mutations in \textit{BRAF} and \textit{NRAS} appear to be rare in canine melanoma (Mayr et al., 2003; Murua Escobar et al., 2004; Shelly et al., 2005). In one study of 16 dogs with melanoma, 2 were found to have activating mutations in \textit{NRAS} at the common 61 codon site (Q61R) (Mayr et al., 2003), and no \textit{BRAF} V600E mutations were found in another 17 canine tumors. However, in this same study constitutive activation of ERK1/2, an important kinase downstream of \textit{BRAF}, was observed in serum starved conditions, implying constitutive activation of ERK signaling through an as-yet undetermined mechanism (Shelly et al., 2005). Additionally, in another study involving 7 canine melanoma cell lines and 31 canine melanoma tumor samples, 50\% had suppression of the PI3K/AKT pathway-associated tumor suppressor PTEN (Koenig et al., 2002). In that same study, p16 was suppressed in 80\% of the samples and p53 was excluded from the nuclear compartment in 78\% of the samples (Koenig et al., 2002). P16 and p53 are tumor suppressors that can regulate cell cycle progression, a downstream effect of multiple signaling pathways including PI3K/AKT and MAPK (Weinberg, 2007a; Weinberg, 2007b). Human melanomas with inactive mutant p53 have a worse prognosis (Ben-Porath and Weinberg, 2005). The loss of tumor suppressors involved in the MAPK and PI3K/AKT pathways appears common in canine melanoma, suggesting that MAPK and PI3K/AKT pathway activation is playing a role in canine melanoma similar to the human disease despite the absence of common activating
mutations seen in humans. The purpose of this study was to compare the activation of the MAPK and PI3K/AKT pathways in both human and canine melanoma via Affymetrix-based gene expression, mutational analysis, and measures of constitutive activation as well as assessing effects of specific inhibitors to these pathways on melanoma cell lines across species.

MATERIALS AND METHODS

Cell culture

Human melanoma cell lines (SKmel2, SKmel28, SKmel31, A375, WM852, MeWo) were purchased from the American Type Culture Collection (ATCC), Manassas, VA. Canine melanoma cell lines were generously provided by Dr. Lauren Wolfe at Auburn University (CML-10C2 and CML-6M), by Dr. David Vail at University of Wisconsin-Madison (17CM98), or were established from primary oral melanomas at the Flint Animal Cancer Center (FACC) of Colorado State University (Jones and Parks). All cells were maintained in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 ug/mL) and incubated at 37°C in a humidified atmosphere of 5% CO$_2$:95% air.

Tumor samples and RNA extraction

12 canine oral melanoma tissue samples and 4 canine normal oral mucosa samples were obtained from the tumor archives of the FACC (Table 3.1). RNA was extracted from the samples through freeze fracture and homogenization with Trizol (Invitrogen, Grand Island, NY), followed by addition of chloroform and isopropanol steps for extraction and precipitation of the RNA, respectively. The RNeasy Cleanup kit (Qiagen, Valencia, CA) was used according to manufacturer’s protocol with an included DNA digestion step to ensure RNA purity. Yield and
Table 3.1 Microarray setup for gene expression analysis

<table>
<thead>
<tr>
<th>Canine Oral Melanoma*</th>
<th>Human Melanoma£</th>
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<tbody>
<tr>
<td>12 canine oral melanoma samples</td>
<td>45 primary malignant melanoma samples</td>
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<td>4 canine melanoma cell lines</td>
<td>7 normal skin samples</td>
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<td>4 canine normal oral mucosa samples</td>
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### Canine melanoma samples

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<th>Breed</th>
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<td>Labrador Retriever</td>
<td>Malignant</td>
<td>Maxilla</td>
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<tr>
<td>447</td>
<td>Mix</td>
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<td>347</td>
<td>Mix</td>
<td>Metastatic, amelanotic</td>
<td>Mandible</td>
</tr>
<tr>
<td>151</td>
<td>Mix</td>
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<td>Oral, tongue</td>
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<tr>
<td>221</td>
<td>Shar-Pei</td>
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<td>Pharyngeal region</td>
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<td>Lip</td>
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<tr>
<td>588</td>
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<td>1222</td>
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<td>Poorly differentiated</td>
<td>Tonsil</td>
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### Canine melanoma cell lines

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<td>Oral melanoma</td>
<td>No</td>
<td>Wisconsin-Madison</td>
</tr>
<tr>
<td>CML-10C2</td>
<td>Cutaneous melanoma</td>
<td>No</td>
<td>Auburn</td>
</tr>
<tr>
<td>Jones</td>
<td>Oral melanoma</td>
<td>Yes</td>
<td>FACC</td>
</tr>
<tr>
<td>Parks</td>
<td>Oral melanoma</td>
<td>Yes</td>
<td>FACC</td>
</tr>
</tbody>
</table>

### Canine oral mucosa samples

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Breed</th>
<th>Tumor location</th>
<th>Stroma location</th>
</tr>
</thead>
<tbody>
<tr>
<td>111</td>
<td>Terrier</td>
<td>Maxilla</td>
<td>Palate</td>
</tr>
<tr>
<td>801</td>
<td>Mix</td>
<td>Maxilla</td>
<td>Maxilla</td>
</tr>
<tr>
<td>745</td>
<td>Labrador Retriever</td>
<td>Mandible</td>
<td>Mandible</td>
</tr>
<tr>
<td>116</td>
<td>Mix</td>
<td>Maxilla</td>
<td>Oral, soft palate</td>
</tr>
</tbody>
</table>

* samples obtained from the archive at the Flint Animal Cancer Center (FACC), Colorado State University
£ Human microarray data obtained from NCBI’s GEO website (GSE3189)
integrity were examined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Asheville, NC) as well as a Bioanalyzer (Agilent, Santa Clara, CA) from the Proteomics and Metabolomics Facility at Colorado State University.

**Gene expression microarray analysis**

RNA samples were hybridized onto Affymetrix GeneChip Canine Genome 2.0 arrays and microarray analysis was performed at the Proteomics and Metabolomics Facility at Colorado State University. After MAS5 preprocessing, Linear Models for Microarray Data (Limma) analysis was performed to identify differentially expressed genes (DEGs) in the tumor samples compared to normal samples with a p-value cutoff of 0.05 and a fold change greater than 2. The resultant list of DEGs was than plugged into Ingenuity Pathway Analysis (IPA) software, where the canonical MAPK/ERK and PI3K/AKT pathways were examined for changes in expression of pathway-specific genes. To compare our data with human melanoma data, we reanalyzed publicly available microarray gene expression data from Talantov et al. (Talantov et al., 2005)(Accession # GSE3189) at NCBI’s Gene Expression Omnibus (GEO) website including 45 primary malignant melanoma samples and 7 normal skin samples. Using the same Limma analysis cutoffs, we identified human DEGs and compared the IPA results between the human and dog. Heatmap generation through unsupervised clustering was done with DEGs in common with the human and dog analyses that were involved in the ERK/MAPK and/or PI3K/AKT signaling canonical pathways in IPA. Expression values were normalized before cluster analysis by subtracting the mean of human or dog samples and dividing by the standard deviation. The average linkage with Euclidean distances setting was used at the CIMMiner website (http://discover.nci.nih.gov/cimminer/index.jsp).
**Mutational analysis of BRAF and NRAS of human and canine melanoma cell lines and canine melanoma tumor samples**

DNA was either extracted or synthesized from extracted RNA samples from 6 human melanoma cell lines, 5 canine melanoma cell lines, 12 canine oral melanoma tumor samples, and 3 canine normal oral mucosal samples with the use of a Quantitect Reverse Transcription kit according to manufactures protocols (Qiagen, Valencia, CA). To evaluate mutations in genomic DNA from human melanoma cells or in complimentary DNA from canine melanoma tumor samples and cell lines, we designed forward and reverse primers to investigate exon 15 in BRAF and exon 2 in the NRAS gene, the locations of the most prevalent BRAF V600E and NRAS Q61R mutations in human melanoma. The human primers lay in the flanking introns of these exons, whereas the canine primers are intron spanning. Primer sequences are shown in Table 3.2. Polymerase chain reaction (PCR) was performed with OneTaq Hot Start 2X Master Mix with Standard Buffer kit according to manufacturer protocols (New England BioLabs, Ipswich, MA). All PCR reactions were electrophoresed on 2% agarose gels and imaged for confirmation of products of

**Table 3.2 Primer sequences for PCR**

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Primer sequences (5’-3’)</th>
<th>Template</th>
</tr>
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<tbody>
<tr>
<td>human*</td>
<td><strong>BRAF</strong> exon 15</td>
<td>F  tcataatgcttgctctgatagga</td>
<td>gDNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R  ggccaaaaatattaacagtgga</td>
<td>gDNA</td>
</tr>
<tr>
<td></td>
<td><strong>NRAS</strong> exon 2</td>
<td>F  gaaccaaatggaaggtcaca</td>
<td>gDNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R  tgggtaaagatgatccgaca</td>
<td>gDNA</td>
</tr>
<tr>
<td>canine£</td>
<td><strong>BRAF</strong> exon 15</td>
<td>F  caacgcaactcaatcatccacaga</td>
<td>cDNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R  cccaaatgctctcctgactgg</td>
<td>cDNA</td>
</tr>
<tr>
<td></td>
<td><strong>NRAS</strong> exon 2</td>
<td>F  agcttgaggtctcttggtctgta</td>
<td>cDNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R  tgtctggtctggtggttctca</td>
<td>cDNA</td>
</tr>
</tbody>
</table>

* primers lay in the flanking introns of exons 15 and 2
£ primers are intron spanning; F=forward primer; R=reverse primer
desired length, and DNA was purified from a Gel Extraction kit (Qiagen, Valencia, CA). Purified DNA was then sent for DNA sequencing via capillary gel electrophoresis at the Proteomics and Metabolomics Facility at Colorado State University. Sequence alignment of sample results with wild-type BRAF or NRAS sequences were performed using ClustalW (Conway Institute UCD, Dublin, Ireland) and BioEdit (Ibis Biosciences, Carlsbad, CA) software.

**Serum starvation assays and Western blotting**

Cells were plated at 700,000 cells per plate in RPMI media with 10% FBS. After 24 hour incubation, media was replaced with serum-free RPMI for the treatment plates, and normal 10% FBS media for the control plates. After 24 hours, cells were harvested by rinsing in 2 mL ice cold PBS, followed by addition of 100 uL lysis buffer (1% TritonX-100, 5% 3 M NaCl, 1% 1 M Tris HCl pH 7.5, 0.2% 100 mM Na-orthovanadate, 0.35% 10 mg/mL phenylmethylsulfonyl fluoride, and 0.2% Protease Inhibitor Cocktail 25X [Sigma P-8340] in dH2O) and 15 min. incubation at 4°C. The plates were then scraped and lysates collected and stored frozen at -80°C until use. Protein concentrations were determined with the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and samples were prepared for loading 40 ug/30 uL into polyacrylamide gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein was then semi-dry transferred to nitrocellulose membranes followed by incubation with blocking buffer (5% bovine serum albumin in Tris-Buffered Saline and Tween 20 (TBST)) for 1 hour. After 3 TBST rinses primary antibody dilutions in blocking buffer were made for the proteins of interest according to manufacture recommendations (Akt Rabbit Ab #9272, P-Akt(S473) Rabbit Ab #9271s, p44/42 MAPK Rabbit #9102, P-p44/42(T202/Y204) Rabbit Ab #9101s, p70 S6 Kinase Rabbit Ab #9202, P-p70 S6 Kinase(T389)(108D2) Rabbit Ab #9234s, Cell Signaling, Danvers, MA), and membranes were incubated with primary antibodies
overnight at 4°C. Membranes were rinsed and incubated with secondary antibody (Goat anti-rabbit IgG HRP-conjugated, Thermo Scientific, Rockford, IL) prepared in fresh blocking buffer for 1 hour. Membranes were imaged with chemiluminescent substrate (SuperSignal West Dura, Thermo Scientific, Rockford, IL) using a Chemi Doc XES+ system (BioRad, Hercules, CA).

Pathway inhibitors AZD6244 and rapamycin

AZD6244 (ARRY-142886) is a selective non-competitive inhibitor of MEK1/2 in the MAPK pathway and was a gift from AstraZeneca (Macclesfield, UK). Rapamycin (Sirolimus) is an inhibitor of mTOR in the PI3K/AKT pathway and was purchased from LKT Laboratories (St. Paul, MN, USA). Stock solutions of each drug were prepared in dimethyl sulfoxide (DMSO), and stored according to manufacturers’ protocols.

Proliferation assays using AZD6244 or rapamycin in human and canine melanoma cells

A resazurin-based bioreductive fluorometric assay was performed to assess cell viability in the presence of pathway inhibitors. Cell lines were plated in 96-well plates at 500 or 1000 cells per well in 100 uL of RPMI media and incubated for 24 hours at 37°C. Serial dilutions of drug along with vehicle controls and media only blanks were prepared and 100 uL added to the respective wells. The plates were then incubated at 37°C. After 72 hours, drug-containing media was aspirated from plates and 200 uL of fresh media with 10% resazurin solution (200 ug/mL of Resazurin Salt in phosphate-buffered saline (PBS)) added to all wells, followed by 2-4 hours of incubation. Fluorescence was then measured using a BioTek Synergy HT plate reader (BioTek, Vermont, USA) with excitation at 530 nm and emission at 590 nm. Each treatment was performed in at least triplicate, and dose response curves were generated in GraphPad Prism (GraphPad Software, La Jolla, CA) software and medial dose values ($D_m$ or $IC_{50}$) were calculated using the Chou-Talalay method (Chou TC, 1984).
**Drug combination assays and synergy calculations**

1:2 serial dilutions of drug were calculated based on the calculated $D_m$ values from the proliferation studies using either AZD6244 or rapamycin alone, starting with a maximum dose of 4X the $D_m$ value. Proliferation assays were performed as described above. CalcuSyn software was used to calculate drug synergy based on the dose response data, resulting in a Combination Index (C.I.) value for the effective doses $ED_{50}$. Assay variability was perpetuated through the synergy analysis with upper and lower 95% confidence interval values tested along with the mean. C.I. value ranges above 0.7 and below 1.3 were considered additive, whereas above 1.3 and below 0.7 were considered antagonistic and synergistic, respectively. Ranges that crossed those boundary values were considered additive/synergistic or antagonistic/additive.

**Cell cycle and apoptosis analysis via flow cytometry**

700,000 cells were plated in 100 mm cell culture-treated plates and incubated for 24 hours at 37°C. Cells were then treated with $IC_{50}$ doses of AZD6244 and/or rapamycin or vehicle control for 24 or 48 hours, depending on growth rate, after which they were then trypsinized, collected, and centrifuged at 300 x g for 5 minutes. Cells were fixed by rinsing with PBS and resuspending in 1.5 mL PBS at $10^6$ cells/mL, followed by adding 3.5 mL of ice-cold 100% ethanol dropwise while gently vortexing the samples. The cells were then placed on ice for 30 minutes or incubated overnight at -20°C. Fixed cells were then centrifuged and rinsed in PBS. Extraction buffer (4% 0.1 M Citric Acid in 0.2 M Na$_2$HPO$_4$ in PBS) and PI-RNAse reagent (50 ug/mL propidium iodide and 125 Worthington U/mL of RNAse in PBS) were added to the samples and incubated at 37°C for 30 minutes. The samples were then analyzed on a Cyan flow cytometer (Beckman Coulter, Brea, CA) and the number of cells in sub-G1, G1, S, and G2
phases were measured with Summit (Beckman Coulter (DakoCytomation), Brea, CA) software. All experiments were done in triplicate.

**Statistical analysis**

A two-tailed unpaired t-test was used to compare means from drug sensitivity studies. One-way ANOVA with Bonferonni post tests for direct comparisons were used for comparing the G1 and sub-G1 peaks between treatments in the cell cycle analysis data. All statistical analyses were performed using GraphPad Prism software.

**RESULTS**

*Human and canine melanoma share similar differential expression patterns of MAPK and PI3K/AKT pathways*

Limma analysis resulted in the identification of 6,414 and 7,262 probesets differentially expressed (DEGs) between tumor and normal tissue samples in the human and canine sets, respectively. When these lists of DEGs were plugged into IPA software and pathways of interest examined, a total of 75 human and 47 canine probesets related to ERK/MAPK pathway proteins, and 49 human and 20 canine probesets related to PI3K/AKT pathway proteins. Comparing human and canine DEGs mapping to the ERK/MAPK and PI3K/AKT pathways, similar expression patterns are observed (**Figure 3.1 & 3.2**). For example in the ERK/MAPK signaling pathway upstream of RAS, proteins such as Growth Factor Receptor-Binding Protein 2 (GRB2), Talin, and Focal Adhesion Kinase (FAK) are upregulated in both the human and canine analysis. Likewise, transcription factors cAMP response element-binding (CREB), Estrogen receptor positive (ER+), E-twenty six (Ets), and the cellular homolog of the myelocytomatosis viral
Figure 3.1 Differential expression of MAPK pathway in human and canine melanoma versus normal tissue. Limma analysis was performed on MAS5 preprocessed gene expression data for both human and canine melanoma and normal control samples. P values < 0.05 and fold change > 2 were cutoffs used to identify differentially expressed genes (DEGs). The DEGs from the human and canine analyses were compared using Ingenuity Pathway Analysis (IPA) software. The ERK/MAPK signaling canonical pathway was specifically examined. A) Differential expression of human melanoma tumors versus normal skin samples in ERK/MAPK signaling. B) Differential expression of canine oral melanoma tumors and cell lines versus normal oral mucosa in the ERK/MAPK signaling pathway. Increasing red and green shading indicates increasing and decreasing gene expression compared to normal, respectively.
Figure 3.2  Differential expression of PI3K/AKT pathway in human and canine melanoma versus normal tissue. A) Differential expression of human melanoma tumors versus normal skin samples in PI3K/AKT signaling. B) Differential expression of canine oral melanoma tumors and cell lines versus normal oral mucosa samples in PI3K/AKT signaling. Increasing red and green shading indicates genes identified to be differentially expressed between tumor and normal samples, with the red and green corresponding to increasing and decreasing gene expression compared to normal, respectively.
oncogene (c-Myc) share the same expression pattern between species (Figure 3.1). In the PI3K/AKT signaling pathway, AKT is upregulated in both analyses, as well as Integrin-linked Kinase (ILK) active upstream of AKT. Interestingly, PI3K and RAS are upregulated in the canine analysis, but not in the human. By contrast, the human analysis alone has specific upregulation of MEK1/2, a therapeutic target in the ERK/MAPK signaling pathway. It is important to note that although the canine analysis did not reveal an upregulation of MEK1/2, both the human and canine analysis showed that phosphatases known to inhibit MEK1/2, Protein Phosphatase 1 (PP1) and MAP Kinase Phosphatase (MKP), are downregulated in tumors compared to normal tissue (Figure 3.2). Thus, the majority of DEGs associated with these signaling pathways lead to pathway activation in both species. Additionally, the downstream transcription factor expression pattern is comparable between human and canine melanoma, suggesting similar outcomes from MAPK and PI3K/AKT signaling changes.

Shown in Figure 3.3 is a heat map generated by unsupervised clustering with a total of 33 DEGs mapping to the ERK/MAPK and/or PI3K/AKT signaling pathways that were common to both species. Human and canine tumor samples intersperse with regard to gene expression and are not segregated by species, suggesting that DEGs in these two pathways are altered in a similar fashion in both the human and dog melanomas. It is interesting to note that there were more MAPK and AKT-specific pathway genes found in the human DEG list than in the canine DEG list. Considering that the normal tissue samples in the canine set were oral mucosal, which normally have higher proliferative levels than skin, it is possible that the highly proliferative genes in the normal samples might have “masked” the high expression in the canine tumor samples. Regardless, these results indicate that human and canine melanoma share similar
patterns of gene expression in signaling pathways known to be important in the human disease, and thus represent attractive sites of intervention for cancer therapy.

Pathway activation in human and canine melanoma

Numerous studies have shown that human melanomas have a high incidence of activating mutations in BRAF or NRAS (Ascierto et al., 2012; Kelleher and McArthur, 2012; Solit et al., 2006). Mutational status of 6 human melanoma cell lines were confirmed (WM852, A375, SKmel5, SKmel28, SKmel2, and SKmel31) using primers to look specifically at exon 15 of
BRAF, the location of the commonly found mutation involving a valine to glutamate substitution at codon 600 (V600E), where 90% of all BRAF mutations have been reported to occur (Flaherty et al., 2010). Additionally, primers were designed to study the most prevalent NRAS mutation in human melanoma, a glutamine to arginine substitution at codon 61 (Q61R). The analysis identified 2 NRAS Q61R mutants (WM852 and SKmel2), 2 BRAF V600E mutants (A375 and SKmel28), 1 heterozygous BRAF V600E mutant (SKmel5), and 1 BRAF and NRAS wildtype (WT) (SKmel31) (Table 3.3). Another human melanoma cell line (MeWo) was purchased later from the ATCC which has been previously reported as being WT for both genes (Lev et al., 2004).

These BRAF and NRAS mutations were also screened for in 12 canine oral melanoma samples, 3 normal canine oral mucosal samples, and the 5 canine melanoma cell lines (Jones, Parks, 17CM98, CML-6M, and CML-10C2). Two mutations were identified, one occurring in the Jones cell line and the other in tumor sample 886. The Jones cell line was identified to be heterozygous for the NRAS Q61R mutation whereas tumor 886 showed a silent mutation at codon 52 (CTG -> TTG, which remains a leucine after translation). The analysis confirmed the remaining cell lines and tumor and normal tissue samples to be WT at the 600 codon site for BRAF and the 61 codon site for NRAS (Table 3.3). The results for both human and canine samples correspond with what has been previously reported (Abi-Habib et al., 2005; Davies et al., 2002; Dumaz et al., 2006; Smalley et al., 2008; Tsai et al., 2008).

Constitutive activation of MAPK and PI3K/AKT pathways was measured by examining the effects of serum starvation on the phosphorylation status of ERK1/2 and AKT, important signaling proteins in the MAPK and PI3K/AKT pathways, respectively. After 24 hours of serum starvation, cells were harvested and total and phosphorylated-protein levels were investigated
<table>
<thead>
<tr>
<th>Sample</th>
<th>Mutational status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human melanoma cells</strong></td>
<td></td>
</tr>
<tr>
<td>WM852</td>
<td>NRAS(Q61R)</td>
</tr>
<tr>
<td>SKmel2</td>
<td>NRAS(Q61R)</td>
</tr>
<tr>
<td>SKmel28</td>
<td>BRAF(V600E)</td>
</tr>
<tr>
<td>A375</td>
<td>BRAF(V600E)</td>
</tr>
<tr>
<td>SKmel5</td>
<td>BRAF wt/mu(V600E)</td>
</tr>
<tr>
<td>SKmel31</td>
<td>WT</td>
</tr>
<tr>
<td><strong>Canine melanoma cells</strong></td>
<td></td>
</tr>
<tr>
<td>CML-6M</td>
<td>WT</td>
</tr>
<tr>
<td>CML-10C2</td>
<td>WT</td>
</tr>
<tr>
<td>17CM98</td>
<td>WT</td>
</tr>
<tr>
<td>Jones</td>
<td>NRAS wt/mu(Q61R)</td>
</tr>
<tr>
<td>Parks</td>
<td>WT</td>
</tr>
<tr>
<td><strong>Canine melanoma tumor</strong></td>
<td></td>
</tr>
<tr>
<td>447</td>
<td>WT</td>
</tr>
<tr>
<td>186</td>
<td>WT</td>
</tr>
<tr>
<td>412-a</td>
<td>WT</td>
</tr>
<tr>
<td>588</td>
<td>WT</td>
</tr>
<tr>
<td>151-b</td>
<td>WT</td>
</tr>
<tr>
<td>1222</td>
<td>WT</td>
</tr>
<tr>
<td>221</td>
<td>WT</td>
</tr>
<tr>
<td>478</td>
<td>WT</td>
</tr>
<tr>
<td>347-b</td>
<td>WT</td>
</tr>
<tr>
<td>918</td>
<td>WT</td>
</tr>
<tr>
<td>1024</td>
<td>WT</td>
</tr>
<tr>
<td>886</td>
<td>NRAS (silent mut. at codon 52)</td>
</tr>
<tr>
<td><strong>Canine normal oral mucosa</strong></td>
<td></td>
</tr>
<tr>
<td>745</td>
<td>WT</td>
</tr>
<tr>
<td>801</td>
<td>WT</td>
</tr>
<tr>
<td>116</td>
<td>WT</td>
</tr>
</tbody>
</table>

WT = wild-type
through western blot analysis. In all four human melanoma lines tested, we observed no difference in phospho-ERK1/2 in the presence or absence of serum (Figure 3.4), strongly suggesting constitutive activation of the MAPK pathway in these cells. The same phenomena were observed in the 4 canine melanoma lines tested, with phospho-ERK1/2 levels unchanged by the absence of serum (Figure 3.4). Evaluation of phospho-AKT levels after serum starvation showed a decrease in all human and canine lines, but some phospho-AKT was still present in 2 of 3 human lines and 3 of 5 canine lines after 24 hours, suggesting some level of constitutive activation in this pathway for both human and canine melanoma (Figure 3.4). Taken together, these data suggest that although human and canine melanomas do not appear to share the same mechanism of pathway activation, constitutive activation of these pathways is present in both species.

Figure 3.4 Constitutive activation of MAPK and PI3K/AKT pathways in human and canine melanoma cell lines. Cells were serum starved for 24 hours followed by Western blot analysis. Total protein levels were examined to ensure proper protein loading. The levels of phosphorylated ERK1/2 and AKT were measured and compared to control conditions. A) 4 human melanoma lines showed no change in phospho-ERK1/2 levels in the presence or absence of serum. B) Phospho-ERK1/2 levels did not decrease in canine melanoma cells after serum starvation. C) Phospho-AKT levels decreased in human melanoma lines after serum starvation, but still present in 2 of 3 lines after 24 hours. D) Phospho-AKT levels decreased in canine melanoma lines, but were still present in 3 of 5 lines after 24 hours.
Human and canine melanoma cell lines are similarly sensitive to MAPK and PI3K/AKT pathway inhibition

Since the discovery of activating mutations in NRAS and more recently BRAF in multiple cancers, including a large subset of human melanomas, drugs have been designed specifically to target either the MAPK and/or PI3K/AKT pathways. Studies have reported that human melanomas are sensitive to these small molecule inhibitors (Chapman et al., 2011; Davies et al., 2007; Flaherty et al., 2012; Jazirehi et al., 2012). AZD6244 (ARRY-142886) is a selective non-ATP competitive inhibitor of MEK1/2 that is now currently being studied in Phase II clinical trials (Davies et al., 2007). MEK1/2 is the only known activator of ERK1/2, a key signaling protein in the MAPK pathway. Rapamycin is an inhibitor of mTOR, a key regulatory protein in the PI3K/AKT pathway, responsible for regulating many processes including cell growth, proliferation, and survival. Rapamycin forms a complex with FK-binding protein 12 (FKBP12) which directly binds to the MTORC1 complex, inhibiting downstream signaling (Willems et al., 2012).

To confirm that AZD6244 and rapamycin were able to inhibit their intended targets in both human and canine melanoma, cells were treated with each drug for 24 hours and evaluated for phosphorylation of the immediate downstream targets of MEK1/2 and mTOR, ERK1/2 and p70S6 kinase (p70S6K). In Figure 3.5, phospho-ERK1/2 decreased in all human and canine melanoma lines treated with AZD6244 compared to vehicle control. Similarly, we observed decreased phospho-p70S6K in all human and canine lines treated with rapamycin compared to control (Figure 3.5), showing that AZD6244 and rapamycin were effective in inhibiting these pathways and were suitable for our remaining comparative studies.
AZD6244 and rapamycin were then assessed for growth inhibition in canine and human cell lines. Table 3.4 shows resulting IC$_{50}$ values from viability assays for both human and canine melanoma cells. Human melanoma lines were sensitive to both AZD6244 and rapamycin, with IC$_{50}$ values ranging from 58-183 nM and 0.037-12 nM, respectively. Likewise, canine
melanoma lines were sensitive to AZD6244 and rapamycin with IC$_{50}$ values ranging from 5.7-391 nM and 0.027-2.6 nM. In Figure 3.5, standardized IC$_{50}$ values for both human and canine groups show comparable ranges of sensitivity and the means of the sensitivity ranges in human and dog were not found to be statistically different (p=0.4687 for AZD6244, p=0.8474 for rapamycin) showing that human and canine melanoma cells respond to AZD6244 and rapamycin in the same dose ranges (Figure 3.5).

Drug combination assays were performed using AZD6244 and rapamycin together. In human melanoma, the drug combination was synergistic in decreasing cell viability at the ED$_{50}$ for SKmel2 and A375 cells, additive/synergistic for MeWo cells, and additive for SKmel28 cells. In canine melanoma, the drug combination was synergistic at the ED$_{50}$ for Jones and 17CM98 cells, and additive/synergistic for CML-6M cells. The combination in CML-10C2 cells could not be called synergistic because of large variability, although the mean value was synergistic (Table 3.4 and Figure 3.6). Taken together, the AZD6244/rapamycin drug combination decreased cell viability in a synergistic manner in both human and canine melanoma cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>AZD6244 IC$_{50}$ (nM)</th>
<th>Rapamycin IC$_{50}$ (nM)</th>
<th>ED$_{50}$ C.I. range$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A375</td>
<td>58</td>
<td>12</td>
<td>0.328-0.647</td>
</tr>
<tr>
<td>SKmel2</td>
<td>142</td>
<td>0.308</td>
<td>0.093-0.026</td>
</tr>
<tr>
<td>SKmel28</td>
<td>183</td>
<td>0.037</td>
<td>0.855-0.890</td>
</tr>
<tr>
<td>MeWo</td>
<td>113</td>
<td>0.042</td>
<td>0.552-0.771</td>
</tr>
<tr>
<td>Canine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jones</td>
<td>105</td>
<td>2.6</td>
<td>0.003-0.096</td>
</tr>
<tr>
<td>17CM98</td>
<td>5.7</td>
<td>0.118</td>
<td>0.063-0.438</td>
</tr>
<tr>
<td>CML-10C2</td>
<td>391</td>
<td>0.184</td>
<td>0.369-3.261</td>
</tr>
<tr>
<td>CML-6M</td>
<td>42</td>
<td>0.027</td>
<td>0.051-1.064</td>
</tr>
</tbody>
</table>

$^\dagger$ Drug synergy was measured for the mean of response and upper and lower 95% confidence intervals using the following parameters: Combination Index (C.I.) value > 1.3 = antagonistic. 1.3 > C.I. value > 0.7 = additive. 0.7 > C.I. value = synergistic. C.I. ranges that crossed these boundaries were called antagonistic/additive or additive/synergistic.
lines, suggesting that targeting both the MAPK and PI3K/AKT pathways in combination is not only a promising strategy in human melanoma, but may prove beneficial to canine melanoma patients as well.

**Effects of AZD6244 and/or rapamycin on cell cycle and apoptosis**

To further explore the mechanisms by which AZD6244 and rapamycin inhibited proliferation in human and canine melanoma cells, cell cycle distribution was measured in the
presence or absence of AZD6244 and/or rapamycin. The results of 3 human melanoma lines, representing a NRAS mutant, BRAF mutant, and WT (SKmel2, SKmel28, MeWo), are shown in Figure 3.7. A significant increase in the percentage of cells in G1 in both the SKmel2 and SKmel28 cell lines with AZD6244 or the combination treatment was observed, suggestive of a G1 cell cycle arrest (p<0.01 and p<0.05, respectively). However, in the WT line (MeWo), only a slight non-significant increase in G1 was observed (Figure 3.7). Rapamycin appeared to slightly increase the percentage of cells in G1 in the NRAS mutant and WT lines, as opposed to the BRAF mutant where no cell cycle effect was observed for rapamycin treatment alone, and no enhancement was observed in the combination when compared to AZD6244 alone. This may be suggestive of oncogene addiction taking place in the SKmel28 cell lines, where it has possibly become heavily dependent on RAF/MEK/ERK signaling for growth and proliferation. For canine melanoma cells, the NRAS mutant and 2 WT lines (Jones, CML-10C2, and 17CM98) were used for the analysis. In Jones cells, a significant increase in G1 cells after treatment with either AZD6244 or rapamycin alone (p<0.05) was measured. Interestingly, the combination significantly increased the percentage of G1 cells when compared to control (p<0.001) or either AZD6244 or rapamycin alone (p<0.05 and p<0.01), suggestive again of the additive/synergistic effect of targeting these pathways together in melanoma cells (Figure 3.7). Surprisingly, results for the 2 canine WT lines differed from each other. Whereas the 17CM98 cells resembled closely the lack of response observed in the human WT line with these drugs, in CML-10C2 cells a strong G1 cell cycle block with the AZD6244 and combination treatments (p<0.001) was observed. Rapamycin increased the percentage of cells in G1, but not significantly (Figure 3.7).

Apoptotic effects of these drugs in human and canine melanoma were assessed by measuring the population of cells in sub-G1 after treatment with these drugs alone and in
Figure 3.7 Cell cycle analysis of human and canine melanoma cells after AZD6244 and/or rapamycin treatment. Human and canine melanoma cells were treated in the presence or absence of AZD6244 and/or rapamycin for 24 or 48 hours depending on growth rate. Cells were harvested, fixed and stained with PI-RNAse followed by cell cycle analysis via flow cytometry. A and B) Graphs showing the percentage of cells in G1,S, or G2 phases in human (A) or canine (B) cell lines after treatment for 24 or 48 hours. C and D) The percentage of cells in sub-G1 was measured to study apoptotic effects of AZD6244 and/or rapamycin in human (C) and canine (D) melanoma cells for 24 or 48 hours. All experiments were performed in triplicate. One way ANOVA was performed to examine changes in the percentage of cells in G1 and sub-G1 in all conditions tested. *, **, and *** corresponds statistically significant observations with p values < 0.05, 0.01, and 0.001, respectively.
combination. As shown in Figure 3.7, most treatments caused an increase of cells in sub-G1, but it was only found to be a significant increase when AZD6244 and rapamycin were used in combination in SKmel2 cells (p<0.05). Taken together, these data suggest that the main mechanism by which AZD6244 works in both human and canine melanoma is through a cell cycle G1 block. Rapamycin caused a significant increase of cells in G1 when used alone and significantly enhanced the G1 block observed with AZD6244 when used in combination in the canine NRAS mutant Jones line. For the remaining human and canine cells, rapamycin appeared to slightly but insignificantly increase the percentage of cells in G1, though to a lesser extent.

**DISCUSSION**

Malignant melanoma is a devastating disease in humans with a dismal prognosis and few effective treatment options resulting in long-term responses. Newer treatment options have focused on the activating mutations in the MAPK pathway commonly found in human melanoma including inhibitors of MEK and BRAF that have shown success in terms of initial responses but whose overall success in terms of increased survival has been limited by emergence of resistance. The use of pet dogs in development of human cancer therapies is a developing paradigm requiring characterization and comparative studies so that study designs and interpretation of results are optimized. The purpose of the studies presented here are to begin a comprehensive comparison of MAPK and PI3K/AKT pathway activation in human and canine melanomas. The histological differences in the malignant form of the disease between species, with the human form being predominantly cutaneous and the canine form being predominantly mucosal, may be viewed as a major hurdle for comparative therapeutics whereas the aggressive
nature, chemotherapy resistance, and immunotherapeutic potential that are shared argue for an equivalency between the diseases. The fact that the MAPK and PI3K/AKT pathways have been major focuses for therapeutic intervention in human malignant melanoma point to characterization of changes in these pathways within canine melanoma a logical next step.

The activating mutations \textit{BRAF} V600E and \textit{NRAS} Q61R account for 65-70\% of cases in human melanoma (Ascierto et al., 2012; Kelleher and McArthur, 2012). The data presented confirm what previous studies have shown with regards to mutations in these codons of \textit{BRAF} and \textit{NRAS} appearing to be rare in canine melanoma. However, the results confirm an earlier report of constitutive activation of these pathways in canine melanoma after serum starvation. What accounts for MAPK and PI3K/AKT activation in the canine tumors assuming that uncharacterized activating mutations in \textit{BRAF} and \textit{NRAS} are not present? Mutations in \textit{CKIT} are a possibility as KIT expression was observed in ~50\% of canine oral melanomas in a recently published study (Newman et al., 2011) although activating mutations in exon 11 of \textit{CKIT} were not observed in 17 canine oral melanomas analyzed (Murakami A, 2011). Another possibility is integrin signaling, which lies upstream and can activate both MAPK and PI3K/AKT pathways. In the gene expression analysis reported here, an upregulation of both ILK and FAK was observed in canine tumors when compared to normal tissue, kinases that link signaling from integrins to the MAPK and PI3K/AKT pathways. A broader all-encompassing pathway analysis using this gene expression microarray data with human and canine melanoma samples compared to normal tissue is currently in process with initial results suggesting an upregulation of multiple integrin signaling genes in canine melanoma compared to normal tissue (unpublished data).

Because of the challenges of cross-talk and redundancy in signaling pathways, strategies to target multiple pathways in combination or even multiple targets in the same pathway have
resulted in greater tumor inhibition and improved clinical outcome (Engelman et al., 2008; Greger et al., 2012; Hoeflich et al., 2012; Mirzoeva et al., 2009; Sos et al., 2009). Targeting the MAPK and PI3K/AKT pathways in combination with a MEK1/2 inhibitor AZD6244 and mTOR inhibitor rapamycin had a synergistic effect in both human and canine melanoma lines. It is interesting to note that similar drug sensitivity was observed in all of the cell lines, regardless of mutational status or species. When both pathways were inhibited in combination synergistic growth inhibition was observed in the WT cells along with the \textit{BRAF} and \textit{NRAS} mutants. Although studies have shown no correlation with mutational status and clinical efficacy in the BRAF inhibitor sorafenib (Mangana et al., 2012), newly designed drugs that are more selective for targets in the MAPK pathway such as MEK or mutated BRAF have shown significantly better responses in patients with a \textit{BRAF} mutation than those with WT BRAF (Bollag et al., 2010; Kirkwood et al., 2012; Patel et al., 2012). Research efforts are now focusing on the optimization of combination therapies for patients with activating mutations who acquire resistance to BRAF or MEK inhibition. Our data suggests the possibility that these emerging combination treatments for resistant BRAF mutant cancers may have a positive effect in WT melanoma as well.

AZD6244 caused a significant increase of cells in the G1 phase of the cell cycle in all of the BRAF and NRAS mutant cell lines in both human and canine, indicative of a G1 block. Interestingly, strong alterations in the cell cycle were also observed in a canine WT line when treated with AZD6244 resulting in a large increase of cells in G1 (\textbf{Figure 3.7}). This is different than the milder non-significant response of the other WT lines in both human and dog to AZD6244, suggesting that melanoma cells that do not contain the specific \textit{BRAF} \textit{V600E} and \textit{NRAS} \textit{Q61R} mutations may still differ from each other in terms of other molecular alterations,
which might make them more or less susceptible to MAPK and/or PI3K/AKT pathway inhibition. AZD6244 did not induce apoptosis in human melanoma cells according to DAPI-based assays performed (unpublished data), which corresponds to the lack of significant increases of cells in subG1 (Figure 3.7). The only exception was the SKmel2 cell line, which has been reported previously to undergo apoptosis when treated with AZD6244 (Yeh et al., 2007). Rapamycin induced a significant G1 block in the canine NRAS mutant line but had smaller effects in the other lines studied. Regardless, significant apoptosis was not observed under our testing conditions, suggesting the main mechanism by which the drugs were working was through a cytostatic G1 block of the cell cycle.

Although only one activating mutation (NRAS Q61R) from our panel of canine melanoma tumors and cell lines was identified, we acknowledge that only the most common exons were examined where mutations are found. In the future, collaborations with other researchers at the FACC are planned to perform a much deeper molecular analysis on a panel of 33 canine cancer cell lines, of which melanomas are represented. Next generation sequencing of the whole BRAF and NRAS genes along with other important cancer-related genes such as TP53, MYC, and PTEN will be performed. Array-comparative genomic hybridization (array CGH) analysis will be run to identify DNA copy number variations and possible gene duplications related to cancer signaling pathways. The better we understand what is going on with canine melanoma on a molecular level, the more useful it can be as a translational model, especially in the veterinary clinical trial setting.

In conclusion, this study shows that canine melanoma shares activation of the same molecular pathways with human melanoma, although most likely by different mechanisms or alternate mutations. Regardless of these different mechanisms, human and canine melanoma are
similarly sensitive to the downstream inhibition of the MAPK and PI3K/AKT pathways, and like has been reported by others and shown by our work, targeting both of these pathways in combination can yield synergistic results in both species. The findings suggest that further comparative studies on the therapeutic benefit of MAPK and PI3K/AKT targeted therapies in canine melanoma are warranted both in terms of translation to the human disease as well as potential therapeutic benefit in pet dogs with cancer.
REFERENCES


Chapter 4

Gene Expression Models for Predicting Drug Response in Canine Osteosarcoma

SUMMARY

Multi-gene prediction models can improve outcome to cancer therapy in human patients. Osteosarcoma is the most common primary bone cancer in dogs. Our purpose is to evaluate the use of prediction models built in an intra- or interspecies manner for the prediction of chemosensitivity in canine osteosarcoma tumors. Models were built using microarray gene expression and drug sensitivity data from a collection of human and canine cancer cell lines, and canine osteosarcoma tumor datasets. The best performing model involved genes identified in human cell lines that were built on a canine osteosarcoma tumor panel, which successfully predicted clinical outcome in an independent test set. Our data show that intra- and interspecies multi-gene prediction models can successfully be used to predict response to treatment in canine cancer, which can serve to improve outcome in dogs as well as serve as pre-clinical validation for similar methods in human cancer research.

INTRODUCTION

Recent breakthroughs in cancer genomics have made the emerging field of personalized medicine not only a plausible but effective alternative to traditional approaches to cancer treatment. The discovery of biomarkers such as the breakpoint cluster region/Abelson

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3 Thanks to Dan Gustafson, Kristen Brown, Deanna Dailey, and Ann Hess for contributions to this chapter.
(BCR/ABL) gene in patients with chronic myeloid leukemia, the Estrogen Receptor (ER) status of breast cancer patients, and the v-Raf murine sarcoma viral oncogene homologue B1 (BRAF) mutational status of melanoma patients has resulted in the development of effective targeted agents and successful patient stratification leading to better clinical outcomes (Dancey et al., 2012). In the case of ER and BRAF, their expression has been found to be prognostic in other tumor types as well, such as ER for non-small-cell lung cancer and the BRAF V600E mutant being observed in 45% of papillary thyroid cancers and 11% of colorectal cancers, showing the potential for predictive molecular characteristics to be extrapolated across histological boundaries (Davies et al., 2002; Kazmi et al., 2012; Tian et al., 2012; Tufano et al., 2012).

Although success of single biomarkers in predicting response to molecularly targeted agents has been observed, this is not the case with traditional chemotherapeutics, which are still heavily used in human and veterinary medicine. Typically a host of genes involved in processes such as drug activation, detoxification, DNA-repair, stress responses, and others are playing a role in determining chemosensitivity. The use of multi-gene prediction signatures have already begun to be used for the accurate prediction of treatment response in many cancers (Chang et al., 2003; Cheng et al., 2010; Manjili et al., 2012; Shen et al., 2012; Takata et al., 2005). One such strategy was recently developed by Lee and Theodorescu and was termed the Co-Expression Extrapolation (COXEN) method (Lee et al., 2007). It combines microarray gene expression and drug sensitivity data from a reference set to determine an initial gene signature, which is correlated to expression data from a second dataset. The genes in the signature that share concordant expression to the second set are then used to predict drug sensitivity in the second set. This method has been used to successfully predict \textit{in vitro} sensitivity as well as clinical outcome in bladder, breast, and non-small cell lung cancers (Ferriss et al., 2012; Havaleshko et
al., 2007; Lee et al., 2010; Lee et al., 2007; Nagji et al., 2010; Smith et al., 2010). It has also been implemented for in silico drug screening and is currently being employed in a prospective human clinical trial (NCT01228942) (Lee et al., 2007; Smith et al., 2011).

In the recent past, clinical trials in human lung and breast cancer with similar strategies of selecting drug treatment based on gene signatures were suspended due to faulty preclinical data and improper validation of predictors, a strong reminder that proper pre-clinical validation is essential for the continued development of such methods in the clinic (Bonnefoi et al., 2011; Potti et al., 2006). What models should be used to provide such validation? Advanced animal models that share highly similar cancer genomics with humans and that are routinely treated for spontaneous tumors with the same anti-cancer drugs in a clinical setting would be an ideal choice for this type of study.

In the United States, 74 million pet dogs are at risk to develop cancer, the number one death-causing disease in adult dogs (O'Donoghue et al., 2010; Paoloni et al., 2009). Canine models of cancer have many advantages for translational studies. Dogs have a higher incidence of cancer than humans, develop tumors in similar sites, and their tumors are biologically more comparable to humans than rodents (Paoloni and Khanna, 2008; Vail DM, 2000). Disease progression in dogs is accelerated compared to humans, allowing study endpoints to be assessed in a much quicker fashion. In recent studies the disease-free time interval needed to assess clinical outcome in canine osteosarcoma and lymphoma patients was 18 months compared to 7 years for human lymphoma patients (Paoloni and Khanna, 2008). Larger body size allows for repeated sampling of fluids and tissue in the same patient throughout the treatment period, and larger tumor size means more available tissue for molecular analysis (Pinho et al., 2012). Veterinary clinical trials are significantly less expensive than human trials and can be performed
in the pre-IND setting. Veterinary patients tend to enter trials with less pre-treatment than humans, and compliance from owners is excellent (Paoloni and Khanna, 2008; Vail DM, 2000).

In terms of cancer genetics, since 2005 when the canine genome became sequenced studies have continued to reveal high similarity between human and canine cancers with respect to genetic homology, molecular alteration of known cancer pathways, and amplification of known oncogenes (Lindblad-Toh et al., 2005). Studies have shown similar molecular pathways being activated in multiple cancers including osteosarcoma, colorectal cancer, soft tissue sarcoma, melanoma, and breast cancer (Rowell et al., 2011; Selvarajah et al., 2009; Tang et al., 2010; Uva et al., 2009). Canine osteosarcoma has molecular alterations in the Wnt, Ras, interleukin, and apoptotic signaling pathways similar to human osteosarcoma (Selvarajah et al., 2009). In another study comparing poor and good responders to therapy, cAMP signaling, chemokines and adhesion and sonic hedgehog pathways were identified in dogs, an observation in human cancers as well (O'Donoghue et al., 2010). Human and canine osteosarcoma patients respond similarly to treatment (Withrow and Wilkins, 2010). In a previous study from our lab, we were able to show that the molecular profile of the MAPK and PI3K/AKT pathways are similar in both human and canine melanoma, and they are both highly sensitive to specific pathway inhibition (Fowles et al., 2013).

The purpose of this study was to investigate the utility of the COXEN method in an interspecies manner between human and canine datasets. The reasons behind this chosen application of COXEN are three-fold: first, a successful application of COXEN across species would show the robustness of this method for cross-platform applications. Secondly, the success of this study would add strength to the growing evidence that pet dogs with cancer make powerful translational models that can greatly impact human research, specifically in this new
genomics era. Lastly, considering the wealth of human genomic and pharmacologic data available to the public, the potential to extrapolate this information into the veterinary setting through molecular comparisons of tumors would be extremely advantageous for the field of canine oncology.

**MATERIALS AND METHODS**

**Cell culture**

A panel of 29 canine cancer cell lines used for drug screening at the Flint Animal Cancer Center (FACC) consists of 2 hemangiosarcomas, 2 histiocytic sarcomas, 1 leukemia, 3 lymphoma, 2 mammary tumors, 1 mast cell tumor, 5 melanomas, 10 osteosarcomas, 1 soft tissue sarcoma, and 2 transitional cell carcinomas, as described in Chapter 2. All cells were maintained as previously described (Fowles et al., 2013).

**Drug sensitivity assays**

Drug sensitivity data was generated via Alamar Blue assays for cisplatin (CIS), carboplatin (CARBO), and doxorubicin (DOX) in the FACC panel as follows: Cells were plated in 96 well plates at a density of 1500 – 5000 cells in 100 uL per well, depending on growth rate. 24 hours after initial plating, serial doses of the drugs in 100 uL of media were added to the plates, including vehicle control wells and blank wells with only media. Drug incubation lasted 48 hours after which old media containing drug was replaced with 200 uL fresh media, and 20 uL of Alamar Blue solution (200 ug resazurin salt / ml in PBS) was added to each well. Following 2-4 hours of incubation, fluorescence was measured on a 96 well plate reader with
emission wavelength parameters of 530 and excitation of 590. Experiments were performed at least in triplicate, and medial dose (Dm) values were calculated.

**RNA extraction and determination of integrity**

**Canine cancer cell lines**: RNA from the ACC30 was extracted using the Qiagen RNeasy Kit (Qiagen, Valencia, CA) according to manufacturer’s protocols. A DNase treatment step using the RNase-Free DNase Set (Qiagen, Valencia, CA) was included to ensure RNA purity.

**Osteosarcoma tumor samples**: Frozen tumor samples were crushed through freeze fracture and then added to 500 uL Trizol (company). Samples were then immediately homogenized and kept on ice for no longer than 30 minutes. Samples were then centrifuged at ~2000 RPM for 30 seconds and supernatant was removed and added to 0.2 mL of chloroform per mL of Trizol. After sample-containing tubes were shaken and incubate at RT for 5 minutes, the samples were centrifuged at ~12000 x g at 4°C for 15 minutes. The top aqueous phase was then transferred to new tubes and RNA was precipitated out with 0.5mL 100% isopropanol /mL Trizol. Samples were incubated at RT for 10 minutes and then centrifuged at ~12000 x g at 4°C for 10 minutes. The white RNA pellet was then washed in 75% ethanol, dried, and resuspended in nuclease free water. The Qiagen RNeasy Cleanup protocol was then followed per manufacturer’s instructions with an included DNA digestion step. Yield and integrity for all RNA samples were calculated using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Asheville, NC) and an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) at the Genomics and Microarray Core at the University of Colorado Denver.

**Microarray gene expression analysis**

RNA samples for the ACC30 were hybridized onto Affymetrix GeneChip Canine Genome 2.0 arrays (Affymetrix, Santa Clara, CA) and microarray analysis was performed at the
Genomics and Microarray Core at the University of Colorado Denver. Resulting CEL files were then imported into Bioconductor (Gentleman et al., 2004) and intensity values were preprocessed with the Robust Multi-Array Average (RMA) algorithm. RMA-preprocessed gene expression data from Affymetrix Human Genome U133A arrays and U133 Plus 2.0 arrays for the human NCI-60 was obtained from NCBI’s publicly available Gene Expression Omnibus website (dataset # GSE5846 and GSE32474, respectively) (Lee et al., 2007). Gene expression data for 16 canine osteosarcoma tumor samples were also publicly available (dataset # GSE24251) (O’Donoghue et al., 2010).

**COXEN method for prediction model building**

*Differential gene expression analysis:* To identify differentially expressed genes (DEGs) in our reference data sets 3 types of testing were performed Significance Analysis of Microarrays (SAM), t-test, and correlation tests. SAM analysis with a false discovery rate cutoff of 0.1 was used in a subset of the most and least sensitive samples for each drug. T-tests were run between sensitive and resistant samples with a q-value cutoff of 0.05 to identify DEGs. If too few DEGs made the q-value cutoff, a p-value cutoff < 0.001 was then used. Correlation tests were used to identify genes that were highly associated with drug sensitivity using a Benjamini Hochberg-adjusted p-value cutoff of 0.05. The correlation test was sometimes added as an additional filter with the SAM or t-test method.

*Probeset matching of gene expression data:* We employed four different strategies for matching probesets selected from either the Human Genome U133A or HT Human Genome U133A array to the GeneChip Canine Genome 2.0 array. **Best sequence homology:** We used the Basic Local Alignment Search Tool (BLAST) to compare the target sequences of the human probesets with their canine orthologs and selected the match with the highest sequence
homology. The blastn algorithm was selected for somewhat similar sequences, with word size = 7 and expected threshold = 100. In instances where no canine ortholog was annotated, an attempt at manual annotation was performed through sequence alignment using Affymetrix, BLAST, and USC genome browser tools. If manual annotation proved unsuccessful, than the human probeset was removed from the list of candidates for COXEN analysis. **Best correlative match:** This strategy was performed by creating correlation matrices for both human and canine probesets separately, and then a 3\textsuperscript{rd} correlation matrix created from each row of the first two matrices. Because the 3\textsuperscript{rd} matrix is created from the 2 original matrices, the original matrices had to be identical in dimensions. To do this, duplicates were added to the human correlation matrix to equal the number of probesets mapping to each gene on the canine matrix. The best match was selected that had the highest concordant expression with other genes in the signature across species. **Data collapsing by averaging method:** We took our list of candidate human PSIDs and their canine orthologs and in cases where multiples existed for a given gene on either the human or canine side, the expression values were averaged. This resulted in collapsing the probeset expression data down to the gene level, reducing the data for each gene to one human and matching canine expression value. Manual annotation was attempted when no annotated canine orthologs were available. **Data collapsing by maximum variance method.** Using the `collapseRows` function from the R package “WGCNA” (Miller A. Jeremy, 2011) we collapsed the gene expression data from the probe level down to gene level before differential gene expression analysis by selecting one representative probe per gene based on maximum variance between samples. After collapsing is performed in both human and canine datasets, genes that were not present on both arrays were filtered out.
Selecting subset of gene signature with strong concordant expression: The selecting of co-expressed genes between the reference and co-expression datasets was based on protocols described by Lee et al. (Lee et al., 2007; Pfister et al., 2009). Briefly, correlation matrices were generated for both the human gene signature and its matching canine probesets separately. Then, a 3rd matrix is created comparing each row of the first two matrices. Genes with correlation values higher than a cutoff (90th percentile from a random null distribution or a p-value cutoff of 0.05) were selected as being strongly co-expressed between the two data sets. This subset of the gene signature was used for prediction model generation.

MiPP algorithm model generation: Prediction models were generated in Bioconductor using the Missclassification-Penalized Posteriors (MiPP) algorithm developed by Soukup, Cho and Lee (Soukup et al.). When both a training and independent test set were included in the modeling process, we ran the sequential selection mode with $n = 3$ and chose the resultant model with the best sMiPP score and lowest error rate for each drug tested. Different classification methods such as linear discriminant analysis (LDA), logistic regression (LOG), linear support vector machines (SVMLIN), and radius-based function support vector machines (SVMRBF) were employed to determine the best models. LDA and SVMLIN were chosen for the majority of our MiPP modeling approaches. When we built prediction models using only a training set, the training set is split randomly several times into training and test sets, and the resultant models are then tested on more random splits of the data to determine robustness. The top 3-5 performing models are then used to predict onto a separate test dataset and the prediction scores are averaged for the final score. For in vivo model building, we identified candidate model genes in cell line datasets, but built the MiPP models on tumor panel data sets that were used in the co-expression step.
Validation of prediction models: To see how well the prediction models correlated with actual sensitivity data, we standardized both the posterior probability scores for each sample and the actual GI\textsubscript{50} data by subtracting the mean and dividing by the standard deviation. A Spearman rank-based correlation was then performed on the two data sets, as well as a binomial test based on random coin tossing for the testing of the models ability to successfully call a cell line “sensitive” or “resistant”. P-values < 0.05 were considered significant. For in vivo models we plotted survival curves based on actual disease free interval data and the class predictions of “responder” or “non-responder”. If the survival curves of the predicted groups were significantly different than each other by Log Rank test, the model was considered significantly accurate.

RESULTS

Human and canine cancer cell lines are similarly sensitive to chemotherapy

The 2 main cell line panels that were used for reference sets in our prediction model development (Figure 4.1) were the human NCI60 panel and the canine FACC panel (Table 4.1). Since drug response and microarray gene expression data were publicly available for the NCI60 panel, we generated drug response data in the FACC panel via Alamar Blue cell viability assays for CARBO, CIS, DOX, LOM, PTX, and VBL. The range of log GI\textsubscript{50} values between the human and canine panels were compared and found to be strikingly similar (Figure 4.2), although the means were significantly different between species except for with CIS (p=0.0942, Mann Whitney test).
Selecting a probeset matching strategy between the human and canine arrays

We next wanted to compare the genomic similarity of human and canine cancer cell lines in the context of drug sensitivity. RNA was extracted from untreated FACC cell lines and microarray gene expression data was generated. 39 differentially-expressed genes (DEGs) were identified in the NCI60 panel by using the 12 most and least sensitive cell lines to DOX in a SAM analysis. Since there are roughly twice as many probesets on the canine microarray chip as

**Figure 4.1 The COXEN method.** Steps 1-3 involve identifying a gene signature that predicts chemosensitivity from the human reference set. Steps 4-5 involve generating gene expression data from the canine test set and identifying a subset of the gene signature that shares strong co-expression with the canine set. The resulting group of genes is used in prediction model development, and the predictions are compared to actual canine chemosensitivity.

**Selecting a probeset matching strategy between the human and canine arrays**

We next wanted to compare the genomic similarity of human and canine cancer cell lines in the context of drug sensitivity. RNA was extracted from untreated FACC cell lines and microarray gene expression data was generated. 39 differentially-expressed genes (DEGs) were identified in the NCI60 panel by using the 12 most and least sensitive cell lines to DOX in a SAM analysis. Since there are roughly twice as many probesets on the canine microarray chip as
### Table 4.1. Datasets used in study

<table>
<thead>
<tr>
<th>Datasets</th>
<th># of samples</th>
<th>Sample type</th>
<th>Tumor types represented</th>
<th>Microarray platform/public ID</th>
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</thead>
<tbody>
<tr>
<td>NCI60</td>
<td>60</td>
<td>Human cancer cell lines</td>
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<tr>
<td>GDSC</td>
<td>10</td>
<td>Human osteosarcoma cell lines</td>
<td>Osteosarcoma</td>
<td>GeneChip HT Human Genome U133A array (E-MTAB-783, ArrayExpress)</td>
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<tr>
<td>ACC29</td>
<td>29</td>
<td>Canine cancer cell lines</td>
<td>Hemangiosarcoma, histiocytosis, leukemia, lymphoma, mammary tumor, mast cell, melanoma, osteosarcoma, soft tissue sarcoma, transitional cell carcinoma</td>
<td>GeneChip Canine Genome 2.0 array</td>
</tr>
<tr>
<td>COS16</td>
<td>16</td>
<td>Canine osteosarcoma tumor samples</td>
<td>Osteosarcoma</td>
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<tr>
<td>COS33</td>
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<td>Canine osteosarcoma tumor samples</td>
<td>Osteosarcoma</td>
<td>GeneChip Canine Genome 2.0 array</td>
</tr>
</tbody>
</table>

**Figure 4.2 Human and canine drug sensitivity is comparable.** GI50 ranges of the human NCI60 panel to 3 chemotherapeutics were compared to the ranges generated in the canine FACC panel via Alamar Blue assays.
the human, and also on both chips there are often multiple probesets mapping to the same gene, it became apparent that a strategy for selecting the best match for the identified DEGs between species was needed. Three strategies were initially implemented and tested for matching up the probesets. The strategies named best sequence homology, data collapsing by averaging, and best correlative match are explained in full in the Methods section and are represented schematically in Figure 4.3. Each strategy was used resulting in a human gene signature with matching canine

Figure 4.3 Selecting a probeset matching strategy. A) “Best Sequence Homology” compares the target sequences for a human PSID and its canine orthologs by BLASTing. The canine PSID with highest sequence homology with the human PSID is selected. B) “Data Collapsing by averaging” is performed by averaging the gene expression intensity values for each sample in cases where there are multiple PSIDS mapping to the same gene. C) “Best Correlative Match” is performed in a similar fashion as step 5 of COXEN, by creating correlation matrices and selecting the canine ortholog that has the highest concordant expression with other genes in the signature across species. D) “Data Collapsing by maximum variance” is performed by collapsing the data to the gene level by selecting one PSID per gene based on maximum variance using the collapseRows function in “WGCNA” R package.
data for both a DOX and VBL analysis. To test which strategy would produce the most predictive models, each were carried through the remaining steps of the COXEN analysis (Figure 4.1). The MiPP algorithm developed by Soukup, Cho, and Lee, which is utilized in the COXEN method, has the ability to use 5 different classification methods: LDA, QDA, LOG, SVMLIN, and SVMRBF. We tested a combination of all 3 probe matching strategies together with the 5 classification methods and generated prediction models. Comparing the resulting sMiPP scores and error rates associated with each model generated, best sequence homology was selected as an appropriate probeset matching strategy. We found multiple classification methods to be comparable in terms of overall performance and selected the commonly used LDA classification method for the remaining of our studies (Table 4.2). During the refining process of developing models, however, we incorporated another probeset matching strategy that was automated and less time intensive than comparing the sequence homology of potential probe matches. This new method involved collapsing both the human and canine array information to the gene level prior to differential gene expression analysis by selecting one probe per gene based on maximum variance between samples (Figure 4.3). It was performed using the collapseRows function in the “WGNCA” R package. We consider both of these methods as viable options for matching genes across platforms and used both in our attempts to optimize our prediction models.
Table 4.2 COXEN models using 5 classification methods and 3 probeset matching strategies

<table>
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<tr>
<th>MiPP rule</th>
<th>drug</th>
<th>probeset matching strategy</th>
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<th>genes in model</th>
<th>sMiPP score</th>
<th>error rate</th>
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<tbody>
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<td>Best homology**</td>
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<td>6</td>
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<td></td>
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</table>

* LDA was selected as the best performing classification method  
** “Best homology” was selected as best performing probeset matching strategy
Human and canine cancer cell lines share comparable genomic profiles of doxorubicin sensitivity

An unsupervised hierarchical cluster analysis was performed using expression data from the 39 NCI60-derived DEGs for DOX in both sensitive and resistant NCI60 and FACC cell lines, and we observed that all the NCI60 samples separated according to DOX sensitivity (Figure 4.4). Also, 67% (8/12) FACC cell lines in the analysis also separated according to sensitivity and interspersed with human samples, suggesting that human-derived DEGs are predictive in canine cancer cell lines (Figure 4.4). When a cluster analysis was performed using NCI60-derived DEGs with the NCI60 panel and a publicly available canine osteosarcoma tumor panel (COS16, Table 4.1) consisting of responder (DFI >300 days) and non-responder (DFI <100 days) patients to DOX, 92% and 100% of the NCI60 and COS16 samples sorted by drug sensitivity, respectively (Figure 4.4). DEGs were then derived from the 6 most and least sensitive samples to DOX in the FACC panel and cluster analysis was performed with FACC and the COS16, 100% and 86% of the FACC and COS16 samples sorted by drug sensitivity (Figure 4.4). Taken together, these data suggest that human and canine cancer cells respond similarly to chemotherapy and genes significantly different in NCI60 sensitive and resistant cell lines are able to separate sensitive and resistant samples in canine cancer cell lines and tumors. These results suggest that the human NCI60 cell line panel and canine FACC cell line panel had predictive potential for developing prediction models in canine osteosarcoma.

Predictivity of human-based COXEN models on chemosensitivity in canine cancer cell lines

Before we developed models to predict clinical outcome in osteosarcoma patients, we evaluated how effective human COXEN models could predict DOX and CARBO sensitivity in canine cancer cell lines. The best NCI60 models for the prediction of FACC cells involved
collapsing the microarray data to the gene level using the max variance method. After data collapsing, DEGs were identified in the NCI60 panel by performing SAM analysis on the 12 most and least sensitive lines. After the co-expression step with the FACC, the remaining candidate genes were used in model building using the MiPP algorithm. The sensitive and resistant NCI60 lines were used as the training set, and the 6 most and least sensitive cell lines in the FACC panel were used as the test set. The MiPP algorithm has the option to generate models with and without an independent test set for final model tuning.

**Figure 4.4 Human and canine cell line gene signatures for doxorubicin accurately sort osteosarcoma samples.** A-B) Differentially expressed genes based on sensitivity of NCI60 samples to doxorubicin were used in most and least sensitive NCI60 and FACC (A) or COS16 (B) samples in unsupervised hierarchical clustering. C) Differentially expressed genes based on sensitivity of FACC samples to doxorubicin were used in most and least sensitive FACC and COS16 samples in unsupervised hierarchical clustering. Light green and pink bars refer to sensitive and resistant samples, respectively. Blue, orange, and yellow bars refer to NCI60, FACC and COS16 samples, respectively.
The model trained on the NCI60 and tuned on the FACC was 75% accurate in predicting sensitivity to DOX (p=0.072, binomial test) (Figure 4.5). Without a tuning set, the training set is randomly split multiple times into training and test sets. The top 3-5 performing models are then tested independently on the test set. Models trained solely on the NCI60 panel were 83% accurate in predicting DOX sensitivity in the FACC (p=0.0193, binomial test) (Figure 4.5).

The model trained on the NCI60 and tuned on the FACC was 75% accurate in predicting sensitivity to CARBO (p=0.072, binomial test) (Figure 4.6). In contrast, models trained solely on the NCI60 were only 58% accurate in predicting CARBO sensitivity in the FACC (p=0.3872, binomial test) (Figure 4.6).

Knowing that the “tuned” multi-gene prediction models are created by adding a gene one at a time based on human reference sets and are programmed to stop adding genes when they stop improving on the error rate in canine test sets, it is impressive that 75% accuracy was achieved with interspecies models for both DOX and CARBO. Even more impressive was the fact that when the canine set was left out of the modeling training process except for the coexpression step, human models achieved significant prediction accuracy in canine cell lines for DOX. CARBO models were less accurate when trained solely on the NCI60 panel. These data are encouraging for our hypothesis that interspecies prediction modeling is plausible between human and canine cancer.

Predictivity of cell line-trained COXEN models on clinical outcome in 49 canine osteosarcoma patients

Our next step was to compare the ability of cell line-based prediction models to accurately predict clinical outcome in canine osteosarcoma patients. Using either the NCI60 or FACC panel as the reference set, identified DEGs for DOX and CARBO were co-expressed with 49
Figure 4.5 In vitro human COXEN models predict canine cell line sensitivity to doxorubicin. A & D) COXEN predictions compared to actual GI50 values from NCI60 model tuned with FACC(A) and un-tuned (D). Dark blue and red bars show resistant and sensitive lines, and light blue and yellow bars show COXEN predictions for resistance and sensitive. Significance of accurate predictions determined by binomial test.
Figure 4.6 In vitro human COXEN models predict canine cell line sensitivity to carboplatin. A & D) COXEN predictions compared to actual GI50 values from NCI60 model tuned with FACC(A) and un-tuned (D). Dark blue and red bars show resistant and sensitive lines, and light blue and yellow bars show COXEN predictions for resistance and sensitive. Significance of accurate predictions determined by binomial test.
canine osteosarcoma tumor samples (COS49, the COS16 and COS33 tumor panels combined). Models were then trained on the corresponding reference set and tested on the COS49. Data from a historic cohort study of 470 dogs treated for osteosarcoma (Selmic et al., 2014) were used to determine cutoffs between “responders” and “non-responders” in the COS49 based on median disease free interval of dogs that received doxorubicin (276 days) or carboplatin (296 days). Significantly accurate models were generated for DOX using both the NCI60 and FACC panels with error rates of 0.3333 and 0.2667, respectively (p = 0.0494 and 0.0081, binomial test) (Table 4.3). However, only the NCI60 model had a significant separation of survival curves (p = 0.0449, Log Rank) and correlation between prediction scores and disease free interval by cox proportional hazards regression (hazard ratio = 0.1989, p-value = 0.00951) (Figure 4.7 and Table 4.3). In contrast, NCI60 and FACC models for CARBO in the COS49 performed poorly, (p = 0.0449, Log Rank) and correlation between prediction scores and disease free interval by cox proportional hazards regression (hazard ratio = 0.1989, p-value = 0.00951) (Figure 4.7 and Table 4.3). In contrast, NCI60 and FACC models for CARBO in the COS49 performed poorly, (p = 0.0449, Log Rank) and correlation between prediction scores and disease free interval by cox proportional hazards regression (hazard ratio = 0.1989, p-value = 0.00951) (Figure 4.7 and Table 4.3). In contrast, NCI60 and FACC models for CARBO in the COS49 performed poorly, (p = 0.0449, Log Rank) and correlation between prediction scores and disease free interval by cox proportional hazards regression (hazard ratio = 0.1989, p-value = 0.00951) (Figure 4.7 and Table 4.3). In contrast, NCI60 and FACC models for CARBO in the COS49 performed poorly, (p = 0.0449, Log Rank) and correlation between prediction scores and disease free interval by cox proportional hazards regression (hazard ratio = 0.1989, p-value = 0.00951) (Figure 4.7 and Table 4.3). In contrast, NCI60 and FACC models for CARBO in the COS49 performed poorly, (p = 0.0449, Log Rank) and correlation between prediction scores and disease free interval by cox proportional hazards regression (hazard ratio = 0.1989, p-value = 0.00951) (Figure 4.7 and Table 4.3). In contrast, NCI60 and FACC models for CARBO in the COS49 performed poorly, (p = 0.0449, Log Rank) and correlation between prediction scores and disease free interval by cox proportional hazards regression (hazard ratio = 0.1989, p-value = 0.00951) (Figure 4.7 and Table 4.3). In contrast, NCI60 and FACC models for CARBO in the COS49 performed poorly,
both resulting in an error rate of 0.4138 (Table 4.4). The plotting of survival curves were found to be not significant (Figure 4.7). These data suggest that our datasets appear better at generating prediction models for DOX than for CARBO, with our human model performing the best with the COS49.

**Figure 4.7** Cell line-trained COXEN models on clinical outcome of COS49. A & C) Survival analysis of a NCI60 trained model co-expressed with COS49 for doxorubicin (A) and carboplatin (C). B & D) Survival analysis of an FACC trained model co-expressed with COS49 for doxorubicin (B) and carboplatin (D). Significant difference between predicted groups determined by Log Rank test.
Table 4.4. COXEN modeling results for carboplatin sensitivity

<table>
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<th>Train set</th>
<th>Tune set</th>
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<th>Cox hazard ratio</th>
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| **In vivo models on tumor response prediction** | | | | | | | | | |
| NCI60         | COS16             | COS16     | NA       | COS33    | 0.4000     | 0.2122         | 0.8240          | 2.0379         | 0.556       |
| FACC          | COS16             | COS16     | NA       | COS33    | 0.3076     | 0.0378         | 0.1504          | 0.1654         | 0.262       |
| GDSCosteo     | COS16             | COS16     | NA       | COS33    | 0.3077     | 0.0378         | 0.5115          | 0.6193         | 0.677       |
| FACCosteo     | COS16             | COS16     | NA       | COS33    | 0.3077     | 0.0378         | 0.5115          | 0.6193         | 0.677       |

* Error rates and tests based on a subset of most and least sensitive samples in the panel.  
  b Carboplatin was not screened in the GDSCosteo panel

**Predictivity of cell line-trained models on clinical outcome of 33 independent canine osteosarcoma patient**

The COS49 models for DOX were encouraging, but the test set could not be considered truly independent, as it was used during the co-expression step of the modeling process. We then separated our osteosarcoma patients into their original panels and evaluated whether accurate models could be generated for the COS33 from cell lines if the COS16 was used as the co-expression set. All of our modeling results are reported in Table 4.3. The NCI60 model had an error rate of 0.3182 compared to 0.3043 for the FACC model (p = 0.0669 and 0.0466, binomial, Table 4.3). The NCI60 model performed better than the FACC model in the survival curve analysis (Table 4.3, Figure 4.8).
We then tested if modeling could be improved if we used osteosarcoma cell lines to better reflect the test set. The 10 osteosarcoma cell lines from the FACC panel were used (FACCosteo), as well as 10 human osteosarcoma lines from the GDSC panel. Interestingly, we observed that human osteosarcoma cell line model for DOX performed poorly with an error rate of 0.4348 (p=0.3388), whereas the FACCosteo model for DOX was equally accurate to the FACC model with an error rate of 0.3043 (p = 0.0466, Table 4.3). Improvement was observed

Figure 4.8 Cell line-trained models on clinical outcome in doxorubicin-treated COS33. A. Survival analysis of a NCI60 trained model co-expressed with COS16. B) Survival analysis of an FACCosteo trained model co-expressed with COS16. Significant difference between predicted groups determined by Log Rank test.
in the survival curve analysis using the FACCoste model, although only approaching significance (p=0.0706, Figure 4.8). Compared with the COS49 models, the performance of human DOX models decreased and the canine DOX models increased when creating a truly independent test set in the COS33.

NCI60 and FACC models of CARBO had error rates of 0.4800 and 0.3462 in predicting sensitivity in the COS33 (p = 0.500 and 0.0843, Table 4.4 and Figure 4.9). The FACCoste

![Figure 4.9](image.png)

**Figure 4.9** Cell line-trained models on clinical outcome in carboplatin-treated COS33. A) Survival analysis of a NCI60 trained model co-expressed with COS16. B) Survival analysis of an FACC trained model co-expressed with COS16. Significant difference between predicted groups determined by Log Rank test.
model was significantly accurate with an error rate of 0.3077 (p =0.0378), however in the survival curve analysis the FACC model was superior to the FACCosteo model (p =0.0482 versus 0.9038, Log Rank) (Table 4.4, Figure 4.9). A human osteosarcoma cell line model could not be performed due to absence of available CARBO sensitivity data in the GDSC panel. These data continue to suggest that CARBO models trained with canine cell line data perform better than when trained on human cell lines.

*Predictivity of tumor-trained models on clinical outcome of 33 independent canine osteosarcoma patient*

A study in 2010 introduced the use of “in vivo COXEN” which implemented cell line panels for the reference set but tumor panels for the model training set (Lee et al., 2010). We tested whether using the COS16 as the model training set would improve our human and/or dog COXEN models for DOX and CARBO in the COS33. The human NCI60 DOX model that was co-expressed and trained on the COS16 performed very well with an error rate of 0.2727 (p = 0.0262, binomial) and a very significant separation of survival curves (p=0.0010, Log Rank) (Table 4.3, Figure 4.10). Additionally, the prediction scores significantly correlated with disease free interval in a cox proportional hazards regression analysis with a hazard ratio of 0.03073 and a p-value of 0.00695 (Table 4.3). In contrast, A human osteosarcoma cell line DOX model trained on the COS16 performed poorly with an error rate of 0.4783 (p = 0.5000) (Table 4.3).

The canine FACC DOX model that was co-expressed and trained on the COS16 performed well but to a lesser extent with an error rate of 0.3043 (p = 0.0466, binomial), and a separation of survival curves that approached significance (p = 0.0714, Log Rank) (Table 4.3, Figure 4.10). The FACCosteo DOX model that was trained on COS16 did not perform as well,
with an error rate of 0.3913 (p = 0.2024) (Table 4.3). These data show that implementing an “in vivo COXEN” method did result in our best human DOX model, and a canine DOX model that performed slightly under the best-performing FACCosteocell line-trained model, suggesting it to be an advantageous strategy.

**Figure 4.10 In vivo COXEN models predict clinical outcome in doxorubicin-treated canine osteosarcoma patients.** A) Survival analysis of a NCI60 model co-expressed and trained on COS16. B) Survival analysis of an FACC model co-expressed and trained on COS16. Significant difference between predicted groups determined by Log Rank test.
The human NCI60 CARBO model trained on the COS16 performed poorly with insignificant results and an error rate of 0.4000 (Table 4.4, Figure 4.11). Both the canine FACC and FACCosteo CARBO models trained on the COS16 had significant accuracies with a shared error rate of 0.307 (p = 0.0378, binomial). However, none of the other tests for model performance were significant (Table 4.4, Figure 4.11), showing that although the CARBO

Figure 4.11 In vivo COXEN models predict clinical outcome in carboplatin-treated canine osteosarcoma patients. A) Survival analysis of a NCI60 model co-expressed and trained on COS16. B) Survival analysis of an FACC model co-expressed and trained on COS16. Significant difference between predicted groups determined by Log Rank test.
models generally performed poorly compared to DOX models, canine CARBO models consistently outperform human CARBO models in our studies.

*Effect of COXEN models on clinical outcome of osteosarcoma patients receiving combination chemotherapy*

The best DOX and CARBO models were selected based on overall performance from statistical testing (Table 4.5). For DOX, the NCI60 model that was co-expressed and trained on the COS16 was selected. Probeset matching between species was done using the best sequence

<p>| Table 4.5 Genes from best COXEN models for doxorubicin and carboplatin response in COS33. |
|---------------------------------|---------------------------------|---------------------------------|</p>
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<th>Model</th>
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<th>Gene Title</th>
<th>Function</th>
</tr>
</thead>
<tbody>
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<td>DOX model</td>
<td>CHKA</td>
<td>Choline kinase alpha</td>
<td>Phospholipid biosynthesis, tumor cell growth</td>
</tr>
<tr>
<td>DOX model</td>
<td>TLE1</td>
<td>Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)</td>
<td>Transcriptional co-repressor; inhibits NF-kappa-B expression and WNT signaling</td>
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<td>NCI60-COS16-COS16-COS33</td>
<td>EIF6</td>
<td>Eukaryotic translation initiation factor 6</td>
<td>Helps ITGBB4 link to cytoskeleton</td>
</tr>
<tr>
<td></td>
<td>TES</td>
<td>Testis derived transcript (3 LIM domains)</td>
<td>Scaffold protein; role in cell adhesion, cell spreading, reorganization of actin cytoskeleton, regulates cell proliferation, may act as a tumor suppressor</td>
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<td>PSMD3</td>
<td>Proteasome (prosome, macropain) 26s subunit, non-ATPase, 3</td>
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<td></td>
<td>cOR4F25</td>
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<td>Transmembrane protein 131-like</td>
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</tbody>
</table>
homology method. 4 genes are involved in the model: Choline kinase alpha (CHKA), Transducin-like enhancer of split 1 (E(sp1)hogmem,Drosophila) (TLE1), Eukaryotic translation initiation factor 6 (EIF6), and Testis derived transcript (3 LIM domains) (TES). CHKA is involved in phospholipid biosynthesis and tumor cell growth. TLE1 is a transcriptional co-repressor known to inhibit NF-kappa-B expression and WNT signaling. EIF6 and TES both are involved with the cytoskeleton; EIF6 helps link ITGB4 to the cytoskeleton, and TES is a scaffold protein that has roles in cell adhesion, cell spreading, reorganization of the actin cytoskeleton, regulates cell proliferation, and may act as a tumor suppressor (Table 4.5). For CARBO, the FACC model that was co-expressed with the COS16 but trained back on the FACC was selected. 6 genes are involved in the model: Oculocutaneous albinism II (OCA2), Hairy and enhancer of split 3 (Drosophila) (HES3), uncharacterized cytochrome C oxidase (LOC100688725), Proteasome (prosome, macropain) 26s subunit, non-ATPase, 3 (PSMD3), cOR4F25 olfactory receptor family 4 subfamily F-like (cOR4F25), and transmembrane protein 131-like (KIAA0922). OCA2 is a transporter of tyrosine within melanocytes. HES3 is a transcriptional repressor of gene requiring bHLH protein for transcription. LOC100688725 has cytochrome C oxidase activity. PSMD3 is involved in ATP-dependent degradation of ubiquinated proteins. cOR4F25 is involved in canine olfactory processes. KIAA0922 is an integral transmembrane protein with possible involvement in immune responses (Table 4.5).

Fifteen dogs from the COS33 panel received combination therapy of DOX and CARBO. Survival curves from dogs who received combination treatment were split into 4 groups and compared: dogs predicted to be sensitive to neither drug, dogs predicted to be sensitive to only CARBO, dogs predicted to be sensitive to only DOX, and dogs predicted to be sensitive to DOX and CARBO. In Figure 4.12 a significant trend was observed between the curves, with the
DOX only sensitive and DOX and CARBO sensitive groups surviving disease-free much longer than the CARBO only sensitive and resistant groups (p = 0.0032, Log Rank trend). These data show that by combining information from our single drug COXEN models the ability to predict response in osteosarcoma patients receiving combination chemotherapy is achievable.

**Effect of COXEN-matching on clinical outcome of canine osteosarcoma patients receiving single agent or combination treatment**

In order to more fully evaluate the benefit of this particular genomic approach of gene expression models to determine treatment in canine osteosarcoma, we classified our COS33 patients into 2 categories: “COXEN matched” and “COXEN unmatched”. We wanted to evaluate patient outcome if they actually received the specific treatment our COXEN models indicated. Our criteria for classification are detailed as follows: For patients treated only with
DOX, if the DOX model predicted them to be sensitive (COXEN score > 0.5), and the CARBO model predicted them to be resistant (COXEN score < 0.5), they were considered “COXEN matched”. For patients treated only with CARBO, if the DOX model score < 0.5 and the CARBO model score > 0.5 they were “COXEN matched”. For patients receiving a combination of DOX and CARBO, both the DOX model and CARBO model score need to be > 0.5 for them to be considered “COXEN matched”.

A survival curve analysis comparing the two groups showed that a larger percentage of COXEN matched dogs survived disease-free at all time points than COXEN unmatched dogs (p = 0.0300, Log Rank) (Figure 4.13). The shapes of the survival curves show a much larger drop in disease-free dogs in the COXEN unmatched dogs before 200 days than the COXEN matched dogs, followed by relatively parallel survival curves at later time points, suggesting that our prediction models may be used as a negative predictor to determine which drug not to treat with.

**Figure 4.13.** Effect of COXEN matching on clinical outcome of canine osteosarcoma patients receiving single agent and combination treatment. A) Survival analysis between dogs that were treated with the best predicted treatment (COXEN matched) and dogs that weren’t (not COXEN matched). Significant differences between predicted groups determined by Log Rank test.
We analyzed the strength of our COXEN modeling as a factor associated with DFI by performing a multivariate Cox proportional hazards regression analysis with “COXEN matched” as a covariate to be compared with 6 other clinical factors: Proximal humeral tumor location, serum alkaline phosphatase levels (ALP), monocyte count, lymphocyte count, body weight, and age at diagnosis. We found that COXEN matched had a hazard ratio of 0.3102 which was significant (p = 0.0124). The other significant factor associated with DFI in our COS33 patients was body weight (hazard ratio = 1.0047, p = 0.0261). The last factor in the model was proximal humeral tumor location (hazard ratio = 2.3974, p = 0.0877) (Table 4.6). Taken together, our studies suggest that gene expression models for drug sensitivity have great potential to be used in an inter- and intraspecies manner for improvement of personalized medicine for canine osteosarcoma patients.

Table 4.6 Factors associated with disease free interval (DFI) of COS33 patients in a multivariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% CI)</th>
<th>P value</th>
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<tbody>
<tr>
<td>COXEN model treatment match</td>
<td>0.3102 (0.1240-0.7762)</td>
<td>0.0124</td>
</tr>
<tr>
<td>Body weight (continuous)</td>
<td>1.0047 (1.0052-1.0857)</td>
<td>0.0261</td>
</tr>
<tr>
<td>Proximal humeral tumor</td>
<td>2.3974 (0.8787-6.5407)</td>
<td>0.0877</td>
</tr>
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</table>

HR, hazard ratio; CI, confidence interval

**DISCUSSION**

The use of gene expression prediction models in personalized medicine is emerging as a potential alternative to traditional treatment strategies. In this study we were able to successfully develop prediction models for DOX and CARBO sensitivity in canine osteosarcoma using both a combination of human and canine datasets as well as canine datasets alone. The best performing DOX model was developed by first identifying DEGs from the human NCI60 panel, followed by co-expression and model training on canine osteosarcoma tumors. The ability for the COXEN
method to extrapolate data from one dataset to another was able to not only be applied from an *in vitro* panel to an *in vivo* dataset, but also from one species to another. Additionally, knowing the NCI60 panel does not contain osteosarcoma cell lines makes this model even more impressive. These results have exciting implications, as it suggests that it may be feasible and beneficial to incorporate human genomic data in the development of gene expression-based predictions of treatment for canine cancer. This would be very advantageous for canine oncology, because available data needed for these types of analyses are currently much more prevalent in human research.

In general, attempts at building prediction models for CARBO sensitivity were not as successful as they were for DOX. Additionally, models developed using only canine datasets for CARBO did consistently better than when the human NCI60 panel was incorporated in the process. A possible explanation for this was that the range of GI$_{50}$ values in the NCI60 was the narrowest for CARBO compared to DOX and CIS, suggesting that genetic differences between “sensitive” and “resistant” groups in the CARBO data may have been minimal or were dominated by unrelated factors (Figure 4.2).

Challenges with this study lies in the relatively small sizes of our datasets. Ideally, larger panels would serve to more fully represent the heterogeneous cancer population, leading potentially to more robust models. Small sample size in our testing sets made it difficult to reach statistically significant predictive power in many of our modeling iterations. We hope to expand the FACC panel in coming years through the establishment of new tumor-derived cell lines as well as through increased collaborations with other institutions. Additionally, as the price for gene expression profiling continues to drop, the opportunity to expand our *in vivo* datasets of canine osteosarcoma as well as other tumor types such as lymphoma will increase. Regardless of
the current obstacles, our best performing models for DOX and CARBO did achieve statistically significant results in a truly independent test set, which is very encouraging.

We used the MiPP algorithm in our model building process, which is designed to select the most parsimonious models without sacrificing predictive efficacy. There are advantages and disadvantages to this. Smaller models could allow for the development of gene expression-based tests similar to ones used in breast cancer diagnosis or even RTPCR protocols that would deliver quick results for patients. However, there is the opinion that prediction models with few genes tend to be less robust when tested against multiple independent datasets. As our models have only been tested in one independent test set, we cannot at present know if they would hold up to validation from additional datasets. In our investigations other model building methods besides using MiPP were employed, including principal component regression and lasso regression, which resulted in larger gene models than when we used MiPP. Unfortunately, these alternative models failed to perform as well as our best MiPP models (data not shown).

Other options for model building that might be explored in the future would be to adopt some machine learning methods with the ability to incorporate multiple types of genomic data into the modeling process. In 2014 an article reporting the results of a community-based contest of 44 competing drug sensitivity prediction algorithms concluded that although gene expression microarrays was consistently the top source for predictive power, performance could be improved with the addition of data such as methylation status of genes, copy number variation, and exome sequencing (Costello et al., 2014). These additional types of genomic data would need to be obtained for the datasets involved in the prediction model process before these new strategies can be investigated, which can be costly.
One of the biggest advantages to this type of approach in canine cancer is the possibility of running future clinical trials in dogs with osteosarcoma. Current protocols in canine osteosarcoma typically call for amputation of the affected limb followed by adjuvant administration of DOX and/or CARBO. A next step for us would be to conduct a clinical trial that would allow us to dictate their chemotherapy strategy based on the predictions from our models. Receiving approval to carry out a genomics trial is challenging in human oncology in the current climate, but less so in the veterinary world. We hope that success of this strategy in dogs could serve as much needed validation for similar methods in human research to move forward.
REFERENCES


Chapter 5

General conclusions and future directions

GENERAL CONCLUSIONS

The purpose of this dissertation is to explore different applications of comparative and translational oncology through the use of the canine cancer model. Canine oncology has grown steadily as a field over the last 20 years, but there is still much to discover in our attempts to better understand cancer processes and develop better treatment strategies for improved clinical outcome. The emergence of genomic data in both human and canine cancer has made possible comparative studies that reveal the strong similarities on a genetic level between the two species. These new insights fit nicely into the ever-growing body of evidence that dogs with cancer make a translational model of human cancer. The use of tissue culture is an invaluable tool in cancer research, and large human cell line panels dedicated to drug screening and molecular profiling has led to new discoveries that has greatly benefited the field of cancer research. Our purpose was to characterize a canine cancer cell line panel we have established at the FACC at Colorado State University, and to use this panel to explore and compare the relationship between gene expression and cancer drug sensitivity in both human and canine cancer.

In Chapter 2 we described the FACC panel, which currently contains 29 canine cancer cell lines representing 11 different tumor types. These cells have been validated and confirmed to be of canine origin and to be unique to each other based on microsatellite analysis. Molecular profiling has been done across the panel, looking at two microarray platforms for mRNA
expression. With this gene expression data, we have shown that the cell lines cluster according to tumor type based on both principal component as well as unsupervised cluster analysis. Performing cluster analysis with known cancer genes revealed some interesting and potentially useful data that could lead to future exploration of certain cancer genes in different canine tumor types. We also showed an example of identifying a gene signature associated with the cellular processes of migration and invasion in osteosarcoma cells, which could lead to potential targets to combat cancer metastasis which is the cause of the inevitable morbidity of this disease in dogs.

In Chapter 3 we wanted to use part of the FACC panel in an in depth comparative analysis of activated pathways in both human and canine melanoma. We were able to show through pathway analysis of DEGs that were identified by comparing tumor and normal tissue samples in both species that although malignant melanoma predominantly occurs in different anatomic sites in humans and dogs, both the MAPK and PI3K/AKT pathways in both cancers shared similar alterations. Knowing that a high percentage of human melanoma is driven by activating mutations in either BRAF or NRAS, we performed mutational analysis at these common sites of mutation in canine melanoma tumors and cell lines. The only mutation discovered was a heterozygous mutation in NRAS for the Jones melanoma cell line, suggesting that for canine melanoma these important pathways are activated by a different mechanism.

After confirming constitutive activation of both the MAPK and PI3K/AKT pathways in human and canine melanoma cell lines through serum starvation assays, we next compared the effects of pathway inhibition through the use of a MEK1/2 inhibitor AZD6244 and the mTOR inhibitor rapamycin. We showed through western blot, cell viability assays, and cell cycle analysis that human and canine melanoma are similarly sensitive to MAPK and PI3K/AKT
pathway inhibition, and the targeting of both pathways in combination led to synergistic blocking of proliferation and the cell cycle. These data suggested that although the common mutations in human melanoma appear rare in canine melanoma, dogs with this disease may benefit from the new pathway targeting strategies being developed for human melanoma. Also, canine melanoma may serve as an important translational model to investigate and develop better pathway targeting protocols.

In chapter 4 we utilized the FACC panel in a different approach to investigate the use of gene expression prediction models and their ability to predict chemosensitivity in canine osteosarcoma. A recently developed method called “COXEN” has been shown to extrapolate predictive gene signatures from one dataset into a target set comprised of an unrepresented histology. Knowing the genetic similarities of human and dog tumors, we hypothesized that this method could be implemented not only across tumor types but also across human and canine cancers in order to develop interspecies models that would be predictive in dogs with osteosarcoma.

Through the use of drug screening and gene expression profiling of the FACC panel, 2 human cell line panels (NCI60 and GDSC), as well as a canine osteosarcoma tumor panel we were able to develop prediction models for both doxorubicin and carboplatin that successfully predicted clinical outcome in an independent canine osteosarcoma tumor dataset. Our best doxorubicin model involved identifying DEGs in the human NCI60 panel followed by co-expression and model training with a canine osteosarcoma tumor panel. Our best carboplatin model involved identifying DEGs in the canine FACC panel followed by co-expression with a canine osteosarcoma tumor panel and training on the FACC. In dogs that were treated with combination doxorubicin/carboplatin adjuvant therapy, we showed that they lived longer
disease-free if they were predicted to be sensitive to both drugs in our top models. Additionally, when we compared dogs that received the drug they were predicted sensitive to (“COXEN matched”) with the dogs that didn’t (“COXEN mismatched”), we saw a significant increase in disease-free survival. In a COX proportional hazards multivariate analysis being a “COXEN matched” patient was a significant factor associated with disease-free survival, followed by body weight and proximal humerus tumor location. These data show that the COXEN method was capable of generating models in both an intra- and interspecies manner that effectively predicted chemosensitivity in an independent set of osteosarcoma patients. These are exciting results because it opens up the possibility for canine oncology to take advantage of the wealth of available human genomic data. Additionally, it opens up the possibility to further test these genomic methods in veterinary clinical trials which could provide pre-clinical validation for these methods to advance in human cancer research.

In conclusion, we have shown that the FACC panel is a valuable tool for comparative and translational studies of cancer. With this resource we were able to show strong similarities of cancer drug sensitivity between human and canine cancer in both a pathway-focused molecular approach in melanoma as well as a non-pathway focused strategy with multi-gene prediction models in osteosarcoma. These studies strongly suggest that the canine cancer model is highly similar on a molecular level to human cancer, which not only makes dogs with cancer a great translational tool to advance human research, but also allows humans with cancer to be a great translational tool to advance canine research. The strong mutual benefits that come from these types of comparative studies calls for a greater investment of resources to more fully integrate canine and human cancer research as we continue to strive towards the common goal to eradicate cancer.
FUTURE DIRECTIONS

We have shown the utility of the FACC panel through multiple projects, all of which can be explored more deeply with future experimentation. In chapter 2 we described the current state of the FACC panel, but acknowledge that it can be further improved as a resource through expansion of the number of cell lines and tumor types. Possible routes to achieve this growth would be either through establishing new lines from fresh tumors available at the FACC, or to collect more from collaborating research labs and institutions. The latter option could potentially lead to the quickest route towards that aim, as there are many difficulties associated with establishing stable cell lines from tumors. Communication and organization would be essential for this type of large-scale collaboration, but multi-institutional collaborations in veterinary oncology such as the Comparative Oncology Trials Consortium (COTC) have proven successful (Gordon et al., 2009).

The FACC panel could also benefit in the future by expanding the types of molecular profiling data available for the cell lines. Next generation sequencing data could help in future investigations of mutation status in canine cancers, and Array CGH data would provide information about copy number alterations in the genome. Much human research has involved these highly sensitive genetic analyses and to translate their findings into veterinary oncology we would need corresponding analyses performed. Additionally, the use of the FACC panel in identifying novel drugs for canine cancer will probably not reach its maximum potential unless some high throughput drug screening methods are developed and employed. This would require major funding for equipment and personnel if were to be developed within the FACC, but
perhaps a partnership with another institution with expertise in this area could be reached that would allow future drug screening to move forward at an accelerated pace.

In Chapter 3 we concluded that canine melanoma would make a good model to investigate novel treatment strategies dealing with MAPK and PI3K/AKT pathway inhibition. The success of selective BRAF V600E inhibitors such as vemurafenib and dabrafenib in human melanoma patients has been considered a long awaited breakthrough for the management of this disease. However, in the last couple of years the problem with acquired and innate resistance to these inhibitors has taken center stage. Unfortunately, most patients who respond will eventually relapse. Secondary mutations, pathway redundancy and up-regulation of upstream signals have all been shown to play a role in reactivation of the MAPK pathway, the main mechanism of acquired resistance in melanomas treated with RAF inhibitors. Although canine melanomas do not appear to have BRAF V600E mutations, it would be interesting to further investigate acquired resistance of MAPK pathway inhibition in canine melanoma, to see if similar mechanisms elucidated in the human disease was reflected in dogs. Experimentally inducing resistance by culturing canine melanoma cell lines with continuous exposure to the MEK1/2 inhibitor AZD6244 would be the first step, followed by ascertaining if the MAPK pathway is indeed re-activated in the presence of MEK inhibition in these resistant cells. If this was found to be true, then this would provide a good translational model to study mechanisms of resistance more fully as well as develop counteractive treatment strategies.

Additionally, there is much left to know about the actual mechanism of pathway activation in canine melanoma. Results from our pathway analysis of DEGs identified by comparing tumor and normal tissue samples not only revealed the similar alterations in the MAPK and PI3K/AKT pathway in human and canine tumors, but also provided potential hints
for what might be the driving force in dogs. One possibility is integrin signaling. Many of the
top differentially expressed pathways in both species were related to cell adhesion and integrin
signaling. Interestingly, the differentially expressed integrins were mostly down-regulated in
human melanoma, but up-regulated in canine melanoma. Since integrins can signal through the
Focal adhesion kinase/steroid receptor coactivator (FAK/Src) complex to activate both MAPK
and PI3K/AKT signaling, this represents a potential target for further research (Bolos et al.,
2010).

In Chapter 4 we developed predictive models for canine osteosarcoma chemosensitivity
despite some difficulties related to the small size of our datasets. However, the testing of our
models on additional canine osteosarcoma samples would serve as further external validation.
Since a couple of years have passed since we last extracted RNA from tumor samples meeting
our criteria for microarray analysis, it would be beneficial to look for newly acquired samples
from patients that have been treated since that time. Although the few other model building
algorithms that we have explored did not produce more accurate models than what we achieved
using the MiPP algorithm, there are still many more methods that we have not yet explored. A
large study that compared 44 competing methods for developing prediction models for drug
sensitivity concluded that the top performing method was based on machine learning to
incorporate multiple types of molecular profiling data (Costello et al., 2014). This strategy was
able to capture the non-linear relationships between genomics, epigenomics, and drug sensitivity.
We have begun collaborations to explore if machine learning methods can perform as well or
better than our MiPP generated models for canine osteosarcoma. In order to thoroughly
investigate this new strategy, the generating of additional genomic data would be beneficial.
Another direction for the future for these prediction models would be to test them prospectively in a veterinary clinical trial. Tumor biopsies would be collected followed by RNA extraction and microarray analysis. The results of our prediction models for their individual tumors would dictate whether they received adjuvant chemotherapy in the form of doxorubicin, carboplatin, or both. Since the approval for clinical trials in dogs is a smoother process and less complicated than in humans, we envision this being feasible. It would provide some very important validation not only for these types of genomic methods being used in the clinic but also help as pre-clinical validation for human trials to move forward as well.
REFERENCES

