CANINE HISTIOCYTIC DISEASES: UNRAVELING THE MECHANISMS OF TUMORIGENESIS

By

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ABSTRACT

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Dog breeds provide unique genetic pools for studying rare disorders that affect both dogs and humans. Understanding the prevalence of these disorders is a key first step in addressing breed-associated diseases and establishing the utility of the dog as a spontaneous model of human disease. Analysis of the community-based assessment of health status of Bernese mountain dogs (BMD) was conducted utilizing an open-access database, the Berner-Garde Database. The Berner-Garde Database was accessed on March 1, 2013 and outputted to SQL, a database management system, for querying. Complete entries that included sex, age, and diagnosis were chosen, resulting in 7,262 individual dogs for analysis. Nearly 86% of these dogs died by ten years of age. Over 44% of diagnoses were categorized as cancer; the most prevalent cancer was histiocytic sarcoma, which comprised 37% of the cancer diagnoses, while lymphoma was the second highest cancer diagnosis at 20%. During the last twenty years cancer has surpassed dysplasias as the most important diagnosis for BMDs. The prevalence of histiocytic sarcoma diagnoses has grown, which may be partially due to improved diagnostic criteria. At the same time, the median age at death for BMDs with histiocytic sarcoma has not improved, but in fact, has decreased over the last decade. Despite improvement in diagnosis of histiocytic sarcoma, improvements in treatment of this disease have not followed.

Histiocytic diseases encompass a spectrum of proliferative diseases in dogs and humans. Human histiocytic diseases are rare diseases that affect children and adults, but in BMDs, these diseases are common. Canine histiocytic diseases range from benign histiocytoma to malignant histiocytic sarcoma (HS), which in the disseminated form has a poor prognosis and does not respond well to chemotherapeutics. The goal of this work is to unravel the mechanisms of tumorigenesis in

canine histiocytic diseases by probing the differential expression of microRNAs (miRNA) along the spectrum of canine histiocytic diseases. As regulators of gene expression, miRNAs will provide insight into the pathways for tumorigenesis in histiocytic malignancies as compared to benign histiocytic diseases. MiRNA profiling of canine histiocytic diseases was conducted on cases of reactive histiocytosis, histiocytic sarcoma, and hemophagocytic histiocytic sarcoma. These samples were compared to normal canine histiocytes derived from peripheral blood and peritoneal fluid. Analysis of the profiling data yielded several miRNAs, which were selected for validation by quantitative reverse transcription PCR (qRT-PCR). Data analysis with unsupervised clustering revealed sets of miRNAs that were overexpressed in BMDs or mixed breed dogs. Validation by qRT-PCR showed significant upregulation of miR-125b and miR-152 in dogs affected with a histiocytic disease. MiRNAs that are critical for tumorigenesis represent targets for manipulation to increase our understanding of these malignancies and discover novel and effective therapies.

As part of a concurrent approach to understanding histiocytic disease, the whole genome of a female spayed BMD with histiocytic sarcoma was sequenced using three genomic DNA samples. The first was a blood sample taken from this dog when she was 4 years of age, prior to diagnosis with histiocytic sarcoma. The second was a sample of a mediastinal tumor mass and the third sample was a cell line derived from an affected abdominal lymph node; both of these samples were taken at necropsy. There were 29 spontaneous nonsynonymous mutations in the tumor that were predicted to be deleterious and four of these variants were in genes associated with cancer including *SSH3*, *ITGB7*, and *FANCM*. Six mutations potentially represented loss of heterozygosity including *FAT1*, *MTUS1*, and *LRP1B*, which are associated with cancer. Over one hundred variants were considered germ line mutations, the majority of which were heterozygous in all the samples and fifty-one were homozygous in all three samples. Using these data, future work will involve evaluating the genes in independent affected and unaffected BMDs to validate the variants as potential driver mutations of histiocytic sarcoma tumorigenesis.

This dissertation is dedicated to my mother, Pamela Gayle Gregory 1956-2014.

ACKNOWLEDGEMENTS

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KEY TO ABBREVIATIONS

BMD = Bernese mountain dog; DIC = disseminated intravascular coagulation; FFPE: formalin-fixed paraffin embedded; GI = gastrointestinal; GIST = Gastrointestinal stromal tumor; GDV = gastric dilatation volvulus; GR = golden retriever; HCM = histiocytoma; HP = hemophagocytic histiocytic sarcoma; HS = histiocytic sarcoma; mix = mixed breed dog; PDGFRA: platelet-derived growth factor receptor, alpha polypeptide; RHC = reactive histiocytosis.

CHAPTER 1

INTRODUCTION

Histiocytic diseases encompass a spectrum of proliferative diseases in both dogs and humans. The clinical presentation, behavior, and response to treatment are quite dissimilar for the different histiocytic diseases. In dogs, these diseases range from the mild proliferative lesion histiocytoma, which can spontaneously regress, to the aggressive neoplastic malignancy histiocytic sarcoma (HS), which in its disseminated form, can lead to death or euthanasia within 2 to 4 months from diagnosis [Zavodovskaya et al., 2006]. Histiocytic diseases are relatively common in dogs. The neoplastic malignancy HS is especially common among certain breeds of dogs including the Bernese mountain dog, golden retriever, flat-coated retriever, and Rottweiler. Approximately 25% of all tumors in the Bernese mountain dog (BMD) breed are diagnosed HS [Abadie et al., 2009]. Unfortunately, there is a dearth of effective treatment options available for histiocytic sarcoma [Skorupski et al., 2007]. HS is usually disseminated at the time of diagnosis in the BMD and response to chemotherapeutics is, at best, short-lived [Hafeman et al., 2010].

Human histiocytic diseases are rare and affect both children and adults. HS accounts for $< 1\%$ of all non- Hodgkin's lymphomas in humans [Ralfkiaer et al., 1990]. Human histiocytic diseases are so rare that research funding and progress are slow due to inadequate sample sizes of human patients to carry out randomized therapy trials. The dog is the only species that spontaneously presents with similar diseases. For example, canine HS is most similar to the human malignancy Langerhans cell histiocytosis (LCH). LCH is the most common of the human histiocytic diseases and is estimated to occur in 1 per 200,000 children and 1-2 per 1,000,000 adults [Egeler and D'Angio, 1995]. Human HS can be a very aggressive disease with a poor prognosis, progressing to death within a year of diagnosis [Schmitz and Favara, 1998; Vos et al., 2005].

Histiocytic diseases arise from the abnormal accumulation of macrophages and dendritic cells leading to tumor formation and potentially to organ damage. Macrophages and dendritic cells are

subsets of a larger family of cells known as histiocytes. Histiocytes develop from the primitive CD34+ hematopoietic stem cell. A schematic of histiocyte lineage is shown in Figure 1.1. As immune cells, the normal function of macrophages is to phagocytize pathogens and cellular debris, while dendritic cells function as antigen-presenting cells. Since histiocytes are normal residents in tissues throughout the body, many organ systems can be affected by histiocytic diseases. In dogs at least four distinct histiocytic proliferative diseases have been recognized. The most benign of the histiocytic diseases are cutaneous histiocytoma and reactive histiocytosis, the latter of which can present as a cutaneous or systemic syndrome. The malignant histiocytic diseases encompass the neoplasms histiocytic sarcoma and hemophagocytic histiocytic sarcoma. Padgett et al. determined that HS is inherited in the Bernese mountain dog, but research groups are still working on finding the precise mutation or set of mutations responsible for disease in this breed [Padgett et al., 1995; Shearin et al., 2012].

More research is needed to understand the biology of histiocytes and the pathogenesis of histiocytic diseases. For this purpose, the dog holds great promise as a model for histiocytic diseases, to the benefit of humans and dogs. Histiocytic diseases in dogs, although not the same as in humans, is similar enough that study of canine HS may permit insights into the pathogenesis of this rare human disease, while providing the sample sizes necessary to provide significant data. In addition, there is a wealth of information that can be gleaned from the stored formalin-fixed, paraffin-embedded (FFPE) samples that are available from cases at Michigan State University Diagnostic Center for Population and Animal Health. Our lab hosts the Bernese mountain dog DNA and Tissue Repository, which provides a unique opportunity to access blood and frozen tumor samples from the highly affected BMD. Fresh samples can be obtained from affected patients at the Veterinary Teaching Hospital here on campus. Therefore, by exploiting these resources in dogs, a study of the regulators of gene expression could be conducted. Investigation of the transcriptional changes among these histiocytic diseases can be studied effectively in FFPE samples. Recent technological advances have provided valuable tools for the interrogation of transcriptomes of various cancers [Barretina et al., 2010]. In this study, microRNA expression will be analyzed to probe

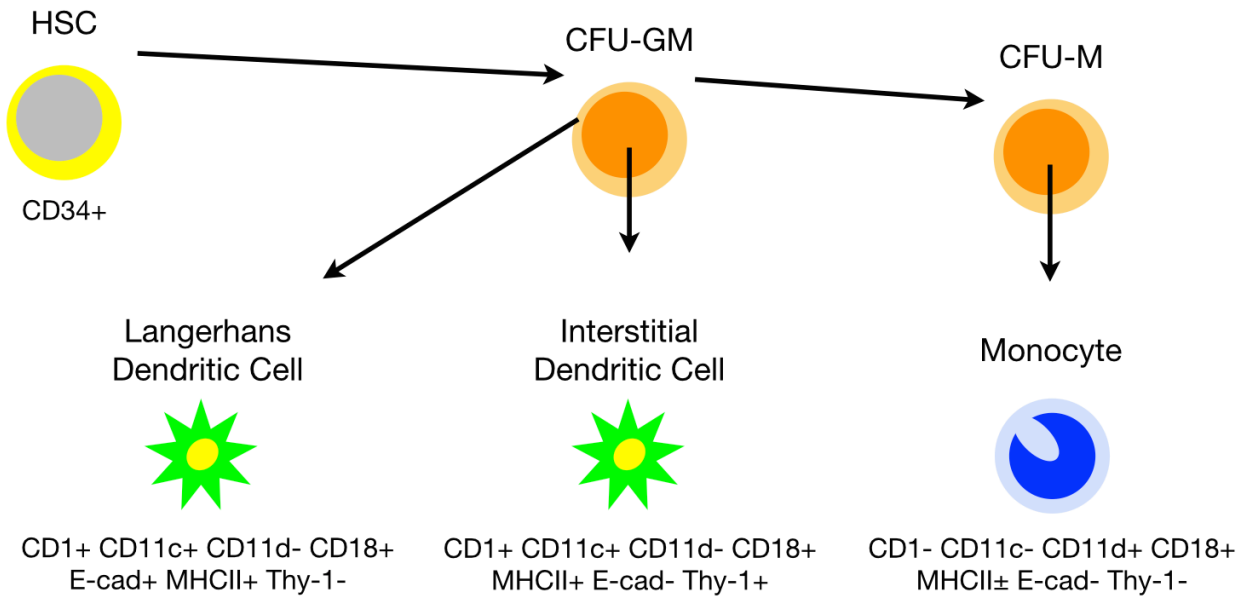

Figure 1.1: **Schematic of histiocyte lineage and their corresponding markers for identification.** Macrophages and dendritic cells develop from hematopoietic stem cells (HSC) in the bone marrow via the granulocyte-macrophage progenitor (CFU-GM = colony forming unit - granulocyte, monocyte). There are two types of dendritic cells that differentiate from this progenitor: Langerhans cells are in the epidermis for cutaneous immunosurveillance, and interstitial dendritic cells are antigen presenting cells in other tissues. The cell surface markers that these cells express, listed below each cell type, are directly related to their function and can be used to distinguish these cells by immunohistochemistry. All leukocytes express $\beta 2$ subunit of integrins (CD18+). Macrophages express αD subunits (CD11d+), which distinguishes them from the dendritic cells which express αX subunit (CD11c+). The two types of dendritic cells can be distinguished by E-cadherin, which is an epithelial cell-cell adhesion protein that allows Langerhans cells to interact with keratinocytes.

the transcriptome of canine histiocytic diseases. Each microRNA regulates as many as 200 to 300 gene targets and have proven to play critical roles in the initiation of many cancers [Calin et al., 2002]. This approach in canine histiocytic diseases will permit the analysis of microRNAs and the pathways they regulate to increase our understanding of both the normal role of histiocytes and the aberrant behavior of histiocytes in these tumors.

Since their relatively recent discovery in 1993 [Fire et al., 1998], microRNAs (miRNAs) have become an active area of research. The miRNAs regulate many cellular processes including proliferation, differentiation, and apoptosis [Kloosterman and Plasterk, 2006]. The miRNAs themselves are small non-coding RNAs of 21 to 25 nucleotides in length. The precursor pri-miRNAs are

frequently transcribed from the introns of protein-coding genes. Within the nucleus, DROSHA functions to process the pri-miRNA into individual pre-miRNAs, which are approximately 70 nucleotides in length folded into a hairpin structure [Du and Zamore, 2005]. The pre-miRNA is then exported from the nucleus where it is cleaved by DICER, a member of the Argonaute family of proteins, to produce the double-stranded miRNAs. One of the strands of the mature miRNA is assembled into the miRNA-induced silencing complex (miRISC) [Peters and Meister, 2007]. This complex acts to inhibit protein action by either degrading the mRNA or inhibiting the translation of mRNA by directly binding to it; in mammals, the predominant action of miRNA is to decrease protein production by the degradation of the mRNA [Guo et al., 2010]. Recent studies have shown that miRNAs can act as tumor suppressors or oncogenes, also known as oncomirs, by their regulation of critical genes [Esquela-Kerscher and Slack, 2006]. Previous work has shown that in FFPE samples miRNAs remain stable due to their small size and can be effectively recovered as compared to the gold standard of RNA recovery from snap- frozen cells [Li et al., 2007]. In addition, several microarray platforms are commercially available that can determine the expression of all known miRNAs simultaneously for certain species including dog.

CHAPTER 2

COMMUNITY-BASED ASSESSMENT OF HEALTH STATUS IN THE BERNESE MOUNTAIN DOG BREED

2.1 Introduction

The Bernese mountain dog (BMD) is considered a large breed of dog and is one of four varieties of Swiss mountain dogs. Originally, the breed worked on farms pulling carts and driving cattle. Today Berners, as they are nicknamed, are often family dogs, due to their gentle, easygoing personality. The breed has become more popular over the last decade, climbing from the American Kennel Club registration ranking from forty-seventh in 2003 to thirty-second in 2013. New owners of BMDs soon become aware of their unfortunate feature: a short lifespan of only six to eight years, which is limited primarily by the high incidence of cancers in this breed, specifically histiocytic sarcoma [Affolter and Moore, 2002].

Histiocytic sarcoma is an aggressive malignancy of dendritic-cell origin. Histiocytic diseases encompass a range of proliferative diseases in both humans and dogs, from the benign histiocytoma to the malignancy, histiocytic sarcoma. Histiocytic diseases are very rare in humans; the most common one, Langerhans cell histiocytosis, has a cumulative incidence of two to ten new diagnoses per one million children each year [Salotti et al., 2009]. It peaks in frequency in children one to three years of age, but it can be diagnosed at any age including adults [Nicholson et al., 1998]. The clinical outcomes are disparate; for example, papular skin disease is generally self-limiting, and histiocytic disease of a single organ system has a 99% survival rate with treatment. However, for children with multi-system disease, the mortality is estimated as high as 35% [Howarth et al., 1999; Gadner et al., 2001; Vos et al., 2005]. The rarity of human histiocytic diseases hinders the development of new therapies due to the inadequate sample sizes of patients. The dog is the only species that spontaneously presents with a similar disease, in this case clinically and pathologically resembling Langerhans cell histiocytosis [Affolter and Moore, 2002]. In dogs, histiocytic diseases

are more common, especially in certain breeds including the BMD where the diseases histiocytic sarcoma and systemic histiocytosis were first described [Moore, 1984; 1986; Rosin et al., 1986; Ramsey et al., 1996]. In a study of dogs affected by histiocytic sarcoma, BMDs were highly overrepresented, only rivaled by Rottweilers, golden retrievers, Labrador retrievers, and flat-coated retrievers [Affolter and Moore, 2002]. In dogs, the diseases of dendritic cells range from the benign histiocytoma to the malignancy, histiocytic sarcoma. Disseminated histiocytic sarcoma at the far end of the spectrum has a poor prognosis and lacks effective treatment options. It can progress rapidly in dogs resulting in death or euthanasia within a few weeks to months. Dog breed associated disorders provide a unique opportunity to study the genetic determinants of diseases and evaluate the potential of novel treatments [Davis and Ostrander, 2014].

To improve the health and longevity of the breed, BMD breeders started the Berner-Garde Foundation to document disease incidence among their dogs and join the data with pedigree information in an open-access computerized database, the Berner-Garde Database. This study provides a community-based assessment of the health status of the BMD breed in congruence with the Berner-Garde Foundation's long-term goal of improving BMD health. Specifically, the database was probed to identify the common disorders affecting the BMD breed and evaluate how the prevalence of these disorders has changed over time.

2.2 Materials and Methods

The Berner-Garde Database is composed of information submitted voluntarily by owners and from public sources such as the Orthopedic Foundation for Animals (OFA) and previously from the Canine Eye Registration Foundation (CERF). All BMD owners and breeders are urged to submit data to the Database via online reporting, starting at birth. The Berner-Garde Foundation was incorporated in 1995 to steward the Database, but it contains data that extend back to the 1970's. The Database can be publicly accessed through the Berner-Garde Foundation's website (www.bernergarde.org). The entire Berner-Garde Database as of March 1, 2013 was outputted to SQL, a database management system. This analysis included database entries with known sex,

age, and diagnosis. The database is organized into individual dogs or entries; one entry contains a recorded event in the dog's life such as a specific diagnosis, and so one dog may have multiple entries. For the purposes of this study, some diagnoses were condensed under similar disease terms. For example, the entries for the previously used diagnosis of malignant histiocytosis were recoded to the contemporary equivalent histiocytic sarcoma. Layman and scientific terms for the same diagnosis were combined, thus "dwarfism" was reclassified to "osteochondrodysplasia". All versions of the diagnosis for bloat were grouped under the diagnosis "gastric dilatation/volvulus." The diagnosis entries for "lymphosarcoma" were grouped together under the general "lymphoma" diagnosis. To facilitate queries, the Berner-Garde Database organizes the diagnoses under general system categories. In addition, the diagnoses were grouped under general categories reflecting the pathological process (Table 2.1). The frequency of histiocytic disease recurring in the same dog was evaluated with recurrent histiocytic disease defined as multiple different histiocytic disease diagnoses of different types, or the same histiocytic disease diagnosis made more than eight months apart unless reported to be the progression of the same disease diagnosis.

2.2.1 Statistical Analysis

Survival plots were generated using the Kaplan-Meier method. Statistical analysis performed using R version 3.1.0. Wilcoxon rank-sum (Mann-Whitney) test was used for determining age differences between males and females. Differences between the lifespan of BMDs without cancer, BMDs with cancer exclusive of histiocytic sarcoma, and BMDs with histiocytic sarcoma were tested for significance using the log-rank or Mantel-Haenszel test and the Peto and Peto modification of the Gehan-Wilcoxon test. The odds ratio with Fisher's Exact Ratio test were used to compare the number of dogs diagnosed with a histiocytic sarcoma to those with recurrent histiocytic disease. A p-value < 0.05 was considered significant.

Table 2.1: **Organization of diagnosis categories in Berner-Garde Database**

Category assigned by the Database	General diagnosis grouping	Specific diagnoses from the Database
General	ACCIDENT/TRAUMA/INJURY	Orthopedic injuries, hit-by-car, poisoning, surgical accidents
Immune System	AUTO IMMUNE DISEASE	Lupus, pemphigus
Behavior Disorders	BEHAVIORAL PROBLEM	Aggressive, anxiety, biting, shy
Cancers/neoplasms/tumors	BENIGN MASS, specific cancers	Non-cancerous and cancerous proliferative disorders
Renal/Urinary	URINARY DISORDER	Stones, crystals, cystitis, obstruction
Cardiovascular	CARDIAC DISORDER	All heart diseases excluding cancer
Cardiovascular	COAGULATION DISORDER	DIC, hemophilia, thrombocytopenia
Reproductive	CONGENITAL DISORDER	Birth defects
Digestive	DENTAL DISORDER	Gum hyperplasia, poor tooth quality
Integumentary	DERMATOLOGICAL DISORDER	Skin diseases excluding cancer
Musculoskeletal	DEVELOPMENTAL GROWTH DISORDER	Defects in growth not including hip and elbow dysplasias
Eye, Ear, Nose	EYE DISORDER	All eye diseases excluding cancer
Digestive	GALL BLADDER DISORDER	Cholecystitis, obstruction, stones, rupture
Reproductive	GESTATIONAL DISORDER	Problems in pregnancy
General	INFECTION	Any infection in any organ
Based on body location	INFLAMMATORY DISORDER	Inflammation "-itis"
Digestive	INTESTINAL DISORDER	GI disorders and obstructions not including GDV or cancer
Renal/Urinary	KIDNEY DISORDER	All kidney diseases excluding cancer
Digestive	LIVER DISORDER	All liver diseases excluding cancer
Respiratory	LUNG DISORDER	All lung diseases excluding cancer
Nervous System	NEUROLOGIC DISORDER	All nervous system disorders excluding cancer
Digestive	PANCREATIC DISORDER	All pancreatic diseases excluding cancer
Reproductive	PROSTATE DISORDER	All prostate diseases excluding cancer
Nervous System	SEIZURE DISORDER	Epilepsy or seizure diseases
Nervous System	SPINAL DISORDER	Orthopedic spinal problems
Cardiovascular	SPLENIC DISORDER	All disorders of the spleen excluding cancer
Endocrine	THYROID DISORDER	All thyroid diseases excluding cancer
Reproductive	UTERINE DISORDER	All uterine diseases excluding cancer

The organization of diagnosis entries in the Berner-Garde Database is summarized in this table. The first column on the left lists the general system categories that were assigned as part of the internal organization of the Berner-Garde Database. The middle column shows the corresponding diagnosis categorization that were assigned by this study to reflect the pathological process. The last column on the right summarizes the specific diagnoses provided in the Database that were included within each category. DIC = disseminated intravascular coagulation; GI = gastrointestinal; GDV = gastric dilatation volvulus.

2.2.2 Ethics Statement

The Berner-Garde Database is an open-access database composed of voluntary submissions of self reported records from BMD owners and breeders. No additional private information other than that obtained from the database was included in this study. No direct animal experimentation was

performed.

2.3 Results

As of March 1, 2013, the Berner-Garde Database contained 92,055 entries belonging to 86,737 individual BMDs (Table 2.2). Selecting for dogs with known sex and date of death yielded 7,262 individual dogs for analysis, which corresponded to 10,690 entries for diagnoses made during the dogs' lives. The sex ratio was nearly 1:1 with 3,616 male (49.8%) and 3,646 female (50.2%). The average age at death was 7.3 years and the median age at death was 7.7 years with a range of 0 to 20.2 years. There was a significant difference ($P < 0.001$) between males (median 7.4 years, range 0 to 14.8 years) and females (median 7.9 years, range 0 to 20.2 years) for age at death. Of these dogs, nearly 86% of BMDs had died by the age of ten years (Figure 2.1).

2.3.1 Diagnoses

There were 470 distinct disease diagnoses recorded in the Berner-Garde Database for the 7,262 selected dogs. The Berner-Garde Database organized the diagnoses into fourteen general system categories. As part of this study the diagnoses were also grouped into 136 general diagnoses based upon the pathophysiology and affected organ system. Among the 10,690 diagnosis entries, 4,680 (44%) were in the general category of neoplasms followed distantly by 1,435 (13%) in the musculoskeletal category, which included developmental growth disorders and injuries to bones or ligaments (Figure 2.2). Excluding unspecific diagnoses like unclassified cancers and tumors, and unspecified deaths described as old age, the most important specific diagnoses included histiocytic sarcoma, lymphoma, and hip and elbow dysplasias (Table 2.3). Among the cancer diagnoses, histiocytic sarcoma represented 37% of cancer diagnoses and lymphoma represented 20% of the cancer diagnoses (Figure 2.3). Together, histiocytic sarcoma and hemophagocytic histiocytic sarcoma composed 38% of the cancer diagnoses (Table 2.4). Among the dogs with cancer, the sex ratio was 0.996 with 1,959 male (49.9%) and 1,967 female (50.1%) and for dogs with histiocytic

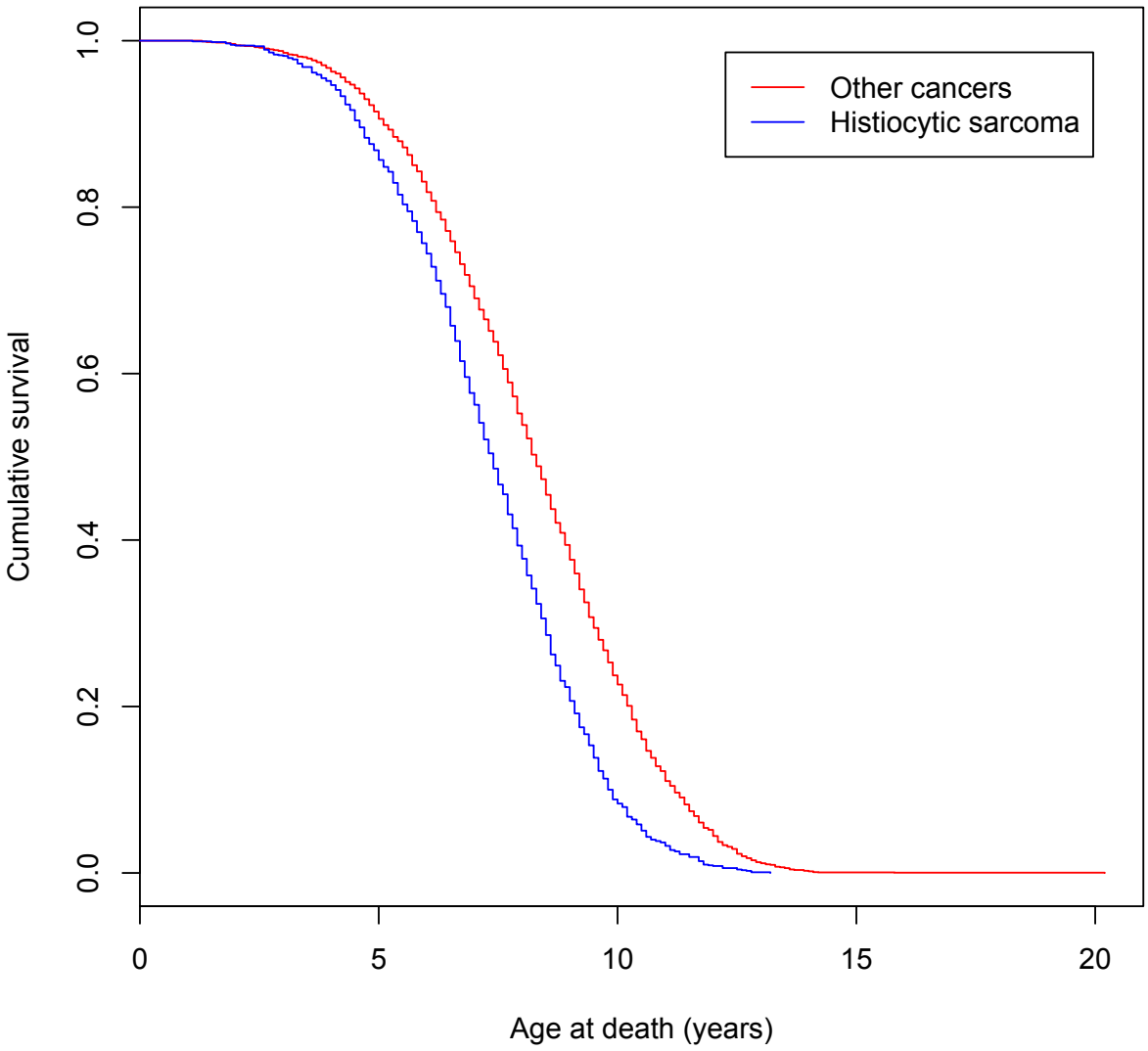

Figure 2.1: **Kaplan-Meier survival plot of the 7,262 selected dogs shows reduced survival for dogs with histiocytic sarcoma.** The cumulative survival of dogs diagnosed with cancers other than histiocytic sarcoma (2,740 of the selected dogs) are shown by the red line, and dogs with histiocytic sarcoma (1,185 of the selected dogs) are shown by the blue line. Cancer impacts the survival of older BMDs who are greater than 5 years of age, however BMDs with histiocytic sarcoma die earlier than BMDs with other types of cancer. The median lifespan of dogs with histiocytic sarcoma is significantly less (median 7.4 years) than dogs with other cancers (median 8.3 years) ($P < 0.001$).

Table 2.2: **Number of entries and dogs in the Berner-Garde Database available for analysis**

All entries in Database	**Entries**	**Proportion of entries**	**Dogs**	**Proportion of dogs**
TOTAL	92,055	100%	86,737	100%
Diagnoses	15,705	17%	10,689	12%
Death date	14,319	16%	10,193	12%
Diagnoses, death date, sex	**10,690**	12%	**7,262**	8%
Cause of death	9,112	10%	7,932	9%
Cancer	4,762	5%	4,352	5%
Specific cancer	3,474	4%	3,132	4%
Histiocytic disease	1,559	2%	1,504	2%
Selected entries	**Entries**	**Proportion of entries**	**Dogs**	**Proportion of dogs**
TOTAL	10,690	100%	7,262	100%
Cause of death	5,490	51%	5,161	71%
Cancer	4,297	40%	3,926	54%
Specific cancer	3,207	30%	2,887	40%
Histiocytic disease	1,412	13%	1,361	19%

The total numbers of entries and individual dogs from the entire Berner-Garde Database for all dogs and all years inclusive are shown in the top half of the table with their corresponding proportions of the total number of entries or dogs. In bold are the number of entries and dogs that met the selection criteria for this study, which included complete entries with known sex, age, and diagnosis. This yielded 7,262 individual dogs that are the focus of the bottom half of the table. Dogs who lacked a recorded date of death, whether due to unknown date of death or the dog was still alive at the time of the Database output, were not selected for this study.

sarcoma, the sex ratio was 1.07 with 602 male (51.6%) and 564 female (48.4%). For age at diagnosis with cancer, there was a significant difference ($P < 0.05$) between males (median 7.6 years, range 0 to 14.1 years) and females (median 7.9 years, range 0.2 to 20.2 years). There was also a significant difference ($P < 0.05$) for age at diagnosis with histiocytic sarcoma between males (median 7.1 years, range 1.1 to 12.6 years) and females (median 7.5 years, range 0.6 to 12.8 years). However, there was no significant difference in survival with histiocytic sarcoma for males (median 0 months, range 0 to 3.3 years) or females (median 0 months, range 0 to 5.2 years). The age distribution for the top five most common cancer diagnoses among the selected BMDs is shown in Figure 2.4. The incidence of diagnosis of histiocytic sarcoma, lymphoma, mast cell tumor, hemangiosarcoma, and osteosarcoma all peak at six years of age (Figure 2.5).

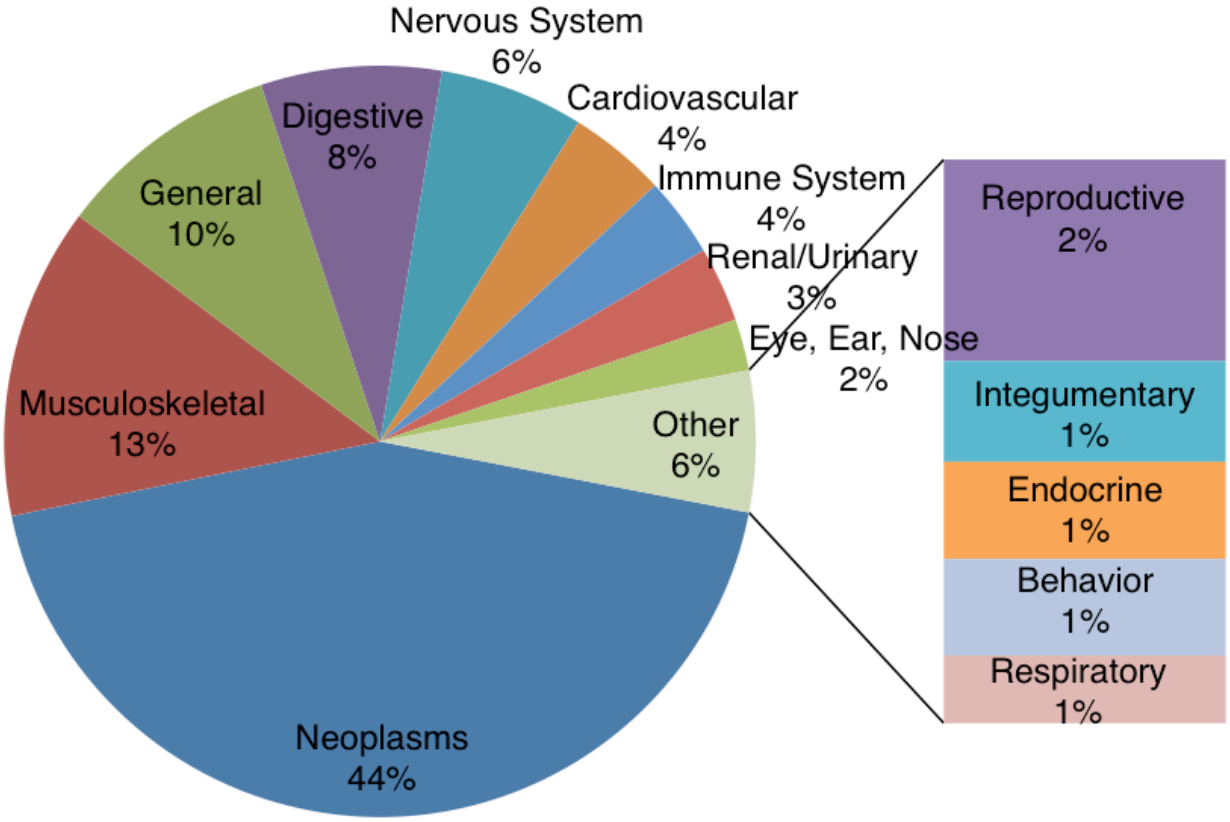

Figure 2.2: **Pie chart of the general categories of diagnoses shows a majority of diagnoses are neoplasms.** There were 470 distinct disease diagnoses recorded in the Berner-Garde Database organized into fourteen general system categories. Nearly half of the diagnoses among the 7,262 selected dogs were categorized as neoplasms which included all cancers, tumors and benign masses. The second prevalent category was the musculoskeletal category, which included developmental growth disorders and injuries to bones or ligaments. The "General" category included infections and accidents.

The three most common congenital or developmental growth disorders in decreasing prevalence were osteochondritis dissecans, neonatal death, and cleft palate (Table 2.5). For dogs that did not reach one year of age, the most common diagnosis besides accidents included neonatal death, cleft palate, and parvovirus (Table 2.6). The most common specified cause of death, other than cancer, for dogs that lived beyond on year of age was gastric dilatation/volvulus.

The Kaplan-Meier survival curve for BMDs with cancer drops precipitously after five years of age, and then is nearly identical to the survival curve of BMDs with any diagnosis over the age of ten years (Figure 2.1). The lifespan of BMDs with histiocytic sarcoma was significantly shortened

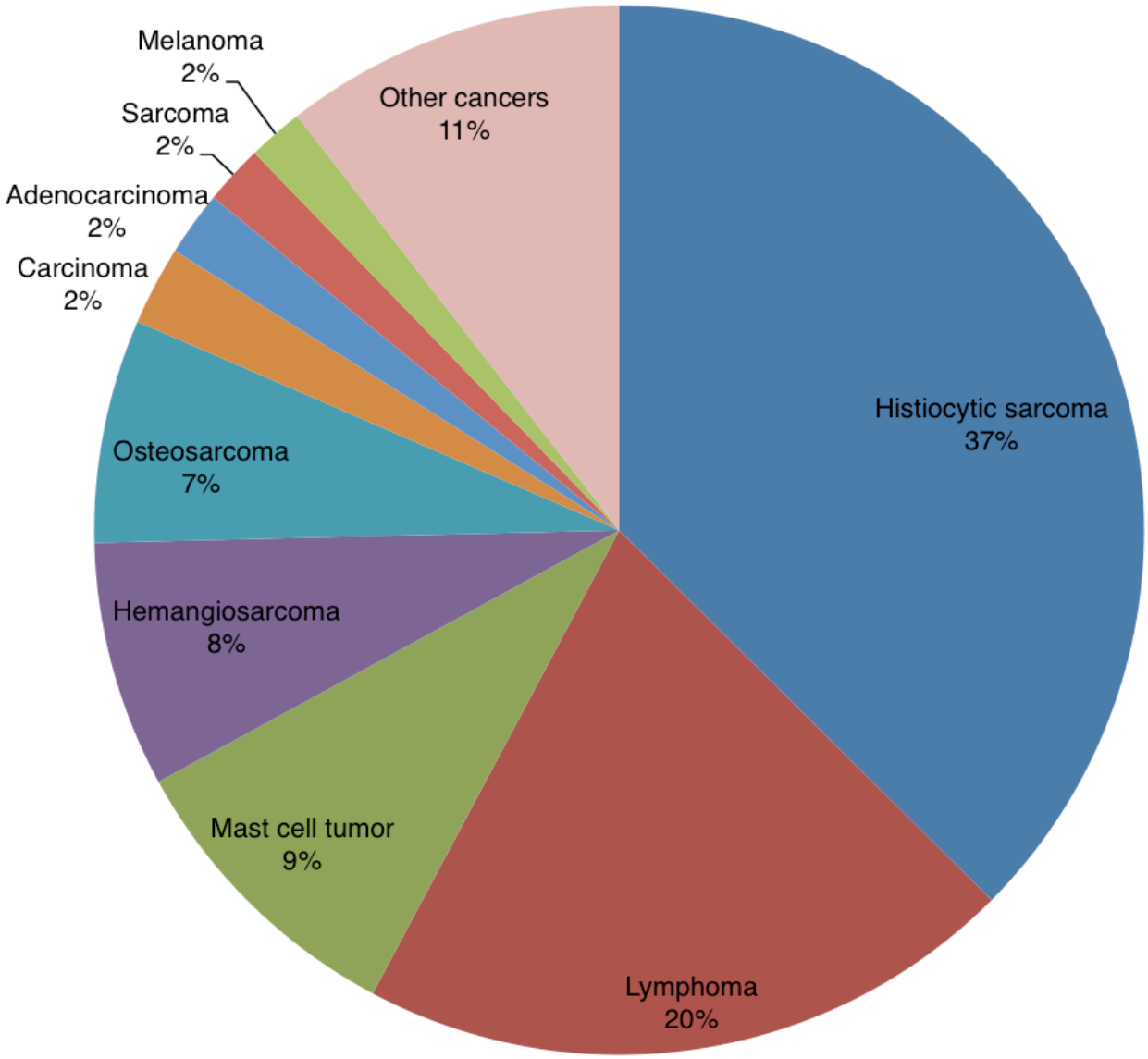

Figure 2.3: **Pie chart focusing on specific cancers shows histiocytic sarcoma is the major cancer diagnosis.** The most important cancer among the 7,262 selected dogs was histiocytic sarcoma followed by lymphoma. The slice referring to “Other cancers" included fibrosarcoma, leukemia, transitional cell carcinoma, hemophagocytic histiocytic sarcoma, and other specified cancers all composing less than 1% each of the total cancer diagnoses.

Table 2.3: **Overall most common diagnoses specified in the Berner-Garde Database**

Diagnosis	Number diagnosed	Proportion of total diagnoses
Histiocytic sarcoma	1,201	11%
Cancer, unspecified	776	7%
Lymphoma	652	6%
Death, unspecified	499	5%
Hip dysplasia	498	5%
Elbow dysplasia	410	4%
Gastric dilatation/volvulus	364	3%
Tumor / mass, unspecified	322	3%
Mast cell tumor	297	3%
Accident/trauma/injury	297	3%
Cruciate ligament rupture	250	2%
Hemangiosarcoma	243	2%
Osteosarcoma	222	2%
Arthritis	155	1%
Myelopathy, degenerative	123	1%

The most prevalent diagnoses among the selected 7,262 dogs are shown in decreasing frequency. Diagnoses that affected less than 100 dogs are not shown. For each diagnosis, the number of entries and the corresponding proportion of all 10,690 diagnosis entries are listed.

(median 7.4 years) compared to survival with other cancers (median 8.1 years) while death not due to cancer remained relatively constant (median 7.1 years). The range and distribution of age at diagnosis for the various histiocytic diseases is shown in Figure 2.6. Histiocytic sarcoma and hemophagocytic histiocytic sarcoma peaked in incidence in dogs five to eight years of age, while histiocytoma affected a younger set of dogs with a peak incidence at one year of age (Figure 2.7). Systemic histiocytosis also tended to be diagnosed earlier with a peak in incidence at four years of age and a second smaller peak at eight years of age (Figure 2.8). The selected dogs were separated into five-year increments based upon the year they were born (Table 2.7). Over the last twenty years, the incidence of hip and elbow dysplasias have decreased while cancer and histiocytic diagnoses have increased in proportion of diagnoses. Qualitatively, this is also shown in Table 2.8, which lists the top three diagnoses and their relative proportions of diagnoses for each time period. Since the late 1980s, cancer has replaced dysplasias as the most important diagnosis for BMDs.

Table 2.4: **Most prevalent cancers listed in the Berner-Garde Database**

Diagnosis	**Number diagnosed**	**Proportion of specified cancers**
Histiocytic sarcoma	1,201	37.4%
Cancer / tumor, unspecified	1,090	-
Lymphoma	652	20.3%
Mast cell tumor	297	9.3%
Hemangiosarcoma	243	7.6%
Osteosarcoma	221	6.9%
Carcinoma	78	2.4%
Adenocarcinoma	64	2.0%
Sarcoma	59	1.8%
Melanoma	55	1.7%
Fibrosarcoma	37	1.2%
Leukemia	36	1.1%
Transitional cell carcinoma	31	1.0%
Hemophagocytic histiocytic sarcoma	22	0.7%
Spindle cell tumor	21	0.7%
Squamous cell carcinoma	21	0.7%
Round cell sarcoma	21	0.6%
Chondrosarcoma	15	0.5%
Synovial cell sarcoma	14	0.4%
Prostate cancer	11	0.3%
Basal cell carcinoma	11	0.3%
Nerve sheath tumor	11	0.3%
Myeloma	7	0.2%
Leiomyosarcoma	6	0.2%
Myxosarcoma	6	0.2%
Mesothelioma	6	0.2%
Mesenchymal neoplasia	6	0.2%
Mammary tumor	5	0.2%

The cancer diagnoses listed for the selected dogs are shown in decreasing frequency. Among the 7,262 selected dogs, 3,926 were diagnosed with cancer at some point in their lives. Cancers that had less than five entries are not shown.

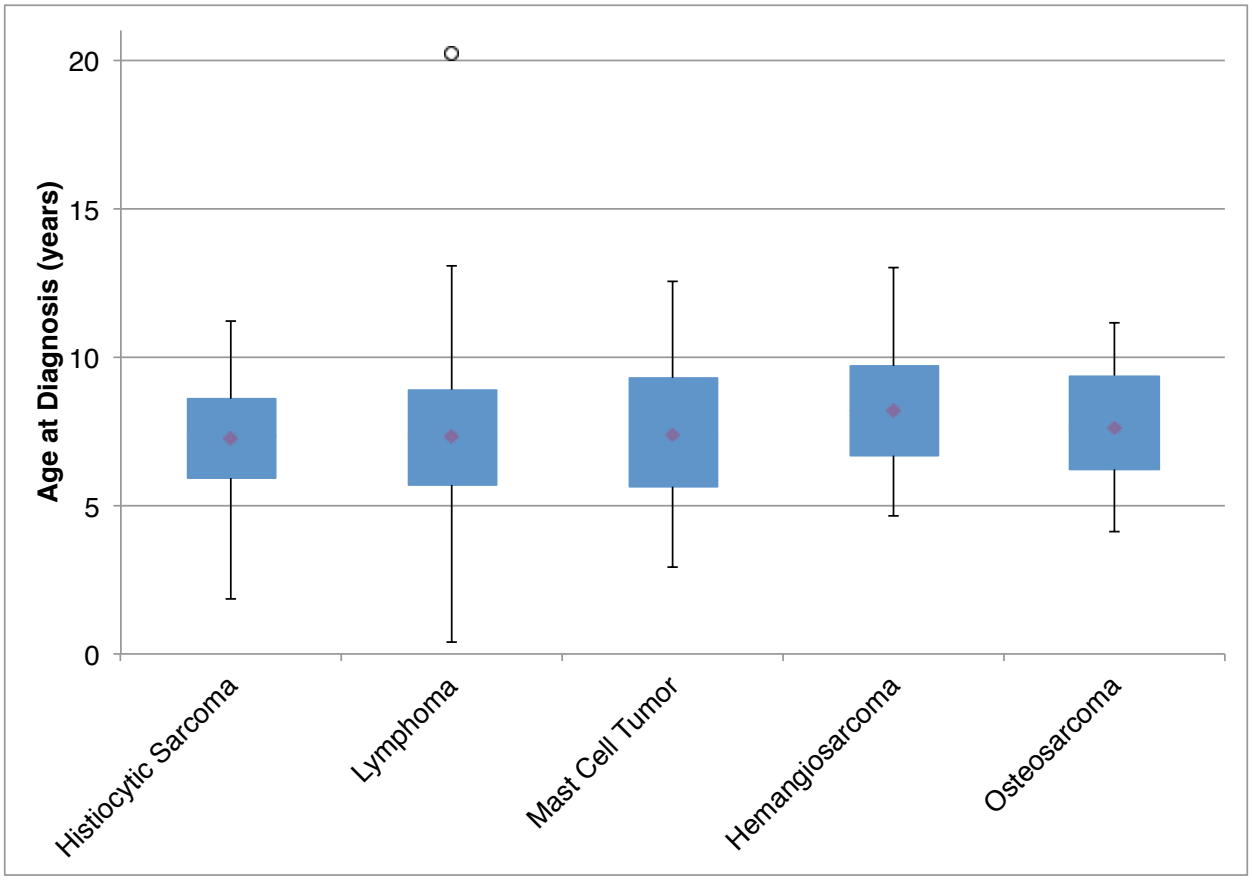

Figure 2.4: **Box and whisker plot of the range and distribution of ages at diagnosis for the top five most prevalent cancers among the 7,262 selected dogs.** Histiocytic sarcoma had a relatively narrow distribution of ages at diagnosis, while the distribution for lymphoma was broader. Hemangiosarcoma and osteosarcoma are more commonly diagnosed in older BMDs than the other cancers. The median age at diagnosis for each cancer is shown by the diamond. Lymphoma had one outlier at 20.2 years of age.

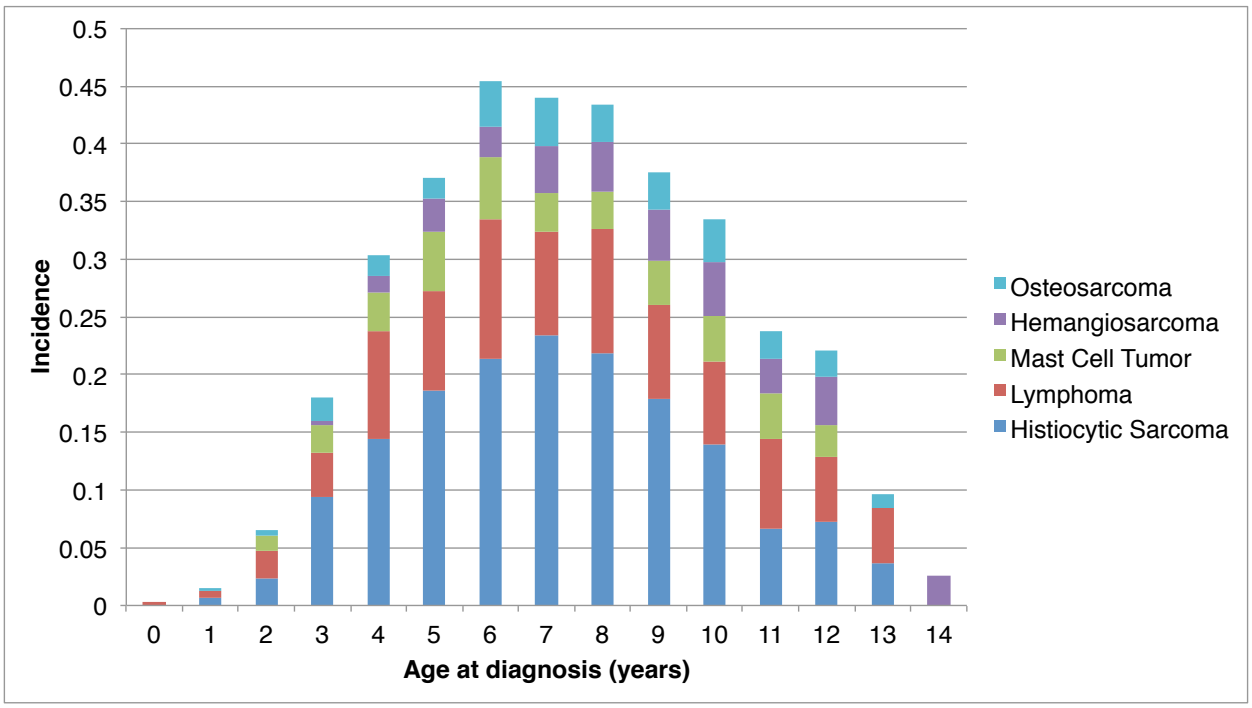

Figure 2.5: **The incidence of cancer diagnoses peaks at six years of age.** The incidences of the top five major cancer diagnoses are stacked across the age at diagnosis. The overall peak incidence for histiocytic sarcoma, lymphoma, mast cell tumor, hemangiosarcoma, and osteosarcoma occurred at six years of age.

Table 2.5: **Congenital and developmental growth disorders in the Berner-Garde Database**

Congenital or developmental disorders	Number diagnosed	Proportion of diagnoses
Osteochondritis dissecans	55	0.51%
Neonatal death	37	0.35%
Cleft palate	31	0.29%
Sub aortic stenosis	31	0.29%
Umbilical hernia	30	0.28%
Shunt, portosystemic	28	0.26%
Panosteitis	20	0.19%
Anomaly	16	0.15%
Persistent pupillary membrane	15	0.14%
Cryptorchid (undescended testicles)	13	0.12%
Carpus valgus	7	0.07%
Size anomalies/birth weights	6	0.06%
Monorchid (one descended testicle)	6	0.06%
Pulmonic stenosis	6	0.06%
Maldevelopment	5	0.05%
Patent ductus arteriosis	4	0.04%
Sterility	3	0.03%
Soft palate	3	0.03%
Hypertrophic osteodistrophy	3	0.03%
Osteochondrodysplasia	3	0.03%
Spina bifida / myelodysplasia	3	0.03%
Patent urachus	2	0.02%
Congenital heart defect unspecified	2	0.02%
Premature growth plate closure	2	0.02%
Hermaphroditism	2	0.02%

There were 350 congenital and developmental growth disorders diagnosed among the 7,262 selected dogs. The most prevalent congenital and developmental growth disorders are shown in decreasing frequency with their corresponding proportions over all 10,690 diagnosis entries. Congenital and developmental growth disorders that had only one diagnosis entry among the selected dogs are not shown.

Table 2.6: **Cause of death for dogs less than one year of age at death**

Cause of death	**Number of dogs**	**Proportion of dogs**
Accident/trauma/injury	66	19.41%
Neonatal death	30	8.82%
Cleft palate	21	6.18%
Hip dysplasia	18	5.29%
Parvo	19	5.59%
Renal disease	13	3.82%
Cerebellar abiotrophy	12	3.53%
Shunt, portosystemic	10	2.94%
Death, unspecified	9	2.65%
Intussusception	9	2.65%
Herpes virus	9	2.65%
Elbow dysplasia	8	2.35%
Pneumonia	7	2.06%
Poison	7	2.06%
Renal dysplasia	6	1.76%
Infection, unspecified	6	1.76%
Intestinal obstruction	6	1.76%
Gastric dilatation/volvulus	5	1.47%
Sub aortic stenosis	5	1.47%
Patent ductus arteriosis	4	1.18%
Anomaly	4	1.18%
Osteochondritis dissecans	4	1.18%
Hemophilia	4	1.18%
Cardiac disease	4	1.18%
Hepatopathy	3	0.88%
Intestinal disorder	3	0.88%
Lymphoma	3	0.88%

The cause of death or reason for euthanasia for the selected group of dogs that died at less than one year of age are shown in decreasing frequency. Among the 7,262 selected dogs, 5,161 dogs had a known cause of death and 340 of these dogs died before reaching one year of age. Each cause of death is listed with the corresponding number of dogs and proportion of the 340 dogs that were less than one year of age at death (right column). Causes of death that affected only one or two dogs are not shown.

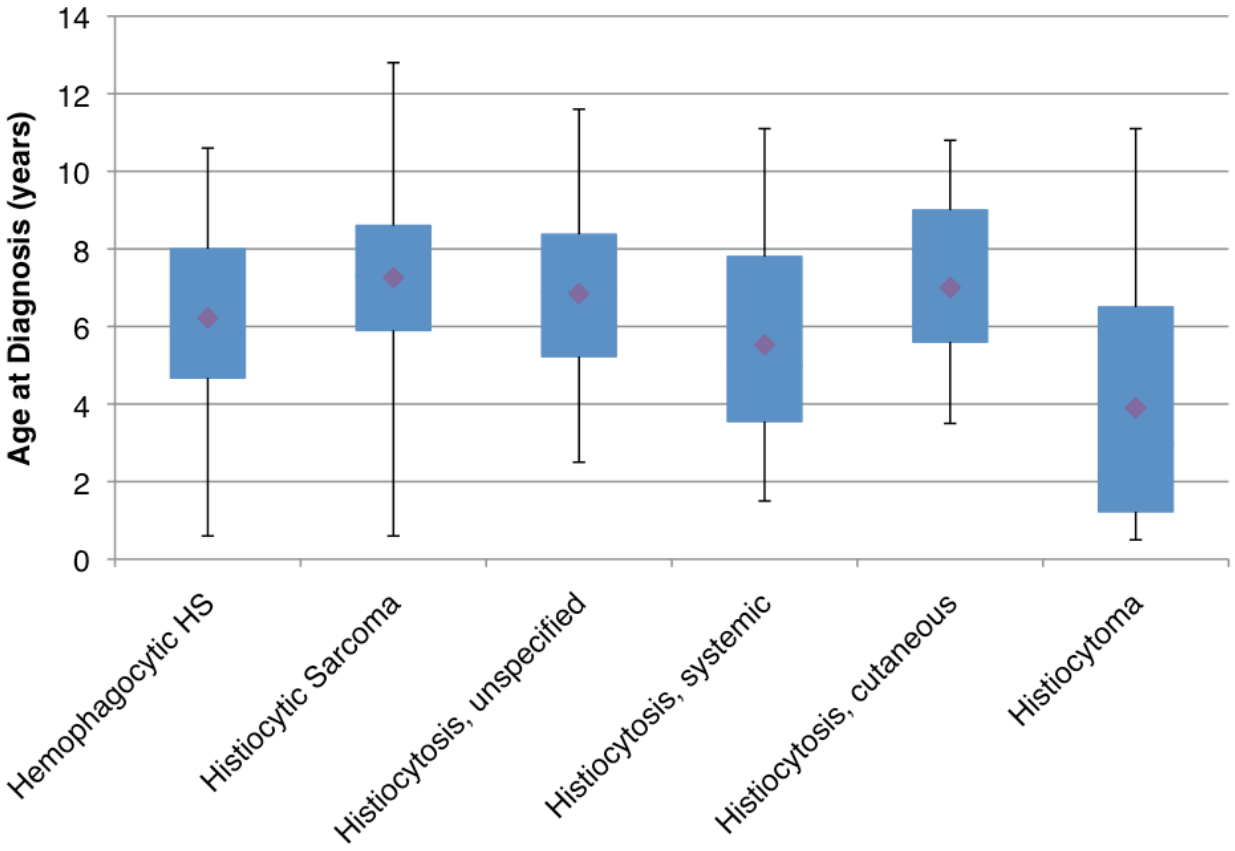

Figure 2.6: **Box and whisker plot of the range and distribution of ages at diagnosis with a histiocytic disease.** Histiocytoma is diagnosed at earlier ages while histiocytic sarcoma is more variable for age at diagnosis. Among the 7,262 selected dogs, histiocytic diseases with known ages at diagnosis accounted for 1,267 dogs. Hemophagocytic histiocytic sarcoma n = 20, histiocytic sarcoma n = 1083, histiocytosis, unspecified n = 78, histiocytosis, systemic n = 51, histiocytosis, cutaneous n = 5, histiocytoma n = 30.

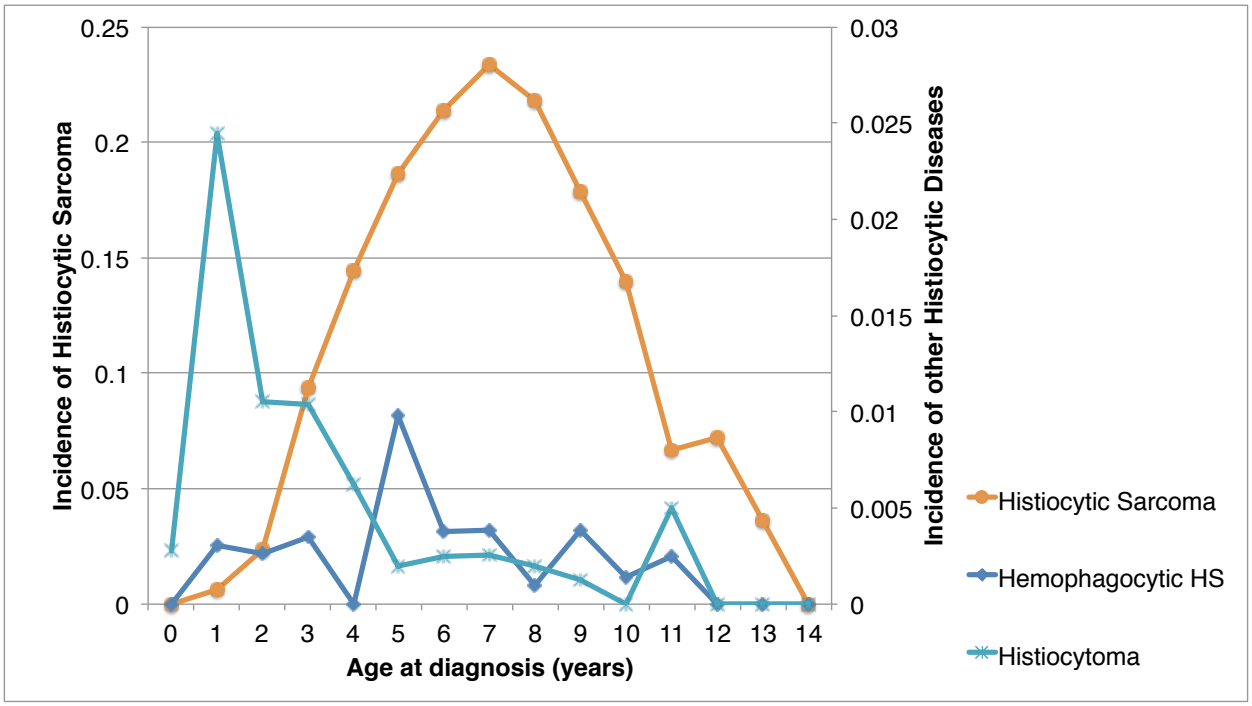

Figure 2.7: **Incidence of histiocytic disease among the 7,262 selected dogs by age, including histiocytic sarcoma, hemophagocytic histiocytic sarcoma, and histiocytoma.** Histiocytic sarcoma (left y-axis) shown with the orange line had a single peak at 8 years of age at diagnosis with 22% of diagnoses being histiocytic sarcoma (219 dogs diagnosed with histiocytic sarcoma at 8 years of age over a total of 1,003 dogs with diagnoses at 8 years of age). Hemophagocytic histiocytic sarcoma (right y-axis) shown with the blue line had an overall lower and more variable incidence without a single obvious peak. Histiocytoma (right y-axis) shown with the teal line peaked at an earlier age of 1 year of age. Hemophagocytic histiocytic sarcoma n = 20, histiocytic sarcoma n = 1083, histiocytoma n = 30.

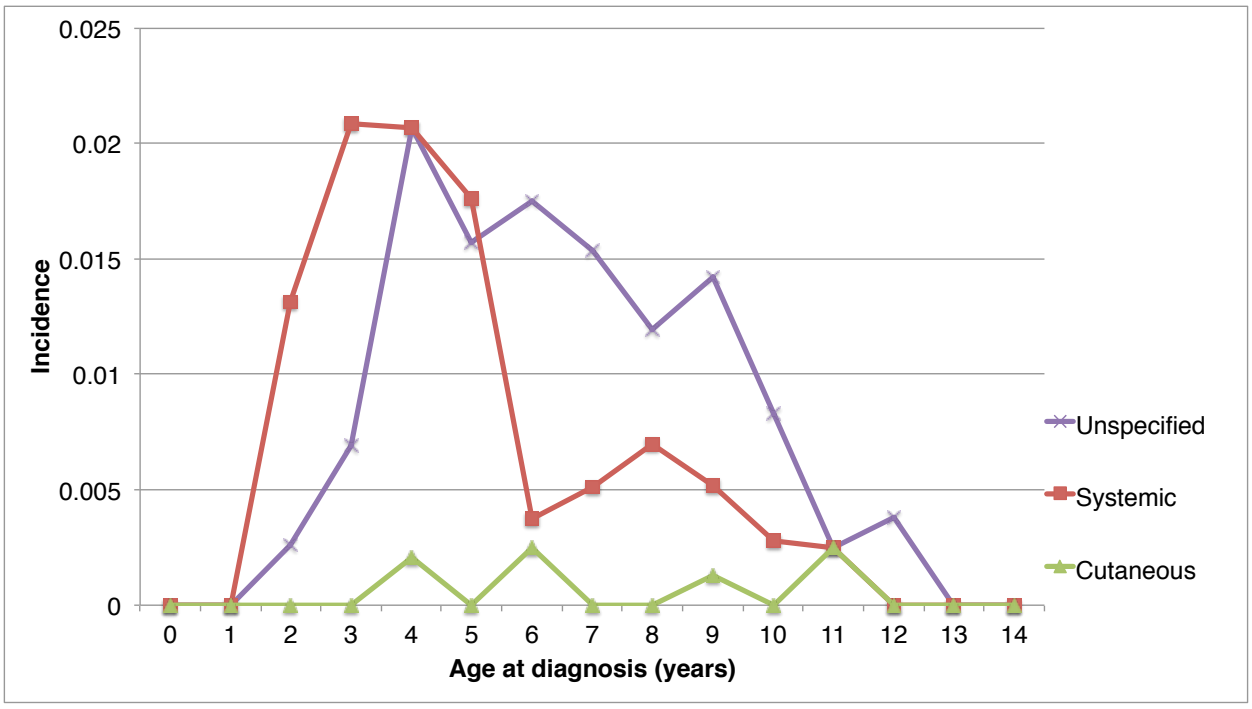

Figure 2.8: **Incidence of histiocytosis disease diagnoses among the 7,262 selected dogs by age, including systemic histiocytosis, cutaneous histiocytosis, and histiocytosis, unspecified.** The systemic histiocytosis, shown with the red line, has two peaks in incidence at 4 years of age and 8 years of age at diagnosis. The cutaneous histiocytosis group, shown with the green line, had a variable incidence, most likely due to the small sample size of only 5 dogs being diagnosed with cutaneous histiocytosis. The unspecified histiocytosis group, shown with the purple line, had a wide peak incidence from 6 to 8 years of age. The unspecified histiocytosis diagnosis may include the previously used diagnosis of malignant histiocytosis, which is now termed histiocytic sarcoma. Histiocytosis, systemic n = 51, histiocytosis, cutaneous n = 5, histiocytosis, unspecified n = 78.

Table 2.7: **Diagnoses stratified into five-year time increments**

5 year increments	Dogs	Age	Diagnoses	Hip dysplasia	Elbow dysplasia	Cancer	Histio	HS	Recurrent histio	Overall survival	Cancer survival	Histio survival
2003 - 2007	1,260	5.8 years	1,805	2%	2%	41%	16%	32%	1%	7 months	1 month	1 month
1998 - 2002	2,376	8.1 years	3,609	3%	3%	45%	15%	30%	1%	10 months	3 months	2 months
1993 - 1997	1,480	8.6 years	2,184	5%	6%	40%	12%	26%	1%	13 months	4 months	3 months
1988 - 1992	770	7.8 years	1,113	6%	4%	33%	10%	25%	2%	13 months	4 months	8 months
1983 - 1987	644	7.4 years	1,003	9%	4%	37%	11%	24%	1%	12 months	5 months	5 months
1978 - 1982	362	8.0 years	514	11%	6%	38%	9%	21%	4%	5 months	3 months	3 months
1973 - 1977	128	8.8 years	166	8%	4%	48%	8%	11%	0%	2 months	3 months	0 months
$<$ **1973**	48	7.9 years	64	19%	3%	30%	6%	16%	0%	5 months	7 months	6 months
All years	7,262	7.6 years	10,690	5%	4%	40%	13%	28%	1%	10 months	3 months	3 month

The 7,262 selected dogs were stratified into five-year time periods based upon their date of birth. The number of dogs, their average age, and the number of diagnosis entries for each time period are shown within each row with the average across all years shown in the bottom row and most recent years in the top row. Specific diagnoses hip and elbow dysplasia and the general category of cancer and histiocytic diseases (Histio) are shown as the proportion of total diagnoses for the given time period. Histiocytic sarcoma (HS) is shown as the proportion of total cancer diagnoses for the given time period. The incidence of recurrent histiocytic disease in the same dog (Reccurent histio) was calculated as the proportion of total histiocytic disease diagnoses for the given time period.

Table 2.8: **The top diagnoses for each five-year time period indicate cancer incidence is increasing and dysplasia is decreasing**

5 year increments	#1 diagnosis	#1 diagnosis %	#2 diagnosis	#2 diagnosis %	#3 diagnosis	#3 diagnosis %
2003 - 2007	histiocytic sarcoma	13.4%	cancer, unspecified	7.8%	lymphoma	6.0%
1998 - 2002	histiocytic sarcoma	13.6%	cancer, unspecified	7.6%	lymphoma	6.4%
1993 - 1997	histiocytic sarcoma	10.5%	cancer, unspecified	6.9%	lymphoma	5.8%
1988 - 1992	histiocytic sarcoma	8.0%	cancer, unspecified	6.0%	hip dysplasia	5.8%
1983 - 1987	hip dysplasia	9.4%	histiocytic sarcoma	8.8%	lymphoma	6.8%
1978 - 1982	hip dysplasia	11.3%	cancer, unspecified	8.8%	histiocytic sarcoma	8.0%
1973 - 1977	cancer, unspecified	15.7%	lymphoma	10.8%	hip dysplasia	8.4%
$<$ **1973**	hip dysplasia	18.8%	cancer, unspecified	10.9%	lymphoma	6.3%
All years	histiocytic sarcoma	11.2%	cancer, unspecified	7.3%	lymphoma	6.1%

The three most frequent diagnoses among the 7,262 selected dogs for each five-year time period are shown with the corresponding proportion of all diagnoses for the time period. Over all years the top three diagnoses were histiocytic sarcoma, unspecified cancers, and lymphoma. Hip dysplasia has been among the top three diagnoses until the late 1980's. Since 1993, cancer diagnoses have encompassed the top three diagnoses in recent years.

2.3.2 Recurrent Histiocytic Disease

The risk of BMDs developing recurrent histiocytic disease was evaluated with the goal of determining if benign proliferative histiocytic disease predisposes a BMD to the more aggressive malignancy histiocytic sarcoma. There were fourteen BMDs that were diagnosed with recurrent histiocytic disease during their lives. Histiocytic sarcoma was listed as the diagnosis for 1,166 dogs, 9 of which had a history of other histiocytic diseases. Of the remaining 6,096 dogs, 186 dogs were diagnosed with some other histiocytic disease besides histiocytic sarcoma during their life. To estimate the risk of a dog developing histiocytic sarcoma given a history of histiocytic disease the odds ratio of 0.26 (95% CI: 0.12-0.51) was calculated with Fisher's Exact Ratio test p-value = $3.658 * 10^{-6}$. One male dog had a cutaneous histiocytoma at 1 year of age that was surgically removed and two years later had another histiocytoma surgically removed and then was euthanized with T-cell lymphoma at ten years of age. Seven dogs, two females and five males, had histiocytoma and were diagnosed with histiocytic sarcoma eight months to nine years later. In one case, a cutaneous histiocytoma was surgically removed from the right elbow of a 7-year-old neutered male dog, then he was diagnosed with histiocytic sarcoma in the same location eight months later. Five dogs were diagnosed with localized histiocytic sarcoma, either subcutaneous or periarticular, and then developed disseminated histiocytic sarcoma six months to one year later. A female dog was diagnosed with systemic histiocytosis and had a subcutaneous histiocytic mass on her neck removed when she was four years of age, with no other masses found. At the time she was treated with cyclosporine and prednisone and appeared to go into remission. Three years later she was diagnosed with systemic histiocytosis in the face, after a wasp sting that resulted in her swollen face and paw. Ultimately, she was unresponsive to treatment and was euthanized.

2.4 Discussion

The BMD has a shortened life expectancy, even compared to other large breeds of dogs like Doberman Pinschers which live to eleven years of age [Adams et al., 2010]. This is due primarily to high

incidence of cancers in BMDs [Brønden et al., 2010]. Due to the relatively regular influx of European BMDs to American BMD breeding stock, it makes sense that many features of the data analyzed in our study are consistent with studies conducted on European BMDs [Quignon et al., 2007]. The data presented here was gleaned from the Berner-Garde database as a community-based assessment of health status, very similar to the Dutch BMD club's reporting system in The Netherlands used in the 2013 study by Erich et al. as opposed to the questionnaire-based studies of Nielsen et al. 2010 [Nielsen et al., 2010; Erich et al., 2013]. The median age at death determined for this group of BMDs was 7.7 years, slightly older than the 7.1 years calculated by Nielsen et al. 2010 but younger than the 8.0 years determined by Erich et al. 2013 and Adams et al. 2010 [Nielsen et al., 2010; Adams et al., 2010; Erich et al., 2013]. Consistent with these previous studies, on average female BMDs lived longer than males, a finding that persisted even when calculated without the female outlier at 20.2 years of age at death [Nielsen et al., 2010; Erich et al., 2013]. In the last decade,we found the average life expectancy of BMDs to be lower in this cohort with 86% of dogs dying by ten years of age, as compared to the Dutch population, where 84% of dogs died by eleven years of age [Erich et al., 2013]. This lifespan is indeed shortened when compared to a recent UK study of multiple breeds of purebred and mixed bred dogs that found 50% of dogs survived beyond 12 years of age [O'Neill et al., 2014]. BMDs recorded as dying of "old age" lived on average to 11.5 years, which is similar to other reports of BMDs succumbing to "old age" at an average of 11 years [Nielsen et al., 2010; Erich et al., 2013]. Those BMDs affected with histiocytic sarcoma had a shortened lifespan on average of 7.3 years, consistent with other reports ranging from 6.3 to 7.7 years for affected BMDs [Abadie et al., 2009; Nielsen et al., 2010; Hedan et al., 2011; Erich et al., 2013]. BMDs with other types of cancer lived almost a year longer to 8.1 years, comparable to the range cited in the literature of living 5 months to 1.5 years beyond their histiocytic sarcoma affected counterparts [Nielsen et al., 2010; Erich et al., 2013].

Cancer affected 44% of the BMDs in our study, comparable to 42.2% of Danish BMDs and 45.7% of UK BMDs [Nielsen et al., 2010; Adams et al., 2010]. The most common cancer affecting BMDs is still histiocytic sarcoma, which comprises 37% of the cancer diagnoses in our study as

compared to 25% described previously [Padgett et al., 1995; Abadie et al., 2009]. This finding is consistent with a 2009 study of another breed predisposed to histiocytic sarcoma, flat-coated retrievers, in which histiocytic sarcoma composed at least 40% of the tumors diagnosed [Dobson et al., 2009]. A recent study on the Dutch BMD population found an even higher proportion of tumors at 64.4% to be histiocytic sarcoma [Erich et al., 2013]. Consistent with these results, Abadie et al. 2009 also did not find a sex predisposition among their group of BMDs affected with histiocytic sarcoma. However, this study found that females were, on average, four months older than males at diagnosis with cancer, which persisted among dogs with histiocytic sarcoma. This sex difference in age at diagnosis did not exist for dogs with the other major cancers, lymphoma, mast cell tumor, or melanoma. Other cancer diagnoses in our study of BMDs included lymphoma, mast cell tumor, and melanoma, comprising 6%, 3%, and 0.5% of all diagnoses, respectively. These results are similar to the Abadie et al. 2009 study of French and European BMDs, which found lymphoma affecting 4%, mast cell tumor 6%, and melanoma 2% [Abadie et al., 2009]. In the current study, cause of death was listed for 5,509 dogs (76%) and 822 (14.9%) of those listed histiocytic sarcoma, which is consistent with the 15.3% found by Erich et al. 2013 [Erich et al., 2013].

Nearly one-quarter of the cancer diagnoses in our study were unspecified cancer or tumors, while previous studies listed unspecified cancers composing half or more of the cancer diagnoses [Nielsen et al., 2010; Erich et al., 2013]. Further investigation of these cases revealed that a more specific diagnosis was not possible due to insufficient diagnostics, limited by owner interest to pursue additional diagnostics or due to physiologic limitations in sampling including the potential for uncontrollable bleeding or inaccessible anatomic location. At least 10% of these unspecified cancers listed histiocytic sarcoma as the top rule-out. Approximately 1% of the dogs with histiocytic disease had distinct recurrent episodes. Our findings indicate that a BMD diagnosed with histiocytic disease is four times more likely to be diagnosed once rather than experiencing recurrent forms of histiocytic disease. These data suggest that the risk of BMDs escalating to more malignant forms of histiocytic disease is relatively unlikely. A recent study of European BMD found that

previous joint disease was associated with the development of peri-articular histiocytic sarcoma in the same joint [Van Kuijk et al., 2013]. Abadie et al. 2009 found 8% of histiocytic sarcoma cases had a history of histiocytoma, and they also stated that although no environmental factor had been identified, they had found two cases of exposure to toxic fertilizers and paint [Abadie et al., 2009]. The possibility of environmental factors interacting with the unique genetic profile of BMDs to cause the high incidence of histiocytic disease in this breed warrants more investigation.

The top five cancers all peak in incidence at six years of age. The incidence of hip and elbow dysplasia has been decreasing, likely due to the orthopedic genetic testing through the Orthopedic Foundation for Animals (OFA) and controlled breeding. A recent study of 74 breeds over the period of 1974 to 2009 found an overall incidence of hip dysplasia to be 0.83% (range 0.07% to 6%) and elbow dysplasia to be 2.08% (range 0.5%-8%) [Hou et al., 2013]. In our study, over the whole study period the incidence of hip and elbow dysplasia was found to be 5% and 4%, respectively, clearly on the higher end of the range found in that study, but, the BMD has appeared to have made genetic improvement to the incidence of these dysplasias similar to the progress other highly affected breeds made [Lavrijsen et al., 2014]. In one decade, while cancer diagnoses increased at a rate of nearly 10%, the proportion of cancer diagnoses due to histiocytic sarcoma increased by over 15%. This increase may be due to increased awareness and improved diagnostic criteria. The median age at death for BMDs with histiocytic sarcoma has decreased over the last decade. On average, BMDs survived one month after diagnosis with histiocytic sarcoma, similar to the mean survival time of 49 days cited in the 2009 study by Abadie et al [Abadie et al., 2009]. In the Abadie et al. 2009 study, less than 10% of the dogs survived more than four months after their diagnosis, while in our study less than 7% of dogs survived to four months [Abadie et al., 2009].

This study is one of the largest sampling of BMDs published so far. These data were derived from a large community-based database, which demonstrate the utility of such community sourced data collection. With the advent of patient-accessible electronic health records, similar databases will be easier to create for human disorders, which will be especially important for tracking inci-

dence and clinical progression for rare diseases. Cancer is an important disease for BMDs and is predominately responsible for their short lifespan. While the incidence of hip and elbow dysplasias are decreasing in this breed, cancer in general and specifically histiocytic sarcoma increased in incidence. Among neonatal diseases, the incidence of osteochondritis dissecans, and cleft palate were among the top diagnoses noted in the BMD database, and can be addressed with further genetic studies. These findings underscore the immediate need for more research to improve the health of the BMD breed. Specifically, the genetic basis for histiocytic sarcoma in this breed needs to be identified, and now with the advances in next-generation sequencing, this may be possible in the near future. In addition, insights gleaned from the BMD regarding the pathophysiology of histiocytic diseases, have the potential to be informative for understanding this rare set of human tumors. Several research teams including our own are working diligently to address this need [Shearin et al., 2012; Boerkamp et al., 2013].

CHAPTER 3

CANINE HISTIOCYTIC DISEASES: USING MIRNAS TO UNRAVEL MECHANISMS OF TUMORIGENESIS

3.1 Introduction

Histiocytic diseases encompass a spectrum of proliferative diseases in both dogs and humans. These diseases arise from the abnormal accumulation of macrophages and dendritic cells leading to tumor formation and potentially to organ damage. The clinical presentation, behavior, and response to treatment are quite dissimilar for the different histiocytic diseases. In dogs at least four distinct histiocytic proliferative diseases have been recognized. The most benign of the histiocytic diseases are cutaneous histiocytoma and reactive histiocytosis, the latter of which can present as a cutaneous or systemic disease. The malignant histiocytic diseases encompass the neoplasms histiocytic sarcoma and hemophagocytic histiocytic sarcoma. Histiocytic sarcoma (HS) in its disseminated form, can lead to death or euthanasia within 2 to 4 months from diagnosis [Zavodovskaya et al., 2006]. While histiocytic diseases are rare in humans, these diseases are relatively common in dogs, especially among certain breeds of dogs including the Bernese mountain dog, golden retriever, flat-coated retriever, and Rottweiler. Approximately 25% of all tumors in the Bernese mountain dog (BMD) breed are diagnosed as HS [Abadie et al., 2009]. The diagnosis of histiocytic diseases is often based on the histopathology of the lesions. Additional confirmation is provided by immunohistochemistry for the leukocyte surface molecules such as the $\beta 2$ integrins CD18, CD11c or CD11d, which are characteristic for the histiocytes present in these diseases. Treatment of HS depends upon the presentation of the disease. Unfortunately, there is a dearth of effective treatment options available for HS for both dogs and humans [Skorupski et al., 2007]. Localized HS or periarticular HS have been cured by early surgical excision [Klahn et al., 2011]. Disseminated HS, on the other hand, is better suited to chemotherapy due to the systemic nature of this disease. The BMD presents more commonly with the disseminated form of HS and response

to chemotherapeutics is, at best, short-lived [Hafeman et al., 2010]. The prognosis for disseminated HS is quite poor, with the disease progressing rapidly resulting in death or euthanasia within weeks to a few months.

The high incidence of HS in BMDs suggests a genetic component to this disease. Although Padgett et al. determined that HS is inherited in the BMD, the genes involved in the tumorigenesis are still unknown [Padgett et al., 1995]. Previous research has evaluated copy number variations in the BMD and flat-coated retriever using genome wide array comparative genomic hybridization and identified deletions of the tumor suppressor genes CDKN2A/B, RB1 and PTEN [Hedan et al., 2011]. The first genome-wide association study of histiocytic sarcoma was conducted in BMD and revealed a susceptibility locus encompassing MTAP and part of CDKN2A [Shearin et al., 2012]. Recently, several genes involved in DNA repair and replication pathways were associated with histiocytic sarcoma through gene expression profiling of affected flat-coated retrievers [Boerkamp et al., 2013].

Human histiocytic diseases are rare and affect both children and adults. HS accounts for $< 1\%$ of all non-Hodgkin's lymphomas in humans [Ralfkiaer et al., 1990]. Human histiocytic diseases are so rare that research funding and progress are slow due to inadequate sample sizes of human patients to carry out randomized therapy trials. The dog is the only species that spontaneously presents with similar diseases. Human HS can be a very aggressive disease with a poor prognosis, progressing to death within a year of diagnosis [Schmitz and Favara, 1998; Vos et al., 2005].

More research is needed to understand the biology of histiocytes, which are important components of allergic and infectious diseases, and the cell of origin for histiocytic malignancies. For this purpose, the dog holds great promise as a model for histiocytic diseases, to the benefit of humans and dogs. Histiocytic diseases in dogs, although not the same as in humans, is similar enough that study of canine HS may permit insights into the pathogenesis of this rare human disease, while providing the sample sizes necessary to provide significant data. In this study, a novel approach used microRNA (miRNA) expression to probe the transcriptome of canine histiocytic diseases. Since their relatively recent discovery in 1993, [Fire et al., 1998] miRNAs have become an active

area of research. The miRNAs can have as many as 200 to 300 gene targets and therefore regulate many cellular processes including proliferation, differentiation, and apoptosis [Kloosterman and Plasterk, 2006]. Recent studies have shown that miRNAs can act as tumor suppressors or oncogenes, also known as oncomirs, by their regulation of critical genes [Esquela-Kerscher and Slack, 2006]. This discovery has led to a growing number of studies in cancer research to evaluate miRNAs as regulators of pathways important to tumorigenesis, biomarkers of cancer, and novel therapeutic targets [Calin et al., 2002]. We hypothesize that along the spectrum of canine histiocytic diseases, differentially expressed miRNAs will provide insight into the critical pathways for tumorigenesis in histiocytic neoplasias as compared to nonmalignant histiocytic proliferative diseases. This approach in canine histiocytic diseases will permit the analysis of miRNAs and the pathways they regulate to increase our understanding of both the normal role of histiocytes and the aberrant behavior of histiocytes in these tumors.

3.2 Materials and Methods

3.2.1 Samples

Canine histiocytic disease samples included formalin-fixed, paraffin-embedded (FFPE) samples from the Michigan State University Diagnostic Center for Population and Animal Health collected between 2006 and 2011. The FFPE samples comprised four canine histiocytic diseases and included cases from Bernese mountain dogs and other breeds: hemophagocytic histiocytic sarcoma, histiocytic sarcoma, reactive histiocytosis, and histiocytoma. Fresh samples were obtained from canine patients at the Michigan State University Veterinary Teaching Hospital. Normal mononuclear cells were isolated from approximately 16 ml fresh blood samples using ACCUSPIN^{TM}Tubes and Histopaque$^{®}$-1077 (Sigma-Aldrich, St. Louis, MO) following the manufacturer's procedure. The isolated cells were washed three times with 10 ml isotonic phosphate buffered saline (PBS) with 1:1000 Gibco$^{®}$ Gentamicin (Life Technologies) added and centrifuged 250xg, 10 minutes, at room temperature. Each sample was then plated into 6-well plates in Gibco$^{®}$ RPMI Media

1640 (Life Technologies, Grand Island, NY) supplemented with 10% Gibco® Fetal Bovine Serum (Life Technologies), 1:1000 Gentamicin, and 1:100 Gibco® Antibiotic-Antimycotic (Life Technologies). After incubating at 37°C, 5% CO_2 for one to two hours, the media and any floating cells were removed and discarded and fresh media was added. For macrophage derivation, cell culture was continued for one week with media refreshed once after three days. For dendritic cell derivation, 24 hours after plating the factors Human Flt3 Ligand Recombinant Protein (25 ng/ml 12.5 μl, Affymetrix 14-8513-80), Human Granulocyte/Macrophage-Colony Stimulating Factor Recombinant Protein (800 U/ml, Sigma-Aldrich SRP3050), and Gibco® IL4 Recombinant Human Protein (500 U/ml 100 μl, Life Technologies PHC0044) were added to promote dendritic cell differentiation and the cells were cultured for one week with the media and factors refreshed once after 3 more days. Fresh tumor samples were dissociated using collagenase. Briefly, the tissue was minced into 3-4 mm pieces, which were washed several times with Hanks' Balanced Salt Solution (HBSS), (Sigma-Aldrich). Collagenase, Type I (Sigma-Aldrich) was added to a final concentration of 200 U/ml in HBSS (Sigma-Aldrich) to the tissue pieces and incubated 37°C, 5% CO_2 for 1 hour. The cell suspension was then filtered through a BD Falcon™Cell Strainer, 40 μm (BD, Franklin Lakes, NJ) and the strainer was washed three times with PBS. The cell suspension was then washed three times with PBS and centrifugation. The final cell pellet was resuspended in RPMI media supplemented with 10% Fetal Bovine Serum, 1:1000 Gentamicin, and 1:100 Antibiotic-Antimycotic. After incubating 37°C, 5% CO_2 for 2 hours, the media and any floating cells were removed and discarded. Histiocyte cell identity for all fresh samples was confirmed by flow cytometry for CD18 and CD11c cellular markers and evaluated for cellular morphology by a board certified veterinary clinical pathologist. Total RNA including miRNA was isolated from the FFPE samples using the QIAGEN™miRNeasy FFPE Kit and from the fresh samples using the Life Technologies™Ambion® mirVana™miRNA Isolation Kit, with phenol per the manufacturer's protocols. The isolated RNA was quantified by a NanoDrop spectrophotometer and RNA quality was evaluated by the Agilent Technologies 2100 Bioanalyzer system.

3.2.2 MiRNA transcriptome profiliing

MiRNA profiling was performed using the Affymetrix® GeneChip® miRNA Array (Santa Clara, CA) which contained probes for 71 organisms including dog, for which it could detect 177 canine miRNAs as acquired from the Sanger miRBase miRNA database v11 (April 15, 2008). The differential expression of miRNAs was compared among three canine histiocytic diseases: hemophagocytic histiocytic sarcoma, histiocytic sarcoma, and reactive histiocytosis. These diseases included FFPE samples from the following four groups: BMD with hemophagocytic HS, BMD with HS, golden retrievers with HS, and reactive cutaneous histiocytosis. A fifth sample was the purchased canine HS cell line, DH82 [Wellman et al., 1988]. All five groups were compared to histiocytes collected from peripheral blood or peritoneal fluid of breed matched unaffected dogs. Table 3.1 details the signalment data for the samples evaluated using the miRNA array.

Table 3.1: **Signalment of 28 samples analyzed by the miRNA array encompass hemophagocytic histiocytic sarcoma, histiocytic sarcoma, and reactive histiocytosis**

Number of samples	Disease	Breed	Location	Age Range
5	Hemophagocytic Histiocytic Sarcoma	Bernese mountain dogs	liver	2-9 years
5	Histiocytic Sarcoma	Bernese mountain dogs	liver, lung, lymph nodes	4-9 years
4	Histiocytic Sarcoma	golden retrievers	lymph nodes, subcutaneous	7-12 years
5	Reactive Histiocytosis	mixed breeds	skin	3-9 years
1	Histiocytic Sarcoma cell line	golden retriever	bone marrow	10 years
4	Normal histiocytes	Bernese mountain dogs	blood	2-12 years
4	Normal histiocytes	golden retriever, mixed breeds	blood, peritoneal effusion	1-12 years

28 canine samples were evaluated for miRNA expression by Affymetrix® GeneChip® miRNA Array. All samples were from FFPE except the cultured cell line and normal histiocytes. Males and females were represented approximately equally in each disease.

The miRNA profiling data was normalized using the Affymetrix® miRNA QCTool and then

analyzed using Ingenuity® Pathway Analysis software (IPA Spring Release 2014). The miRNA data was paired with mRNA data to visualize miRNA-mRNA relationships using the microRNA Target Filter feature of IPA. The mRNA data came from other work in the lab using Affymetrix® GeneChip® Canine Genome 2.0 Array (Santa Clara, CA) on a set of hemophagocytic histiocytic sarcoma and histiocytic sarcoma diagnosed samples of Bernese mountain dogs and other breeds [Dervisis et al., 2012]. Gene targets that had the opposite expression pattern to the miRNA (i.e. the gene target had decreased expression relative to normal samples while the corresponding miRNA had increased expression or vice versa) were selected for further pathway exploration. The selected gene list was inputted into DAVID Bioinformatics (http://david.abcc.ncifcrf.gov/) to identify functional clusters of pathways in these samples [Da Wei Huang and Lempicki, 2008].

3.2.3 Validation by qRT-PCR

Fourteen miRNAs were selected for validation by Quantitative Reverse Transcription PCR (qRT-PCR) using the TaqMan® MicroRNA Assays available from Life Technologies™. The canine miRNAs cfa-let-7c, cfa-miR-19a, cfa-miR-28, cfa-miR-29b, cfa-miR-34a, cfa-miR-125b, cfa-miR-143, cfa-miR-146a, cfa-miR-146b, cfa-miR-148a, cfa-miR-148b, cfa-miR-152, cfa-miR-155, and cfa-miR-200c were selected based upon their differential expression from the miRNA array data and their role in cancer as predicted by a review of the literature. Consistent with many miRNAs, these miRNAs are highly conserved among mammals (Table 3.2 especially between the dog and human [Lagos-Quintana et al., 2001; Meunier et al., 2013]. Samples for validation included the samples used for the microarray, and additional FFPE samples for all diseases and included an additional disease group of histiocytoma samples. A second canine histiocytic sarcoma cell line (designated BD) derived in lab from a BMD with HS was also evaluated. Primary cultured cells derived from fresh HS tumors of various breeds were included in the qRT-PCR analysis. Table 3.3 details the signalment data for all samples validated using qRT-PCR. All reactions were run in triplicate with U6 snRNA used as the endogenous control and a negative water control included on every plate. MiRNA expression was quantified by the comparative threshold cycle method ($\Delta\Delta$CT)

using a breed-matched normal histiocyte sample to compare to where possible (i.e. BMD disease samples compared to the average of the four BMD normal histiocyte samples and other breeds disease samples compared to the average of four mix breed and golden retriever normal histocyte samples).

Table 3.2: **MiRNAs are generally conserved among mammals**

miRNA	Dog	Cat	Cow	Human	Mouse	Rabbit	Rat	Sheep
let-7c	100%	82%	100%	100%	82%	84%	82%	82%
miR-19a	100%	100%	100%	100%	99%	99%	98%	100%
miR-28	100%	99%	99%	99%	93%	95%	92%	99%
miR-29b	100%	100%	100%	100%	99%	88%	99%	100%
miR-34a	100%	100%	83%	91%	92%	80%	92%	83%
miR-125b	100%	68%	97%	98%	85%	69%	97%	97%
miR-143	100%	100%	100%	100%	100%	100%	100%	100%
miR-146a	100%	100%	95%	97%	91%	97%	91%	93%
miR-146b	100%	92%	85%	80%	84%	82%	82%	85%
miR-148a	100%	100%	100%	96%	94%	93%	93%	100%
miR-148b	100%	100%	100%	100%	94%	100%	94%	100%
miR-152	100%	100%	95%	94%	86%	74%	93%	93%
miR-155	100%	100%	98%	95%	92%	98%	92%	98%
miR-200c	100%	100%	100%	85%	91%	91%	55%	91%

Among the fourteen selected miRNAs, the percent of the dog miRNA sequence that matches the miRNA homolog in other mammals are listed. MiR-19a, miR-28, miR-143, miR-146a, miR-148a, miR-148b, and miR-155 were all highly conserved with more than 90% identity between the dog sequences and the miRNA homologues in the mammals evaluated.

3.2.4 Statistical Analysis

The miRNA array data was visualized with principal component analysis (PCA) using Qlucore Omics Explorer v.3.0, (Lund, Sweden). Heat mapping with unsupervised and supervised hierarchical clustering was conducted using using R version 3.1.0 to visualize the differences between the samples and the normal histiocytes. This difference was calculated as the average of the breed-matched normal canine histiocytes subtracted from the normalized sample intensity. One-way ANOVA was used to determine statistically significant differences between the miRNA profiles of the disease groups.

3.3 Results

3.3.1 Samples

A total of 28 samples representing hemophagocytic histiocytic sarcoma, histiocytic sarcoma, and reactive histiocytosis were evaluated by Affymetrix® GeneChip® miRNA Array and compared to normal breed-matched histiocytes. An expanded set of 138 samples representing hemophagocytic histiocytic sarcoma, histiocytic sarcoma, reactive histiocytosis, and histiocytoma were analyzed by qRT-PCR for a selected set of 14 miRNAs. Among the FFPE samples an average of 24 μg (range: 1 μg-74 μg) of RNA was isolated with quality estimated by the RNA Integrity Number averaging 2 (range: 1-2.4). For the fresh samples an average of 10 μg of RNA (range: 0.27 μg-85 μg) was obtained, with the RNA Integrity Number averaging 7.7 (range: 2.3-9.9).

3.3.2 MiRNA transcriptome profiliing

The miRNA expression profiles of six groups of cases representing hemophagocytic histiocytic sarcoma in BMD, histiocytic sarcoma in BMD, histiocytic sarcoma in golden retrievers, the histiocytic sarcoma cell line DH82, reactive histiocytosis, and normal histiocytes were evaluated by Affymetrix® GeneChip® miRNA Array for the expression of 177 canine miRNAs. In total, 148 (84%) canine miRNAs were detectable in the samples and 55 of these miRNAs had greater than two-fold differences in the diseased samples relative to the normal canine histiocytes.

The application of principal component analysis (PCA) to the miRNA array data resulted in three distinct groupings among the samples: normal histiocytes from mixed breeds, normal histiocytes from BMDs, and all of the disease samples together (Figure 3.1). PCA of just the disease samples did not result in any apparent sub groupings (Figure 3.2). These data were also analyzed with unsupervised hierarchical clustering, which revealed sets of miRNAs that were overexpressed in Bernese mountain dogs or mixed breed dogs (Figure 3.3). The first cluster includes the normal histiocytes grouping together regardless of breed. The affected dogs form two additional clusters dependent on breed. The affected Bernese mountain dogs have a identifiable subset of overex-

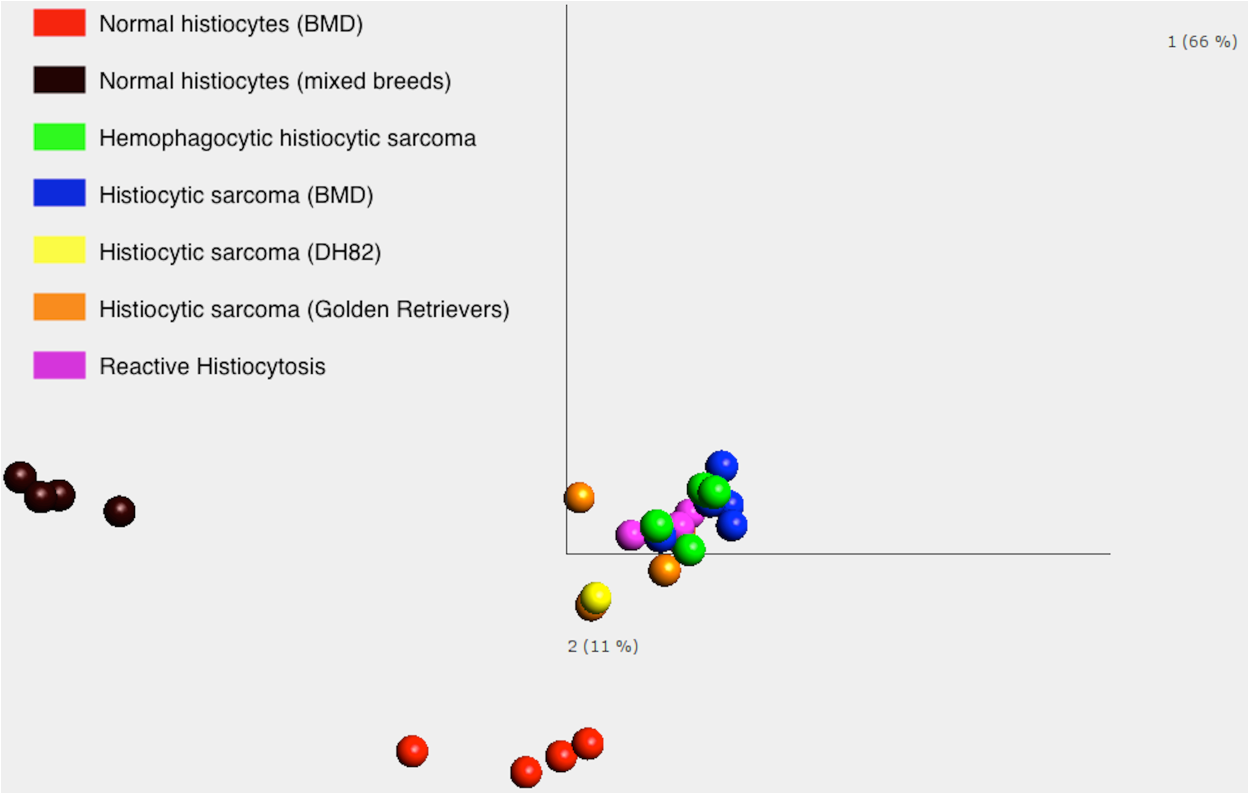

Figure 3.1: **Principal component analysis of the microRNA array data shows three groupings among the samples based upon their miRNA expression.**The axes represent the first (x-axis) and second (y-axis) principal components and indicate the direction of the greatest amount of variance among the samples. The samples were color coded according to their disease type. The four normal histiocyte samples from mixed breeds are shown in brown as one group. The four normal histiocyte samples from BMD are shown in red forming a second group. The disease samples together form a third grouping.

pressed miRNAs (Table 3.4). Other breeds of affected dogs, including golden retrievers and mixed breeds form another cluster with a different subset of overexpressed miRNAs. There is also a subset of overexpressed miRNAs that are shared among affected dogs regardless of breed. Supervised clustering resulted in a similar expression pattern.

A list of gene targets was generated from the miRNA profiling data and corresponding mRNA data using Ingenuity® Pathway Analysis (IPA). Those gene targets with inverse expression to their miRNAs were put into DAVID Bioinformatics to find functional clusters of genes and identify common gene families among the miRNA targets (Table 3.5). The canine histiocytic sarcoma cell line DH82 was a common sample between the miRNA profiling and the mRNA data and thus the

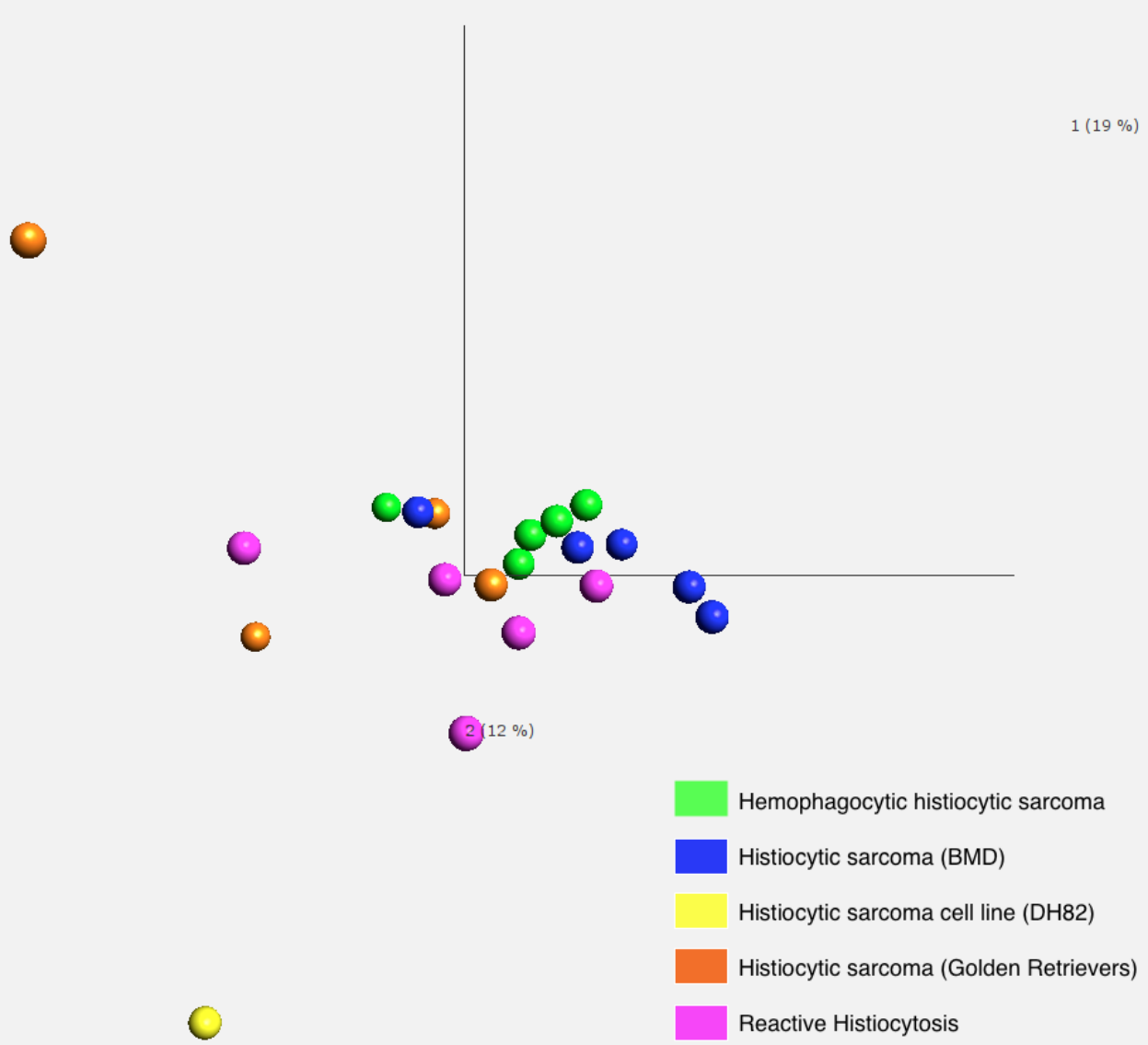

Figure 3.2: **Principal component analysis of the microRNA array data for just the disease samples fails to display any subgroups.** The axes represent the first (x-axis) and second (y-axis) principal components. The normal histiocyte samples were removed and PCA was conducted on only the disease samples. No subgroups within the disease samples were found.

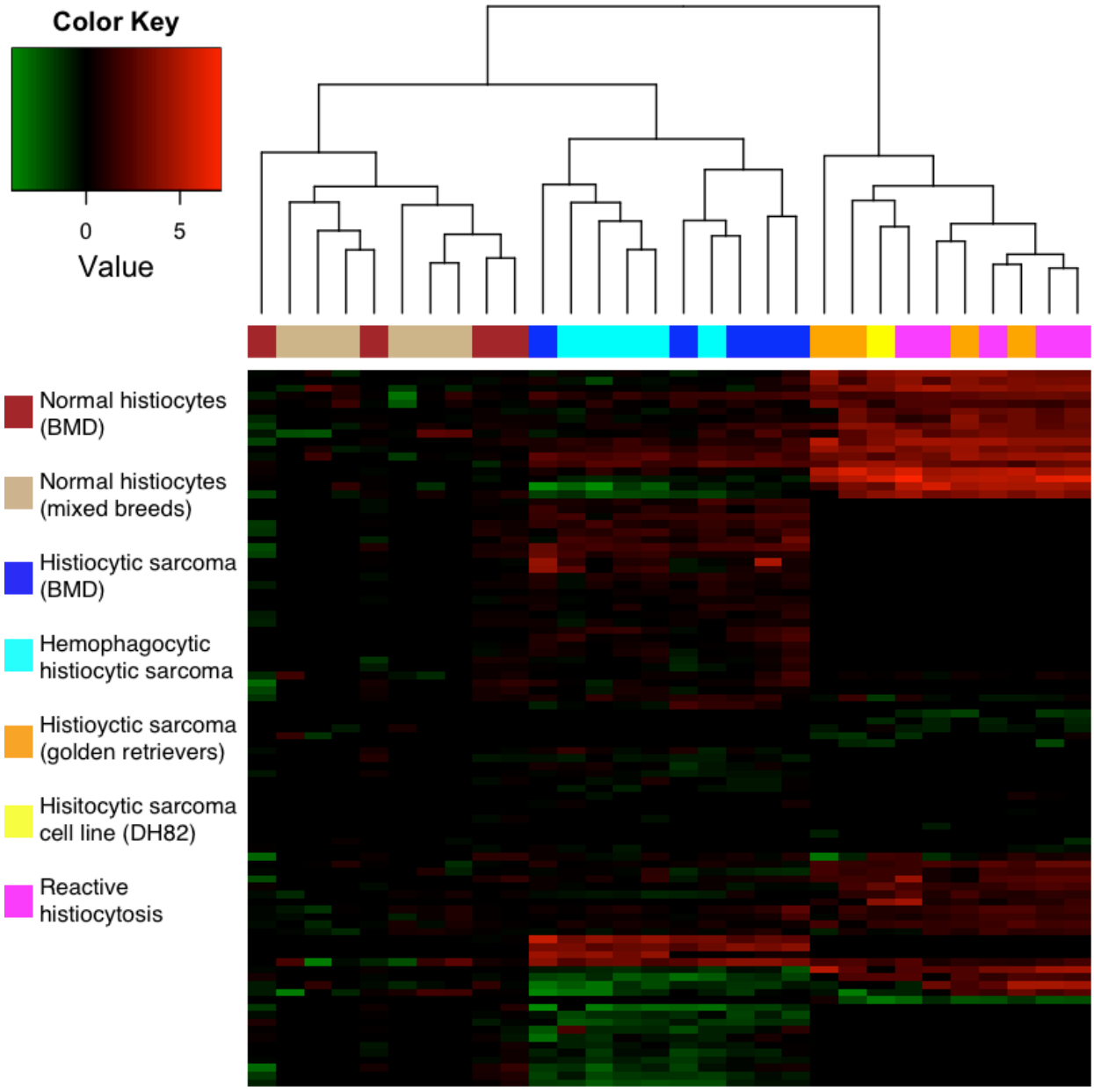

Figure 3.3: **Heat map generated using unsupervised clustering shows the differential expression of the 148 detectable canine microRNAs from the array data.** Red signifies increased expression and green signifies decreased expression of microRNAs. The miRNAs cluster based upon breed and disease phenotype. On the far left the normal histiocytes cluster together. The affected Bernese mountain dogs form a cluster in the middle. Other breeds of affected dogs, including golden retrievers and mixed breeds clustered together on the far right.

focus of the IPA microRNA Target Filter. This resulted in 477 highly predicted or experimentally observed gene targets of 47 miRNAs. The functional annotation clustering algorithm determined 36 functional clusters among these genes, the most significant of which encompassed apoptosis, growth factors, kinases, and several signaling pathways such as ERBB. The gene target list was also classified into nine gene groups or families. These gene groups included the fibroblast growth factors, WNT family members, kinases, SMAD family members, mitogens, ribosomal protein kinases, calcium/calmodulin-dependent protein kinases, other growth factors, and integrins. A subset of the paired expression data that has an inverse expression specifically in the BMD samples revealed five miRNAs and nine gene targets as shown in Figure 3.4.

Table 3.3: **Signalment of 138 samples used for miRNA validation by qRT-PCR encompass hemophagocytic histiocytic sarcoma, histiocytic sarcoma, reactive histiocytosis, and histiocytoma**

Number of samples	Disease	Breed	Location	Age Range
10	Hemophagocytic Histiocytic Sarcoma	Bernese mountain dogs	spleen, liver, lymph node, lung	2-9 years
5	Hemophagocytic Histiocytic Sarcoma	golden retrievers	spleen, liver	6-9 years
8	Hemophagocytic Histiocytic Sarcoma	Labrador retrievers, Rottweilers, mixed breeds	spleen, liver, lymph node, lung	5-12 years
23	Histiocytic Sarcoma	Bernese mountain dogs	spleen, liver, lung, lymph nodes	4-13 years
12	Histiocytic Sarcoma	golden retrievers	spleen, lymph nodes, subcutaneous	4-12 years
9	Histiocytic Sarcoma	Labrador retrievers, Rottweilers, flat-coated retriever, mixed breeds	spleen, lymph nodes, subcutaneous	6-11 years
2	Reactive Histiocytosis	Bernese mountain dogs	skin	4-9 years
3	Reactive Histiocytosis	golden retrievers	skin	2-8 years
23	Reactive Histiocytosis	Various breeds, mixed breeds	skin	2-12 years
25	Histiocytoma	Various breeds, mixed breeds	skin	1-12 years
1	Histiocytic Sarcoma cell line	golden retriever	bone marrow	10 years
1	Histiocytic Sarcoma cell line	Bernese mountain dog	lymph node	8 years
7	Histiocytic Sarcoma primary cultured cells	Bernese mountain dog, golden retrievers, various breeds	lymph node, blood, subcutaneous	6-12 years
4	Normal histiocytes	Bernese mountain dogs	blood	2-12 years
5	Normal histiocytes	golden retriever, mixed breeds	blood, peritoneal effusion	1-12 years

Expanded set of 138 canine samples evaluated for miRNA expression by qRT-PCR using Life Technologies TaqMan® MicroRNA Assays. All samples were from FFPE except the cultured cell line, primary cells, and normal histiocytes. Males and females were represented approximately equally in each disease.

Table 3.4: **MiRNAs with significant differential expression in the disease groups**

miRNAs	**BMDs**	**Others**
mir-574	decrease #	increase #
mir-150	decrease #	increase *
mir-155	decrease *	increase #
mir-23a	decrease *	increase #
mir-181a	decrease #	increase #
mir-125b	decrease *	increase #
mir-103	increase *	increase #
mir-20	increase *	increase #
mir-93	increase #	increase #
let-7a	increase #	increase #
mir-26a	increase #	increase #
mir-99b	increase #	increase #
let-7e	increase #	increase *

miRNAs	**BMDs**
mir-146b	decrease #
mir-23b	decrease #
mir-1307	decrease #
mir-146a	decrease *
mir-27a	decrease *
mir-99a	decrease #
mir-27b	decrease *
mir-532	increase *
mir-193a	increase *
mir-30b	increase *
mir-503	increase *
mir-106b	increase *
mir-130b	increase *
mir-30c	increase *
mir-423a	increase #
mir-25	increase #
mir-151	increase #
mir-30a	increase #

miRNAs	**Others**
mir-191	increase *
mir-107	increase #
mir-24	increase #
mir-22	increase #
mir-16	increase #
mir-30d	increase #
mir-34a	increase *
let-7c	increase #
mir-320	increase #
mir-502	increase #
mir-140	increase #
mir-221	increase #
mir-185	increase #
let-7f	increase #
mir-106a	increase #
mir-92a	increase #
mir-378	increase #

Among the detectable miRNAs there were subsets of miRNAs that were differentially expressed in the disease samples in a breed dependent manner as identified on the heat map. The miRNAs are listed down the columns in order of increasing difference relative to normal histiocytes. The first segment of the table on the left lists significantly differentially expressed miRNAs that are shared among affected dogs regardless of breed. The corresponding direction of the differential expression is indicated for the BMDs and other breeds of dog as increase being increased expression relative to normal histiocytes and decrease being decreased expression relative to normal histiocytes. The subset of miRNAs defining the affected Bernese mountain dogs are listed in the middle segment of the table. The last table segment on the right lists the significantly overexpressed miRNAs in other breeds of affected dogs, including golden retrievers and mixed breeds. Symbols denote significance as determined by two-tailed, random variance T-test: * = $P < 0.05$, # = $P < 0.005$.

Table 3.5: **Functional clusters of miRNA gene targets include immune signaling, migration, and proliferation**

Term	Count	P-Value	Genes
Annotation Cluster 1	**Enrichment Score: 2.6**		
Jak-STAT signaling pathway	8	6.38E-05	*IL2RA, GRB2, CREBBP, IFNG, CSF2RB, PIK3R5, JAK3, PIK3R2*
Natural killer cell mediated cytotoxicity	7	6.45E-05	*CASP3, GRB2, LCK, IFNG, PIK3R5, FAS, PIK3R2*
Chemokine signaling pathway	6	7.96E-03	*IL8, GRB2, GNG2, PIK3R5, JAK3, PIK3R2*
T cell receptor signaling pathway	5	8.40E-03	*GRB2, LCK, IFNG, PIK3R5, PIK3R2*
Fc epsilon RI signaling pathway	4	1.65E-02	*GRB2, PIK3R5, MAP2K6, PIK3R2*
ErbB signaling pathway	4	2.48E-02	*GRB2, TGFA, PIK3R5, PIK3R2*
Annotation Cluster 2	**Enrichment Score: 2.5**		
Focal adhesion	6	1.32E-02	*GRB2, PDGFRB, PIK3R5, PDGFD, PIK3R2, KDR*
Regulation of actin cytoskeleton	6	1.65E-02	*FGF7, PDGFRB, PIK3R5, PDGFD, FGF1, PIK3R2*
Annotation Cluster 3	**Enrichment Score: 1.9**		
Regulation of cell proliferation	4	1.10E-02	*IL2RA, CDKN1B, FGF7, KDR*

The most significant pathways that resulted from DAVID Bioinformatics functional clustering are listed. For the miRNAs that were potentially biologically significant (greater than 2-fold difference from the normal samples), their corresponding gene targets were inputted to DAVID and three significant functional gene clusters resulted. Annotation Cluster 1 encompassed several immune system signaling pathways. The second cluster included genes that regulate cell migration. The third cluster included genes responsible for the regulation of cell proliferation.

3.3.3 Validation by qRT-PCR

From the array data, fourteen miRNAs were selected by validation by qRT-PCR. Among the selected miRNAs, miR-19a, miR-29b, miR-148a, and miR-148b were overexpressed in the cluster of affected BMDs according to the array data. The miRNAs let-7c, miR-28, miR-34a, miR-125b, miR-146a, miR-146b, and miR-155 were overexpressed in other affected breeds of dogs. The miRNAs miR-143, miR-152, and miR-200c were overexpressed in all affected dogs on the array regardless of breed. Validation was performed on an expanded set of samples representing multiple breeds affected with hemophagocytic histiocytic sarcoma, histiocytic sarcoma, reactive histiocytosis, and histiocytoma, which were all compared to breed-matched normal histiocytes. Along with the histiocytic sarcoma cell line DH82, the samples for qRT-PCR included a new cell line derived in our lab from a BMD with histiocytic sarcoma. Figure 3.5 displays the qRT-PCR results for the miRNA expression as the fold-change relative to breed-matched normal canine histiocytes.

Most disease groups showed upregulation relative to normal histiocytes for each miRNA evaluated as shown in Table 3.6. The largest fold changes were seen for miR-143, which had increased expression in all disease groups regardless of breed, for an overall average of 10.5 fold increase in the disease samples. MiR-152 expression was increased an average of 4.1 fold in all disease groups regardless of breed. MiR-200c had increased expression more than 2-fold in most disease samples except in the HS cell lines and primary cell cultures where the fold change was less than 2-fold. Let-7c had more than 2-fold increased expression in the hemophagocytic HS and HS samples only regardless of breed. MiR-125b had an average of 6.9-fold increase in expression in all affected breeds of dog, except for the BMD, which had an average of 3-fold decrease in expression. MiR-148a had increased expression more than 2-fold in hemophagocytic HS, HS and histiocytoma samples regardless of breed. Hemophagocytic HS samples in all breeds and in the HS samples from BMD. MiR-155 was increased 3.9-fold in most affected breeds of dog, except for the BMD, which had an average of 1.4 fold decreased expression. MiR-148b was increased more than 2-fold in BMD with hemophagocytic HS and in the HS primary cell cultures. MiR-34a was increased more than 2-fold in breeds with hemophagocytic HS and histiocytoma, but not in

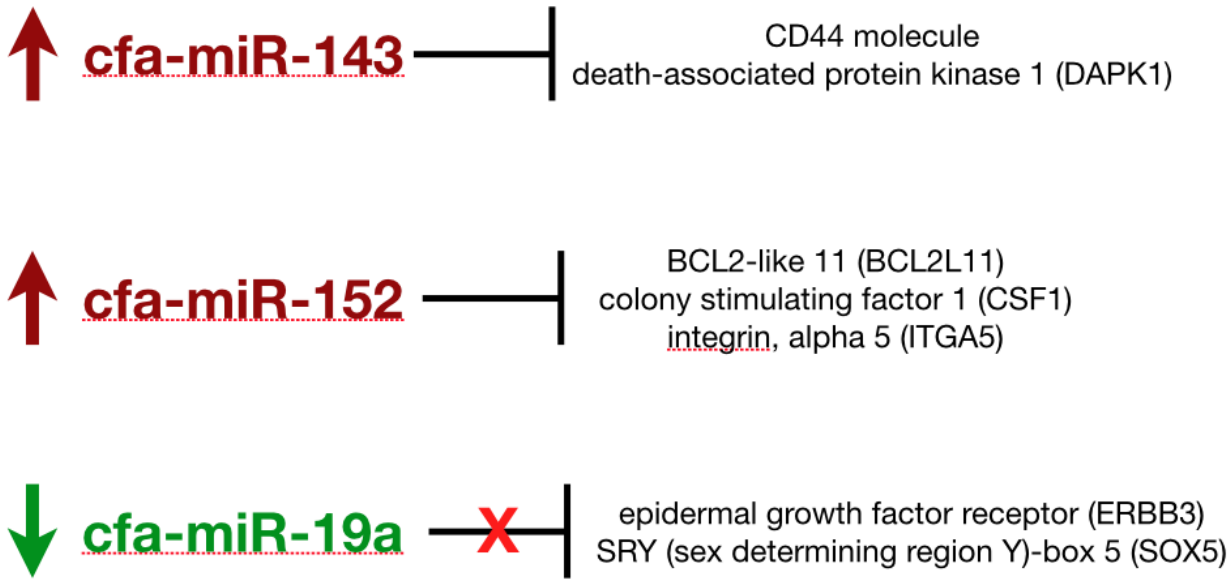

Figure 3.4: **Pathways regulated by miRNAs predicted to be important for histiocytic disease.** MiR-143 is predicted to target CD44 molecule, which is associated with miRNA induced migration, and death-associated protein kinase 1, a positive mediator of programmed cell death. MiR-152, is of particular interest because it has been shown to play an integral role in the differentiation of dendritic cells, which are the cell of origin for histiocytic disease. MiR-152 was also over expressed and is predicted to target BCL2-like 11, an apoptosis facilitator, colony stimulating factor 1 in macrophages, and integrin, alpha 5 (fibronectin receptor, alpha polypeptide), an integrin that if lost can result in immune dysfunction. MiR-19a was had lower than normal expression in the reactive histiocytosis samples and is predicted to target the epidermal growth factor receptor (ERBB3) and SRY (sex determining region Y)-box 5, which promotes cell survival.

BMD, which had more than 2-fold decrease in expression in the reactive histiocytosis samples. MiR-146a was decreased more than 2-fold in BMD with hemophagocytic HS and in the cultured cells, but was increased in other breeds of dog with hemophagocytic HS. MiR-19a was increased 2-fold in the hemophagocytic HS samples but decreased in the BMD HS cell line and in reactive histiocytosis samples in dogs other than BMD. MiR-146b was decreased more than 2-fold in the BMD HS cell line and increased reactive histiocytosis samples in dogs other than BMD. MiR-29b was increased more than 2-fold in BMD cases of hemophagocytic HS and HS but decreased in reactive histiocytosis samples in dogs other than BMD. MiR-28 was decreased more than 2-fold in the histiocytoma samples only.

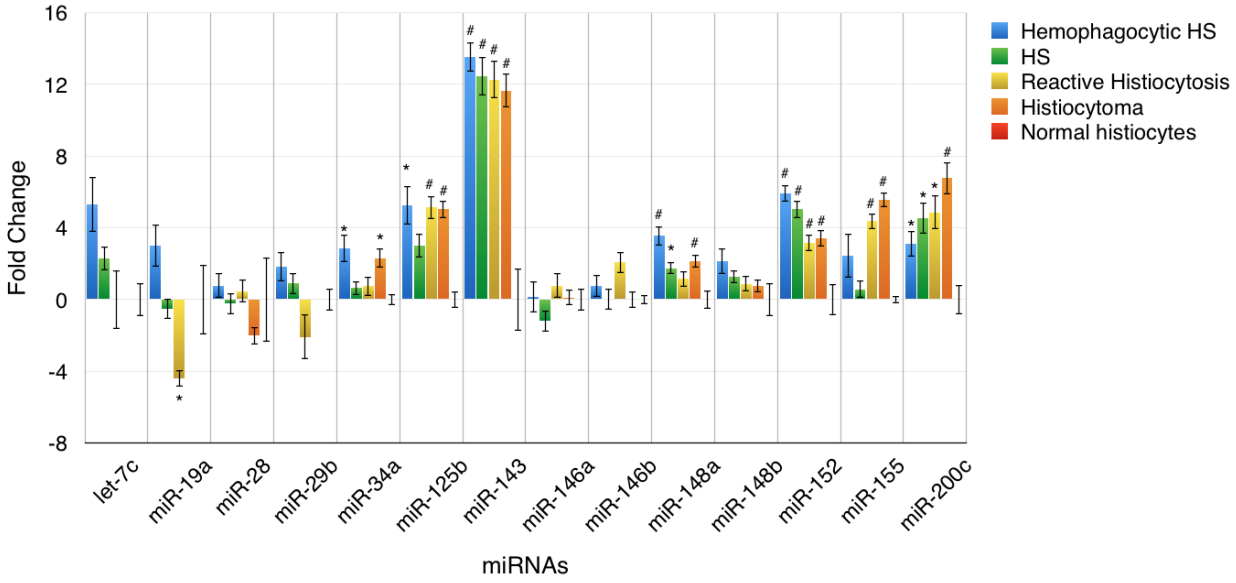

Figure 3.5: **Results from qRT-PCR of selected canine miRNAs validates the upregulation of most miRNAs consistent with the array data.** Values are fold change relative to breed-matched normal canine histiocytes. Symbols denote the groups which are significantly different from normal as determined by two-tailed, random variance T-test, Bonferroni corrected: * = $P < 0.05$, # = $P < 0.005$. HS = histiocytic sarcoma.

Table 3.6: **Results of qRT-PCR validation of miRNAs on samples of hemophagocytic histiocytic sarcoma, histiocytic sarcoma, reactive histiocytosis, and histiocytoma**

Group	miR-143	miR-152	miR-200c	let-7c	miR-125b	miR-148a	miR-155	miR-148b	miR-34a	miR-146a	miR-19a	miR-146b	miR-29b	miR-28
BMD HP	**12.42**	**5.25**	2.22	4.39	-1.00	**3.92**	-1.62	2.52	-0.62	-3.39	2.56	-0.39	**2.18**	-0.36
Other HP	**14.57**	**6.32**	**4.16**	8.44	**9.15**	**3.75**	**5.99**	1.58	**4.76**	2.72	6.43	1.75	-1.77	1.04
BMD HS	**14.02**	**4.94**	**4.61**	3.07	-0.53	2.31	-1.16	1.34	-0.51	-1.47	1.33	0.75	2.81	-0.44
Other HS	**13.27**	**4.35**	**6.11**	3.25	**6.68**	1.53	**3.29**	0.92	1.61	0.80	-1.43	-0.25	0.49	-0.41
BMD cell line	5.24	0.38	1.48	-0.29	-6.73	1.05	**-4.05**	-0.12	-0.47	-2.12	-2.02	-3.33	-0.11	-1.79
Other cell lines	-1.00	**7.92**	-1.35	0.51	**8.05**	0.47	-0.16	2.58	1.82	-3.81	-0.87	-0.65	0.02	1.56
BMD RHC	**12.19**	1.29	3.32	NP	**-3.84**	0.17	**1.35**	1.13	-2.98	-0.89	NP	1.50	NP	1.12
Other RHC	**12.33**	**3.29**	**5.91**	0.02	**5.82**	1.23	**4.59**	0.87	1.02	1.00	**-4.38**	2.11	-2.08	0.43
Other HCM	**11.66**	**3.42**	**6.78**	NP	**5.03**	**2.12**	**5.55**	0.75	2.32	0.12	NP	-0.02	NP	-2.00

The average fold-change for all miRNAs validated by qRT-PCR are shown for each disease group (listed vertically) and miRNA (listed horizontally at top). The disease groups are also divided by breed: BMDs and other breeds of dogs. The miRNAs are in order of overall fold-change across all groups, decreasing from left to right. Bolded numbers indicate $P < 0.05$ as determined by two-tailed, random variance T-test. Other = all other breeds of dog beside BMD, HP = Hemophagocytic HS, Other cell lines = primary cell cultures from breeds of dog besides the BMD, RHC = Reactive Histiocytosis, HCM = histiocytoma, NP = not enough sample for analysis to be performed.

3.4 Discussion

The cases of histiocytic disease used in this study were isolated from FFPE and fresh samples. While our histiocyte derivation protocol did limit the number of cells isolated from fresh samples and therefore the quantity of RNA, the RNA quality was acceptable with an average RNA Integrity Number of 7.7. The FFPE samples yielded more RNA on average, but the quality was substantially lower with an average RNA Integrity Number of 2, which was expected from this type of sample. Several studies have demonstrated that FFPE samples correlate well with matched fresh frozen samples for miRNA expression [Li et al., 2007; Xi et al., 2007; Doleshal et al., 2008; Zhang et al., 2008; Glud et al., 2008; Hui et al., 2009; Mortarino et al., 2010]. The cross-linking that occurs during the processing of FFPE samples is well-known to inhibit total RNA yield and quality. However, miRNAs appear to remain stable due to their small size and can be easily recovered from FFPE samples as compared to the gold standard of RNA recovery from snap-frozen cells [Li et al., 2007]. Therefore, despite the low RNA quality, these samples were considered suitable for miRNA expression profiling.

For many of the miRNAs the array data and qRT-PCR data were in concordance with one another and varied mostly in the magnitude of differential expression relative to the normal canine histiocytes. In general, the reactive histiocytosis group showed higher increased expression by the array data than by qRT-PCR. Although both the Affymetrix GeneChip® miRNA array and the TaqMan® MicroRNA Assay use the same probe sequence targeting these miRNAs, there are mechanistic differences between the two methods resulting in these disconcordant data. This discrepancy between array and qRT-PCR data is not an uncommon finding in the literature [Rajeevan et al., 2001]. The miRNA array was intended to be used for the discovery of potentially interesting miRNAs and the qRT-PCR results are considered the gold standard.

The PCA results revealed a strong relationship between the miRNA expression and the diseased samples relative to the normal histiocytesdxfs. The heat map confirmed this relationship and further identified breed-dependent differential miRNA expression among the disease samples. Several miRNAs were selected for validation by qRT-PCR, nine of which were determined bio-

logically significant as predicted by a greater than two-fold change. The miRNAs with the highest fold-change relative to normal histiocytes include miR-143, miR-152, and miR-125b. In the literature, miR-143 is most commonly associated with being a tumor suppressor miRNA, however it is predicted by miRDB (www.http://mirdb.org) and TargetScan (http://www.targetscan.org/) to target several tumor suppressors in the dog including *IGFBP5* and *TSHR*, thus potentially fulfilling an oncomir role. MiR-152, an immune regulatory miRNA, is of particular interest because it has been shown to play an integral role in the differentiation of dendritic cells, which are the cell of origin for histiocytic diseases. MiR-152 is predicted by miRNA prediction algorithms to target the tumor suppressor gene *CCDC6*. MiR-125b, is overexpressed in several cancers, including prostate cancer, leukemia, urothelial carcinomas and gastric cancer. It inhibits several important tumor suppressor genes including *BAK1* and *E2F2*. Of the validated miRNAs, miR-125b, miR-146a, miR-155, miR-29b, and miR-34a have been associated with cancer in human studies [Lewis et al., 2005; Wang et al., 2008]. MiR-125b, which had increased expression in nearly all disease samples, also had decreased expression of the gene targets *BAK1*, *CYP24*, *EGFR*, and *TP53* from the mRNA array data. This pattern has been associated with migration, invasion and epithelial-mesenchymal transition of prostate cancer and therapy resistance in breast cancer [Shi et al., 2007; Zhou et al., 2010]. Increased expression of miR-155 leading to decreased expression of *SOCS1* has a role in cancer initiation in breast cancer [Iorio et al., 2005].Tumor suppressor miR-34a under expression and corresponding *SIRT1* over expression may have a role in early tumorigenesis of the prostatic epithelial cell [Fujita et al., 2008].

The pathway analysis using Ingenuity® and DAVID resulted in several interesting clusters among the miRNA gene targets. Genes involved in apoptosis and growth clearly play a role in cancer development. The clusters of integrin genes and those involved in T cell receptor signaling and cytotoxicity also have a functional role in immune cells such as histiocytes, the cell of origin for histiocytic diseases. Examination of the paired expression data with inverse expression among the BMD samples revealed nine interesting gene targets, including several cancer associated genes such as *DAPK1*, *CD44*, *SOX5*, and *ITGA5*. *DAPK1* is a positive mediator of programmed cell

death and has been shown to be decreased in several highly aggressive metastatic tumors similar to the decreased expression observed in the BMD hemophagocytic histiocytic sarcoma samples [Inbal et al., 1997]. In a human breast cancer cell line, *CD44* expression was inversely correlated with metastasis as part of a miRNA induced migration phenotype [Huang et al., 2008]. Interestingly, the BMD hemophagocytic histiocytic sarcoma samples in this study exhibited decreased CD44 expression similar what was described for the breast cancer cell line, which suggests that *CD44* may also play a role in the highly aggressive phenotype of hemophagocytic histiocytic sarcoma. *SOX5* is a transcriptional regulator that when amplified, was correlated with reduced apoptosis in human testicular seminomas [Zafarana et al., 2002]. *SOX5* exhibited over expression in nearly all diseased BMD samples analyzed and perhaps reduced apoptosis is also playing a role in these tumors. Previous work has demonstrated that the loss of integrins on leukocytes results in immune dysfunction, a phenomenon that may also be occurring in the the histiocytic sarcoma samples in our study which exhibited decreased expression of *ITGA5* [Travis et al., 2007]. Interestingly, miR-125b and miR-181a are predicted to target the p14/p19ARF tumor suppressor gene, also known as CDKN2A, which has previously been associated with histiocytic disease in dogs [Hedan et al., 2011; Shearin et al., 2012]. Identification of the pathway members with aberrant expression patterns will provide insight into the pathways responsible for tumorigenesis in histiocytic malignancies. MiRNAs that are critical for tumorigenesis would potentially represent targets for manipulation to facilitate the development of novel and more effective cancer therapies. By further exploring the pathways regulated by these miRNAs, we expect they will prove to be instrumental in understanding the tumorigenesis of histiocytic diseases for the benefit of dogs and humans.

CHAPTER 4

WHOLE GENOME SEQUENCING OF A BERNESE MOUNTAIN DOG WITH HISTIOCYTIC SARCOMA

4.1 Introduction

Every dog breed can be easily identified by the physical traits defining that breed. Careful selection and often inbreeding have been used for thousands of generations to create the dog breeds of today. A specific set of physical and behavioral characteristics are determined as breed standard based upon the historic use of the dog breed, for example hunting or herding or as a lap dog. However, in the pursuit of the ideal breed specimen, sometimes rare genetic mutations and subsequent health problems are inadvertently amplified in these restricted gene pools. While the health implications of deleterious inherited traits must be addressed, dog breeds also provide a unique opportunity to study what are otherwise rare genetic diseases in dogs and humans. The advent of relatively inexpensive whole genome sequencing is a powerful new scientific tool that will facilitate this sort of genetic study. Through the accrual of more genome sequences from carefully curated samples, the genetic basis for inherited diseases in these dog breeds will be identifiable. In this work, we present the whole genome sequencing of a Bernese mountain dog with histiocytic sarcoma.

Histiocytic sarcoma (HS) is an aggressive neoplasia arising from interstitial dendritic cells. Approximately 25% of all tumors in the Bernese mountain dog (BMD) breed are diagnosed as HS [Abadie et al., 2009]. HS is known to be familial in BMDs, and has been hypothesized to be a polygenic trait [Padgett et al., 1995; Abadie et al., 2009]. Recent studies have utilized comparative genomic hybridization, genome-wide association, and gene expression profiling to identify the genetic component of histiocytic sarcoma in BMDs and other highly affected breeds like the flat-coated retriever. Hedan et al. 2011 evaluated copy number variations in the BMD and flat-coated retriever using genome wide array comparative genomic hybridization. This study identified deletions of the tumor suppressor genes CDKN2A/B, RB1 and PTEN present in affected dogs of both

breeds [Hedan et al., 2011]. The first genome-wide association study of histiocytic sarcoma was conducted in BMDs of American and European origin and revealed a susceptibility locus encompassing MTAP and part of CDKN2A, one of the tumor suppressors also identified by the Hedan et al. 2011 study [Shearin et al., 2012]. One of the latest research papers on this subject conducted gene expression profiling of affected flat-coated retrievers and found several genes involved in DNA repair and replication pathways associated with histiocytic sarcoma in these dogs [Boerkamp et al., 2013]. Here we present the results of the first study using whole genome sequencing to probe the genetic variations present in a Bernese mountain dog with histiocytic sarcoma.

4.2 Materials and Methods

4.2.1 Samples

The samples were derived from a spayed female Bernese mountain dog diagnosed with histiocytic sarcoma in the lungs at 8 years of age. Initially, she was treated with the chemotherapeutic agent Actinomycin D. She survived for an additional year before succumbing to disseminated histiocytic sarcoma of the lung, liver, stomach, adrenal gland, mediastinum, omentum, and retroperitoneum at 9 years of age. Three samples from the same dog were submitted for whole genome sequencing. Genomic DNA was isolated from a blood sample taken from this dog when she was 4 years of age, four years before her diagnosis with histiocytic sarcoma. A tissue sample was taken from a mediastinal mass at necropsy and snap-frozen in liquid nitrogen and kept at $-80\,^{\circ}$C until genomic DNA isolation. In addition, a cell line was derived from a sample of an abdominal lymph node taken during necropsy. From the blood sample, genomic DNA was isolated from 200μl of whole blood using 400μl lysis buffer (50 mM Tris pH 8.0, 0.5% Tween20, 1 mM ethylenediaminetetraacetic acid) and 40μl proteinase K (15 mg/ml). Cell lysis and protein digestion occurred overnight at $42\,^{\circ}$C. The following day the proteinase K was inactivated by incubating at $95\,^{\circ}$C for ten minutes. The DNA was extracted using a phenol:chloroform:isoamyl alcohol protocol. The frozen tumor tissue was ground using a mortar and pestle and then genomic DNA isolation proceeded similar to

the blood sample. The cell line was established in-house and characterized as spontaneously immortalized CD11c positive histiocytes [Takada et al., 2014]. The cells were washed with phosphate buffered saline and lysed directly on the 10 cm cell culture plate using 1000μl lysis buffer (50 mM Tris pH 8.0, 0.5% Tween20, 1 mM ethylenediaminetetraacetic acid) and 100μl proteinase K (15 mg/ml). The cells were released with a disposable rubber spatula and the lysate was pipetted into a tube. Cell lysis and protein digestion occurred overnight as previously described and genomic DNA isolation proceeded as described for the blood sample. The microsatellite markers of the cell line were determined using the primers listed in Table 4.1 and evaluated using gel electrophoresis [Neilan et al., 1997; Irion et al., 2003].

Table 4.1: **Primer sequences used to verify the identity of BD cell line as a unique cell line**

Marker	Primer	Sequence	Motif	Reference size	Location
1	F	TTGGACCTGAGTTGTGGATG	(TTTC)n	299	chr4:66543470-66543646
	R	CTGGGCTCCACTACATGGAA			
2	F	GGGTTGATGTCAGGTCAGG	(GAAA)n	372	chr5:61523795-61524073
	R	GGGTCCTCACAAGAGAGTATGG			
3	F	CAAAAAATATGCAATGAAAAAGAGG	(GAAA)n	390	chr9:37808480-37808650
	R	GGACAAGGATAGGAGGCAGAC			
4	F	CCAAGGGAAGGAAGTTTAAGG	(TCTA)n	239	chr14:21564592-21564721
	R	TTTTCTCTTTCTGTCTTTCTTGATG			

Primer sequences used to verify the microsatellite markers of the BD cell line are distinct from other cell lines concurrently grown in the lab. An M13 tail (AGGGTTTTCCCAGTCACGAC) was added to each F primer for a universal labeling. Motif is obtained from the RepeatMasker track of the UCSC Genome Browser. Reference size (reference genome size) is based upon an in silico PCR from CanFam3 and includes the length of the M13 tail. Location is for the tetraSTR as identified by RepeatMasker in CanFam3.

4.2.2 Analysis

Whole genome sequencing was performed on the three samples using Illumina's HiSeq 2000 sequencing system (San Diego, CA) with paired-end reads of 100 base pairs in length to give an average coverage of 30X. Quality trimming of the reads and removal of the adaptor sequence was performed by Trimmomatic v.0.30 [Bolger et al., 2014]. The reads were aligned against the canine boxer reference genome (CanFam 3.1.69) using the Burrows-Wheeler Aligner (bwa v.0.7.5a-r405) [Li and Durbin, 2009]. The resulting variant call format (vcf) file was generated using SAMTools v.0.1.19 [Li et al., 2009]. The predicted effect of variants on protein function was annotated using the SIFT 4G v.1.0 software [Ng and Henikoff, 2001; 2002; 2003; Kumar et al., 2009]. The vcf files were imported into a MySQL database for filtering and prioritizing. The list of variants for each sample type were filtered to those that are novel in this Bernese mountain dog by removing those common with the Single Nucleotide Polymorphism Database (dbSNP) and with other unaffected breeds of dogs that were sequenced as part of other studies in the lab. Then the filtered list for each sample were compared to each other to find variants that meet criteria for spontaneous mutations in the tumor tissue, loss of heterozygosity mutations, and germ line mutations. Spontaneous mutations in the tumor tissue were identified as variants that were present in the cell line and may be present in the tumor tissue but were not found in the blood sample. The loss of heterozygosity mutations were variants that were heterozygous in the blood sample and homozygous in the cell line and were present in the tumor tissue. Germ line mutations are variants that were shared among all three samples. Perturbations in common pathways were determined by inputing the gene lists into DAVID (http://david.abcc.ncifcrf.gov/).

4.3 Results

The pedigree of the selected Bernese mountain dog was investigated using the Berner-Garde Foundation Database (http://bernergarde.org/db/). No other dogs affected with a histiocytic disease were found in this dog's pedigree as far back as three generations (great-grandparents). Prior to

sequencing, the microsatellite markers of the cell line were analyzed to differentiate this cell line from others concurrently grown in the lab. Gel electrophoresis confirmed that the microsatellite motifs of the cell line were distinct from other canine cell lines and matched the motif sizes of the blood and tumor tissue from the same dog as expected.

Prior to filtering, the blood, tumor tissue, and cell line samples had a total of 3.80 million, 3.84 million, and 3.86 million variants, respectively, resulting in 1 change every 632 bases, 625 bases, and 622 bases, respectively (Table 4.2). Chromosomes X and 20 had the highest number of changes for all three sample types. CD molecules composed the gene family with the highest number of variants. Other gene families with a high number of variants included the immunoglobulin superfamily and cadherins.

Table 4.2: **Summary of variant analysis from whole genome sequencing**

	Blood	**Tumor Tissue**	**Cell Line**
Number of variants	3,803,849	3,841,485	3,862,932
Change rate	1 change every 632 bases	1 change every 625 bases	1 change every 622 bases
X Chromosome with highest change rate	1,142	1,105	1,158
Chromosome 20 with next highest change rate	728	732	793
Single-nucleotide polymorphism (SNP)	3,142,837	3,191,720	3,228,985
Multiple-nucleotide polymorphism (MNP)	11	12	18
Insertion	342,641	338,221	320,146
Deletion	318,360	311,532	313,783
Missense	14,276	12,674	12,726
Nonsense	269	130	150
Silent	18,112	17,696	16,284

Summary of variant analysis for blood, tumor tissue, and cell line samples. The reads were aligned against the canine boxer reference genome. Across the three samples from this dog, an average of 3.8 million variants were identified resulting in a change rate of 1 change every 626 bases. Chromosomes X and 20 had the highest change rate for all three sample types. The majority of variants were single nucleotide polymorphisms with inserts and deletions responsible for about 300,000 variants each. The maximum deletion size was 42 bases and the maximum insertion size was 29 bases.

For the purposes of this study, the focus was on high impact variants. These were variants in protein coding regions of known genes with predicted deleterious effects on the protein. There were 29 variants that met the criteria for spontaneous mutations in the tumor (Table 4.3). Two of these variants are in genes associated with cancer: *SSH3* and *ITGB7*. 1% of the variants were identified to be within the ECM-receptor interaction pathway. Six mutations show loss of heterozygosity (Table 4.4), and among these, three of the variants are in genes associated with cancer: *FAT1*, *MTUS1*, and *LRP1B*. Over one hundred variants met the criteria for germ line mutations (Table 4.5). The majority were heterozygous in all the samples and fifty-one were homozygous in all three samples.

Table 4.3: **Spontaneous nonsynonymous mutations in tumor and cell line**

Chr	Pos	Ref	Alt	AA	Gene	Qual	GT	Gene function
1	99577380	C	T	R305C	*ZNF837*	222	2/2	transcriptional regulation
2	21723758	A	C	H520P	*DCLRE1C*	53	1/2	V(D)J recombination
2	71919034	C	T	D277N	*TRNAU1AP*	94	1/2	selenocysteine biosynthesis
3	40432632	T	C	F373S	*LINS*	36	1/2	development
3	61335881	A	C	K175Q	*NOP14*	85	1/2	nucleolar processing of pre-18S rRNA
4	19158759	C	T	G166D	*HNRNPA1L2*	117	1/2	packaging pre-mRNA into hnRNP particles
4	72771154	G	T	P946T	*SPEF2*	170	2/2	axoneme development
7	64513295	C	G	L1084F	*LAMA3*	50	1/2	attachment, migration, and organization
9	4394424	G	A	G522S	*QRICH2*	49	1/2	glutamine-rich protein-coding
9	25027641	CAAAA	CAAAAA	A277S	*TTLL6*	142	2/2	mediates tubulin polyglutamylation
9	42583941	G	A	P24S	*IFT20*	73	1/2	intraflagellar transport particles
9	55311831	G	T	D421Y	*CIZ1*	41	1/2	subcellular localization of CIP/WAF1
10	19808586	G	A	G1605R	*CELSR1*	25	1/2	cell/cell signaling
11	17927599	G	A	D257N	*SLC27A6*	30	1/2	translocation of long-chain fatty acids
12	1560882	G	A	D268N	*EGFL8*	15.1	1/2	calcium ion binding
12	19306115	C	T	V3873M	*PKHD1*	37	1/2	regulation of centrosome duplication
14	26363540	T	C	D1358G	*THSD7A*	66	1/2	endothelial cell migration
17	8318138	T	G	I1675M	*GREB1*	32	1/2	estrogen-stimulated cell proliferation
18	50130506	C	G	Q316H	*SSH3*	126	1/2	Protein tyrosine phosphatase
18	55493519	A	G	I87T	*MS4A15*	52.1	1/2	signal transduction
20	4917889	G	C	E862D	*FGD5*	222	2/2	activates CDC42, of the Ras family
20	44758332	C	T	R1301C	*KIAA1683*	139	2/2	protein-coding gene
27	1994136	C	G	R759G	*ITGB7*	16.1	1/2	adhesion molecule - lymphocyte migration
27	2342172	C	T	A315V	*KRT76*	113	1/2	terminal cornification
28	40673991	G	A	A172V	*TTC40*	87	1/2	tetratricopeptide repeat protein-coding gene
33	24961845	A	G	V2604A	*GOLGB1*	52	1/2	intercisternal cross-bridges of the Golgi
33	24969068	G	T	S548Y	*GOLGB1*	142	1/2	intercisternal cross-bridges of the Golgi

Chr = chromosome, Pos = position based upon CanFam3.1.69, Ref = reference allele, Alt = alternate allele, AA = amino acid change, Qual = quality score of cell line, GT = genotype of cell line where 1 = reference allele and 2 = alternate allele thus 1/2 = heterozygous variant and 2/2 = homozygous variant.

Table 4.4: **Loss of heterozygosity variants resulting in nonsynonymous mutations**

Chr	Pos	Ref	Alt	AA	Gene	Qual blood	GT blood	Qual cell line	GT cell line	Gene function
16	41067620	G	T	P401T	*MTUS1*	55	1/2	95	2/2	inhibit ERK2 and cell proliferation
16	44131177	C	G	P925R	*FAT1*	14.2	1/2	82.1	2/2	cellular polarization
19	42832330	G	A	S2899F	*LRP1B*	42	1/2	13.2	2/2	bind and internalize ligands
21	42006008	C	T	T585M	*NAV2*	133	1/2	222	2/2	helicase and exonuclease activity
36	10135919	G	A	A208V	*SLC38A11*	176	1/2	96.3	2/2	amino acid/proton antiporter
X	55633452	C	T	A36V	*ITGB1BP2*	65	1/2	65.5	2/2	maturation, organization of muscle cells

There are six variants that show loss of heterozygosity, i.e. heterozygous in the blood and homozygous in the cell line and tumor tissue. Among these, three of the variants, *MTUS1*, *FAT1*, and *LRP1B*, are nonsynonymous mutations in genes associated with cancer. Chr = chromosome, Pos = position based upon CanFam3.1.69, Ref = reference allele, Alt = alternate allele, AA = amino acid change, Qual = quality score, GT = genotype where 1 = reference allele and 2 = alternate allele thus 1/2 = heterozygous variant and 2/2 = homozygous variant.

Table 4.5: **Germ line variants resulting in nonsynonymous mutations in cancer associated genes**

Chr	Pos	Ref	Alt	AA	Gene	Qual	GT	Gene function
1	108127452	C	T	A393V	*LIG1*	140	2/2	DNA ligase
1	111816850	G	A	V125M	*CEACAM28*	57	1/2	carcinoembryonic antigen-related cell adhesion molecule
1	111816940	T	G	S155A	*CEACAM28*	11.3	1/2	carcinoembryonic antigen-related cell adhesion molecule
1	111907507	A	G	N155I	*CEACAM1*	114	2/2	cell-cell adhesion molecule
1	111917369	C	G	Q496E	*CEACAM1*	140	2/2	cell-cell adhesion molecule
3	54114371	G	A	R1813Q	*ALPK3*	74	1/2	kinase, cardiomyocyte differentiation
4	57765782	G	A	E763K	*FAT2*	140	2/2	migration of epidermal cells
6	5751604	G	A	T556M	*GTF2I*	36.1	1/2	formation of complex at C-FOS promoter
6	55023326	G	A	S1099N	*ARHGAP29*	117	2/2	Rho GTPase activating protein
9	25310309	T	G	V88G	*PHB*	95	1/2	Prohibitin inhibits DNA synthesis
9	52519537	C	T	R257C	*RAPGEF1*	37	1/2	transduces signals to activate RAS
10	43245707	C	T	R1063C	*REV1*	106	1/2	Deoxycytidyl transferase involved in DNA repair
14	40338217	G	A	L201F	*HOXA9*	217	2/2	sequence-specific transcription factor
16	1139218	C	T	R305Q	*ADCY1*	24	1/2	calmodulin-sensitive adenylyl cyclase
16	41067620	G	T	P401T	*MTUS1*	95	2/2	inhibit ERK2 and cell proliferation
16	44131177	C	G	P925R	*FAT1*	82.1	2/2	cellular polarization
18	52324714	A	G	M360V	*CDC42BPG*	222	2/2	effector of CDC42 in cytoskeletal reorganization
19	42832330	G	A	S3036F	*LRP1B*	13.2	2/2	bind and internalize ligands
22	58549714	G	A	P395L	*COL4A1*	109	2/2	Type IV collagen, inhibits angiogenesis and tumors
24	20493619	C	T	R115H	*NRSN2*	55	1/2	maintenance and/or transport of vesicles
38	1679038	T	A	S597C	*DSTYK*	61	2/2	caspase-dependent apoptosis and cell death
38	23319736	G	A	T11M	*CD1B*	185	1/2	antigen-presenting protein

List of variants in genes associated with cancer that met the criteria for potential germ line nonsynonymous mutations. Chr = chromosome, Pos = position based upon CanFam3.1.69, Ref = reference allele, Alt = alternate allele, AA = amino acid change, Qual = quality score, GT = genotype where 1 = reference allele and 2 = alternate allele thus 1/2 = heterozygous variant and 2/2 = homozygous variant.

4.4 Discussion

This Bernese mountain dog was selected for sequencing because the lab had obtained multiple high quality samples of sufficient quantity from her and, most importantly, a cell line had been successfully derived from her tumor. Unfortunately, not enough information was known about the health of this dog's pedigree to determine if there were other relatives affected with histiocytic sarcoma. However, interestingly, an unrelated BMD that shared the household was affected with HS at approximately the same time and also did not have a known history of affected relatives in the pedigree. The possibility of an environmental trigger of histiocytic sarcoma in these dogs cannot be ruled out.

Filtering of the variants was based upon several assumptions. One was that the blood sample was considered a normal sample for this Bernese mountain dog and variants identified in this sample were germ line mutations in this dog. The second assumption was that the tumor tissue sample was actually a mix of neoplastic cells and normal stromal cells and thus the allele status of the variants may reflect that mixed population. The cell line was considered to be purely neoplastic cells due to the clonal derivation utilized in the production of the cell line. While cell lines can accrue spontaneous mutations during repeated subculturing, this cell line was less than a year old and unlikely to have a substantial number of these mutations.

Chromosomes X and 20 had the highest base change rate among the chromosomes. Interestingly, the gene families with the highest number of variants included CD molecules, immunoglobulins, and cadherins - all gene families that are associated with the major facets of immune function through signaling, function, or mobility. Nearly 10% of the genes associated with cancer did not have variants identified and presumably are normal in the Bernese mountain dog. These genes belong to over one hundred different gene families.

A 2011 study of BMDs and flat-coated retrievers suggested that deletions of tumor suppressors on certain chromosomes including 2 and 16 may play a role in histiocytic sarcoma [Hedan et al., 2011]. Our study revealed frameshift mutations in *UBIAD1*, a tumor suppressor on chromosome 2, and *CSMD1*, a tumor suppressor on chromosome 16. Recent work using genome wide association

studies has found histiocytic sarcoma susceptibility associated with the MTAP-CDKN2A locus [Shearin et al., 2012]. In our study we found fifteen variants shared between all three samples that were within the same region of interest and two additional variants that were present only in the tumor and cell line 4.6. These variants ranged in quality scores from 21 to 222 (corresponding to a phred quality score of 13 to 23) with a depth coverage ranging from 5 to 23. However, all of these variants were intergenic and the effect on the protein function of *CDKN2A* or *MTAP* was not determined.

Table 4.6: **Variants found in the MTAP-CDKN2A locus**

Chr	Pos	Reference	Alternate	Qual	Depth	GT blood	GT tissue	GT cell line
11	44179537	CA	CAA	70.7	7	2/2	2/2	2/2
11	44180236	T	G	119	11	0/0	2/2	2/2
11	44187367	CTTTTTTT	CTTTTTT	18.8	5	2/2	2/2	2/2
11	44193532	G	A	120	12	2/2	1/2	2/2
11	44200738	TGGG	TGG	114	8	2/2	2/2	2/2
11	44200767	G	A	146	13	2/2	2/2	2/2
11	44207948	TG(AG)12	TG(AG)11	44	15	2/2	2/2	2/2
11	44208197	G	A	95.1	6	2/2	2/2	2/2
11	44214225	GAAAA	GAAA	18.5	9	1/2	1/2	1/2
11	44222716	C	T	94	12	1/2	1/2	1/2
11	44223736	T	C	98	14	1/2	1/2	1/2
11	44223794	C	T	26	6	0/0	1/2	1/2
11	44223907	G	T	23	11	1/2	1/2	1/2
11	44223919	G	T	39	9	1/2	1/2	1/2
11	44229492	A	G	45	14	1/2	1/2	1/2
11	44231570	C	G	24	11	1/2	1/2	1/2
11	44236979	A	G	152	8	2/2	2/2	2/2

List of variants in the region of interest from other studies. All variants were intergenic. Chr = chromosome, Pos = position based upon CanFam3.1.69, Ref = reference allele, Alt = alternate allele, AA = amino acid change, Qual = quality score, GT = genotype where 1 = reference allele and 2 = alternate allele thus 1/2 = heterozygous variant and 2/2 = homozygous variant.

The causative genetic mutation for histiocytic sarcoma in Bernese mountain dogs has yet to be identified. Future work will require experimental validation of the variants identified in this study. Validated variants will be investigated to determine their association with histiocytic sarcoma and their functional role in the tumorigenesis. The six variants that show loss of heterozygosity by

the whole genome sequencing can be validated quickly. Additional variants of interest include the frameshift mutations in *UBIAD1* and *CSMD1*. The whole genome sequencing has provided a tremendous amount of data and is an important first step that will ultimately lead to the identification of the mutation that predisposes Bernese mountain dogs to histiocytic sarcoma.

CHAPTER 5

CONCLUSIONS

This work demonstrates the utility of dog breeds as unique genetic pools for studying rare disorders that affect both dogs and humans. The focus here is on histiocytic diseases, a rare disease in humans for which the dog may facilitate research on the genetic basis of these disorders. Historically, the BMD is highly overrepresented among dog breeds affected by histiocytic diseases. By capitalizing on a community sourced database of BMD health information, a contemporary assessment of the health status of the BMD breed was conducted. The average life expectancy of a large breed dog is around ten years of age, however, this analysis revealed that an overwhelming majority of BMDs have a shortened lifespan of seven to eight years. Cancer is the major factor in the shortened lifespan of BMDs, and among the cancers, histiocytic sarcoma was the most frequent diagnosis. This investigation confirmed that histiocytic diseases remain an important disease for the BMD, thus warranting further studies to improve the life expectancy of BMDs with this cancer, and inform human studies where the rarity of the disease hinders the development of treatments. Furthermore, histiocytic sarcoma can provide insight into the biology of dendritic cells, from which these cancers arise.

To unravel the mechanisms of tumorigenesis in histiocytic diseases, a novel approach involving miRNAs was utilized. The differential expression of miRNAs along the spectrum of canine histiocytic diseases was evaluated. MicroRNA expression was clearly different between the normal histiocytes and the histiocytic diseases. Among the histiocytic disease phenotypes, the miRNA expression was more influenced by breed than specific disease diagnosis. Continued analysis of the pathways (e.g., see Figure 5.1) regulated by these microRNAs may pinpoint critical targets in the tumorigenesis of histiocytic sarcoma and allow us to design novel cancer therapies that are more effective in humans and dogs.

The advent of relatively inexpensive next-generation sequencing has extensive applications,

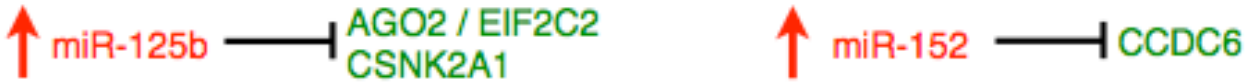

Figure 5.1: **MiRNA regulated pathways.** Predicted tumor suppressor mRNA targets of cfa-miR-125b and miR-152.

not the least of which has been in investigating the genetic basis of rare diseases. These studies can now be more effectively conducted using whole genome sequencing. Three samples from one BMD diagnosed with histiocytic sarcoma were submitted for whole genome sequencing. Several terabytes of data were generated yielding an abundance of information that will continue to be mined for answers to new questions in canine genetics. Results from this approach were compared to the pathways regulated by the miRNAs identified by the miRNA array analysis. Several interesting gene targets were identified, including several cancer associated genes such as *DAPK1*, *CD44*, *SOX5*, and *ITGA5*.

The importance of continued research into rare orphan diseases, such as histiocytic diseases, cannot be overstated. These cancers may hold the key for discovering the mechanism of tumorigenesis in these cancers and potentially others as well.

CHAPTER 6

FUTURE WORK

In the course of this work, several miRNAs were identified that may have a critical role in the pathology of histiocytes. These miRNAs will need to be evaluated as potential targets for down-regulation, for example in histiocytic sarcoma, to determine if knockdown of these critical microRNAs to normal levels will reverse the oncogenic phenotype in affected cells. Work along these lines was attempted and merits further attention. Although the miRNAs studied here did not display a disease specific signature, the widespread miRNA upregulation in histiocytic diseases suggests there are miRNAs playing a critical role in the pathology. Continued investigation of the pathways regulated by these miRNAs may trace back to the core genetic perturbations responsible for the development of disease. A few interesting cancer-associated gene targets were identified by comparing the miRNA results with gene expression data obtained through another study in the lab and the gene variants identified in the whole genome sequencing data. These include *DAPK1*, a positive mediator of programmed cell death, *CD44*, which is associated with miRNA induced migration, *SOX5*, a transcriptional regulator correlated with reduced apoptosis, and *ITGA5*, an integrin that if lost can result in immune dysfunction. Work is ongoing to verify the aberrant expression of these genes, among others, in histiocytic disease samples. Histiocytic diseases have been studied in humans and dogs for decades with no specific genetic mutation found despite the clear inheritance in BMDs. By undertaking a multi pronged investigation such as this, the confluence of data are more likely to identify the critical players in these complicated diseases. Bioinformatic approaches in combination with experimental analysis will be necessary to progress our understanding of these diseases.

APPENDIX

APPENDIX A

CANINE AND HUMAN GASTROINTESTINAL STROMAL TUMORS DISPLAY SIMILAR MUTATIONS IN *C-KIT* EXON 11

Published in BMC Cancer, 2010 [Gregory-Bryson et al., 2010].

A.1 Abstract

Background: Gastrointestinal stromal tumors (GISTs) are common mesenchymal neoplasms in the gastrointestinal tract of humans and dogs. Little is known about the pathogenesis of these tumors. This study evaluated the role of *c-KIT* in canine GISTs; specifically, we investigated activating mutations in exons 8, 9, 11, 13, and 17 of *c-KIT* and exons 12, 14, and 18 of platelet-derived growth factor receptor, alpha polypeptide (*PDGFRA*), all of which have been implicated in human GISTs.

Methods: Seventeen canine GISTs all confirmed to be positive for KIT immunostaining were studied. Exons 8, 9, 11, 13 and 17 of *c-KIT* and exons 12, 14, and 18 of *PDGFRA*, were amplified from DNA isolated from formalin-fixed paraffin-embedded samples.

Results: Of these seventeen cases, six amplicons of exon 11 of *c-KIT* showed aberrant bands on gel electrophoresis. Sequencing of these amplicons revealed heterozygous in-frame deletions in six cases. The mutations include two different but overlapping six base pair deletions. Exons 8, 9, 13, and 17 of *c-KIT* and exons 12, 14, and 18 of *PDGFRA* had no abnormalities detected by electrophoresis and sequencing did not reveal any mutations, other than synonymous single nucleotide polymorphisms (SNPs) found in exon 11 of *c-KIT* and exons 12 and 14 of *PDGFRA*.

Conclusions: The deletion mutations detected in canine GISTs are similar to those previously found in the juxtamembrane domain of *c-KIT* in canine cutaneous mast cell tumors in our laboratory as well as to those reported in human GISTs. Interestingly, none of the other *c-KIT* or *PDGFRA* exons showed any abnormalities in our cases. This finding underlines the critical

importance of *c-KIT* in the pathophysiology of canine GISTs. The expression of KIT and the identification of these activating mutations in *c-KIT* implicate KIT in the pathogenesis of these tumors. Our results indicate that mutations in *c-KIT* may be of prognostic significance and that targeting KIT may be a rational approach to treatment of these malignant tumors. This study further demonstrates that spontaneously occurring canine GISTs share molecular features with human GISTs and are an appropriate model for human GISTs.

A.2 Background

Gastro**i**ntestinal **s**tromal **t**umors (GISTs) are one of the most common mesenchymal tumors that arise from the wall of the gastrointestinal tract. Gastrointestinal stromal tumors occur in many species including humans, dogs [LaRock and Ginn, 1997; Frost et al., 2003; Kumagai et al., 2003], and horses [Del Piero et al., 2001; Hafner et al., 2001]. GISTs can metastasize to the liver and peritoneal cavity, warranting a very poor prognosis. In humans, approximately 70% of GISTs occur in the stomach and 20% occur in the small intestine [Miettinen et al., 2002; Agaimy et al., 2008b], whereas in dogs the reverse is true with 76% of GISTs occurring in the small intestine and colon, while 19% occur in the stomach [Frost et al., 2003].

The majority of GISTs are diagnosed by the demonstration of the expression of KIT (CD117), a type III tyrosine kinase receptor encoded by the proto-oncogene *c-KIT* [Taniguchi et al., 1999; Rubin et al., 2001; Croom and Perry, 2003; Frost et al., 2003; Wong et al., 2003]; although a small proportion of GISTs do not exhibit CD117 immunoreactivity [Debiec-Rychter et al., 2004]. KIT has critical roles in cell differentiation, proliferation and migration, especially in hematopoietic, neural crest, and germ cell lineages [Ma et al., 1999b]. In addition, KIT along with its ligand, stem cell factor (SCF), also known as steel factor, is necessary for the development of melanocytes, mast cells, and interstitial cells of Cajal [Hulzinga et al., 1995]. It has been suggested that GISTs may originate from the interstitial cells of Cajal, which are pacemaker cells responsible for regulating peristalsis in the gastrointestinal tract [Kindblom et al., 1998; Steigen and Eide, 2009].

The KIT receptor is a cell surface receptor consisting of an extracellular domain, a transmem-

brane domain, and a cytoplasmic domain, which includes the juxtamembrane and kinase domains [Yarden et al., 1987; Qiu et al., 1988]. The juxtamembrane domain (amino acid residues 543-580) [Ma et al., 1999b] is a highly conserved region of KIT located between the transmembrane domain (amino acid residues 521-543) [Yarden et al., 1987] and kinase domain (amino acid residues 581-936) [Ma et al., 1999b]. The KIT juxtamembrane domain is primarily coded for by exon 11 of *c-KIT*, while the split kinase domain are coded for by exons 12-18 of *c-KIT* [London et al., 1999]. The juxtamembrane domain regulates the enzymatic activity of KIT by preventing relative movement of the protein and thus inhibiting receptor dimerization [Roskoski, 2005]. In normal cells, binding of the SCF ligand to the KIT receptor results in receptor homodimerization and subsequent activation of the KIT receptor via cross phosphorylation of tyrosine residues on the opposite KIT homodimer partner [Roskoski, 2005]. The phosphotyrosines become binding sites and activators of several cell-signaling proteins including JAK2 and PI3K [Heinrich et al., 2002]. These particular proteins are the start of the JAK-STAT and JNK pathways, leading to a potent intracellular signal for the cell to proliferate[Blechman et al., 1993; Heinrich et al., 2002]. Gain-of-function mutations in KIT among human GISTs have demonstrated that the constitutive activation of KIT in the absence of its ligand and without dimerization may play a critical role in GIST tumorigenesis [Hirota et al., 1998].

In humans, mutations in *c-KIT* have been reported in more than 65% of GIST cases [Hirota et al., 1998; Taniguchi et al., 1999; Roskoski, 2005; Agaimy et al., 2008a], and in GISTs with wild-type c-KIT, mutations of platelet-derived growth factor receptor, alpha polypeptide (*PDGFRA*) were found in 35% of those cases [Heinrich et al., 2003]. *PDGFRA* codes for a transmembrane type III tyrosine kinase receptor for members of the platelet-derived growth factor family, which are mitogens for cells of mesenchymal origin. GISTs with *c-KIT* or *PDGFRA* mutations have similar downstream signaling pathways, suggesting that *PDGFRA* mutations serve as an alternative tumorigenic mechanism to *c-KIT* in GISTs [Heinrich et al., 2003]. Mutations have been found in exons 11, 9, 13, and 17 of *c-KIT* in sporadic GISTs, with exon 11, the juxtamembrane domain, being the most frequent site of mutations [Miettinen and Lasota, 2006; Ostrowski et al., 2009;

Steigen and Eide, 2009], comprising up to 90% of all *c-KIT* mutations [Longley et al., 2001]. Exon 8 of *c-KIT* has been reported to have mutations in other types of neoplasias. Most GISTs are sporadic, but familial GIST syndromes presenting with multiple GISTs have been reported in humans [Nishida et al., 1998]. Affected family members often harbor germline mutations of the *c-KIT* gene in their tumors and leukocytes [Nishida et al., 1998; Hirota and Isozaki, 2006] and there is a report of one family with a germline mutation in *PDGFRA* [Chompret et al., 2004]. The familial GIST syndrome has been recapitulated in two knock-in mouse models, one designed with a V558 deletion mutation in exon 11 of *c-KIT*, and the other carrying a K-to-E amino acid mutation at position 641 in exon 13 of *c-KIT* [Sommer et al., 2003; Rubin et al., 2005].

All of the reported mutations in *c-KIT* could potentially lead to the activation of KIT in the absence of its ligand. Constitutively activated KIT would then give rise to the development and/or progression of gastrointestinal stromal tumors in dogs. A comparison of the currently documented mutations found in *c-KIT* in humans and canines is presented in Figure A.1. The reported canine *c-KIT* mutations have been associated with mast cell tumors [Zemke et al., 2002; Webster et al., 2006a] as well as GISTs [Frost et al., 2003]. The purpose of this study was to evaluate the role of *c-KIT* and *PDGFRA* in canine GISTs. While KIT immunopositivity has been demonstrated in canine GISTs in two previous studies [Frost et al., 2003; Kumagai et al., 2003], only the study by Frost et al. has explored mutations in *c-KIT* to date, where four archived canine GISTs were examined, revealing mutations in exon 11 of *c-KIT* in two of the cases. Our present study investigates exons 8, 9, 11, 13, and 17 of *c-KIT* and exons 12, 14, and 18 of *PDGFRA* for mutations in a larger sample set of canine GISTs, providing information on *c-KIT* mutational status in seventeen cases.

A.3 Methods

A.3.1 Cases

Forty-six cases of canine gastrointestinal smooth muscle tumors were submitted to the Michigan State University Diagnostic Center for Population and Animal Health from 1991 to 2006. All

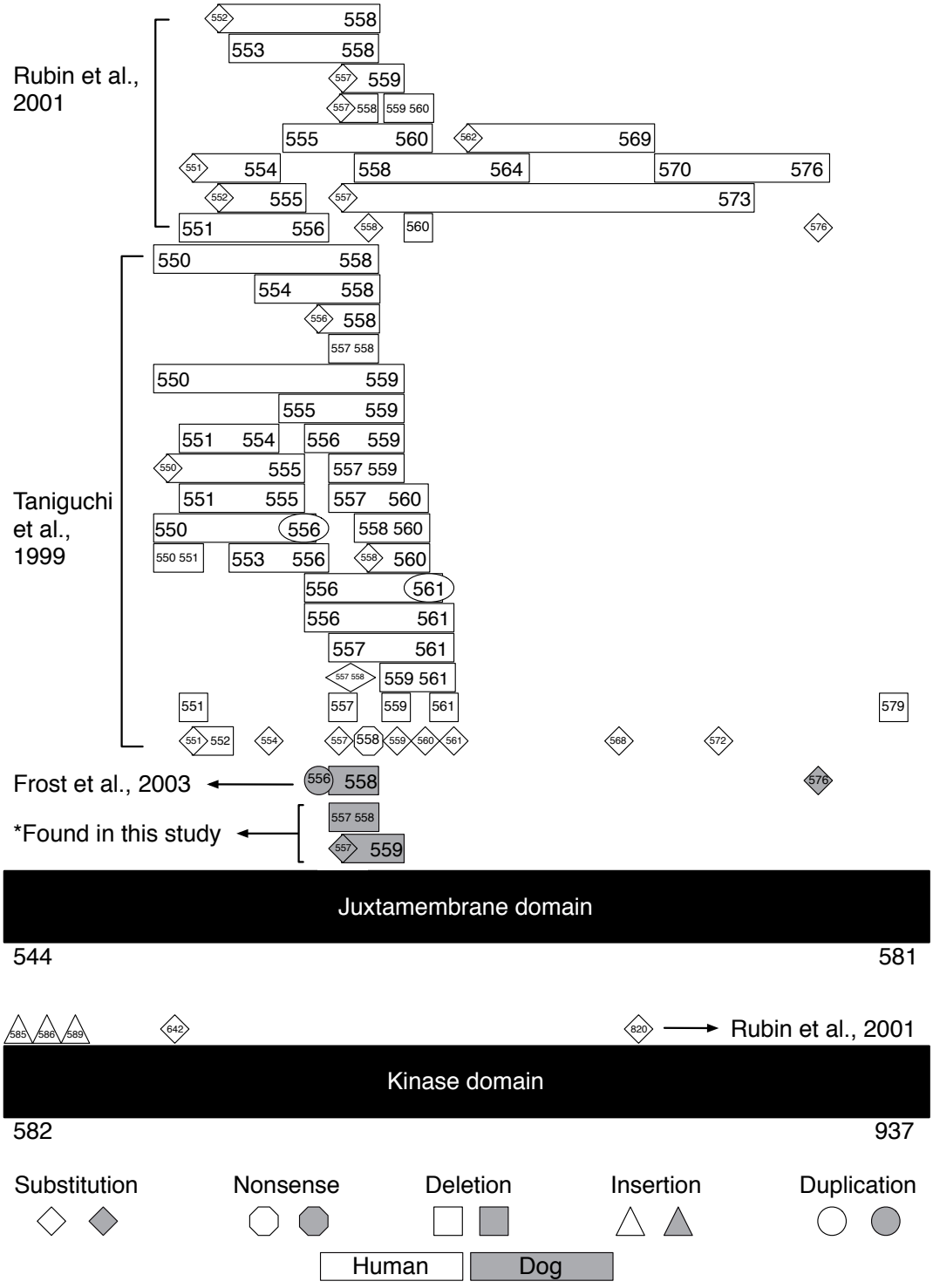

Figure A.1: **Comparison of *c-KIT* mutations in human and canine GISTs.** Human mutations are in white; dog mutations are indicated in gray [Frost et al., 2003; Kumagai et al., 2003; Taniguchi et al., 1999; Rubin et al., 2001; Heinrich et al., 2002]. Codon numbering is based upon human amino acid sequence [GenBank:NP_000213]. *These are the two deletion mutations found in this study.

tumors originated from surgical biopsies that were immediately fixed in 10% neutral buffered formalin and embedded in paraffin within 24-48 hours, following routine protocols. From this pool of cases eighteen tumors were diagnosed as GISTs confirmed by characteristic histomorphology and positive KIT staining by immunohistochemistry and were included in this study (Figure A.2). The age of the dogs in this study ranged from 4 to 15 years with a mean age of 10.9 years. Various purebred and mixed bred dogs were included with a gender ratio of 72% female to 28% male dogs. Tumor sites were distributed throughout the gastrointestinal tract from the stomach to the cecum as indicated in Table A.1. A histologically normal, non-neoplastic tissue sample from each dog was also analyzed to determine the *c-KIT* mutation status in constitutive DNA.

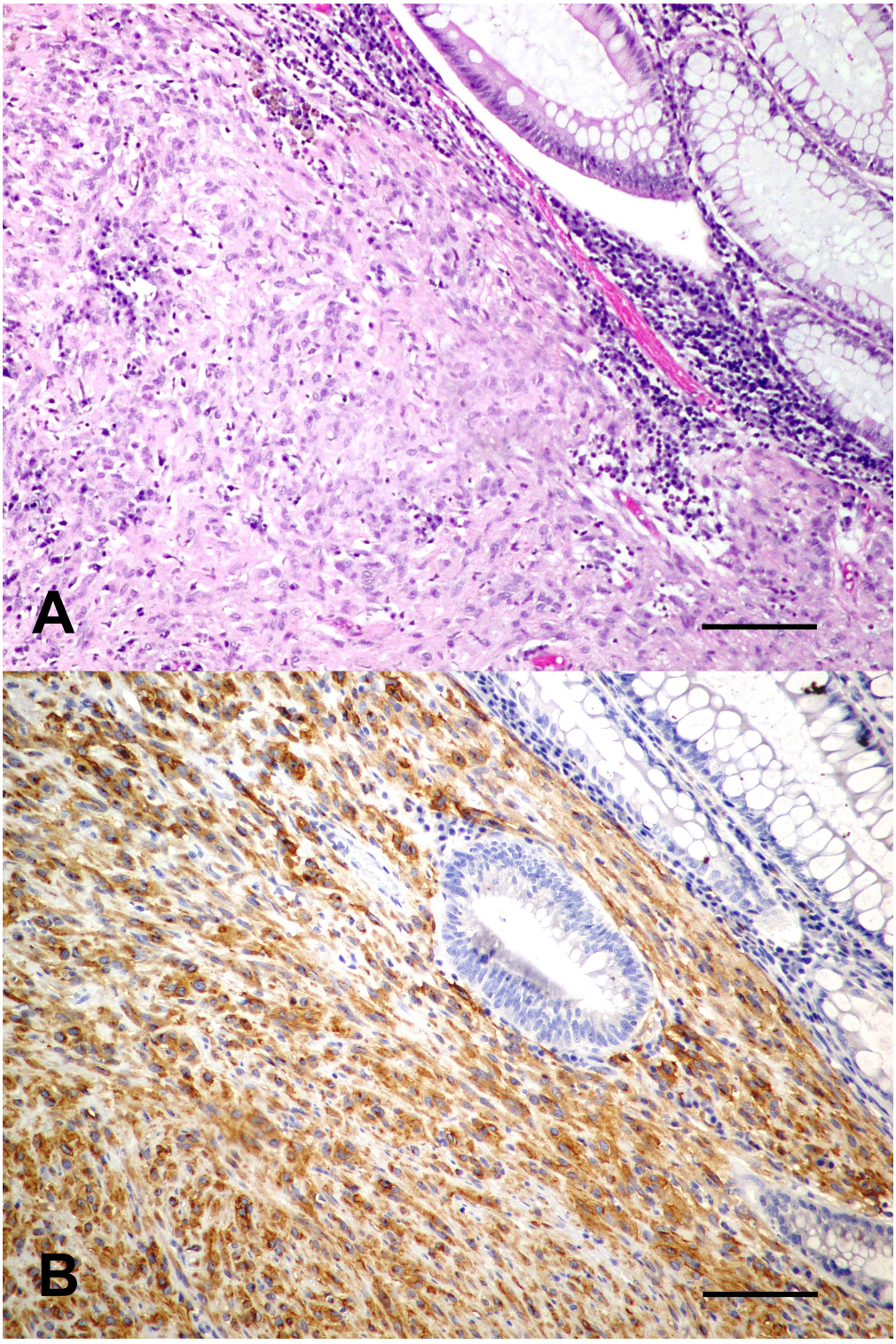

Figure A.2: **Histopathology of GIST. A** Hemotoxylin and eosin stain of a GIST. Section from a gastrointestinal stromal tumor analyzed in this study. **B** Immunohistochemical staining for KIT. KIT (brown deposits) in a section of a gastrointestinal stromal tumor.

Table A.1: **Samples**

Case	Breed	Age	Sex	Tumor Site	*c-KIT* Mutations	*PDGFRA* Mutations
1	Cockapoo	13	FS	stomach	exon 11 deletion and SNP	normal
2	German Wirehaired Pointer	10.5	FS	cecum	exon 11 deletion	exon 12 and 14 SNPs
3	Cocker Spaniel	12	FS	small intestine	normal	normal
4	Labrador Retriever	4	FS	duodenum	normal	normal
5	American Pit Bull Terrier	15	FS	cecum	exon 11 deletion	normal
6	Golden Retriever	13	F	small intestine	normal	normal
7	Collie	12	M	jejunum	exon 11 SNP	normal
8	Boxer	10	M	jejunum	normal	normal
9	Cocker Spaniel	US	FS	small intestine	exon 11 SNP	normal
10	Irish Setter	11	FS	small intestine	normal	normal
11	Golden Retriever	10.5	MN	cecum	exon 11 SNP	normal
12	Springer Spaniel	10.6	FS	cecum	exon 11 deletion	normal
13	Labrador Retriever	8.5	FS	jejunum	normal	normal
14	German Shorthaired Pointer	9	FS	small intestine	normal	exon 12 SNP
15	Labrador Retriever cross	10	MN	ileocecocolic junction	exon 11 deletion	normal
16	Mixed	9	FS	cecum	exon 11 deletion	exon 12 and 14 SNPs
17	Mixed	15	MN	jejunum	normal	normal

Signalment and site of canine gastrointestinal stromal tumor cases analyzed in this study. FS = female spayed, MN = male neutered, US = unspecified.

A.3.2 DNA isolation from formalin-fixed paraffin-embedded (FFPE) sections

Neoplastic tissue, less than 1 mm^3, was excised from each FFPE block to retrieve sections corresponding to KIT positive immunostaining areas. Similarly, sections of histologically normal tissue, negative for KIT immunostaining were also collected from each case. From these tissue sections DNA was isolated as described previously [Banerjee et al., 1995; Zemke et al., 2002]. The tissue section was placed in 400 μl of digestion buffer (50 mM Tris, pH 8.5, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.5% Tween) and heated to 95 °C for 10 minutes to melt the paraffin. The tissue solutions were then subjected to high power microwave irradiation twice for 30 seconds each with vortexing after each heating step. After cooling, 5 μl of 15 mg/ml proteinase K was added to each solution and incubated overnight at 42 °C. Following protein digestion, proteinase K was inactivated at 95 °C for 10 minutes. The solutions were then centrifuged and 150 μl was aliquoted to be used as DNA template in subsequent polymerase chain reaction (PCR).

A.3.3 Amplification of *c-KIT* juxtamembrane and kinase domains

Exon 11, coding for the juxtamembrane domain of KIT, and exon 17, coding for the kinase domain of KIT, were amplified from these tissue sections via PCR using primers and conditions optimized in earlier studies [Jones et al., 2004; Webster et al., 2006b]. Exon 8, 9, and 13 of *c-KIT* and exons 12, 14, and 18 of *PDGFRA* were also amplified (Table A.2). The PCRs were set-up in 25 μl total reaction volume consisting of 50 ng of DNA template prepared as described above: 5 pmol of each primer, 0.5 U of Taq polymerase (Invitrogen, Carlsbad, CA) and final concentrations of 80 μM deoxynucleoside triphosphate, and 2 mM $MgCl_2$. Cycling conditions for the PCR were 94 °C for 4 minutes; 40 cycles of 94 °C for 1 minute, annealing temperatures averaging 58 °C for 1 minute, and 72 °C for 1 minute; followed by a final elongation step at 72 °C for 5 minutes. PCR products were then subjected to electrophoresis on 2% agarose gels and visualized under ultraviolet light after ethidium bromide staining.

Table A.2: **Primer sets**

Amplified Region	**Forward**	**Reverse**
c-KIT exon 8	5'-CAGCAGTCTGACCTATGGC -3'	5'-GCTCAGCTCCTGGACAGAAA-3'
c-KIT exon 9	5'-GATTGATTGATTGATTTTCCTAG-3'	5'-GCAGGCAGAGCCTAAACATC-3'
c-KIT exon 11	5'-CATTTGTTCTCTACCCTAAGTGCT-3'	5'-GTTCCCTAAAGTCATTGTTACACG-3'
c-KIT exon 13	5'-CTGATTAAGTCGGATGCGGC-3'	5'-CAAGCACTGTCGCAATGG-3'
c-KIT exon 17	5'-ATAGCAGCATTCTCGTGTTG-3'	5'-AACTAAAATCCTTCACTGGACTG-3'
PDGFRA exon 12	5'-TTAATGGCTCTGATTGCTCAC-3'	5'-CACCCAGTGCTCATAACCTC-3'
PDGFRA exon 14	5'-ACTGGTTTTGGTTCCCACAG -3'	5'-CAATGATTCGCAGCAACG-3'
PDGFRA exon 18	5'-TAGCTCAGCCGTGGGTATG-3'	5'-CACATGAGCAGAGATGTCAGG-3'

Primer sets used for the amplification of the indicated exons of *c-KIT* and *PDGFRA* during PCR.

A.3.4 Sequencing

Amplified fragments from all tissue sections were characterized by automated sequencing. The PCR product for each section was submitted in 5 μl quantities with 30 pmol of the appropriate primer to Michigan State University's Genomics Technology Support Facility. This facility utilizes the automated direct sequencing technique, which incorporates fluorescently labeled dideoxynucleotides during cycle sequencing and separates the resulting products by capillary electrophoresis for detection on an ABI 3700 sequence analyzer (Foster City, CA).

A.4 Results

Of the eighteen KIT immunopositive cases, seventeen cases yielded amplification products. The remaining case did not yield amplification products with any of the *c-KIT* or *PDGFRA* primer sets or with primers for unrelated canine genes.

For exon 11 of *c-KIT*, six of these seventeen cases of canine GISTs displayed an aberrant banding pattern upon gel electrophoresis of the PCR product (Figure A.3). The remaining eleven cases displayed a band similar to the positive control on electrophoresis, and analysis confirmed the sequence was identical to the wild-type exon 11 of *c-KIT* except for a single nucleotide polymorphism (SNP) located at base pair 50110905 C>T [GenBank:NC_006595.2] detected in four cases. However, sequence analysis of the aberrant six cases uncovered a mixture of normal and mutant alleles. Further examination identified short in-frame deletions (Figure A.4). The mutations included two different, but overlapping 6 base pair deletions, which translated to a deletion of two amino acids in two of the cases and an amino acid change and a deletion of two amino acids in the other four cases. The first mutation (canine codons 556-557) occurred in two of the cases and consisted of the deletion of the sequence AGTGGA located at base pairs 50110838 to 50110843 of the canine genomic DNA [GenBank:NC_006595.2]. This mutation translated to a deletion of two amino acids, tryptophan and lysine, at codons 556 and 557 of canine KIT, respectively [GenBank:NP_001003181]. The second mutation (canine codons 556-558) was discovered in four of

the cases and results in the deletion of the sequence GGAAGG located at base pairs 50110841 to 50110846 of the canine genomic DNA [GenBank:NC_006595.2]. This second mutation translated to a deletion of two amino acids, lysine and valine, at codons 557 and 558 of canine KIT, respectively [GenBank:NP_001003181]. The deletion of the last two guanines of the codon 556 in this mutation combined with deletion of the next 4 nucleotides resulted in an amino acid change from the tryptophan at codon 556 to a phenylalanine (Table A.3). In these six cases, analysis of the normal tissue obtained from these dogs revealed sequences that were identical to the wild-type exon 11 of *c-KIT*.

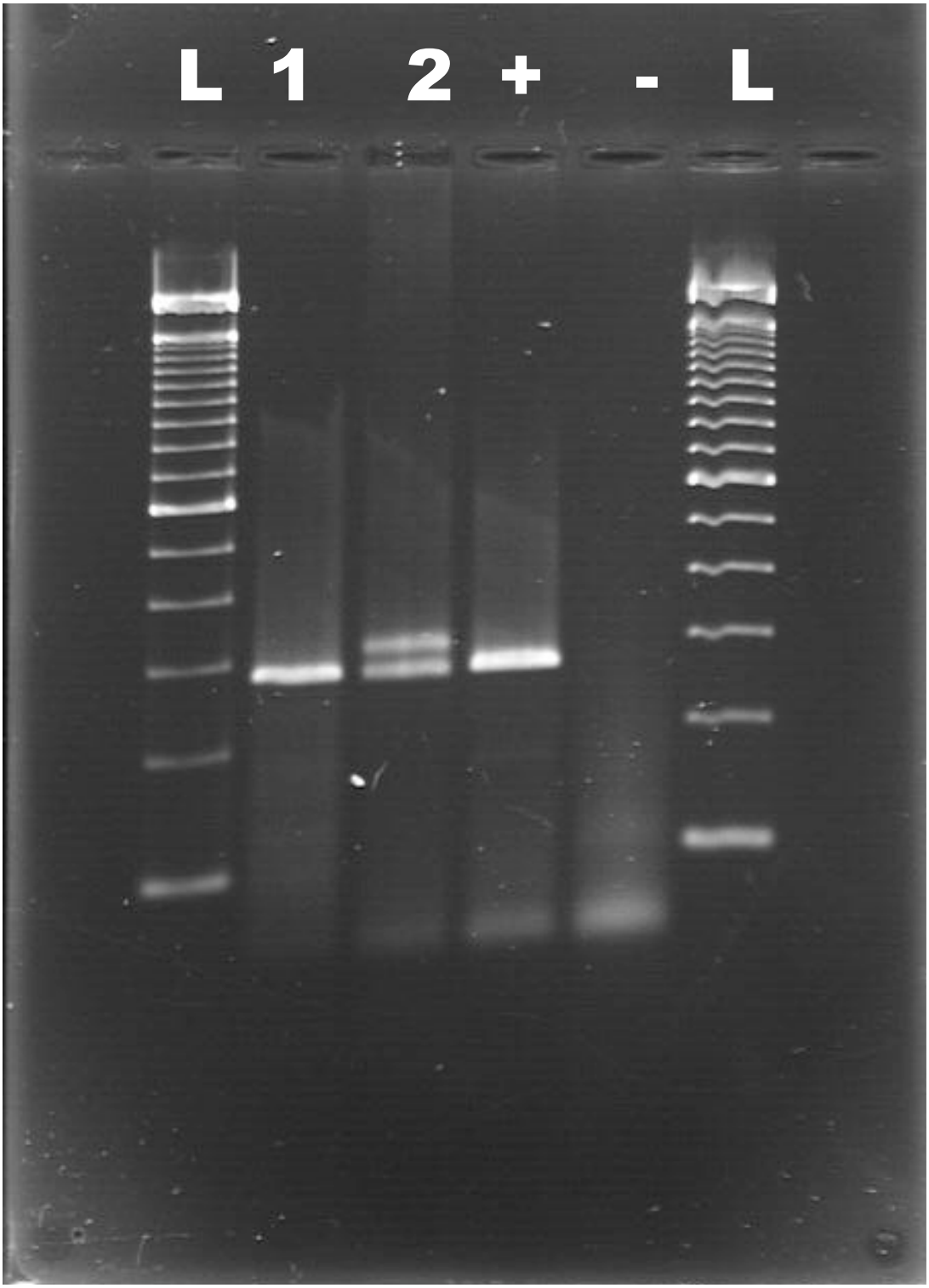

Figure A.3: **Electrophoresis of PCR product of exon 11 of *c-KIT*, juxtamembrane domain.** L = 100 bp ladder, 1 = case with normal exon 11 of *c-KIT*, confirmed by sequencing, 2 = case with aberrant banding pattern, + = positive control (normal dog spleen), - = negative control (water)

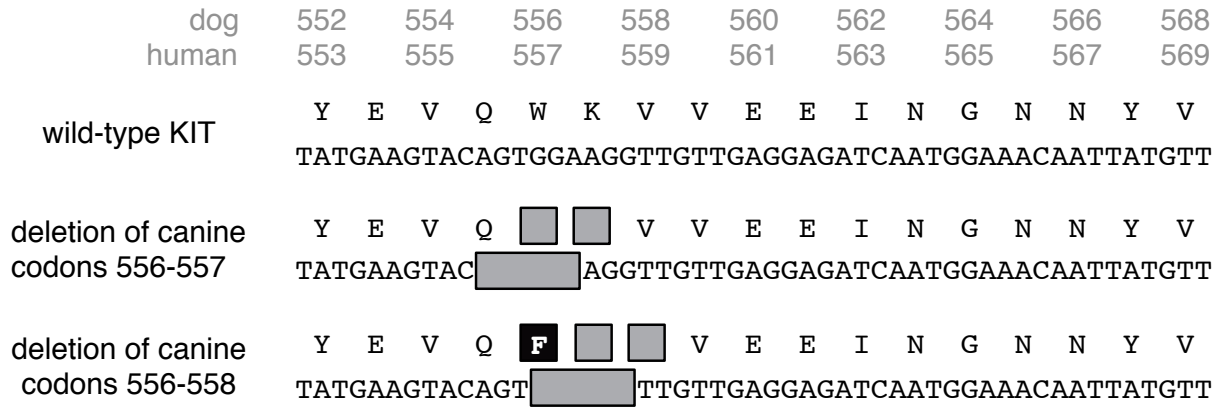

Figure A.4: **Deletion mutations found in exon 11 of c-KIT.** Sequence analysis results for the six cases with the aberrant banding pattern illustrating the two deletion mutations present in these cases. This region of exon 11 of canine *c-KIT* [GenBank:NP_001003181] differs by only one codon from humans [GenBank: NP_000213].

Table A.3: **Deletion mutations found in canine GISTs in this study**

Case	Codon	DNA Mutation	Amino Acid
2	556-557	GTA CAG TGG AAG GTT GTT > GTA C AG GTT GTT	VQWKVV > VQVV
16	556-557	GTA CAG TGG AAG GTT GTT > GTA C AG GTT GTT	VQWKVV > VQVV
1	556-558	GTA CAG TGG AAG GTT GTT > GTA CAG T TT GTT	VQWKVV > VQFV
5	556-558	GTA CAG TGG AAG GTT GTT > GTA CAG T TT GTT	VQWKVV > VQFV
12	556-558	GTA CAG TGG AAG GTT GTT > GTA CAG T TT GTT	VQWKVV > VQFV
15	556-558	GTA CAG TGG AAG GTT GTT > GTA CAG T TT GTT	VQWKVV > VQFV

The codon numbering is based upon the canine KIT amino acid sequence. The genomic DNA mutations were experimentally determined by direct sequencing and the altered sequence is underlined in column three. The expected amino acid changes in the protein are provided in column four. [GenBank:NC_006595.2, GenBank:NP_001003181].

Exon 11 of *c-KIT*

```
50110819   AAACCCATGT ATGAAGTACA GTGGAAGGTT GTTGAGGAGA   50110858
50110859   TCAATGGAAA CAATTATGTT TACATAGACC CAACACAGCT   50110898
50110899   TCCTTAYGAT CACAAATGGG AGTTTCCCAG AAACAGGCTG   50110938
50110939   AGCTTTG                                      50110945
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Exon 12 of *PDGFRA*

```
49690336   GTCGGATCCT GGGATCTGGT GCGTTTGGGA AAGTGGTTGA   49690375
49690376   AGGAACTGCC TATGGATTAA GCCGCTCCCA GCCGGTCATG   49690415
49690416   AAAGTCGCRG TGAAGATGCT GAAAC                  49690440
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Exon 14 of *PDGFRA*

```
49691320   GCCCCATTTA CATCATCACC GAGTACTGCT TCTATGGGGA   49691359
49691360   TTTGGTCAAC TATTTGCATA AGAATAGRGA TAGCTTCCTG   49691399
49691400   AGCCGCCACC CRGAGAAGCC AAAGAAAGAG TTGGACATTT   49691439
49691440   TTGGATTGAA CCCTGCTGAT GACAGCACAC GGAG         49691473
```

Figure A.5: **Single nucleotide polymorphisms (SNPs).** SNPs, shown in boxed letters, found in exon 11 of *c-KIT* and exons 12 and 14 of *PDGFRA* [GenBank: NC 0006595.2] in the canine GIST samples (R = A or G; Y = C or T).

All seventeen cases were also amplified for exons 8, 9, 13, and 17 of *c-KIT*. Only the expected single band, similar to the positive control, was observed after gel electrophoresis. Sequencing of all PCR products obtained revealed no mutations in these GIST samples for exons 8, 9, 13, or 17 of *c-KIT*. Similarly, amplification of exons 12, 14, and 18 of *PDGFRA* in these GIST samples revealed clear, single bands on electrophoresis and the PCR products were directly sequenced. Three of the cases had a SNP located at base pair 49690424 A>G [GenBank:NC_006595.2] in exon 12 of *PDGFRA*. Two of the cases also had a SNPs located at base pair 49691387 A>G and 49691411 G>A [GenBank:NC_006595.2] of exon 14 of *PDGFRA* (Figure A.5).

A.5 Discussion

This study was able to ascertain *c-KIT* and *PDGFRA* mutational status of seventeen of eighteen KIT positive canine gastrointestinal stromal tumors, representing a good amplification success rate of 94% from FFPE tissues. Significantly, the study identified two distinct but overlapping mutations in exon 11 of *c-KIT* in the juxtamembrane domain. This region appears to be a mutational

hotspot with an overall incidence of 35.3% in our study population of canine GISTs. The only other study of *c-KIT* mutations in canine GISTs reported mutations in two of four (50%) GISTs [Kumagai et al., 2003]. Human GISTs have higher incidences of *c-KIT* mutations ranging from 65% to 92% across exons 8, 9, 11, 13, and 17, a majority of which occur in the juxtamembrane domain [Hirota et al., 1998; Taniguchi et al., 1999; Rubin et al., 2001; Tabone et al., 2005]. In our study, no mutations were identified in exons 8, 9, 13, and 17 of *c-KIT*. None of our cases showed mutations in *PDGFRA*. Only a single amplification product was noted from the corresponding normal tissue of each GIST case, with sequencing verifying the presence of only the wild type allele in the normal tissue. These results indicate that all mutations observed arose somatically in each tumor.

Interestingly, these deletion mutations are similar to those previously found in the juxtamembrane domain of *c-KIT* in canine cutaneous mast cell tumors in our laboratory [Zemke et al., 2002] and others [Ma et al., 1999b]. In a previous study of 21 canine GISTs, DNA suitable for amplification was recovered from only four cases and then amplified for the KIT exon 11 of *c-KIT*, juxtamembrane domain, and sequenced [Frost et al., 2003]. Sequencing revealed mutations in two of the four canine GISTs, one with a 6 base pair deletion, TGGAAG, and insertion of CAG, predicted to translate to a deletion of tryptophan and lysine and an insertion of glycine at codon 556 [Frost et al., 2003]. This deletion is quite similar to the mutation at canine codons 556-557 discovered in the canine GISTs in our study. The second mutation discovered by Frost et al. was a substitution of T with C predicted to replace codon 575 leucine with proline [Frost et al., 2003]. We did not detect a similar mutation in our study population. The mutations observed in our study population of GISTs were clustered at codons 556-558 of *c-KIT*. No gender predilection has been reported in human GISTs, and the observation of 73% female to 27% male ratio in our study is interesting, but its significance needs further evaluation.

A simple deletion identical to the mutation at canine codons 556-557 in our study has also been reported in multiple cases of human GISTs [Taniguchi et al., 1999; Agaimy et al., 2008a]. In the study by Taniguchi et al., a deletion and point mutation similar to the mutation at canine codons

556-558 in our study was also detected in one of the cases they analyzed [Taniguchi et al., 1999]. Rubin et al., found the same mutation as the deletion of canine codons 556-557 in our canine GISTs in 2 of 48 human GISTs, and they reported the same deletion of canine codons 556-558 in 1 of the 48 cases [Rubin et al., 2001]. In a study of human familial GISTs, germline deletion mutations were discovered in the same region as the previously mentioned deletions [Nishida et al., 1998].

All four of the SNPs found in our canine GIST samples were silent mutations, with no change predicted in the translated protein. The SNP in exon 12 of *PDGFRA* at genomic base pair 49690424 [GenBank:NC_006595.2], has been reported previously [Lindblad-Toh et al., 2005].

In humans, GISTs are rare neoplasms. The age-adjusted incidence of gastric mesenchymal tumors was 0.31 per 100,000 population in 2002, of which 82% were classified as GISTs [Perez et al., 2006]. The population incidence of GISTs is difficult to determine in dogs. Frost et al. commented that in dogs, gastrointestinal neoplasias account for 12-120 cases per 10,000 neoplasia cases, and in our study GISTs accounted for 39% of the total number of gastrointestinal tumors collected during the study period.

Heterozygosity with regard to mutations in the tumor sections resulted in an easily detectable aberrant banding pattern on agarose gels. While the gel electrophoresis used in this study does not resolve the normal versus the mutant alleles, which differ by only six base pairs, the normal and mutant alleles formed a heteroduplex, which contained a bubble created by the longer normal allele. The heteroduplex structure is predicted to generate a drag during gel electrophoresis giving rise to the higher band and allowing easy detection of this relatively small deletion [Bhattacharyya and Lilley, 1989]. We cannot be absolutely certain that the tumor cells are heterozygous with respect to the mutation, as the tumor sections contained some non-neoplastic components such as blood vessels. Regardless, the aberrant banding is a useful screening tool for this set of mutations.

A.6 Conclusions

These data substantially expand the number of canine gastrointestinal stromal tumors evaluated for mutations in *c-KIT* by previous studies [Frost et al., 2003]. The mutations we have found

are clustered and consistent with those shown to be activating mutations in the *c-KIT* gene of human tumors [Hirota et al., 1998]. Based on these data, we can conclude that the nature of *c-KIT* mutations in GISTs in dogs is similar to that observed in humans.

The juxtamembrane domain of the KIT gene is a highly conserved region among mammals [Ma et al., 1999b]. This juxtamembrane domain acts as a negative regulator of KIT activation and thus, when this particular domain is mutated, the autoinhibition is removed, allowing KIT to be activated in the absence of the KIT ligand [Mol et al., 2004]. The residues we found to be deleted in our cases of canine GISTs are the very same that Ma et al. determined to increase basal receptor phosphorylation when mutated in *c-KIT* [Ma et al., 1999a]. The expression of KIT and the presence of these mutations in *c-KIT* implicate KIT in the pathogenesis of these tumors pointing to spontaneous GISTs in the dog being a relevant model for the human disease. Our results also indicate that mutations in KIT may be of prognostic and therapeutic significance in canine GISTs as they are in canine cutaneous mast cell tumors.

Numerous small molecule inhibitors that target specific tyrosine kinases, tyrosine kinase inhibitors (TKIs), have successfully been used for the treatment of human and canine cancers with mutations in KIT [London, 2009; Pytel et al., 2009]. Imatinib mesylate (Gleevec, Novartis, Basel, Switzerland), has been utilized for its ability to inhibit protein tyrosine kinases since the Food and Drug Administration approved it in 2001 for the treatment of Chronic Myelogenous Leukemia (CML) [Savage and Antman, 2002]. Early trials demonstrated imatinib was also highly effective against GISTs [Croom and Perry, 2003]. Imatinib caused marked tumor response rates and dramatically increased survival times in most patients [Hornick and Fletcher, 2007] and has now become the standard of care in the treatment of patients with advanced GISTs [Schnadig and Blanke, 2006]. However, since with prolonged treatment clinical resistance can develop, most likely due to secondary *c-KIT* mutations, a new generation of TKIs, such as sunitinib, have been successfully introduced [Hornick and Fletcher, 2007; Rutkowski et al., 2008]. Research is ongoing to treat GISTs with resistance to imatinib and sunitinib [Nilsson et al., 2009]. In dogs, the TKIs Palladia (toceranib), Kinavet (masitinib), and Gleevec (imatinib) have been successfully used in numerous

neoplastic diseases [London, 2009] and toceranib (Palladia, Pfizer, New York, NY, USA) and masitinib (Kinavet, AB Science, Short Hills, NJ, USA) have been registered for the use in dogs with cutaneous mast cell tumors [Gleixner et al., 2007; Isotani et al., 2008; Marconato et al., 2008]. In a randomized trial, dogs with KIT mutations were much more likely to respond to Palladia than those without KIT mutations [London et al., 2009]. To our knowledge there are no published data on the treatment of canine GISTs with TKIs. Based upon the data presented here, we propose that targeting KIT may be a rational approach to treatment of canine GISTs as well. In addition, we put forward that canine GISTs are a relevant and accessible model for human GISTs, with shared molecular pathways that can be targeted for therapy.

BIBLIOGRAPHY

BIBLIOGRAPHY

Jerome Abadie, Benoit Hedan, Edouard Cadieu, Clotilde De Brito, Patrick Devauchelle, Catherine Bourgain, Heidi G. Parker, Amaury Vaysse, Patricia Margaritte-Jeannin, Francis Galibert, Elaine A. Ostrander, and Catherine Andre. Epidemiology, pathology, and genetics of histiocytic sarcoma in the Bernese Mountain Dog breed. *Journal of Heredity*, 100(suppl 1):S19–27, Jul 2009.

VJ Adams, KM Evans, J Sampson, and JLN Wood. Methods and mortality results of a health survey of purebred dogs in the uk. *Journal of Small Animal Practice*, 51(10):512–524, 2010.

V. K. Affolter and P. F. Moore. Localized and disseminated histiocytic sarcoma of dendritic cell origin in dogs. *Vet Pathol*, 39(1):74–83, Jan 2002.

A Agaimy, S Dirnhofer, PH WUnsch, LM Terracciano, L Tornillo, and MP Bihl. Multiple sporadic gastrointestinal stromal tumors (GISTs) of the proximal stomach are caused by different somatic KIT mutations suggesting a field effect. *Am J Surg Pathol*, 32(10):1553–1559, 2008a.

A Agaimy, PH Wunsch, S Dirnhofer, MP Bihl, LM Terracciano, and L Tornillo. Microscopic gastrointestinal stromal tumors in esophageal and intestinal surgical resection specimens: A clinicopathologic, immunohistochemical, and molecular study of 19 lesions. *Am J Surg Pathol*, 32(6), 2008b.

S Banerjee, W Makdisi, A Weston, S Mitchell, and D Campbell. Microwave-based dna extraction from paraffin-embedded tissue for pcr amplification. *Biotechniques*, 18(5):768–770, 1995.

Jordi Barretina, Barry S Taylor, Shantanu Banerji, Alexis H Ramos, Mariana Lagos-Quintana, Penelope L DeCarolis, Kinjal Shah, Nicholas D Socci, Barbara A Weir, Alan Ho, Derek Y Chiang, Boris Reva, Craig H Mermel, Gad Getz, Yevgenyi Antipin, Rameen Beroukhim, John E Major, Charles Hatton, Richard Nicoletti, Megan Hanna, Ted Sharpe, Tim J Fennell, Kristian Cibulskis, Robert C Onofrio, Tsuyoshi Saito, Neerav Shukla, Christopher Lau, Sven Nelander, Serena J Silver, Carrie Sougnez, Agnes Viale, Wendy Winckler, Robert G Maki, Levi A Garraway, Alex Lash, Heidi Greulich, David E Root, William R Sellers, Gary K Schwartz, Cristina R Antonescu, Eric S Lander, Harold E Varmus, Marc Ladanyi, Chris Sander, Matthew Meyerson, and Samuel Singer. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. *Nat Genet*, 42:715–721, Aug 2010.

A Bhattacharyya and DM Lilley. The contrasting structures of mismatched dna sequences containing looped-out bases (bulges) and multiple mismatches (bubbles). *Nucleic Acids Res*, 17(17): 6821–6840, 1989.

J.M. Blechman, S. Lev, D. Givol, and Y. Yarden. Structure-function analyses of the kit receptor for the steel factor. *Stem Cells*, 11(2):12–21, July 1993.

Kim M. Boerkamp, Marieke van der Kooij, Frank G. van Steenbeek, Monique E. van Wolferen, Marian J. A. Groot Koerkamp, Dik van Leenen, Guy C. M. Grinwis, Louis C. Penning, Erik

A. C. Wiemer, and Gerard R. Rutteman. Gene expression profiling of histiocytic sarcomas in a canine model: The predisposed Flatcoated Retriever Dog. *PLoS ONE*, 8(8):e71094 EP –, 08 2013.

Anthony M Bolger, Marc Lohse, and Bjoern Usadel. Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics*, page btu170, 2014.

L. B. Brønden, S. S. Nielsen, N. Toft, and A. T. Kristensen. Data from the danish veterinary cancer registry on the occurrence and distribution of neoplasms in dogs in denmark. *Veterinary Record*, 166(19):586–590, 05 2010.

George Adrian Calin, Calin Dan Dumitru, Masayoshi Shimizu, Roberta Bichi, Simona Zupo, Evan Noch, Hansjuerg Aldler, Sashi Rattan, Michael Keating, Kanti Rai, Laura Rassenti, Thomas Kipps, Massimo Negrini, Florencia Bullrich, and Carlo M. Croce. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA*, 99(24):15524–15529, Nov 2002.

A Chompret, C Kannengiesser, M Barrois, P Terrier, P Dahan, T Tursz, G Lenoir, and B Bressac-De Paillerets. Pdgfra germline mutation in a family with multiple cases of gastrointestinal stromal tumor. *Gastroenterology*, 126:318–321, 2004.

K Croom and C Perry. Imatinib mesylate: In the treatment of gastrointestinal stromal tumours. *Drugs*, 63(5):513–522, 2003.

Brad T Sherman Da Wei Huang and Richard A Lempicki. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols*, 4(1):44–57, 2008.

Brian W Davis and Elaine A Ostrander. Domestic dogs and cancer research: A breed-based genomics approach. *ILAR Journal*, 55(1):59–68, 2014.

M Debiec-Rychter, B Wasag, M Stul, A Hagemeijer, and R Sciot. Gastrointestinal stromal tumours (GISTs) negative for KIT (CD117 antigen) immunoreactivity. *J Pathol*, 202(4):430–438, 2004.

F. Del Piero, B. A. Summers, J. F. Cummings, G. Mandelli, and E. A. Blomme. Gastrointestinal stromal tumors in equids. *Veterinary Pathology*, 38(6):689–697, 2001.

Nikolaos G Dervisis, Shawna L Klahn, Matti Kiupel, Maciej Parys, Emmalena Gregory-Bryson, and Vilma Yuzbasiyan-Gurkan. Localized and disseminated histiocytic sarcoma complex: A canine model [abstract number 5092]. In *Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research*, Chicago, Illinois, Mar 31-Apr 4 2012. AACR, Philadelphia (PA).

J Dobson, T Hoather, TJ McKinley, and JL Wood. Mortality in a cohort of flat-coated retrievers in the UK. *Vet Comp Oncol*, 7(2):115–121, Jun 2009.

Martina Doleshal, Amber A. Magotra, Bhavna Choudhury, Brian D. Cannon, Emmanuel Labourier, and Anna E. Szafranska. Evaluation and validation of total RNA extraction methods for microRNA expression analyses in formalin-fixed, paraffin-embedded tissues. *J Mol Diagn*, 10(3):203–211, 2008.

Tingting Du and Phillip D. Zamore. microPrimer: the biogenesis and function of microRNA. *Development*, 132(21):4645–4652, Nov 2005.

R. Maarten Egeler and Giulio J. D'Angio. Langerhans cell histiocytosis. *The Journal of Pediatrics*, 127(1):1–11, 7 1995.

Suzanne A Erich, Gerard R Rutteman, and Erik Teske. Causes of death and the impact of histiocytic sarcoma on the life expectancy of the Dutch population of Bernese mountain dogs and Flat-coated retrievers. *The Veterinary Journal*, 198(3):678–683, 2013.

Aurora Esquela-Kerscher and Frank J. Slack. Oncomirs — microRNAs with a role in cancer. *Nat Rev Cancer*, 6(4):259–269, Apr 2006.

A Fire, S Xu, M K Montgomery, S A Kostas, S E Driver, and C C Mello. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, 391(6669): 806–11, Feb 1998.

D. Frost, J. Lasota, and M. Miettinen. Gastrointestinal stromal tumors and leiomyomas in the dog: A histopathologic, immunohistochemical, and molecular genetic study of 50 cases. *Vet Pathol*, 40(1):42–54, 2003.

Yasunori Fujita, Keitaro Kojima, Nanako Hamada, Riyako Ohhashi, Yukihiro Akao, Yoshinori Nozawa, Takashi Deguchi, and Masafumi Ito. Effects of mir-34a on cell growth and chemoresistance in prostate cancer pc3 cells. *Biochemical and biophysical research communications*, 377(1):114–119, 2008.

Helmut Gadner, Nicole Grois, Maurizio Arico, Valerie Broadbent, Adriana Ceci, Ake Jakobson, Diane Komp, Jörg Michaelis, Stacy Nicholson, Ulrike Pötschger, et al. A randomized trial of treatment for multisystem Langerhans cell histiocytosis. *The Journal of pediatrics*, 138(5): 728–734, 2001.

K Gleixner, L Rebuzzi, M Mayerhofer, A Gruze, E Hadzijusufovic, K Sonneck, A Vales, M Kneidinger, P Samorapoompichit, T Thaiwong, W Pickl, V Yuzbasiyan-Gurkan, C Sillaber, M Willmann, and P Valent. Synergistic antiproliferative effects of KIT tyrosine kinase inhibitors on neoplastic canine mast cells. *Exp Hematol*, 35(10):1510–1521, 2007.

Martin Glud, Mikkel Klausen, Robert Gniadecki, Maria Rossing, Nina Hastrup, Finn C Nielsen, and Krzysztof T Drzewiecki. MicroRNA expression in melanocytic nevi: The usefulness of formalin-fixed, paraffin-embedded material for miRNA microarray profiling. *J Invest Dermatol*, 129(5):1219–1224, Nov 2008.

Emmalena Gregory-Bryson, Elizabeth Bartlett, Matti Kiupel, Schantel Hayes, and Vilma Yuzbasiyan-Gurkan. Canine and human gastrointestinal stromal tumors display similar mutations in c-KIT exon 11. *BMC Cancer*, 10(1), 2010.

Huili Guo, Nicholas T. Ingolia, Jonathan S. Weissman, and David P. Bartel. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*, 466(7308):835–840, Aug 2010.

Scott Hafeman, Cheryl London, Robyn Elmslie, and Steven Dow. Evaluation of liposomal clodronate for treatment of malignant histiocytosis in dogs. *Cancer Immunol Immunother*, 59(3): 441–452, Mar 2010.

S. Hafner, B. G. Harmon, and T. King. Gastrointestinal stromal tumors of the equine cecum. *Veterinary Pathology*, 38(2):242–246, 2001.

Benoit Hedan, Rachael Thomas, Alison Motsinger-Reif, Jerome Abadie, Catherine Andre, John Cullen, and Matthew Breen. Molecular cytogenetic characterization of canine histiocytic sarcoma: A spontaneous model for human histiocytic cancer identifies deletion of tumor suppressor genes and highlights influence of genetic background on tumor behavior. *BMC Cancer*, 11(1), 2011.

M Heinrich, C Corless, A Duensing, L McGreevey, C Chen, N Joseph, S Singer, D Griffith, A Haley, A Town, G Demetri, C Fletcher, and J Fletcher. Pdgfra activating mutations in gastrointestinal stromal tumors. *Science*, 299(5607):708–710, 2003.

Michael C. Heinrich, Brian P. Rubin, B. Jack Longley, and Jonathan A. Fletcher. Biology and genetic aspects of gastrointestinal stromal tumors: KIT activation and cytogenetic alterations. *Human Pathology*, 33(5):484–495, 2002.

Seiichi Hirota and Koji Isozaki. Pathology of gastrointestinal stromal tumors. *Pathology International*, 56(1):1–9, 2006.

Seiichi Hirota, Koji Isozaki, Yasuhiro Moriyama, Koji Hashimoto, Toshirou Nishida, Shingo Ishiguro, Kiyoshi Kawano, Masato Hanada, Akihiko Kurata, Masashi Takeda, Ghulam Muhammad Tunio, Yuji Matsuzawa, Yuzuru Kanakura, Yasuhisa Shinomura, and Yukihiko Kitamura. Gain-of-function mutations of c-KIT in human gastrointestinal stromal tumors. *Science*, 279 (5350):577–580, 1998.

J Hornick and C Fletcher. The role of KIT in the management of patients with gastrointestinal stromal tumors. *Hum Pathol*, 38(5):679–687, 2007.

Yali Hou, Yachun Wang, Xuemei Lu, Xu Zhang, Qian Zhao, Rory J Todhunter, and Zhiwu Zhang. Monitoring hip and elbow dysplasia achieved modest genetic improvement of 74 dog breeds over 40 years in usa. *PLoS One*, 8(10):e76390, 2013.

Douglas M. Howarth, Gerald S. Gilchrist, Brian P. Mullan, Gregory A. Wiseman, John H. Edmonson, and Paula J. Schomberg. Langerhans cell histiocytosis. *Cancer*, 85(10):2278–2290, 1999.

Qihong Huang, Kiranmai Gumireddy, Mariette Schrier, Carlos Le Sage, Remco Nagel, Suresh Nair, David A Egan, Anping Li, Guanghua Huang, Andres J Klein-Szanto, et al. The microrna mir-373 and mir-520c promote tumour invasion and metastasis. *Nature cell biology*, 10(2): 202–210, 2008.

Angela B Y Hui, Wei Shi, Paul C Boutros, Naomi Miller, Melania Pintilie, Tony Fyles, David McCready, Derek Wong, Kate Gerster, Igor Jurisica, Linda Z Penn, and Fei-Fei Liu. Robust

global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. *Lab Invest*, 89(5):597–606, Mar 2009.

Jan D. Hulzinga, Lars Thuneberg, Michael Kluppel, John Malysz, Hanne B. Mikkelsen, and Alan Bernstein. W/kit gene required for interstitial cells of cajal and for intestinal pacemaker activity. *Nature*, 373(6512):347–349, 1995.

Boaz Inbal, Ofer Cohen, Sylvie Polak-Charcon, Juri Kopolovic, Ezra Vadai, Lea Eisenbach, and Adi Kimchi. Dap kinase links the control of apoptosis to metastasis. *Nature*, 390(6656):180–184, 1997.

Marilena V Iorio, Manuela Ferracin, Chang-Gong Liu, Angelo Veronese, Riccardo Spizzo, Silvia Sabbioni, Eros Magri, Massimo Pedriali, Muller Fabbri, Manuela Campiglio, et al. Microrna gene expression deregulation in human breast cancer. *Cancer research*, 65(16):7065–7070, 2005.

D. N. Irion, A. L. Schaffer, T. R. Famula, M. L. Eggleston, S. S. Hughes, and N. C. Pedersen. Analysis of genetic variation in 28 dog breed populations with 100 microsatellite markers. *Journal of Heredity*, 94(1):81–87, 01 2003.

M Isotani, N Ishida, M Tominaga, K Tamura, H Yagihara, S Ochi, R Kato, T Kobayashi, M Fujita, Y Fujino, A Setoguchi, K Ono, T Washizu, and M Bonkobara. Effect of tyrosine kinase inhibition by imatinib mesylate on mast cell tumors in dogs. *J Vet Intern Med*, 22(4):985–988, 2008.

C Jones, R Grahn, M Chien, L Lyons, and C London. Detection of c-KIT mutations in canine mast cell tumors using fluorescent polyacrylamide gel electrophoresis. *J Vet Diagn Invest*, 16 (2):95–100, 2004.

L.G. Kindblom, H.E. Remotti, F. Aldenborg, and J.M. Meis-Kindblom. Gastrointestinal pacemaker cell tumor (gipact): gastrointestinal stromal tumors show phenotypic characteristics of the interstitial cells of cajal. *American Journal of Pathology*, 152(5):1259–1269, May 1998.

Shawna L. Klahn, Barbara E. Kitchell, and Nikolaos G. Dervisis. Evaluation and comparison of outcomes in dogs with periarticular and nonperiarticular histiocytic sarcoma. *Journal of the American Veterinary Medical Association*, 239(1):90–96, 2011/07/02 2011.

Wigard P. Kloosterman and Ronald H. A. Plasterk. The diverse functions of microRNAs in animal development and disease. *Dev Cell*, 11(4):441–450, Oct 2006.

Kazuyoshi Kumagai, Kazuyuki Uchida, Toru Miyamoto, Takahiro Ushigusa, Syusaku Shinohara, Ryoji Yamaguchi, and Susumu Tateyama. Three cases of canine gastroinstestinal stromal tumors with multiple differentiations and c-KIT-expression. *Journal of Veterinary Medical Science*, 65 (10):119–1122, 2003.

Prateek Kumar, Steven Henikoff, and Pauline C Ng. Predicting the effects of coding non-synonymous variants on protein function using the sift algorithm. *Nature protocols*, 4(7):1073–1081, 2009.

Mariana Lagos-Quintana, Reinhard Rauhut, Winfried Lendeckel, and Thomas Tuschl. Identification of novel genes coding for small expressed RNAs. *Science*, 294(5543):853–858, 10 2001.

R. G. LaRock and P. E. Ginn. Immunohistochemical staining characteristics of canine gastrointestinal stromal tumors. *Veterinary Pathology*, 34(4):303–311, 1997.

ICM Lavrijsen, HCM Heuven, BP Meij, LFH Theyse, RC Nap, PAJ Leegwater, and HAW Hazewinkel. Prevalence and co-occurrence of hip dysplasia and elbow dysplasia in dutch purebred dogs. *Preventive veterinary medicine*, 114(2):114–122, 2014.

Benjamin P Lewis, Christopher B Burge, and David P Bartel. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, 120(1):15–20, Jan 2005.

Heng Li and Richard Durbin. Fast and accurate short read alignment with burrows–wheeler transform. *Bioinformatics*, 25(14):1754–1760, 2009.

Heng Li, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, Richard Durbin, et al. The sequence alignment/map format and samtools. *Bioinformatics*, 25(16):2078–2079, 2009.

Jinghuan Li, Paul Smyth, Richard Flavin, Susanne Cahill, Karen Denning, Sinead Aherne, Simone Guenther, John O'Leary, and Orla Sheils. Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells. *BMC Biotechnol*, 7(1):36, 2007. ISSN 1472-6750.

K Lindblad-Toh, CM Wade, TS Mikkelsen, EK Karlsson, DB Ja e, M Kamal, M Clamp, JL Chang, EJ Kulbokas, MC Zody, E Mauceli, X Xie, M Breen, RK Wayne, EA Ostrander, CP Ponting, F Galibert, DR Smith, PJ deJong, E Kirkness, P Alvarez, T Biagi, W Brockman, J Butler, CW Chin, A Cook, J Cu, MJ Daly, D DeCaprio, S Gnerre, M Grabherr, M Kellis, M Kleber, C Bardeleben, L Goodstadt, A Heger, C Hitte, L Kim, KP Koepfli, HG Parker, JP Pollinger, SMJ Searle, NB Sutter, R Thomas, C Webber, Broad Institute Genome Sequencing Platform, and ES Lander. Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature*, 438(7069):803–819, 2005.

CA London. Tyrosine kinase inhibitors in veterinary medicine. *Top Companion Anim Med*, 24(3): 106–112, 2009.

Cheryl A. London, Stephen J. Galli, Toshifumi Yuuki, Zhi-Qing Hu, Stuart C. Helfand, and Edwin N. Geissler. Spontaneous canine mast cell tumors express tandem duplications in the proto-oncogene c-KIT. *Experimental Hematology*, 27(4):689–697, 4 1999.

Cheryl A. London, Phyllis B. Malpas, Stacey L. Wood-Follis, Joseph F. Boucher, Anthony W. Rusk, Mona P. Rosenberg, Carolyn J. Henry, Kathy L. Mitchener, Mary K. Klein, John G. Hintermeister, Philip J. Bergman, Guillermo C. Couto, Guy N. Mauldin, and Gina M. Michels. Multi-center, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SU11654), a receptor tyrosine kinase inhibitor, for the treatment of dogs with recurrent (either local or distant) mast cell tumor following surgical excision. *Clinical Cancer Research*, 15(11): 3856–3865, 6 2009.

B. Jack Longley, Maria Jose Reguera, and Yongsheng Ma. Classes of c-KIT activating mutations: proposed mechanisms of action and implications for disease classification and therapy. *Leukemia Research*, 25(7):571–576, 2001.

Y Ma, BJ Longley, X Wang, JL Blount, K Langley, and GH Caughey. Clustering of activating mutations in c-KIT's juxtamembrane coding region in canine mast cell neoplasms. *J Invest Dermatol*, 112(2):165–170, 1999a.

Yongsheng Ma, B Jack Longley, Xiaomei Wang, John L Blount, Keith Langley, and George H Caughey. Clustering of activating mutations in c-KIT's juxtamembrane coding region in canine mast cell neoplasms. *Journal of Investigative Dermatology*, 112(2):165–170, 1999b.

L Marconato, G Bettini, C Giacoboni, G Romanelli, A Cesari, A Zatelli, and E Zini. Clinicopathological features and outcome for dogs with mast cell tumors and bone marrow involvement. *J Vet Intern Med*, 22(4):1001–1007, 2008.

Julien Meunier, Frédéric Lemoine, Magali Soumillon, Angélica Liechti, Manuela Weier, Katerina Guschanski, Haiyang Hu, Philipp Khaitovich, and Henrik Kaessmann. Birth and expression evolution of mammalian microrna genes. *Genome Research*, 23(1):34–45, 01 2013.

M. Miettinen and J. Lasota. Gastrointestinal stromal tumors: review on morphology, molecular pathology, prognosis, and differential diagnosis. *Archives of Pathology and Laboratory Medicine*, 130(10):1466–1478, Oct 2006.

Markku Miettinen, Wa'El El-Rifai, Leslie H, and Jerzy Lasota. Evaluation of malignancy and prognosis of gastrointestinal stromal tumors: A review. *Human pathology*, 33(5):478–483, 2002.

C Mol, D Dougan, T Schneider, R Skene, M Kraus, D Scheibe, G Snell, H Zou, B Sang, and K Wilson. Structural basis for the autoinhibition and STI-571 inhibition of c-KIT tyrosine kinase. *J Biol Chem*, 279(30):31655–31663, 2004.

Peter F. Moore. Systemic histiocytosis of Bernese mountain dogs. *Vet Pathol*, 21(6):554–563, Nov 1984.

PF Moore. Utilization of cytoplasmic lysozyme immunoreactivity as a histiocytic marker in canine histiocytic disorders. *Vet Pathol*, 23(6):757–762, Nov 1986.

M. Mortarino, G. Gioia, M.E. Gelain, F. Albonico, P. Roccabianca, E. Ferri, and S. Comazzi. Identification of suitable endogenous controls and differentially expressed microRNAs in canine fresh-frozen and FFPE lymphoma samples. *Leuk Res*, 34(8):1070 – 1077, 2010. ISSN 0145-2126.

B A Neilan, A N Wilton, and D Jacobs. A universal procedure for primer labelling of amplicons. *Nucleic Acids Research*, 25(14):2938–2939, 07 1997.

Pauline C Ng and Steven Henikoff. Predicting deleterious amino acid substitutions. *Genome research*, 11(5):863–874, 2001.

Pauline C Ng and Steven Henikoff. Accounting for human polymorphisms predicted to affect protein function. *Genome research*, 12(3):436–446, 2002.

Pauline C Ng and Steven Henikoff. Sift: Predicting amino acid changes that affect protein function. *Nucleic acids research*, 31(13):3812–3814, 2003.

H. Stacy Nicholson, R. Maarten Egeler, and Mark E. Nesbit. The epidemiology of langerhans cell histiocytosis. *Hematology/Oncology Clinics of North America*, 12(2):379–384, 4 1998.

L. Nielsen, S. N. Andreasen, S. D. Andersen, and A. T. Kristensen. Malignant histiocytosis and other causes of death in Bernese mountain dogs in Denmark. *Vet Rec*, 166(7):199–202, Feb 2010.

B Nilsson, O Nilsson, and H Ahlman. Treatment of gastrointestinal stromal tumours: imatinib, sunitinib - and then? *Expert Opin Investig Drugs*, 18(4):457–468, 2009.

Toshirou Nishida, Seiichi Hirota, Masahiko Taniguchi, Koji Hashimoto, Koji Isozaki, Harumi Nakamura, Yuzuru Kanakura, Tomoyuki Tanaka, Arimichi Takabayashi, Hikaru Matsuda, and Yukihiko Kitamura. Familial gastrointestinal stromal tumours with germline mutation of the kit gene. *Nat Genet*, 19(4):323–324, 1998.

Dan G. O'Neill, David B. Church, Paul D. McGreevy, Peter C. Thomson, and Dave C. Brodbelt. Prevalence of disorders recorded in dogs attending primary-care veterinary practices in England. *PLoS ONE*, 9(3):e90501 EP, 03 2014.

J Ostrowski, M Polkowski, A Paziewska, M Skrzypczak, K Goryca, T Rubel, K Kokoszynska, P Rutkowski, Z Nowecki, A Dobosz, D Jarosz, W Ruka, and L Wyrwicz. Functional features of gene expression pro les differentiating gastrointestinal stromal tumours according to kit mutations and expression. *BMC Cancer*, 9, 2009.

GA Padgett, BR Madewell, ET Keller, L Jodar, and M Packard. Inheritance of histiocytosis in Bernese mountain dogs. *J Small Anim Pract*, 36(3):93–98, Mar 1995.

EA Perez, AS Livingstone, D Franceschi, C Rocha-Lima, DJ Lee, N Hodgson, M Jorda, and LG Koniaris. Current incidence and outcomes of gastrointestinal mesenchymal tumors including gastrointestinal stromal tumors. *J Am Coll Surg*, 202(4):623–629, 2006.

Lasse Peters and Gunter Meister. Argonaute proteins: Mediators of RNA silencing. *Mol Cell*, 26 (5):611–623, Jun 2007.

D Pytel, T Sliwinski, T Poplawski, D Ferriola, and I Majsterek. Tyrosine kinase blockers: new hope for successful cancer therapy. *Anticancer Agents Med Chem*, 9:66–76, 2009.

F H Qiu, P Ray, K Brown, P E Barker, S Jhanwar, F H Ruddle, and P Besmer. Primary structure of c-KIT: relationship with the CSF-1/PDGF receptor kinase family-oncogenic activation of v-KIT involves deletion of extracellular domain and C terminus. *EMBO Journal*, 7(4):1003–1011, April 1988.

Pascale Quignon, Laetitia Herbin, Edouard Cadieu, Ewen F. Kirkness, Benoit Hédan, Dana S. Mosher, Francis Galibert, Catherine André, Elaine A. Ostrander, and Christophe Hitte. Canine population structure: Assessment and impact of intra-breed stratification on snp-based association studies. *PLoS ONE*, 2(12):e1324 EP –, 12 2007.

Mangalathu S. Rajeevan, Daya G. Ranamukhaarachchi, Suzanne D. Vernon, and Elizabeth R. Unger. Use of Real-Time Quantitative PCR to validate the results of cDNA array and differential display PCR technologies. *Methods*, 25(4):443–451, 12 2001.

E Ralfkiaer, G Delsol, NT O'Connor, P Brandtzaeg, P Brousset, GL Vejlsgaard, and DY Mason. Malignant lymphomas of true histiocytic origin. A clinical, histological, immunophenotypic and genotypic study. *J Pathol*, 160(1):9–17, Jan 1990.

IK Ramsey, JS McKay, H Rudorf, and JM Dobson. Malignant histiocytosis in three Bernese mountain dogs. *Vet Rec*, 138(18):440–444, May 1996.

A Rosin, Peter F. Moore, and Richard R. Dubielzig. Malignant histiocytosis in Bernese Mountain dogs. *J Am Vet Med Assoc*, 188(9):1041–1045, May 1986.

Robert Roskoski, Jr. Signaling by KIT protein-tyrosine kinase–the stem cell factor receptor. *Biochemical and Biophysical Research Communications*, 337(1):1–13, 2005.

Brian P. Rubin, Samuel Singer, Connie Tsao, Anette Duensing, Marcia L. Lux, Robert Ruiz, Michele K. Hibbard, Chang-Jie Chen, Sheng Xiao, David A. Tuveson, George D. Demetri, Christopher D. M. Fletcher, and Jonathan A. Fletcher. KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. *Cancer Research*, 61(22):8118–8121, 2001.

Brian P. Rubin, Cristina R. Antonescu, James P. Scott-Browne, Melissa L. Comstock, Yansong Gu, Munir R. Tanas, Carol B. Ware, and Jessica Woodell. A knock-in mouse model of gastrointestinal stromal tumor harboring KIT K641E. *Cancer Research*, 65(15):6631–6639, 2005.

P Rutkowski, M Symonides, M Zdzienicki, and JA Siedlecki. Developments in targeted therapy of advanced gastrointestinal stromal tumors. *Recent Pat Anticancer Drug Discov*, 3(2):88–99, 2008.

J A Salotti, V Nanduri, M S Pearce, L Parker, R Lynn, and K P Windebank. Incidence and clinical features of Langerhans cell histiocytosis in the UK and Ireland. *Archives of Disease in Childhood*, 94(5):376–380, 05 2009.

D Savage and K Antman. Imatinib mesylate-a new oral targeted therapy. *N Engl J Med*, 346(9): 683–693, 2002.

Laura Schmitz and Blaise E. Favara. Nosology and pathology of Langerhans Cell Histiocytosis. *Hematol Oncol Clin N Am*, 12(2):221–246, 1998.

I Schnadig and C Blanke. Gastrointestinal stromal tumors: imatinib and beyond. *Curr Treat Options Oncol*, 7(6):427–437, 2006.

Abigail L. Shearin, Benoit Hedan, Edouard Cadieu, Suzanne A. Erich, Emmett V. Schmidt, Daniel L. Faden, John Cullen, Jerome Abadie, Erika M. Kwon, Andrea Gröne, Patrick Devauchelle, Maud Rimbault, Danielle M. Karyadi, Mary Lynch, Francis Galibert, Matthew Breen, Gerard R. Rutteman, Catherine André, Heidi G. Parker, and Elaine A. Ostrander. The MTAP-CDKN2A locus confers susceptibility to a naturally occurring canine cancer. *Cancer Epidemiology Biomarkers & Prevention*, 05 2012.

Xu-Bao Shi, Lingru Xue, Joy Yang, Ai-Hong Ma, Jianjun Zhao, Ma Xu, Clifford G Tepper, Christopher P Evans, Hsing-Jien Kung, and Ralph W deVere White. An androgen-regulated miRNA suppresses Bak1 expression and induces androgen-independent growth of prostate cancer cells. *Proceedings of the National Academy of Sciences*, 104(50):19983–19988, 2007.

KA Skorupski, CA Clifford, MC Paoloni, A Lara-Garcia, L Barber, MS Kent, AK LeBlanc, A Sabhlok, EA Mauldin, FS Shofer, CG Couto, and KU Sørenmo. CCNU for the treatment of dogs with histiocytic sarcoma. *J Vet Intern Med*, 21(1):121–126, Jan-Feb 2007.

Gunhild Sommer, Valter Agosti, Imke Ehlers, Ferdinand Rossi, Selim Corbacioglu, Judith Farkas, Malcolm Moore, Katia Manova, Cristina R. Antonescu, and Peter Besmer. Gastrointestinal stromal tumors in a mouse model by targeted mutation of the KIT receptor tyrosine kinase. *Proceedings of the National Academy of Sciences*, 100(11):6706–6711, 2003.

Sonja E. Steigen and Tor J. Eide. Gastrointestinal stromal tumors (GISTs): a review. *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, 117(2):73–86, 2009.

S Tabone, N Theou, A Wozniak, R Saffroy, L Deville, C Julie, P Callard, A Lavergne-Slove, M Debiec-Rychter, A Lemoine, and J Emile. KIT overexpression and amplification in gastrointestinal stromal tumors (GISTs). *Biochim Biophys Acta*, 1741(1-2):165–172, 2005.

Marilia Takada, Maciej Parys, Emmalena Gregory-Bryson, and Vilma Yuzbasiyan-Gurkan. A novel canine histiocytic sarcoma cell line provides a potential path to effective treatments with relevance for translational and comparative studies in humans [abstract number 3931]. In *Proceedings of the 105th Annual Meeting of the American Association for Cancer Research*, San Diego, CA, Apr 5-9 2014. AACR, Philadelphia (PA).

Masahiko Taniguchi, Toshirou Nishida, Seiichi Hirota, Koji Isozaki, Toshinori Ito, Taisei Nomura, Hikaru Matsuda, and Yukihiko Kitamura. Effect of c-KIT mutation on prognosis of gastrointestinal stromal tumors. *Cancer Research*, 59(17):4297–4300, 1999.

Mark A Travis, Boris Reizis, Andrew C Melton, Emma Masteller, Qizhi Tang, John M Proctor, Yanli Wang, Xin Bernstein, Xiaozhu Huang, Louis F Reichardt, et al. Loss of integrin alpha-V/beta-8 on dendritic cells causes autoimmunity and colitis in mice. *Nature*, 449(7160):361–365, 2007.

L Van Kuijk, K van Ginkel, JP de Vos, MJ Brearley, J Butinar, Ingrid Gielen, E van Garderen, Koen Chiers, and PS Verhoeven. Peri-articular histiocytic sarcoma and previous joint disease in Bernese mountain dogs. *Journal of Veterinary Internal Medicine*, 27(2):293–299, 2013.

Jeffrey A Vos, Susan L Abbondanzo, Carol L Barekman, JoAnn W Andriko, Markku Miettinen, and Nadine S Aguilera. Histiocytic sarcoma: a study of five cases including the histiocyte marker CD163. *Mod Pathol*, 18(5):693–704, Jan 2005.

Xiaohong Wang, Shuang Tang, Shu-Yun Le, Robert Lu, Janet S. Rader, Craig Meyers, and Zhi-Ming Zheng. Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. *PLoS ONE*, 3(7):e2557–, Jul 2008.

Joshua D Webster, Matti Kiupel, and Vilma Yuzbasiyan-Gurkan. Evaluation of the kinase domain of c-KIT in canine cutaneous mast cell tumors. *BMC Cancer*, 6(85), April 2006a.

Joshua D. Webster, Vilma Yuzbasiyan-Gurkan, John B. Kaneene, RoseAnn Miller, James H. Resau, and Matti Kiupel. The role of c-KIT in tumorigenesis: Evaluation in canine cutaneous mast cell tumors. *Neoplasia*, 8(2):104–111, February 2006b.

ML Wellman, S Krakowka, RM Jacobs, and GJ Kociba. A macrophage-monocyte cell line from a dog with malignant histiocytosis. *In Vitro Cell Dev Biol*, 24(3):223–229, Mar 1988.

NACS Wong, R Young, RDG Malcomson, AG Nayar, LA Jamieson, VE Save, FA Carey, DH Brewster, C Han, and A Al-Nafussi. Prognostic indicators for gastrointestinal stromal tumours: a clinicopathological and immunohistochemical study of 108 resected cases of the stomach. *Histopathology*, 43(2):118–126, 2003.

Yaguang Xi, Go Nakajima, Elaine Gavin, Chris G. Morris, Kenji Kudo, Kazuhiko Hayashi, and Jingfang Ju. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. *RNA*, 13:1668–1674, 2007.

Y Yarden, W J Kuang, T Yang-Feng, L Coussens, S Munemitsu, T J Dull, E Chen, J Schlessinger, U Francke, and A Ullrich. Human proto-oncogene c-KIT: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO Journal*, 6(11):3341–3351, November 1987.

Gaetano Zafarana, Ad JM Gillis, Ruud JHLM van Gurp, Per G Olsson, Fons Elstrodt, Hans Stoop, José Luis Millán, J Wolter Oosterhuis, and Leendert HJ Looijenga. Coamplification of DAD-R, SOX5, and EKI1 in human testicular seminomas, with specific overexpression of DAD-R, correlates with reduced levels of apoptosis and earlier clinical manifestation. *Cancer research*, 62(6):1822–1831, 2002.

Regina Zavodovskaya, Albert T. Liao, Cameron L. R. Jones, Becky Yip, May B. Chien, Peter F. Moore, and Cheryl A. London. Evaluation of dysregulation of the receptor tyrosine kinases Kit, Flt3, and Met in histiocytic sarcomas of dogs. *Am J Vet Res*, 67(4):633–641, Nov 2006.

D. Zemke, B. Yamini, and V. Yuzbasiyan-Gurkan. Mutations in the juxtamembrane domain of c-KIT are associated with higher grade mast cell tumors in dogs. *Veterinary Pathology*, 39(5): 529–535, 2002.

Xiao Zhang, Jiamin Chen, Tom Radcliffe, Dave P. LeBrun, Victor A. Tron, and Harriet Feilotter. An array-based analysis of microRNA expression comparing matched frozen and formalin-fixed paraffin-embedded human tissue samples. *J Mol Diagn*, 10(6):513–519, 2008.

Ming Zhou, Zixing Liu, Yuhua Zhao, Yan Ding, Hao Liu, Yaguang Xi, Wei Xiong, Guiyuan Li, Jianrong Lu, Oystein Fodstad, et al. MicroRNA-125b confers the resistance of breast cancer cells to paclitaxel through suppression of pro-apoptotic Bcl-2 antagonist killer 1 (Bak1) expression. *Journal of Biological Chemistry*, 285(28):21496–21507, 2010.