ABSTRACT

TSAI, PEI-CHIEN. Canine Lymphoma- the Prognostic Significance of Cytogenetic Changes. (Under the direction of Dr. Matthew Breen.)

Canine lymphoma accounts for nearly one quarter of all cancers in dogs. Although it is treatable with chemotherapy, as is true for NHL in humans, additional predictive factors are needed to generate more refined schemes that can offer reliable prognostic information. In recent years, cytogenetic evaluation has been used increasingly in the diagnosis and assessment of human NHLs. To determine the prognostic value of chromosomal aberrations identified in canine lymphoma, in Chapter 2 we analyzed 160 archival lymphoma specimens derived from a clinical trial, using interphase fluorescence *in situ* hybridization (FISH) analysis with genome integrated bacterial artificial chromosome contigs as probes for targeting nine recurrent chromosomal copy number aberrations. By the splitting-sample approach, the data obtained from 121 cases using regression analysis indicate that copy number aberration in one of the nine loci evaluated is significantly associated with disease-free intervals of dog patients with lymphoma. A validation cohort of 39 cases was used to confirm the findings.

To provide reliable data in our subsequent gene expression analysis, required identification and validation of suitable canine reference genes. In Chapter 3, we used array comparative genomic hybridization (aCGH) data to guide the selection of new candidate reference genes and applied these genes to different tumor types, including canine lymphoma, histiocytic sarcoma and osteosarcoma. The stability of candidate reference genes and four conventional reference genes was accessed by three different programs. *LOC611555* was identified as the most stable reference gene across the three tumor types. Comparison of gene stability between newly identified candidate genes and previously used housekeeping genes in multiple tumors, demonstrated a greater level of stability of the proposed new reference genes. In addition, our analysis also provided the most suitable combination of reference genes for multiple genes normalization for each type of tumor.

Moreover, to identify potential target genes located in these recurring aberrations identified in canine lymphoma, 11 genes were selected for further study based on their known tumor-associated roles and history of deregulation in human lymphomas. We characterized the gene expression patterns of these 11 tumor-associated genes in canine lymphomas and found that *MYC* was upregulated in lymphoma samples, whereas *MEOX-2* and *KIT* were downregulated. We observed that *KIT* was highly expressed in several T-cell lymphoma cases, which suggests that these cases may be potential targets for KIT-specific treatment using tyrosine kinase inhibitors. Furthermore, by combining data of gene expression with cytogenetic changes, three gene-dosage regulated candidates were identified, including *CDKN2A*, *TSC2* and *STAT1*. Deregulation of these genes may interfere with cell cycle control and signaling pathways that are involved in the pathogenesis of canine lymphoma.

MEOX-2 is a transcription factor whose involvement in many aspects of vascular cellular processes has been recognized, in particular as a regulator controlling the angiogenic transition in vascular endothelial cells. Due to the impact of MEOX-2 in development and cellular behavior, we were interested in investigating its expression pattern in tumors. A preliminary study in Chapter 4 showed that *MEOX-2* expression was downregulated in all studied lymphoma cases, with no corresponding genetic deletion detected. In Chapter 5, we present evidence supporting our previous findings and further demonstrate that the CpG island of *MEOX-2* was hypermethylated in all studied lymphoma cases, but not in normal lymph nodes. These findings suggest that *MEOX-2* expression might be silenced by DNA methylation. Further studies are necessary to elucidate the significance of loss of *MEOX-2* expression in canine lymphoma. The tumor-specific methylation pattern of *MEOX-2* may have potential use as an epigenetic marker for molecular diagnosis of canine lymphoma.

Canine Lymphoma- the Prognostic Significance of Cytogenetic Changes

by
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DEDICATION

This dissertation is dedicated to my parents for their support and love.

BIOGRAPHY

Pei-Chien Tsai was born on September 30, 1979, in Chiayi, Taiwan. She grew up and spent her earlier school years in Taipei. She received a bachelor degree in Agricultural Chemistry from National Taiwan University (NTU), Taiwan in June 2002. She enrolled in the Microbiology program in NTU at the same year and completed her master's degree, working on protein engineering of a biodegradable polyester synthetase (PHA synthetase) in 2004. She was employed as a research assistant in NTU hospital and focused on investigating a blood coagulation-related protein and its role in human lung cancer invasion and metastasis. In August of 2005, she came to the United State to pursue her Ph.D. degree in the Comparative Biomedical Science program with a cell biology concentration. She began to work with Dr. Matthew Breen at the same year.

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CHAPTER 1: Research Background

Introduction: Canine lymphoma and its prognostic dilemma

Lymphoma is one of the most common neoplasms in dogs, with an estimated age-adjusted annual incident rate of 107 cases per 100,000 dogs (Edwards et al 2003). Lymphoma is diagnosed in up to 24% of all dog neoplasms and 83% of all hematopoietic neoplasms in dogs (MacEwen 1990, Moulton and Harvey 1990). Although lymphomas affect dogs of all ages, they are most predominant in middle-aged to older dogs (Modiano et al 2005, Priester and McKay 1980), with a median age of 6 to 9 years (Backgren 1965, Parodi et al 1968). Most reports show no gender predilection in dog lymphomas (Haga et al 1988, Parodi et al 1968), but a lower risk in female dogs has been reported (Priester and McKay 1980). In addition, higher risk is associated with certain breeds, such as boxers, beagles, Airedale terriers, bull mastiffs, basset hounds, Saint Bernards, Scottish terriers, bulldogs, and golden retrievers, whereas breeds such as dachshunds and Pomeranians are at lower risk (Edwards et al 2003, Priester and McKay 1980). Furthermore, familial aggregation of lymphoma has been observed in bull mastiffs, Rottweilers, and otter-hounds (Onions 1984, Teske et al 1994a). These breed-dependent differences in the incidence of lymphoma suggests that lymphoma is a genetic disease or associated with certain inherited characteristics (Priester 1967).

Classification and clinical signs

Canine lymphoma has been classified in several schemes. The oldest one characterizes lymphoma on the basis of anatomic location. In this scheme, lymphoma is classified as multicentric, gastrointestinal, mediastinal, cutaneous, and extranodal (eyes, central nervous system, bone, testes, bladder, heart, and nasal cavity) (Vail and Young 2007). Multicentric lymphoma is the most common form and accounts for approximately 80% of all canine lymphomas (Madewell and Theilen 1987). Multicentric lymphoma is distinguished by the presence of painless, discrete superficial lymphadenopathy. Hepatosplenomegaly is a common manifestation associated with an advanced stage (Vail and Young 2007). Clinical signs of multicentric lymphoma are often nonspecific, such as weight loss, vomiting, diarrhea,

polydipsia, polyuria, weakness, ascites, fever, or difficulty breathing. Gastrointestinal lymphoma is a less prevalent form (5% to 7%) and occurs multifocally and diffusely throughout the small intestine (Madewell and Theilen 1987). Similar to the multicentric form, dogs with gastrointestinal lymphoma commonly exhibit nonspecific gastrointestinal signs, such as vomiting, diarrhea, weight loss or decreased appetite. Mediastinal lymphoma accounts for 5 % of cases and is characterized by enlargement of the craniomediastinal lymph nodes and/or the thymus (Madewell and Theilen 1987). Dogs with mediastinal lymphoma usually have respiratory distress which may be due to the space-occupying lesions themselves, exercise intolerance, and pleural effusion. Cutaneous lymphoma is commonly multifocal or generalized and can be classified as either an epitheliotropic or nonepitheliotropic form. Lesions can occur as nodules, plaques, ulcers, erythremic or exfoliative dermatitis, scaling, alopecia, and pruritus. Oral lesions and lymph node involvement also may occur. In most cases, the canine epitheliotropic cutaneous lymphoma originates from T lymphocytes (Moore et al 1994). In contrast, B-cell cutaneous lymphoma affects the deeper dermal layers. Dogs with primary central nervous system lymphoma may show solitary or multifocal involvement and may present with neuropathy signs, such as seizures, paralysis, and paresis. Ocular lymphoma is caused by infiltration of lymphoma cells or uveitis. Other ocular changes include thickening of the iris, synechia, hypopyon, glaucoma and hyphema (Swanson 1990).

Canine lymphoma may also be associated with paraneoplastic syndromes. Anemia is the most frequently reported paraneoplastic syndrome associated with lymphoma (Madewell and Feldman 1980). Hypercalcemia is also common in dogs with lymphoma and has been reported to occur in 10% to 40% of canine lymphomas (Chew and Meuten 1982, Greenlee et al 1990). Polydipsia and polyuria are particular signs for dogs with hypercalcemia. The fundamental cause of cancer-induced hypercalcemia is increased bone resorption with calcium mobilization into the extracellular fluid. It is now evident that a parathyroid hormone-related peptide, elaborated by cancerous cells, is the cause of lymphoma-induced hypercalcemia through increases in bone resorption and distal renal tubular calcium resorption in most cases (Rosol et al 1992); however, other humoral factors, such as

interleukin-1 and -6 (IL-1, 6), tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β), are also inducers of bone resorption and may mediate hypercalcemia (Barri and Knochel 1996, Ishimi et al 1990, Yoneda et al 1993).

Histologic grading schemes

Several histologic classification schemes have been used to classify canine lymphoma. The World Health Organization (WHO) has published a classification scheme of hematopoietic tumors of domestic animals, which is based on the same principles of disease definition as the Revised European American Lymphoma (REAL) system (Valli et al 2002). This scheme incorporates tumor morphology, immunophenotype, genetic features, and clinical manifestations. Table 1 shows a comparison of common non-Hodgkin's lymphoma (NHL) subtypes in human and dogs according to the WHO classification (Modiano et al 2007, Valli et al 2011). The low incidence of follicular lymphoma in dogs is one of the most marked differences between canine and human lymphoma (Lieberman et al 1986, Moulton and Harvey 1990). In the dog a follicular architecture is seen only in few cases. In contrast, approximately 20% to 25% of the human NHLs are follicular lymphomas (Lieberman et al 1986).

In addition, the National Cancer Institute Working Formulation (NCI-WF) and the updated Kiel system are also commonly accepted classification systems (Table 2). In the NCI-WF scheme, tumors are described in terms of architectural pattern (diffuse or follicular) and nuclear type (small cleaved cell, large cell, or immunoblastic) (Vail and Young 2007). The Kiel classification defines tumor cells based on morphology of the cell (centroblastic, centrocytic, or immunoblastic), and immunophenotype (B or T-cell) (Vail and Young 2007). In both schemes, the tumors can be classified as low, intermediate (NCI-WF only), and high grade malignancies. Only a minority of canine lymphomas were classified as low grade lymphoma (i.e., 5 to 16 % in the NCI-WF; 24 to 29% in the Kiel classification) (Carter et al 1986, Greenlee et al 1990, Teske et al 1994b). Diffuse large cell lymphomas and diffuse centroblastic lymphomas were the most commonly encountered lymphoma in the NCI-WF scheme and the Kiel system, respectively (Teske et al 1994c).

From the clinical perspective, low grade lymphomas have a low mitotic rate and may only partially respond to chemotherapy. However, low grade lymphomas generally progress slowly and dogs containing low grade lymphoma may live longer without treatment. High grade lymphomas typically spread rapidly but are more responsive to chemotherapy. In addition, dogs with T-cell lymphoma have shown shorter remission and survival times than dogs with B-cell lymphoma (Ruslander et al 1997, Teske et al 1994b, Vail et al 1996). B-cell lymphoma is the predominant type in canine lymphoma. In veterinary studies, 60% to 80% of canine lymphoma cases have a B-cell origin; T cell lymphomas range from 10% to 38%; mixed B- and T-cell lymphomas account for up to 22% (Culmsee et al 2001, Fournel-Fleury et al 1997a, Fournel-Fleury et al 2002, Greenlee et al 1990, Ruslander et al 1997, Teske et al 1994b, Vail et al 1996, Wilkerson et al 2005). It is important to note, there is a significant variation in the relative prevalence of B- and T-cell lymphomas in different breeds (Modiano et al 2005). For example, Labrador Retrievers are more likely to develop B-cell lymphoma comparatively than when considering all breeds as one population, while Boxers are more likely to develop T-cell lymphoma, and Golden Retrievers show approximately equal incidence of both B- and T-cell lymphomas. Figure 1 shows an example of the immunophenotype distribution of different breeds.

Diagnosis and staging

Canine lymphoma is diagnosed through a combination of diagnostic tests. A minimum database consists of physical examination, complete blood count, serum biochemistry profile, and urinalysis. Histologic or cytologic evaluation of the affected lymphoid tissues and cells is essential for a definitive diagnosis of lymphoma. In most cases, lymphoma diagnosis can be made through lymph-node fine-needle aspiration. For accurate determination of histologic grade, lymph node excision should be performed and submitted to a pathologist. After the initial diagnosis is made, molecular diagnostic techniques can be used to facilitate diagnosis of lymphoma in the diagnosis of difficult cases or to further categorize lymphoma. For example, diagnosis of lymphoma and the differentiation of malignant and reactive lymphocytosis can be difficult in some cases based on standard histologic methods. In this

situation, molecular assays are needed to confirm a diagnosis. Clonality is the characteristic mark of malignancy, theoretically, each lymphoma should be a clonal expansion of a single lymphocyte that has a unique sequence in the variable region (CDR3) of both the immunoglobulin receptor gene (IGR) and the T-cell receptor (TCR) gene. Conversely, in benign reactive lymphocytosis, the cells are polyclonal in that each cell possesses an unique antigen receptor. Using this characteristic, a PCR test for antigen receptor gene rearrangement (PARR) technique which amplifies the variable region of the TCR and IGR genes was developed to detect clonality in dog samples (Avery and Avery 2004, Burnett et al 2003, Keller et al 2004). For accurate determination of the immunophenotype, specific antibodies against marker molecules on lymphocytes can be applied to tissues or cells derived from lymph nodes or other sites through immunohistochemical, immunocytochemical, or flow cytometric techniques (Culmsee et al 2001, Fournel-Fleury et al 1997a, Wilkerson et al 2005). For B-cell, the markers are CD79a and CD21; for T-cell, the markers are CD3 (pan T), CD4 (helper T) and CD8 (cytotoxic T) (Workman and Vernau 2003). In addition, other molecular diagnostic tests, such as evaluation of cell proliferation (e.g., expression of Ki-67, proliferating cell nuclear antigen [PCNA], and argyrophilic nucleolar organizer regions [AgNORs]), also play an increasingly important role in aiding the diagnosis, clinical staging, and prognosis (Dobson et al 2001, Fournel-Fleury et al 1997b, Hung et al 2000, Vail et al 1996, Vandesompele et al 2002). After a diagnosis has been established, additional tests such as bone marrow aspirate and biopsy, thoracic and abdominal radiographs, abdominal ultrasonography, and ultrasound-guided aspirate of the liver and spleen are used to determine clinical stage of canine lymphoma. The clinical staging allows evaluation for the extent of disease. The World Health Organization (WHO) clinical staging system for canine lymphoma is described in Table 3. Most canine lymphomas (more than 80%) are represented in an advanced stage (stage III, or IV) (Vail and Young 2007).

Treatment of lymphoma

The prognosis of untreated dogs with lymphoma is poor. Most dogs die within 4 to 6 weeks of diagnosis (MacEwen et al 1977). Since canine lymphoma is generally considered a systemic neoplasm, systemic chemotherapy is therefore the most important treatment of choice. Chemotherapy is effective for killing cells that are rapidly dividing. The chemical agents target cellular pathways required for cell division, DNA synthesis, or protein synthesis, halting cell growth and resulting in cell death. Although chemotherapeutic agents often prove effective in managing cancer growth, the efficacy of chemotherapy might be affected by a number of factors. A major factor that influences the efficacy is the duration of tumor exposure to an effective concentration of drug. Methods of increasing the exposure time, such as continuous infusion, increasing the frequency of treatment, or enhancing the drugs' circulation time, theoretically would enhance tumor killing . In addition, drug resistance can also affect the efficacy of chemotherapy. Drug resistance can occur in cancer patients with previous exposure to chemotherapy drugs and usually is associated with acquiring genetic mutations that inactivate the drug within the cancer cells through either moving the drug out of the cancer cell or preventing the drug from entering the cell. A common form of acquired drug resistance is associated with overexpression of pleiotropic glycoprotein (p-glycoprotein; P-gp) which acts as a transmembrane efflux pump that removes drug from the tumor cells. P-glycoprotein is the product of the multiple drug resistance gene (MDR) (Kartner et al 1983, Miller et al 1991). Other factors such as upregulating of drug inactivating enzyme, upregulating of cellular detoxicification pathways, and deregulation of apoptotic or cell proliferation pathways are also considered to be associated with chemotherapy efficacy (Chun et al 2007).

Currently, the most commonly used chemotherapy agents for the treatment of lymphoma include doxorubicin, cyclophosphamide, vincristine, prednisone, and L-asparaginase (Vail and Young 2007). Doxorubicin is an anthracycline antibiotic and works by inhibiting the activity of topoisomerase through stabilizing the topoisomerase complex after it has broken the DNA chain for replication, preventing the DNA strands from being resealed and thereby stopping the process of replication. This drug is commonly regarded as one of the most

effective chemotherapy drugs. Cyclophosphamide is an alkylating agent that attaches the alkyl group to the guanine base of DNA. This impairs DNA and may form DNA crosslinks between and within DNA strands, leading to cell death. Vincristine is a member of the vinca alkaloid class of chemotherapy drugs and acts as a mitotic inhibitor. It binds to tubulin dimers, inhibiting assembly of microtubule structures and leading to mitotic arrest. Prednisone is a corticosteroid drug. As a chemotherapeutic medication, its mechanism is not full understood. It may work by inducing cell death of certain cancerous blood cells, and as an anti-inflammatory medicine that can relieve swelling around tumors. L-asparaginase is an enzyme that catalyzes the hydrolysis of asparagine to aspartic acid. It kills cancer cells by depleting asparagine which is necessary for survival and growth of cancer cells.

Over the past 20 years, many chemotherapy protocols for dogs have been developed and demonstrated effective for the treatment of canine lymphoma. Most chemotherapy protocols comprise multiple drugs and rotate drugs frequently. The use of multiple drugs or drug rotation can reduce the possibility of the drug resistance. Also, the drug rotation can help reduce the risk of side effects of cumulative use of a particular drug. Therefore, more complex combination chemotherapy protocols are more expensive, more time-consuming, and more likely to result in increased risk for toxicity than a simpler or single agent protocol. However, complex protocols offer dog patients a superior response rate and longer remission and survival times (the treatment terminology is summarized in Table 4). Dogs that respond to chemotherapy and achieve complete remission are usually free of clinical signs and maintain a good quality of life. Unfortunately, lymphomas are rarely cured in dogs, and the goals of chemotherapy are to achieve durable remission and prolong survival. In veterinary medicine, most complex combination protocols are derived from CHOP protocols used for humans. CHOP is the combination of cyclophosphamide (C), doxorubicin (H), vincristine (O), and prednisone (P). Protocols based on CHOP generally induce complete remission in 80 to 90% of dogs, with median survival time of 12 months (Vail and Young 2007). For example, the modified University of Wisconsin-Madison chemotherapy protocol (UW-25) has a median survival time of 13 months with a complete remission in 92% (Garrett et al 2002); the modified University of Wisconsin -Madison 19-week protocol (UW-19) has a

median survival time of 10 months with a complete remission in 83% (MacDonald et al 2005). The UW-19 protocol is listed in Table 5. Because a high percentage of treated dogs have a complete remission and tolerate chemotherapy well, studies evaluating clients' perceptions indicate that clients were positive regarding treatment of lymphoma; few clients felt regretful about treating their dogs with a multidrug chemotherapy protocol (Bronden et al 2003, Mellanby et al 2003). Other treatment choices include single agent doxorubicin, which can result in a complete remission rate ranging from 75% to 85% and a median survival time of 6 to 8 months (Mutsaers et al 2002, Page et al 1992, Valerius et al 1997). Prednisone can achieve a short-term remission of one to two months. The commonly used combination or single agent protocols are summarized in Table 6.

Prognosis and its dilemma

Compared with the human counterpart, human NHL, determination of the prognosis for canine lymphoma is more challenging. For the prognosis of human NHLs, histopathologic grade and clinical stage provide a basis for the choice of treatment and prediction of survival. A predictive model, the International Prognostic Index (IPI), has been established to predict overall survival for aggressive NHLs in NCI-WF categories (1993). The IPI is based on clinical features of tumor progression, patient's response to the disease, and patient's ability to tolerate chemotherapy and has become a commonly used prognostic model for intermediate-grade lymphoma. However, in dogs, lymphoma is usually not noticed until it has reached the later stages of the disease; by the time of getting a veterinarian diagnosis, most canine lymphoma cases represent similar histopathologic grade and clinical stage. As we mentioned previously, most canine lymphoma presents as intermediate or high grade according to NCI-WF, and more than 80% are in advanced stages (III or IV). Because there is less diversity of lymphoma tumors, traditional histopathologic schemes of prognostic significance in human NHL have played a less important role in predicting the survival or managing the treatment of canine lymphoma.

To improve the clinical management of canine lymphoma, there have been many efforts to identify factors of prognostic significance in canine lymphoma. Factors that have been shown

to influence survival include immunophenotype (Dobson et al 2001, Ruslander et al 1997, Teske et al 1994b), WHO substage (Greenlee et al 1990, Jagielski et al 2002, Keller et al 1993, Vail et al 1996), response to therapy (Dobson et al 2001, Jagielski et al 2002), anatomic site (MacEwen 1990), proliferative index (Fournel-Fleury et al 1997a, Kiupel et al 1999), vascular endothelial growth factor (VEGF) (Gentilini et al 2005), and hypercalcemia (Greenlee et al 1990, Weller et al 1982). Table 7 summarizes the prognostic features of these factors. The factors which have associated consistently with patient survival in different studies include immunophenotype, and WHO substage. Several studies have confirmed that dogs with T-cell lymphoma are associated with shorter remission and survival times (Dobson et al 2001, Ruslander et al 1997, Teske et al 1994b); Dogs without systemic signs of disease (WHO substage a) have better prognosis than dogs with signs (WHO substage b) (Greenlee et al 1990, Jagielski et al 2002, Keller et al 1993, Vail et al 1996). These factors remain useful for the current prognosis of canine lymphoma. However, in all of these prognostic factors, there was considerable residual heterogeneity in outcome. This suggests that the incorporation of appropriate prognostic markers into the current prognostic system is required to generate more refined schemes for patient prognosis.

The genetic and molecular mechanisms underlying tumorigenesis are increasingly recognized. Besides being of biological value, they might also be of prognostic and therapeutic importance. Recently, in human lymphoma studies, the application of a range of different microarray-based technologies, such as array-based comparative genomic hybridization (aCGH), cDNA microarray, and methylation array, has increased the understanding of lymphomagenesis and, more importantly, has generated a large number of candidate molecular markers with potential clinical value. Since humans and dogs have similar pathophysiologic characteristics and share extensive genome homology (Breen et al 1999, Yang et al 1999), it is likely that these types of markers might be useful in prognosis of canine lymphoma. In the following sections, we discuss the potential of several types of molecular markers with regard to prognostic aspects and review the findings in the field of lymphoma research. We initially summarize genetic markers identified in cytogenetic studies investigating prognostic significance of specific chromosomal or genetic changes, followed

by a comprehensive overview of epigenetic studies investigating the clinical value of DNA methylation markers. Then, we briefly review the important discoveries made by gene expression profiling in the diagnosis and prognosis of lymphomas. Finally, we address the research goals of this thesis.

Cytogenetics: An aid to diagnosis and prognosis of canine lymphoma

Chromosomal aberrations are a key feature of tumors and may result in altered expression of genes residing within the affected genome regions (Hanahan and Weinberg 2000). Detailed analyses of these segments in the tumor genome allow researchers to identify important oncogenes or tumor suppressor genes. Identification of recurrent chromosomal aberrations and the target genes involved allows a better understanding of cancer development and more importantly provide more sophisticated approaches for clinical management of cancer, such as diagnosis, prognosis, and therapy.

Approaches

Fluorescence in situ hybridization (FISH)

FISH is a technique that uses specific DNA probes of known chromosomal location to evaluate alterations at a specific locus on chromosome. DNA probes are often derived from fragments of genomic DNA and either labeled with fluorophores or with reporter molecules bound to a hapten (e.g. biotin or digoxigenin). The target DNA can be in the form of denatured interphase or metaphase chromosome preparations fixed on a glass slide. Denatured probe is then applied to the target DNA. After a sufficient time for annealing to occur, the probe hybridizes to the chromosome carrying the complementary sequence. Several wash steps are required to remove all excess probes. The sites of hybridization are then visualized using fluorescence microscopy. If the probe has been labeled with biotin or digoxigenin, an additional step is needed for visualization of the nonfluorescent hapten that uses fluorescently-tagged streptavidin or anti-digoxigenin antibodies for detection, respectively.

Two types of probes are generally used for FISH analysis of canine lymphomas. Whole chromosome paint probes are collections of sequences stretching over the entire length of the specific chromosome, derived by labeling DNA derived from flow-sorted chromosomes or microdissected chromosomes. Chromosome paint probes are usually used for identification of chromosomal rearrangements in metaphase cells. Single locus probes are designed to hybridize to specific sequences and are often derived by labeling genomic DNA fragments cloned into a suitable vector and propagated in a bacterial host. The size of the insert is generally proportional to the size of the hybridization signal and so while phage and cosmid clones were used for many years, the use of larger insert clones, such as bacterial artificial chromosomes (BAC) clones is now the preferred option. Single locus specific probes are very versatile, allowing the detection of translocations, deletions, and duplication of a given locus, using metaphase and/or interphase cells.

FISH is very useful for detailed karyotypic assessment of structural chromosomal aberrations and for fine mapping genetic alterations in very small specimens. The development of fluorochromes that fluoresce at different wavelengths (Gray et al 1991), combined with multi-color fluorescence imaging systems, made possible examination of multiple chromosome aberrations simultaneously within individual nuclei. In addition, over the past decade, there have been a number of protocols available for applying FISH on paraffin-embedded specimens by either hybridizing to thin sections of tissue or to individual cells which are extracted from thick sections of tissue. These approaches allow the use of sample materials from archival cases with known clinical outcome, thus facilitating the identification of chromosomal aberrations associated with response to therapy and prognosis in cancers.

Comparative genomic hybridization

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that provides a means to detect imbalanced genomic aberrations in tumors, without the need to generate tumor chromosome preparations (Kallioniemi et al 1992). It allows a genome-wide assessment of copy number changes in a tumor sample compared with a normal sample. In

its conventional form, total DNA samples from a tumor and a normal control tissue are differentially labeled with fluorochromes and cohybridized to normal metaphase chromosomes. Differences in the tumor to normal fluorescence ratio provide information on the relative copy numbers in the tumor genome as compared with the normal genome of the control sample. In 2000, Dunn et al. optimized CGH technology for application to the dog (Dunn et al 2000). CGH is useful for providing a global assessment of the genomic aberrations that lead to gain or loss of genetic material. Balanced chromosomal changes such as translocation and inversion are not shown. Another limitation of CGH is the resolution. It does not allow detection of any imbalance smaller than approximately 5 to10 Mb in size (except high-level amplification).

Subsequently array-based CGH (aCGH) was first developed in 1997 by Solinas-Toldo et al. (Solinas-Toldo et al 1997). aCGH is similar to conventional CGH except that it uses arrayed genomic sequences as hybridization targets instead of the metaphase chromosomes. Hybridization onto such assays greatly improves the resolution of conventional CGH. The first aCGH platform was BAC-based format which comprises DNA fragments cloned in BAC vectors. In 2003, Thomas et al. introduced the first canine BAC aCGH (Thomas et al 2003a). The resolution and performance of BAC aCGH is dictated by several factors, such as the density of the arrays and the optimization of the BAC clone sets. In 2008, a high resolution canine BAC array was described which consists of 2,097 BAC clones covering the dog genome at intervals of approximately 1 Mb (Thomas et al 2008).

Recently, oligonucleotide-based arrays have been introduced as an alternative platform for higher resolution aCGH. The oligonucleotides can be designed for any genome target of interest and any organism with a completely sequenced genome. For canine research, Agilent Technologies offer a commercial 180K oligonucleotide array CGH (oaCGH) platform (http://www.agilent.com). The median probe spacing is 13 kb. Another commercial oaCGH platform is provided by NimbleGen, which offers a canine 385K whole genome CGH array with a median probe spacing of 4.6 kb (http://www.nimblegen.com).

Clinical significance of chromosomal aberrations in lymphoma

In 1982, Dalla-Favera et al. discovered the genetic secrets of the translocation t(8; 14), which characterizes human Burkitt's lymphoma (Dalla-Favera et al 1982). Since that time, considerable efforts have been made to identify regions with chromosomal aberrations and genes involved in human lymphomas. Chromosomal aberrations are nonrandom in human lymphoma and a variety of recurrent aberrations have been characterized as molecular markers for particular subtypes of human lymphoma. For example, the translocation events t(14; 18)(q32;q21), t(11; 14)(q13; q32), and t(8;14)(q24;q32) are associated with follicular lymphoma, mantle cell lymphoma, and Burkitt's lymphoma respectively, while T-cell lymphoma such as anaplastic large cell lymphoma is associated with aberrations involving t(2;5)(p23;q25). Table 8 summarizes cytogenetic changes associated with particular subtypes of human NHLs. In addition, several homozygous deletions that harbor tumor suppressor genes have been detected in human lymphomas. An overview of regions of homozygous loss in human lymphoma is shown in Table 9.

A proportion of these recurrent chromosomal aberrations have been associated with clinical behavior. For example, clinical progression of follicular lymphoma (FL) to transformed diffuse large B-cell lymphoma (DLBCL) occurs in approximately 25 to 60% of FL patients (Lossos and Levy 2003). Genetic events reportedly correlated with the progression of FL to DLBCL include *MYC* locus rearrangement (Yano et al 1992), amplification of 2p13-16 involving *REL* (Goff et al 2000), and deletion at 9p21 involving CDNK2A/B (Elenitoba-Johnson et al 1998). Specific aberrations have also been linked with certain pathological tumor features. As an example, the translocation t(14;18)(q32; q21) involving *BCL2* is predominantly seen in germinal centre B cell-like (GCB) DLBCL. Furthermore, chromosomal aberrations have been shown to have prognostic value. For instance, a study found that loss of 9p21 (*CDNK2A*) was strongly associated with aggressive features and an inferior survival of patients with human DLBCL (Tagawa et al 2005b). In mantle cell lymphoma (MCL), Rubio-Moscardo et al. reported that deletions of 9p21.3 (involving *CDNK2A*), 17p13.1 (involving *TP53*), as well as 9q21-22 (involving *CDC14B* and *FANCC*) were associated with poor outcomes, whereas deletion of 1p21 was associated with

prolonged survival (Rubio-Moscardo et al 2005). Table 10 summarizes cytogenetic markers with prognostic significance in human NHLs. Finally, chromosomal aberrations have been associated with response to treatment. For example, the translocation t(11;18)(q21;q21), which involves the apoptotic inhibitor *AP12* at 11q21 and the *MALT1* gene at 18q21, is detected in 30 to 50% of mucosa-associated tissue (MALT) lymphomas. For gastric MALT lymphoma, the presence of this translocation has been found to be associated with non-response to anti-*Helicobacter pylori* antibiotic therapy (Liu et al 2002).

These cytogenetic marker studies provided valuable additional clinical and prognostic information to human lymphomas and have increased current knowledge on the biological mechanisms underlying lymphomagenesis. However, a number of studies have yielded conflicting results, leading to the limited role in clinical work-up of these cytogenetic markers as reliable prognostic criteria. For example, the translocation t(3; v)(q27; v) affecting *BCL6* are commonly observed in human DLBCL. Offit et al. reported that 3q27 translocations were associated with a favorable clinical outcome (Offit et al 1994). However, subsequent studies failed to show supportive evidence for improved survival in patients with 3q27 translocations (Barrans et al 2002, Bastard et al 1994, Jerkeman et al 2002, Kramer et al 1998, Pescarmona et al 1997, Vitolo et al 1998). In addition, Barrans et al. have found that DLBCL with translocation t(14;18)(q32;q21) had a significantly shorter overall survival (Barrans et al 2003), whereas other reports showed no difference in overall survival between t(14; 18)(q32;q21) positive and negative cases (Hirose et al 2005, Iqbal et al 2004). These might be caused by the extensive heterogeneity of human populations, within which the highly diverse and complex disease phenotypes are observed, making these cytogenetic markers unable to reflect the heterogeneity of tumor cells accurately.

This problem might be partially solved by parallel investigations of comparable cancers in a more genetically homogeneous biomedical model system. The purebred domestic dog represents an ideal model system for studying genetic factors that are clinically relevant in lymphoma because of the limited genetic diversity within dog breeds. As we mentioned above, canine lymphomas share many biological and clinical similarities with human NHLs. Furthermore, with the release of a high-quality, annotated 7.6x genome sequence assembly

for the domestic dog (Lindblad-Toh et al 2005), the dog is now amenable to comparative genomic studies. Researchers are able to easily translate informative findings of human lymphoma cytogenetics into the dog to see if the equivalent problems occur, and vice versa. Thus, the canine lymphoma population offers a unique opportunity to identify evolutionarily conserved genomic aberrations that are more closely associated with lymphoma pathogenesis, using the dog to reduce the background "noise" in human genome that confound human lymphoma studies (Thomas et al 2011).

In our previous studies, we demonstrated that recurrent chromosomal aberrations are also present in canine lymphomas and have identified aberrations that are associated with specific tumor subtypes (Modiano et al 2005, Thomas et al 2003b, Thomas et al 2011). Our group has documented gain of dog chromosome (CFA) 13 and 31 and loss of CFA 11 and 14 as the most common aberrations in a group of 25 cases analyzed (Modiano et al 2005, Thomas et al 2003b). More recently, a larger series of 150 lymphoma cases were analyzed using aCGH analysis (Thomas et al 2011). The aberrations most frequently found in B-cell lymphoma were gains in CFA 13 and 31, and losses in CFA 14 and CFA 26q24. In T-cell lymphoma, recurrent aberrations include gains of CFA 6, 9, 13, 20, 29, 31, and 36 and losses of CFA 11, 17, 22, 28, and 38. Loss of CFA 11 was restricted to T-cell lymphoma, whereas deletion of CFA 26q24 was seen exclusively in B-cell lymphoma. In addition, small regions of deletion were identified in CFA 11q16 and 26q24, which spanned *CDKN2A* and *IGLλ* loci, respectively (Fosmire et al 2007, Thomas et al 2011). Furthermore, comparative analysis between canine and human cytogenetic profiles indicated that DLBCL exhibits conserved gain of CFA 13q/HSA 8q (including the *MYC* locus) and CFA 31q/HSA 21q. In peripheral T-cell lymphomas (PTCLs), copy number gain of HSA 1q, 2, 4, 8q (including *MYC*), 16p, and 17q and loss of HSA 9p (including *CDNK2A*), 10q and 13q are conserved in the dog counterpart. Moreover, we have shown previously that *MYC-IGH* fusion also occurs in canine Burkitt's lymphoma (Modiano et al 2005). Other evolutionarily conserved chromosomal aberrations that are shared by human and canines with the same hematological malignancies include BCL-ABL fusion in chronic myelogenous leukemia, and deletion of RB in chronic lymphocytic leukemia (Breen and Modiano 2008). These observations suggest

that humans and dogs share a conserved pathogenetic basis for cancer (Breen and Modiano 2008) . Targeted investigation of these shared regions may speed progress of identifying the fundamental underlying genetic mechanisms of lymphomagenesis in both species. The information derived from these comparative studies may contribute to diagnosis, prognosis, and the development of target-specific therapies for both human and canine patients.

DNA methylation-based biomarker for hematopoietic malignancies

Cancer is characterized not only by the presence of chromosomal or genetic defects but also by epigenetic lesions (Esteller and Herman 2002, Herman and Baylin 2003). The most studied epigenetic lesion in neoplasms is the DNA methylation of promoter CpG islands of tumor suppressor genes, which is associated with the transcriptional inactivation of the gene (Bird 1996). Thus, in addition to mutations and deletions, aberrant DNA methylation has been recognized as an alternative mechanism that inactivates tumor suppressor genes, contributing to the multiple step process of malignant transformation of normal cells. There are two established mechanisms by which DNA methylation can affect the transcription of a target gene. One is that methylated DNA can directly interfere with the binding of transcription factors (Tate and Bird 1993). The other is that methylated DNA can recruit methyl-CpG binding proteins (e.g., MeCP, MBD), blocking access of other factors (Lewis et al 1992). Additionally, the methyl-CpG binding proteins can also recruit histone deacetylase (HDAC)(Jones et al 1998), which would result in histone deacetylation, leading to tighter coiling of DNA and reduced access of transcription factors to their cognate sites (Robertson and Wolffe 2000).

A large number of genes that are known to be essential for fundamental cellular processes have been inactivated by aberrant DNA methylation in human cancers (Esteller et al 2001, Herman and Baylin 2003, Jones and Baylin 2002). Although some tumor suppressor genes are affected in multiple tumor types, such as CDKN2A, increasing evidence has shown that the pattern of hypermethylation in malignant cells is not random but rather tumor type specific (Costello et al 2000, Esteller et al 2001). These properties of DNA methylation in cancer have opened up new opportunities to utilize DNA methylation as biomarkers for

cancer classification, early diagnosis, and prognosis (Kagan et al 2007, Sepulveda et al 2009, Shi et al 2007).

Approaches

Methods for studying DNA methylation can be classified into those precisely determining the methylation status of a particular gene and those allowing genome-wide assessment of variation in DNA methylation pattern. The methods to determine the methylation status of particular genes are generally based on the sodium bisulfite treatment of DNA, which can convert unmethylated cytosine to uracil and leave methylated cytosine unaffected. Thus, in a methylation specific PCR (MSP), PCR primers can be designed to amplify specifically either the methylated or unmethylated targets. Other techniques for analysis of methylation status of single genes are summarized in Table 11. Microarray-based DNA methylation profiling allows the analysis of a large number of genes simultaneously or even a genome-wide screening of the methylome. Currently, a commercial canine oligonucleotide DNA methylation microarray is available from NimbleGen (http://www.nimblegen.com).

Clinical relevance of DNA methylation in lymphoma

In recent years, with the advances in microarray-based platforms for studying DNA methylation, an increasing number of genes with aberrant methylation changes have been identified and mapped in a wide range of tumor types. In human NHL, genes that have been frequently methylated include *DARK, CRBP1, p57KIP2, CDKN2A/B, p73, SHP1, RARB2, HIC1,* and *MGMT* (Esteller 2003, Hayslip and Montero 2006). It has become apparent that each tumor is unique in methylation profile. Thus, these tumor type specific methylation genes may have potential as biomarkers for cancer detection. For example, Esteller et al. reported that the use of a panel of three to four methylation markers can define an abnormality in 70 to 90% of tumor cases (Esteller et al 2001). Wang et al. also described a methylation-based method using the genes *DLC-1, PCDHGA12* and *RPIB9* to detect B-cell neoplasms (Wang et al).

Furthermore, methylation markers can also be used to distinguish tumor subtypes (Paz et al 2003). Guo et al. identified a group of genes whose methylation profiles differed between subtypes of human small B-cell lymphomas (SBCL), including MCL, FL, and B-cell chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), which approximately represent the pre-germinal center, germinal center and post-germinal center lymphocyte differentiation stages, respectively (Guo et al 2005). A subsequent study with a larger sample size developed a panel of methylation markers that allows effective differentiation of SBCL subtypes (Rahmatpanah et al 2006).

Another potential use of the DNA methylation marker is in the assessment of prognosis. A number of studies have demonstrated the link between aberrant methylation and patient outcome in a variety of cancers. For example, Esteller et al. reported that the presence of O-6-methylguanine-DNA methyltransferase (*MGMT*) gene hypermethylation was associated with increased patient survival in human DLBCL treated with alkylation therapy (Esteller et al 2002). The MGMT protein is a DNA repair enzyme responsible for repairing alkylation damage of the DNA base guanine. This base is a target of alkylating agents, such as BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) and cyclophosphamide-based chemotherapy. When the function of MGMT is lost, the malignant cells become sensitive to the effects of alkylating agents, since the DNA damages cannot be repaired and results in programmed cell death. Thus, *MGMT* hypermethylation is a favorable prognostic marker in lymphoma treated with alkylation therapies. A similar scenario was observed in gliomas (Esteller et al 2000). LMNA is another example of a predictive marker for human DLBCL (Agrelo et al 2005). Inactivation of the nuclear lamin LMNA by aberrant methylation has been associated with poor outcome in DLBCL (Agrelo et al 2005). Furthermore, aberrant methylation of tumor suppressor genes is a common event in cancer cells and might be of prognostic significance. For example, hypermethylation of Death Associated Protein Kinase 1 (*DAPK1*) and *CDKN2A* have been linked to a poor outcome in human patients with multiple myeloma (MM) (Mateos et al 2002, Ng et al 2001). Aberrant methylation of *CDKN2B* has been associated with poor outcome in human acute myelogenous leukemia (AML) (Chim et al 2001). A recent study reported an extensive analysis of the methylation profile of 15 tumor

suppressor genes in 251 patients with human acute lymphocytic leukemia (ALL) (Roman-Gomez et al 2004). This study showed an adverse effect in the number of methylated tumor suppressor genes on the outcome of the ALL patients.

In veterinary studies, limited information has been published on the examination of methylated genes in canine lymphomas. Until now, only one gene *DLC-1* has been reportedly hypermethylated in canine lymphoma (Bryan et al 2009). Aberrant methylation of *CDKN2A* is commonly observed in human NHL but is rare in canine lymphoma (Fosmire et al 2007). The methylation status of *FHIT* has been examined in canine lymphoma cell lines, but no aberrant methylation was observed (Hiraoka et al 2009). Additional methylation studies are required to establish the diagnostic/prognostic markers or the methylation patterns associated with specific subtypes of canine lymphomas.

Prognostic significance of gene expression profiling in lymphomas

Cancer is a heterogeneous disease. During the tumorigenic process, different genetic or epigenetic lesions may occur that can result in distinct transcriptome, which is associated with a distinct tumor phenotype. Gene expression profiling (GEP) using microarray platforms represents a powerful tool to explore the expression of thousands of genes simultaneously. In the context of cancer, GEP has been used to accurately classify tumors or define tumor subtypes. The molecular signatures derived from gene expression profiling might have an impact on diagnosis, prognosis, and therapy selection.

Approaches

Microarray

Array-based GEP can be performed on two main types of microarrays, including cDNA platform and oligonucleotide platform. cDNA microarrays have PCR-amplified cDNA fragments deposited onto a solid array surface (e.g., glass slide), whereas oligonucleotide microarrays are fabricated using either a photolithographic process that directly synthesizes oligonucleotide sequence onto the array surface or deposition of oligonucleotides onto the array surface (Relogio et al 2002, Yeatman 2003). Both types of microarray are hybridized

with cDNA samples derived from tissues of interest to assess expression changes in mRNA expression levels. In comparison with cDNA microarrays, oligonucleotide microarrays represent higher specificity and enable detection of alternatively spliced mRNAs. There are a number of commercial available oligonucleotide microarrays. For canine research, the providers include Affymetrix Inc (http://www.affymetrix.com) and Agilent Technologies (http://www.agilent.com).

Real-time quantitative PCR (RT-qPCR)

Gene expression profiling methods such as microarrays generate candidate genes that need further validation. RT-qPCR is the method of choice for validation of results derived from array-based analyses. In RT-qPCR, a target gene can be amplified and simultaneously quantified. Currently, four common approaches are available for RT-qPCR, including TaqMan (Applied Biosystems), Molecular Beacons, Scorpions and SYBR Green. In all of these approaches PCR products are detected through the generation of a fluorescent signal. In the SYBR Green method, fluorescent dye bound to double-stranded DNA is measured to quantify the amount of PCR products. In the other three systems, the fluorescence resonance energy transfer (FRET) oligonucleotide probe is used as the reporter system to quantitate the PCR product through a fluorescent dye molecule coupled to the 5' end and a quencher molecule coupled to the 3’ end of an oligonucleotide substrate.

Clinical relevance of gene expression profiling in lymphoma

In the past decade, the application of gene expression profiling (GEP) has contributed to the identification of unique gene expression signatures for the major groups of human NHLs. For example, within human DLBCL, GEP studies defined two distinct molecular subtypes: the germinal center B-cell-like (GCB) subtype and the activated B-cell-like (ABC) subtype (Alizadeh et al 2000). GCB DLBCL expresses genes that are characteristic of normal germinal center B-cells, whereas ABC DLBCL expresses genes induced during mitogenic stimulation of peripheral blood B-cells. These two subtypes represented a significant difference in patient outcome following a standard multi-agent chemotherapy regimen

(Alizadeh et al 2000). The GCB DLBCL subtype showed a favorable clinical outcome. These observations were further confirmed in several more recent GEP studies performed by different microarray platforms and in the different context of chemotherapies (Bea et al 2005, Monti et al 2005, Rosenwald et al 2002, Shipp et al 2002, Wright et al 2003). Furthermore, the survival predictor genes defined by GEP can be used for constructing survival prediction models. Rosenwald et al. established a survival prediction model for human DLBCL that comprised the expression level of 17 genes that represent four prognosis signatures ('GCB cell', 'MHC class II', 'lymph node', and 'proliferation') (Rosenwald et al 2002). Shipp et al. also derived another 13-gene predictive model for human DLBCL using the GEP from 58 patients (Shipp et al 2002). To provide a technically simple model for clinical use, a six-gene model (*LMO2, BCL6, FN1, CCND2, SCYA3* and *BCL2*) has recently been described for the prognosis of human DLBCL (Lossos et al 2004).

Notably, some of the genes comprising the predictive model were among the single prognostic markers in human DLBCL, such as *BCL6* and *BCL2*. For example, *BCL2* expression has been associated with poor disease free survival (DFS), overall survival (OS), or relapse-free survival (RFS) in ABC DLBCL (Gascoyne et al 1997, Hermine et al 1996, Hill et al 1996, Iqbal et al 2004, Kramer et al 1996); *BCL6* expression has been associated with longer overall survival in human DLBCL (Lossos et al 2001, Winter et al 2003).

In veterinary studies, GEP analysis in canine lymphoma is currently underway, which, once complete, will allow the identification of informative expression signatures for each subtype. Currently, few publications have addressed the clinical relevance of specific genes. Sato et al. have examined the prognostic significance of *BCL6* expression in canine DLBCL (Sato et al 2011). They found insignificant correlation observed between *BCL6* mRNA expression and DFS or OS, which is inconsistent with the findings reported in human DLBCL. In addition, they reported that the BCL6 protein cannot be detected in canine DLBCL using immunohistochemistry.

Thesis research goals

With knowledge generated by different platforms on various types of human lymphoid malignancies, the application of molecular markers seems to bring a new dimension to lymphoma diagnosis and prognosis. However, due to the extensive heterogeneity of human populations, the translation of these potential molecular diagnostic and prognostic markers into clinical tests and towards the development of targeted therapies has proved more difficult than originally expected. As mentioned above, a comparative approach might provide a means to alleviate this challenge and benefit both human and veterinary medicine. Currently, limited information has been published on the identification of molecular markers in canine lymphoma tissues. To improve the clinical management of dog patients and provide opportunities for comparative studies, the general aim of this work, is to identify molecular markers with potential clinical value in canine lymphoma.

Cytogenetic markers have long been used in the diagnosis and assessment of human NHLs. In canine lymphoma studies, our lab has previously identified several recurrent chromosomal aberrations; however, investigation of the prognostic significance of these recurrent aberrations has been hampered by the lack of large numbers of patients with clinical follow-up data. Therefore, the first goal of this study is to evaluate the potential clinical value of these recurrent chromosomal aberrations using fluorescence *in situ* hybridization (FISH) analysis in a cohort of 160 archival paraffin-embedded specimens derived from dogs enrolled in a multi-center clinical trial, all receiving the same treatment. Our hypothesis is that these chromosomal aberrations in canine lymphoma are of prognostic significance.

With a complete high quality canine genome sequence assembly and available tools for studying gene expression, there is increasing interest in examining canine tumors at the molecular level. However, it has become clear that the commonly used reference genes such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta actin (*ACTB*) may not be expressed consistently across all tissue types and disease states. Normalization of data using these reference genes without proper validation can result in inaccurate conclusions in the study findings. In order to increase the accuracy and sensitivity of our following gene expression experiments, our second goal, is to validate suitable reference genes for real-time

quantitative PCR in canine experimental systems. The idea is to compile canine aCGH data generated from the evaluation of three different canine tumor studies and identify regions considered to be highly stable in DNA copy number across these cancers. Candidate reference genes are selected from these regions. The hypothesis is that genes from relatively copy number stable regions of the genome demonstrate greater expression stability across multiple tissue types. The stability of these candidate genes is evaluated in canine lymphoma, histiocytic sarcoma and osteosarcoma using three different algorithms to determine suitable reference genes for each of canine disease state.

To gain more insight into the genetic mechanisms underlying tumor development and disease progression in canine lymphoma, our third goal, is to identify potential target genes residing within these recurrent chromosomal aberrations identified in canine lymphoma. To achieve this, we select a series of candidate genes mapped in these affected genomic regions based on their established roles in tumor development and history of altered gene expression in human NHLs. Such genes are subjected to gene expression analysis as well as to cytogenetic analysis in a series of fresh canine lymphoma samples and corresponding non-neoplastic tissues to identify gene-dosage regulated genes that might be potentially important in canine lymphoma pathology.

Furthermore, the homeobox gene *MEOX-2* is a transcription factor that controls many aspects of vascular cellular processes and has been recognized as a negative regulator of angiogenesis; however, the expression and functional role of *MEOX-2* in a disease state has remained largely unknown. A preliminary study presented in Chapter 4 demonstrates that *MEOX-2* expression is uniformly silenced in all studied lymphoma cases. Therefore, our final goal is to elucidate further how homeobox gene *MEOX-2* is deregulated in canine lymphoma To achieve this, approaches for investigation of epigenetic**,** genetic**,** and gene expression status are conducted to access the molecular alterations of *MEOX-2* in canine lymphoma.

Table 1. Comparison of common NHL subtypes in humans and dogs according to the WHO classification

	Comments	
Tumor classification	human	Dog
B-cell NHL	More than 85-90% of cases	Approximately 60% of cases
Follicular lymphoma	Accounts for 20-25% of cases	Fewer than 5% of cases
Diffuse Large B-cell lymphoma	Most common subtype; accounts for up to 40% of cases	Most common subtype; accounts for up to 45% of cases
Mantle cell lymphoma	Accounts for approximately 6%	Rare (less than 1%)
Marginal zone lymphoma	Most commonly diagnosed as a splenic tumor, about 2% occur in lymph nodes	Most common indolent B-cell NHL subtype in dogs
Burkitt's lymphoma	Most common form is endemic (EBV-associated), sporadic form not associated with EBV	Only occur as sporadic form, a gamma-herpesvirus of dogs has not been identified
Small lymphocytic lymphoma	Solid counterpart of CLL	Solid counterpart of CLL, more often has T-cell phenotype
T-cell lymphoma	Fewer than 10%	Approximately 40%
Precursor T-lymphoblastic lymphoma	Associated with poor prognosis	Associated with poor prognosis, may be more common in dogs than in human
Peripheral T-cell lymphoma-not otherwise specified	Associated with poor prognosis	Associated with poor prognosis, may be more common in dogs than in human
Angioimmunoblastic lymphoma	Mature T-cell NHL subtype with poor prognosis	Rare in dogs
Anaplastic large cell lymphoma	Mature T-cell NHL subtype with best prognosis, forms include $CD30^+$ and $CD30^-$, associated with inappropriate expression of ALK	Can have B-cell phenotype, expression of CD30 and ALK as yet undefined.; uncommon in dogs (<2%)
T-zone lymphoma	Among few types of indolent T-cell lymphoma	Most common type of indolent T-cell lymphoma (accounts up to 37%)
Small lymphocytic lymphoma	T-cell phenotype is rare	Most common solid counterpart to canine CLL, indolent progression and good prognosis

Abbreviations: ALK, activated lymphocyte kinase; CLL, chronic lymphocytic leukemia; EVB, Epstein-Barr virus; NHL, non-Hodgkin's lymphoma.
(Modified from Modiano JF, Breen M, Valli VE, Wojcieszyn JW, Cutter GR (2007). Predictive value of p16 or Rb inactivation in a model of naturally occurring canine non-Hodgkin's lymphoma. Leukemia 21: 184-187)

Table 2. Classification of canine lymphoma according to National Cancer Institute Working Formulation (NCI-WF) and updated Kiel system

Grade	NCI-Working Formulation	Updated Kiel system
Low grade	Small lymphocytic	Lymphocytic
	Follicular small cleaved	Lymphoplasmacytic
	Follicular mixed small cleaved	Centrocytic
		Centroblastic-centrocytic
Intermediate grade	Follicular large cell	
	Diffuse small cleaved cell	
	Diffuse mixed small and large	
	Diffuse large cell	
Low grade	Diffuse immunoblastic	Centroblastic
	Diffuse lymphoblastic	Lymphoblastic T-cell
	Diffuse small noncleaved	Lymphoblastic B-cell
		immunoblastic

Table 3. World Health Organization Clinical Staging System for canine lymphoma

Stage	Location
I	Involvement limited to a single node or lymphoid tissue in a single organ
II	Involvement of many nodes in a regional area or on the same side of the diaphragm
III	Generalized lymph node involvement
IV	Liver and/or spleen involvement (with or without stage III disease)
V	Manifestation in the blood and involvement of bone marrow and/or other organ systems (with or without stages I to IV disease
Substage	
A	Without systemic signs
B	With systemic signs

Table 4. Commonly used treatment terminology in this study

Terms	Definition
Complete remission	Complete resolution of clinical signs and measurable tumor based on physical examination, hematological or biochemical monitoring, or diagnostic imaging
Partial remission	Under the RECIST criteria, partial remission is defined as a reduction in the sum of the longest diameter of all measurable lesions of 30% or greater, with no new lesions development
Stable disease	Stable disease is to describe a tumor that is neither growing nor shrinking
Progressive disease	Progressive disease is defined as an increase in the sum of the longest diameter of all measurable lesions by 20% or greater, or the development of new lesions.
Relapse	Recurrence of clinical signs or measurable tumor
Disease free interval	The time complete remission was achieved to the time of progression

Table 5. Modified University of Wisconsin-Madison canine lymphoma treatment protocol (UW-19)

	Week															
Drug	1	2	3	4	6	7	8	9	11	12	13	14	16	17	18	19
L-asparaginase [a]	○															
Vincristine 0.7mg/m^2, IV	○		○		○		○		○		○		○		○	
Cyclophosphamide 250mg/m^2 PO or IV [b]		○				○				○				○		
Doxorubicin 30mg/m^2 IV [c]				○				○				○				○
Prednisone 2.0 mg/kg PO SID	○															
Prednisone 1.5 mg/kg PO SID		○														
Prednisone 1.0 mg/kg PO SID			○													
Prednisone 0.5 mg/kg PO SID				○												

[a.] L-asparaginase has now been removed from this protocol
[b.] Furosemide (1mg/kg) is given intravenously concurrently with cyclophosphamide to reduce the incidence if sterile hemorrhagic cystitis.
[c.] In dogs that weigh less than 15 kg, a doxorubicin dose of 1mg/kg is substituted for 30 mg/m^2
IV, Intravenously; PO, orally; SID, once a day.

Table 6. Summary of the most commonly used combination or single agent chemotherapy protocols for canine lymphoma

Protocol	Complete remission rate (%)	Median survival time (months)	Note
CHOP+L/CHOP	80-90%	12	1. Several studies have confirmed that the addition of L-asparaginase does not affect the remission rate, first remission duration. 2. 25% of dogs can survive longer than 2 years 3. The shortest protocol takes 19 weeks
COP	60-70%	6-7	Low toxicity
Single agent doxorubicin	75-85%	6-8	30mg/m^2 given intravenously every three weeks, totally 6 treatments
Prednisone	50%	1-2	1. 2mg/kg/given orally daily 2. More likely to induce multiple drug resistance

L: L-asparaginase; C: cyclophosphamide; H: doxorubincin; O: vincristine; P: Prednisone

Table 7. Prognostic factors for lymphoma in dogs

Factor	Comments
WHO clinical substage	Substage b associated with unfavorable survival
Histopathology	High/intermediate grade associated with high chemotherapy response rate but reduced survival if left untreated
Immunophenotype	T-cell phenotype is associated with unfavorable survival; one of the most important independent factors
Hypercalcemia	Negative factor of associated with T-cell subtype
Proliferation index AgNOR	Increased AgNOR counts associated with shorter remission duration and survival time
p-glycoprotein expression	May be associated with poor response rate and shortened remission; chemotherapy drugs can be removed from tumor cell intracellular space through this efflux pump; associated with multiple drug resistance
Anatomic sites	Leukemia, diffuse cutaneous and alimentary, and hepatosplenic forms are associated with unfavorable prognosis
Serum VEGF	High level of VEGF has been associated with substage b and worse disease free interval
Response to treatment	Complete remission is associated with significantly longer survival time
Pretreatment with steroid	Less likely to response to combination chemotherapy and associated with shorter survival; may cause multiple drug resistance

Table 8. Cytogenetic markers associated with particular subtypes of human NHL

Diagnosis	Cytogenetic markers	Genes involved
Follicular lymphoma	t(14;18)(q32;q21)	*IGH* and *BCL2*
	t(3;v)(q27; v)	*BCL6* and numerous partners
MALT lymphoma	t(11;18)(q21;q21)	*API2* and *MALT1*
	t(14;18)(q32;q21)	IGH and *MALT1*
Mantle cell lymphoma	t(11;14)(q13;q32)	*CCND1* and *IGH*
Diffuse large B-cell	t(14;18)(q32;q21)	*IGH* and *BCL2*
lymphoma	t(3;v)(q27; v)	*BCL6* and numerous partners
Burkitt's lymphoma	t(8;14)(q24;q32)	*MYC* and *IGH*
	t(2;8)(p12;q24)	*MYC* and *IGK*
	t(8;22)(q24; q11.2)	*MYC* and *IGL*
Anaplastic large cell	t(2;5)(p23;q35)	*ALK* and *NPM*
lymphoma	t(2;v)(p23;v)	*ALK* and numerous partner

Table 9. Summary of the regions showing homozygous loss detected by aCGH in human NHLs

Lymphoma	Region of homozygous loss	Candidate genes	Ref.
Diffuse large B-cell lymphoma	3p14.2	*FHIT*	(Tagawa et al 2004)
	9p21.1	*CDKN2A*	
Primary mediastinal B-cell lymphoma	16p13.13	*SOCS1*	(Mestre et al 2005)
Mantle cell lymphoma	1p32.3	*CDKN2C*	(Mestre-Escorihuela et al 2007, Rubio-Moscardo et al 2005, Tagawa et al 2005a, Taylor et al 2006)
	2q13	*BIM*	
	9p21.3	*CDKN2A*	
	11p12-p14	*ATM*	
	11q14-q23	*RB1*	
	13q14.2	*KAL*	
	Xp22.3		
Nodular lymphocyte predominance Hodgkin'slymphoma	17q24		(Atayar et al 2006)
Burkitt's lymphoma	2q13-q21	*BIM*	(Mestre-Escorihuela et al 2007)
	4q35.1		
	18q21.3	*NOXA*	

Table 10. Cytogenetic markers with prognostic significance in human NHL.

Chromosomal region	Candidate genes	Prognostic indication	Lymphoma subtype	Ref.
Deletion of 1p31.1	*PTGFR. IFI44L, IFI44*	Favorable outcome	DLBCL	(Chen et al 2006)
Deletion of 2p25	*RNASEH1, RPS7, CLOEC11, ALLC*	Poor outcome	DLBCL	(Chen et al 2006)
Deletion of 9p21.3	*CDKN2A*	Poor outcome; aggressive disease	DLBCL	(Tagawa et al 2005b)
Deletion of 16p11	*FLJ43855, TP53TG3*	Poor outcome	DLBCL	(Chen et al 2006)
t(14;18)(q32; q21)	*BCL2*	Shorter survival	DLBCL	(Barrans et al 2003)
t(3;v)(q27; v)	*BCL6*	Favorable outcome	DLBCL	(Offit et al 1994)
Deletion of 13q and gain of 1q, 7q		Negative factor	BL	(de Souza et al , Garcia et al 2003, Lones et al 2004, Poirel et al 2009)
Loss of 6q and 17p		Unfavorable survival	FL	(Hoglund et al 2004, Viardot et al 2002)
Deletions of 9p21.3	*CDKN2A*	Poor outcome	MCL	(Rubio-Moscardo et al 2005)
Deletion of 17p13.1	*TP53*	Poor outcome; disease progression	MCL	(Rubio-Moscardo et al 2005), (Allen et al 2002) , (Au et al 2002, Maravelaki et al 2004)
Deletion of 9q21-22	*CDC14B and FANCC*	Poor outcome	MCL	(Rubio-Moscardo et al 2005)
Deletion of 1p21		Prolong outcome	MCL	(Rubio-Moscardo et al 2005)
Additional copy of X		Unfavorable outcome	MALT	(Schlegelberger et al 1996)

Abbreviations: DLBCL: Diffuse large B-cell lymphoma; BL: Burkitt's lymphoma; FL: follicular lymphoma; MCL: mantle cell lymphoma; MALT: mucosa-associated tissue (MALT) lymphoma

Table 11. Techniques for analysis of DNA methylation status of single genes

Technique	Methylation detection	Platform	Ref.
Methylation specific PCR	Bisulfite treatment	Electrophoresis	(Herman et al 1996)
COBRA	Bisulfite treatment and enzymatic digestion	Electrophoresis	(Xiong and Laird 1997)
Bisulfite sequencing	Bisulfite treatment	Sequencing	(Frommer et al 1992)
MS-SNuPE	Bisulfite treatment	Electrophoresis	(Gonzalgo and Jones 1997)
MethyLight	Bisulfite treatment	Real time PCR	(Eads et al 2000)
MS-MCA	Bisulfite treatment	Real time PCR	(Worm et al 2001)
MS-DGGE	Bisulfite treatment	Electrophoresis	(Aggerholm et al 1999)
MS-SSCA	Bisulfite treatment	Electrophoresis	(Maekawa et al 1999)

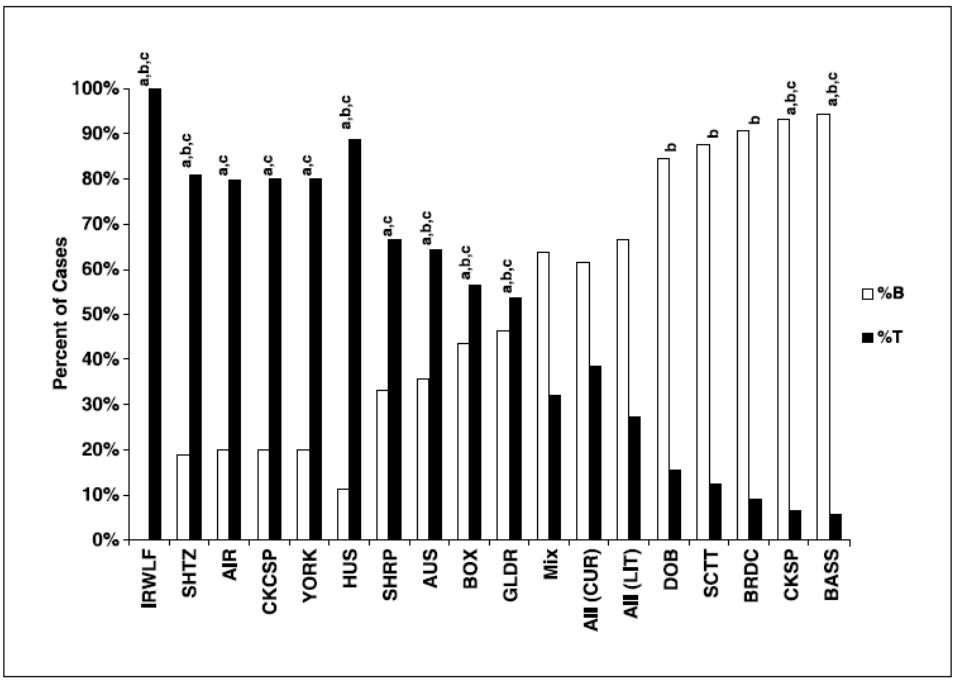

Figure 1. Phenotype distribution of breeds that are significantly different from reference populations. Frequency distribution of B-cell and T-cell immunophenotypes for LPD. Mixed-breed dogs and the reference populations for all dogs in the current data set and all dogs from previous reports in the literature are shown for comparison. IRWLF, Irish Wolfhound; SHTZ, Shih Tzu; AIR, Airedale Terrier; CKCSP, Cavalier King Charles Spaniel; YORK, Yorkshire Terrier; HUS, Siberian Husky; SHRP, Chinese Shar-Pei; AUS, Australian Shepherd; BOX, Boxer; GLDR, Golden Retriever; DOB, Doberman Pinscher; SCTT, Scottish Terrier; BRDC, Border Collie; CKSP, Cocker Spaniel; BASS, Basset Hound; Mix, mixed-breed dogs; ALL (CUR), all dogs in this study; ALL (LIT), all dogs reported previously in the literature. a, significantly different from mixed-breed dogs in current data set by χ^2 test; b, significantly different from all other dogs in current data set by χ^2 test; c, significantly different from all other dogs reported previously set by χ^2 test.
(From Modiano JF, Breen M, Burnett RC, Parker HG, Inusah S, Thomas R *et al* (2005). Distinct B-cell and T-cell lymphoproliferative disease prevalence among dog breeds indicates heritable risk. *Cancer Res* **65:** 5654-5661.)

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CHAPTER 2: Canine lymphoma- the Prognostic Significance of Cytogenetic Changes

Abstract

Canine lymphoma accounts for nearly one-quarter of all cancers in dogs. Although it is treatable with chemotherapy, response to treatment and survival times remain unpredictable. In our previous studies, the most recurrent chromosomal aberrations identified in canine lymphoma included regions of CFA 1, 6, 11, 13, 14, 18, 31, 37, and 38. However, the significance of these aberrations in canine lymphoma is unknown. To determine the prognostic value of chromosomal aberrations identified in dogs with lymphoma, we analyzed 160 archival pre-treated lymphoma specimens derived from a cohort of 322 canine patients recruited to a multi-center clinical trial, all receiving the same treatment: doxorubicin plus L-asparaginase. Using genome integrated bacterial artificial chromosome (BAC) contigs as probes in interphase fluorescence *in situ* hybridization (FISH) analysis of cells isolated from paraffin-embedded biopsy specimens, cases were evaluated for the copy number of each of the nine loci of interest. Based on the splitting-sample approach, the data obtained using regression analysis from a training set comprising 121 cases showed that the mean copy number of A1 locus (located on CFA 1) was positively related to disease-free interval (DFI) of dog patients with lymphoma. A validation cohort of 39 cases was used to confirm the findings. These results indicate that progressive increase in copy number of A1 correlates with prolonged DFI. Further studies are required to further validate these observations and to define the potential roles of such factors as predictors of prognosis.

Introduction

Lymphoma (LSA) is the most common life-threatening cancer in dogs, accounting for up to 24% of all canine neoplasia and over 80% of all canine hematopoetic cancers (MacEwen 1990, Moulton and Harvey 1990). Untreated dogs usually die within 4 to 6 weeks (MacEwen et al 1977), but the disease is treatable with chemotherapy; dogs that achieve complete remission are usually free of clinical signs of the disease and subsequently return to a good quality of life (Hansen and Khanna 2004). Many chemotherapeutic protocols for dogs have

been studied and reported over the years. Protocols based on CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone, and with or without L-asparaginase) induce complete remission in 80% to 90% of cases, with median survival time of 12 months according to the protocol used (Vail and Young 2007). Other choices, such as single agent doxorubicin, have a complete remission rate of 75% to 85% with a median survival time of 6 to 8 months (Mutsaers et al 2002, Page et al 1992, Valerius et al 1997). Prednisone alone can result in short-lived remission of 1 to 2 months (Vail and Young 2007). In general, complete remission can be achieved in 60% to 90% of cases treated with conventional chemotherapy; the median survival is 6 to 12 months, depending on the protocol chosen. However, among treated cases receiving the same diagnosis and chemotherapy, there is considerable variation in the extent of response to treatment and survival time (Teske 1994, Teske et al 1994). Especially in those cases that fail to achieve remission and have a short survival time, the use of chemotherapy becomes economically difficult to justify.

To help the client and clinician make key decisions about treatment, there have been many efforts to identify factors of prognostic significance in canine lymphoma. Unlike human non-Hodgkin's lymphoma (NHL), a limited number of prognostic factors have been consistently identified as prognostically important in dogs with lymphoma in different studies. These have included immunophenotype and clinical substage (Dobson et al 2001, Greenlee et al 1990, Jagielski et al 2002, Keller et al 1993, Ruslander et al 1997, Teske et al 1994, Vail et al 1996). However, in all of these prognostic factors, there was considerable residual heterogeneity in the response of therapy and clinical outcome, suggesting that the incorporation of appropriate prognostic markers into the current prognostic system is required to generate more refined schemes for patient prognosis. These variations in patient outcome might be attributed to the heterogeneity of genetic and molecular abnormalities that occur during tumor development and progression. Thus, a better understanding of the genetic and molecular mechanisms underlying the diverse clinical manifestations of lymphomas is not only biologically important but also of prognostic significance.

In human patients, molecular cytogenetic analysis such as comparative genomic hybridization (CGH) has provided a comprehensive view of copy number alterations in a

variety of NHL subtypes. Fluorescence *in situ* hybridization (FISH) has long been used to characterize lymphoma-related chromosomal abnormalities in cases for which CGH has proven uninformative; it is now a routine part of the molecular diagnosis of a significant number of lymphoid malignancies (Campbell 2005, Chaganti et al 2000). The complex patterns of genetic abnormalities observed in these lymphomas reflect their biological and clinical diversity. As mentioned in the previous chapter, there is increasing evidence that cytogenetic evaluation of human NHL provides the clinician with important diagnostic and prognostic information that is not available through traditional pathology and clinical staging systems. As dogs and humans are physiologically similar and share extensive genome homology, we suggest that canine lymphoma should also present recurrent chromosomal aberrations and that these aberrations might be of prognostic significance (Breen et al 1999, Breen et al 2001, Breen et al 2004, Guyon et al 2003, Yang et al 1999).

Previously, our group identified several recurrent chromosomal aberrations, including gain of dog chromosome (CFA) 13 and 31 and loss of CFA 11 and 14 (Modiano et al 2005, Thomas et al 2003). Including 25 lymphoma cases reported by Thomas et al. 2003 (Thomas et al 2003), an additional 15 cases were subsequently evaluated by CGH analysis, bringing the data set to a total of 40 cases of lymphoma. From the cytogenetic profile of these 40 cases, we identified nine recurrent chromosomal aberrations in canine lymphoma, including aberrations located on CFA 1, 6, 11, 13, 14, 18, 31, 37, and 38 (unpublished data). More recently, a larger series of 150 lymphoma cases were analyzed using aCGH analysis (Thomas et al 2011). The aberrations most frequently found in B-cell lymphoma (n=113) were gains of CFA 13 and 31 and losses of CFA 14 and CFA 26q24. In T-cell lymphoma (n=37), recurrent aberrations include gains of CFA 6, 9, 13, 20, 29, 31, and 36 and losses of CFA 11, 17, 22, 28, and 38. Several of the aberrations were strongly associated with a particular immunophenotype or with dog breed. For example, loss of CFA 11 was restricted to T-cell lymphoma, whereas deletion of CFA 26q24 was seen exclusively in B-cell lymphoma; copy number aberrations of CFA 6, 12, 20, and 31 were significantly more common in Boxers with T-cell lymphoma. However, investigation of the prognostic significance of recurrent

chromosomal aberrations has been hampered by the lack of large series of patients with standardized treatment and reliable clinical follow-up data.

Paraffin-embedded material is the most readily available source of a number of well-annotated tumor samples, including those of patients in clinical trials (Shipley and Fisher 1998). Molecular cytogenetic techniques for monitoring specific chromosomal aberrations associated with malignancies, such as interphase FISH, has proven applicable in paraffin-embedded materials (Cook 2004, Godon et al 2004, Summersgill et al 2008, Ventura et al 2006, Wehle et al 2008). Thus, the current study was designed to use interphase FISH to evaluate the prognostic significance of nine selected recurring chromosome aberrations in patients comprising a cohort of 160 archival paraffin-embedded specimens, all derived from dogs that were included in a multi-center clinical trial, all receiving the same treatment. Here, we first describe a new DNA probe design for interphase FISH assay in which up to five contiguous bacterial artificial chromosome (BAC) clones covering up to 800 kbp were selected to represent each locus of interest. The designed probes were then used to assess the copy number changes of nine recurring aberrations located on CFA 1, 6, 11, 13, 14, 18, 31, 37, and 38 in a population of 160 paraffin-embedded canine lymphoma specimens, which were divided into the training set and validation set. We correlated the FISH results obtained from the training set with the disease-free interval (DFI) of each patient to identify chromosomal aberrations with prognostic significance. The findings were subsequently validated in a validation set.

Materials and methods

Archival tissue specimens

To ensure a sufficient sample size of suitable cases for this study, a study population comprising 322 cases of canine lymphoma was recruited from dogs that were used as part of a multi-institutional clinical trial based at Colorado State University (CSU). All samples were lymphoma presentation cases, diagnosed between 1998 and 2002, and treated with the single agent doxorubicin plus L-asparaginase. Dog patients received doxorubicin at a dose of 30mg/m^2 every three weeks for a total of five treatments. Patients also received L-

asparaginase weekly for 3 weeks after the first dose of doxorubicin. At the conclusion of chemotherapy, the patients were randomized to receive either an investigational drug or placebo. Within this sample population, seventy-two dogs were considered as early failures, those that did not complete initial chemotherapy and did not receive investigational drug or placebo. The remaining patients (250 dogs) were randomized to receive either an investigational drug or placebo. At the end of this trial, the statistical analysis of the data showed that there was no significant difference in disease free interval (DFI) between dogs receiving placebo and dogs receiving investigational drug, and concluded that all patients of 250 dogs could be classified as a single population. Lymph node biopsy specimens from each case were taken at the time prior to treatment, fixed in zinc formalin fixative, and embedded in paraffin. For each case, data are available for age, gender, breed, histologic grade, DFI, and remission status at the end of the trial (partial remission, complete remission, stable remission and progressive disease).

A total of 160 cases were selected randomly from the sample population for analysis in this study. The median DFI of the 160 cases was 196 days (~6.5 months). The histological subtypes were classified according to National Cancer Institute Working Formulation as diffuse mixed small and large (12.2%); immunoblastic (37.8%); large cell (14.7%); lymphoblastic (30%); diffuse small noncleaved (2.6%), diffuse small cleaved (1.3%); and small lymphocytic (1.3%) (Figure 2A). Overall, 70.5% were high grade tumors, 28.2% were intermediate grade tumors, and only 1.3% were low grade tumors. The average age at diagnosis across these 160 cases was 7.7 years, (range 2 to15 years). Regarding dog breeds, 79% of the 160 cases were derived from 43 breeds. Breeds with more than five individuals include Golden Retriever (16 cases, 9%), Labrador Retriever (12 cases, 7%), Cocker Spaniel (10 cases, 6%), Rottweiler (9 cases, 5%), Basset Hound (6 cases, 4%), and German Shepherd (6 cases, 4%) (Figure 2B). The remaining 21% of the 160 cases were mixed breeds. Within the cohort of 160 cases, 87 cases were immunophenotyped and comprised 83 B-cell lymphoma cases, and 4 T-cell lymphoma cases. The demographic and clinical characteristics of the original sample cohort (322 cases) and our studied cohort (160 cases) are presented in Table 12.

Interphase fluorescent in situ hybridization

DNA probes description. The recurrent chromosomal aberrations identified for this study were derived from a previous study of 40 canine lymphoma cases using CGH analysis (unpublished data). For each of the nine recurrent chromosome aberrations, we first defined their minimal common region (MCR) based on evaluation of the combined CGH profiles of all 40 cases. For probe design, in each of the nine loci tested, up to five contiguous bacterial artificial chromosome (BAC) clones were selected in the middle of the MCR with the help of the UCSC dog genome browser. These BAC clones were obtained from the CHORI-82 canine BAC library (http://bacpac.chori.org/library.php?id=253). The overalpping BAC clones resulted in an effective probe size of up to 800 Kbp; Figure 3 shows a schematic representation of the probe design. The BAC clones used in this study are listed in Table 13. In order to validate that each BAC has a unique cytogenetic location as well as a normal copy number (n=2) in non-neoplastic cells, all clones were hybridized first onto metaphase chromosome preparations of lymphocytes from a panel of five clinically normal dogs using conventional FISH. The procedures of the conventional FISH are described later.

The BAC DNA was extracted from 100 ml of bacterial cultures using a standard alkaline lysis method. The isolated BAC DNA was stored as aliquoted stocks frozen at -20°C until used. The quality of DNA was checked by agarose gel electrophoresis prior to performing the probe labeling. For each probe, equal amounts (150 ng) of each BAC clone DNA were pooled, labeled with either Spectrum Red-dUTP, Spectrum orange-dUTP, or Spectrum Green-dUTP fluorochromes (Vysis, Des Plains, USA) using standard nick translation in which the DNAse-I concentration has been adjusted to generate fragments that are in the range of 100 to 300 base pairs, a size that allows the probes to penetrate the fixed cell nuclei. The probes were then purified by nucleotide removal kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Nuclei isolation and slide preparation. Paraffin-embedded biopsies of lymph node tissue were used to obtain 25 μm thick sections. Regions for cutting were identified based on their pathology in standard hematoxylin and eosin-stained sections evaluated by CSU CVM

pathology service. One to three sections were placed in a sterile 1.5 ml microcentrifuge tube depending on the size of the tumor, and then de-waxed using five successive baths of 1 ml of xylene in 10-minute intervals at room temperature. The tissues were rehydrated by sequential, 5-minute incubations in 1 ml of 100%, 70%, and 50% ethanol and then rinsed twice in 1x phosphate-buffered saline (PBS). Next, the tissue fragments were treated with 0.5% pepsin (ready-to-use, pH=2, Dako, Carpinteria, CA) at 37°C for 20 minutes, rinsed in 1xPBS, and then incubated in new 0.5% pepsin at 4°C for 36 to 48 hours. After proteolytic digestion, cells were rinsed in 1x PBS, harvested by centrifugation, and fixed with 3:1 methanol-glacial acetic acid. Isolated nuclei were dropped on glass slides and air-dried prior to being dehydrated through an ethanol series (70%, 90%, 100%). The slides were then fixed in 3:1 methanol-glacial acetic acid fixative buffer for 45 min, air-dried, and subsequently pre-treated using the Dako Histology FISH Accessory Kit (Dako, Carpinteria, CA) according to the manufacturer's instructions. Briefly, the slides were heated in a 2-Morpholinoethanesulphonic acid (MES) containing pretreatment solution at 95°C for 10 minutes. After giving the slides a rinse in a Tris/HCl buffer, a proteolytic digestion step using 0.5% pepsin was applied to the slides at 37°C for 5 minutes.

In situ hybridization. The hybridization procedures were performed according to the method of Hyytinen et al. (Hyytinen et al 1994), but with some modifications. Briefly, purified, differentially labeled probes were mixed (200 ng of each), precipitated in the presence of 40 μg sonicated dog genomic DNA as competitor and resuspended in 40μl of hybridization buffer containing 50% deionized formamide, 10% dextran sulfate, and 2x standard saline citrate (SSC). The hybridization reaction was then added to the slides under a sealed coverslip and co-denatured at 80°C for 2 minutes. Hybridization was then performed overnight at 38°C in a humid chamber. After hybridization, the coverslips were removed, and the slides were washed in 2xSSC containing detergent (0.3% NP-40) at 73°C for 3 minutes and rinsed twice in a 2xSSC bath. Slides were counterstained with 80 ng/ml 4',6-diaminidino-2-phenylindole (DAPI) and mounted in an antifade solution (Vectashield mounting medium, Vector Laboratories, Burlingame, CA). Images were collected and processed using a multicolor FISH station comprising a Zeiss AxioPlan 2 ie fluorescence

microscope (Carl Zeiss, Thornwood, NY) equipped with narrow pass fluorescence filter sets and a CoolSnapHQ cooled CCD camera (Photometrics, Tucson, AZ). The system is controlled by SmartCapture 3 software (Digital scientific, Cambridge, UK). The average copy number of each of the nine loci was evaluated from counts of up to 30 nuclei per case. The four control specimens from normal lymph node yielded an average copy number of 2 for each probe. A schematic flowchart of the FISH process used is shown in Figure 4.

Conventional fluorescence *in situ* hybridization

DNA probe preparation. The BAC DNA was isolated using a standard alkaline lysis method. Isolated DNA was differentially labeled with fluorochromes for FISH analysis using standard nick translation to generate labeled fragments in the range 200-500 bp. Differentially labeled probes were mixed (25 ng of each), precipitated in the presence of 15 μg sonicated dog genomic DNA as competitor and resuspended in 15 μl of hybridization buffer containing 50% deionized formamide, 10% dextran sulfite, and 2x saline sodium citrate (SSC).

Chromosome preparation and slide preparation. Canine metaphase chromosomes were prepared for FISH by mitogenic stimulation of peripheral lymphocytes in RPMI 1640 supplemented with 20% fetal bovine serum and 100 mM L-glutamine. The cells were then treated with colcemid (0.2 μg/ml), and conventionally harvested using hypotonic treatment and 3:1 methanol/glacial acetic acid fixation, and dropped on glass slides. The slides were air-dried for one day prior to being used for FISH.

In situ hybridization. The DNA probe and competitor mixtures were denatured at 70°C for 10 minutes, and allowed to preanneal at 37°C for 30 minutes. The chromosome preparation slide was denatured in 70% formamide/2xSCC at 65°C for 2 minutes, quenched in ice cold 70% ethanol, and dehydrated through an ethanol series (70%, 90%, 100%). Preannealed probe was added to the denatured slides under a coverslip that was sealed with rubber cement. Hybridization was performed overnight at 38℃ in a humid chamber. After hybridization, the coverslips were removed, and the slides were washed in three exchanges of 50% formamide/2xSSC at 42℃ for 3 minutes and rinsed three times in a 2xSSC bath. Slides were

counterstained with 80 ng/ml 4',6-diaminidino-2-ohenylindole (DAPI) and mounted in an antifade solution (Vectashield mounting medium, Vector Laboratories, Burlingame, CA). Images were collected and processed using a multicolor FISH station comprising a Zeiss AxioPlan 2 ie fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with narrow pass fluorescence filter sets and a CoolSnapHQ cooled CCD camera (Photometrics, Tucson, AZ). The system is controlled by SmartCapture 3 software (Digital scientific, Cambridge, UK).

Statistical analysis

The 160 canine lymphoma cases were randomly assigned to either a training set (n=121) or a validation set (n=39). The demographic and clinical characteristics of these two cohorts are presented in Table 14. Categorical data were compared using Fisher's exact test or chi-square test, while numerical data were analyzed using Student's t test. The copy number status of each locus of interest was assessed. Univariate linear regression was used to screen which recurring aberrations were associated with disease free interval (DFI). In this trial, DFI was defined from the time of diagnosis to the time of first relapse. The relapse was determined by staging procedures, including complete history, physical exam, complete blood count, biochemistry profile, urinalysis, chest and abdominal radiographs, and Karnofsky's scores. Colinearity between nine aberrations was evaluated by examining pairwise correlations. Correlation between individual copy number aberrations and histologic grade were examined using contingency table analysis (Pearson 1904). Two-sided *P* values of less than 0.05 were considered to indicate statistical significance. Statistical analysis was conducted using JMP software (JMP 8, SAS, Cary, NC).

Results

DNA probe design and analysis

In general, stronger signals are obtained using probes with a larger target sequence (Summersgill et al 2008). To improve the signal size and intensity, three to five BAC clones containing overlapping genomic fragments that cover up to 800 kbp were used to design the

DNA probes. As shown in Figure 5, we compared signals between cells originating from fresh cell cultures (interphase and metaphase) with those obtained from paraffin-embedded tissues. Moreover, the effect of probe design (a single BAC clone or three to five contiguous BAC clones) was also added to the comparison. Analysis of the hybridization patterns reveals that the use of contiguous BAC clones provides larger, more robust fluorescent signals than a single BAC clone in both cell culture and paraffin-embedded derived cell nuclei. In addition, to confirm the normal copy number (n=2) and the genomic specificity of each designed probe, we hybridized the labeled probes representing each of the nine loci onto metaphase chromosome preparations from a clinically normal dog. As shown in Figure 6, on the normal control lymphocytes tested, all probes showed the normal copy number (n=2) and hybridized solely to the expected locations on their corresponding chromosomes.

Correlation between copy number alterations and DFI of the patients

After the probe validation, the interphase FISH assay was performed on paraffin-embedded lymphoma specimens to access the copy number status for all nine loci. Figure 7 shows examples of hybridization signals for several lymphoma cases and normal control. The 160 archival lymphoma specimens were randomly assigned to a training set (n = 121) or a validation set (n = 39). The clinical characteristics of the original sample cohort (n=322) versus the studied cohort (n=160) and the training set (n=121) versus the validation set (n=39) are provided in Tables 12 and 14, respectively. No differences were observed between the original sample cohort and the studied cohort regarding the distribution of age, weight, gender, and histologic grade (Table 12). The studied cohort have longer average DFI compared to the original sample cohort (P=0.026). The difference in average DFI might be due to the extreme values of DFI. The median DFI of the studied cohort was 178 days, which is comparable with 196 days for the original sample cohort. In addition, no differences in age, gender, histologic grade, and average DFI were observed between the training and the validation sets (Table 14). The validation set included patients with higher body weight compared to the training set (P=0.045).

The data for copy number status obtained from the training set (121 cases) were first compiled, and the mean value was calculated for each case. Univariate linear regression analysis was then performed to investigate the association between the mean copy number of each individual locus of interest and the DFI of the dog patients. Each of the loci was independently analyzed in a regression model. Table 15 summarizes the regression results for the nine individual loci tested. The mean copy number of A1 locus (located on CFA 1) and C2 locus (located on CFA 6) was positively associated with the duration of DFI ($P<0.0001$, $P=0.0103$, respectively). After a Bonferroni correction for multiple testing, only A1 kept its significance ($P< 0.001$). Dog patients with increased copy number of A1 locus were associated with prolonged DFI. The scatter plots of the mean copy number of each of the nine loci versus DFI based on the training data set are shown in Figure 8.

To confirm the results obtained from the training set, we carried out the same cytogenetic and statistical evaluation procedures in another subset of cases (validation set; 39 cases). Similar to the findings from the training set, the mean copy number of A1, C2, and C3 (located on CFA 37) was positively associated with DFI ($P=0.0018$, $P=0.0461$, $P= 0.0021$, respectively) as shown in Table 15. After a Bonferroni correction, A1 and C3 were still significant ($P=0.0162$, $P=0.0189$, respectively), but C2 was not. The scatter plots of DFI versus the copy number of each of the nine loci based on the validation data set are presented in Figure 9. We also repeated the regression analysis on all 160 cases (including both training and validation data sets) as shown in Table 15. Mean copy number of A1 and C2 was significantly associated with DFI, even after applying Bonferroni correction ($P<0.001$; $P=0.0369$). There results indicate a strong, replicable association between A1 copy number and DFI.

Furthermore, we examined the collinearity between nine loci to explore whether there is a linear relationship between these copy number aberrations. The correlation matrix for the nine loci based on 160 cases is shown in Table 16. Significant correlations are marked by an asterisk. The result indicates significant correlation between A3 (located on CFA 18) and C1 (located on CFA 38), between C1 and C2, as well as between C2 and C3. Moreover, the copy number status of each of the nine loci was also compared with the histologic grade. As

shown in Table 17, after Bonferroni correction, there were no significant differences in copy number aberration status based on histologic grade. The entire cytogenetic data set is available in Table 28 of the Appendix.

Discussion

FISH allows the detection of various numerical and structural chromosome changes in tumor samples and has become a firmly established technique in the diagnosis and assessment of human lymphoid malignancies (Campbell 2005, Chaganti et al 2000). Although reports of its use on paraffin-embedded materials have appeared for quite some time, FISH analysis of paraffin-embedded materials has proven to be more difficult than the analysis of fresh materials (DiFrancesco et al 2000, Hyytinen et al 1994, McKay et al 1997, Qian et al 1996). Indeed, problems observed in our initial hybridization tests include poor probe penetration, excessive background, and uncertain signals (see examples in Appendix, Figure 18). In an attempt to obtain better hybridization efficiency and more robust hybridization signals on our specimens, we investigated the effects of the probe design and demonstrated the advantage of using contiguous BACs probes instead of single BAC probe. Using these designed probes, we subsequently conducted an extensive cytogenetic study on a cohort of 160 canine lymphoma specimens, which were divided into training and validation sets. We first applied the univariate linear regression analysis to the training data set (121 cases) to find cytogenetic changes that correlated with the DFI. Among all nine chromosomal aberrations analyzed, only the copy number status of A1 locus was significantly associated with DFI. The increase of C2 copy number had a trend toward better DFI, although this did not achieve significance after Bonfferoni correction. These results were confirmed in an additional series of 39 cases (i.e. the validation data set). However, an unexpected finding in the validation data set was that the copy number status of C3 was significantly associated with DFI. Considering the small number of the study cohort, this finding could be due to chance, particularly since this marker was not significant in the larger cohort. Moreover, in addition to A1 locus, the copy number status of C2 was also associated with DFI after Bonfferoni correction when patients from two data sets were combined. As just mentioned,

the C2 locus appeared significant in both training and validation data set before adjustment, but it was not significant after Bonfferoni correction for multiple comparisons. These observations suggest a marginally or almost significant association between C2 and DFI. Independent work will be necessary to confirm a clinically useful predictive role for C2. Taken together, the results of these analyses demonstrate a strong positive association between A1 and DFI, i.e. patients with increased mean copy number of A1 showed a longer duration of DFI. These suggest that additional copy number aberrations may play an important role in the pathogenesis and prognosis of canine lymphoma.

The MCR of aberration on CFA 1 (harbors A1) shares extensive regions of conserved synteny with human chromosome (HSA) 19q. Copy number gain of 19q has been observed in human NHLs (Arranz et al 1996, Kaneko et al 1982, Ko et al 2010). In human diffuse large B-cell lymphoma (DLBCL), gain of 19q appears to be associated with activated B-cell-like (ABC) subtype (Lenz et al 2008, Tagawa et al 2005). A candidate oncogene *SPIB* has been implicated as a target gene at 19q, and its overexpression is associated with a gain of 19q in ABC DLBCL (Lenz et al 2008). These human findings are contradictory to our data from canine patients (since ABC DLBCL has been thought to have a more unfavorable clinical outcome than GCB DLBCL in human patients (Alizadeh et al 2000)), but still provides some clues for potentially important genes involved in A1 locus. *SPIB* plays a role in DLBCL, but the canine cohort is a mixture of NHL subtypes. Maybe that is part of the reason for the difference in direction of copy number aberration. The expression of *SPIB* in canine lymphoma will be discussed further in Chapter 4. In addition, amplification of oncogene *AKT2* has been observed in NHLs bearing gains at 19q (Arranz et al 1996). Because *AKT2* is frequently amplified in human solid tumors and overexpression of *AKT2* in transgenic mice has been reported to lead to spontaneous T-cell lymphomas (Altomare and Testa 2005, Staal and Hartley 1988), we suggest that *AKT2* may also be a candidate target gene of the A1 region. Moreover, other abnormalities involving 19q such as loss of 19q, loss of heterozygosity (LOH) of 19q, and t(14;19)(q32;q13) translocation involving *BCL3* and *IGH*, have also been reported in human NHLs (Mao et al 2002, Michaux et al 1997, Takeuchi et al 1995). The t(14;19)(q32;q13) translocation results in *BCL3* overexpression

and has been associated with rapid progression and poor prognosis in human CLL (Michaux et al 1997). *BCL3* is a recurrent translocation partner of *IGH* locus in human B-cell lymphomas (Szymanowska et al 2008). It is interesting to further explore its role and expression in canine lymphoma in the future.

The MCR of aberration on CFA 6 (harbors C2) shares conserved synteny with HSA 16p. Abnormalities such as gain of 16p, loss of 16p, rearrangements involving 16p, and loss of heterozygosity (LOH) of 16p have been reported in human mantle cell lymphoma (Obukhova et al 2004, Rinaldi et al 2006, Rudolph et al 2004), DLBCL (Karnan et al 2004, Katzenberger et al 2003) and follicular lymphoma (O'Shea et al 2009, Ross et al 2007). LOH of 16p has been associated with unfavorable survival in human follicular lymphoma (O'Shea et al 2009). Furthermore, the C2 locus harbors the canine *TSC2*. *TSC2* is a known tumor suppressor gene and has been shown to act as a negative regulator of cell growth and G1-S transition in the cell cycle (Astrinidis and Henske 2005, Soucek et al 1997). Loss of *TSC2* has been reported to accelerate the onset and severity of *MYC*-induced lymphomagenesis in mouse model (Mills et al 2008). To explore the possible relationship between *TSC2* and canine lymphoma and the potential target genes on the C2 region, *TSC2* expression will be further examined in canine lymphomas to determine whether gain or loss of C2 locus affects the expression of *TSC2* (see Chapter 4).

In addition, by reviewing the cytogenetic data of all 160 canine lymphoma cases, gain of B1 (located on CFA 31) was the most common aberration observed, followed by loss of C1 (located in CFA 38), loss of A3 (located in CFA 18), loss of B3 (located in CFA 14), gain of B2 (located in CFA 13), and loss of C3 (located on CFA 37) (see Appendix, Figure 19). However, the incidence of these aberrations showed some differences with other studies. Thomas et al. reported that gain of CFA 13 was the most common aberration observed, followed by gain of CFA 31 and loss of CFA 14 (Thomas et al 2003, Thomas et al 2011). In our series, B2 gains (on CFA 13) were found less frequently than B1 gains (on CFA 31) and B3 losses (on CFA 14). Loss of C1, A3, and C3 seems far more common than reported in other studies (Thomas et al 2003, Thomas et al 2011). CFA 31 is evolutionarily related to HSA 21 which is amplified (e.g., trisomy 21) in a variety of hematopoietic malignancies (e.g.,

childhood acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and acute megakaryoblastic leukemia (AMKL)), although not specifically NHL. Genes that confer oncogenic properties of HSA 21 have been widely discussed but remain incompletely understood. This may prompt us to carry out comparative investigation of the genetic material shared by CFA 31 and HSA 21 to accelerate progress in defining the underlying molecular pathogenesis in both species. CFA 13 is evolutionarily linked to two sites on HSA 8qdist and HSA 4pprox-qprox. HSA 8qdist harbors the oncogene *MYC* and HSA 4pprox-qprox the oncogene *KIT*. Activation of *MYC* or *KIT* has been implicated in a number of human NHLs and thus it is important to investigate the potential involvement of *MYC* and *KIT* in canine lymphomagenesis. The expression of *MYC*/*KIT* in canine patients will be discussed further in Chapter 4. CFA 14 shares conserved synteny with two sites on HSA 7p21-12 and HSA 7q11.22-32. Deletions of HSA 7q22-32 have been reported in human NHL, with a 40% incidence in splenic marginal zone B-cell lymphomas (MZBCL) and a 7% incidence in other forms of NHL (Brunning et al. 2001). In addition, CFA 38, CFA 37 and CFA 18 are evolutionarily linked to HSA 1q (scattered on 1q), HSA 2q33-37, and HSA 11(11p14-11 and 11q11-13.3), respectively. Although aberrations of these chromosomal regions seem not to be common in human lymphoma, these canine-specific abnormalities may also play a significant role in the pathogenesis and must be given equal attention.

Furthermore, in the present work, most of the lymphoma cases showed a complex pattern of copy number changes; over 90% of the cases carried more than one aberration. The highly variable cytogenetic pattern reflects a heterogeneous group of tumors with different responses to therapy and clinical outcome.

In conclusion, we investigated the clinical significance of recurrent chromosomal aberrations by interphase FISH in lymphoma specimens from a large cohort of dog patients. The results suggest that the copy number status of A1 (on CFA 1) is associated with DFI in lymphoma patients treated with doxorubicin plus asparaginase, supporting our hypothesis that recurring chromosomal aberrations are of prognostic significance in canine lymphoma. This finding may help develop a cytogenetic screening test that would enable clinicians to identify a subset of patients with more favorable clinical outcome. The chromosomal

aberrations identified in this study that have prognostic significance of canine lymphoma may give us clues about target genes that may be relevant in the pathogenesis of lymphoma. The identification of these target genes for such an outcome-associated region would likely provide new information on cancer development and new treatments. In addition, because the canine lymphoma cases evaluated in the present study had been treated with single agent doxorubicin plus asparaginase, it would be interesting to evaluate the prognostic value of A1 locus in the context of multiagent, CHOP-based chemotherapy. Moreover, by using comparative genomic analysis, conserved chromosome segments of the human genome that shared with aberration A1 can be defined. Further assessment of the presence of corresponding changes in human lymphoma samples may provide valuable data regarding the prognostic value of evolutionarily conserved DNA copy number aberrations shared between human and dog.

Table 12. Demographic and clinical characteristics of the original sample cohort and the studied cohort

Clinical characteristic	Original sample cohort (n=322)	Studied cohort (n=160)	*P* value
Age (mean ± SD) (years)	7.9 ± 2.9	7.7 ± 2.6	0.491
Weight (mean ± SD) (kg)	30.0 ± 13.0	29.1 ± 12.5	0.326
Gender			0.844
Male	141 (44.2%)	72 (45.6%)	
Female	178 (55.8%)	86 (54.4%)	
Histologic grade			0.527
High grade	214 (67.1%)	110 (70.5%)	
Intermediate grade	103 (32.3%)	44 (28.2%)	
Low grade	2 (0.6%)	2 (1.3%)	
DFI			
mean ± SD (days)	230.6 ± 253.9	293.2 ± 301.2	0.026
median (days)	178	196	

Table 13. CHORI-82 canine BAC clones used to generate the FISH probes used in this study

Probe name (canine genome location)	BAC address	Position in canine genome assembly
A1 (CFA1:116.8-117.6Mb)	470-C12	1:116839835-117080644
	007-M12	1:117060755-117226554
	336-J10	1:117213561-117419798
	122-O16	1:117410180-117635991
A2 (CFA 11: 44-44.7Mb)	043-A07	11:44061103-44283223
	325-C12	11:44256301-44428212
	004-K11	11:44393311-44622268
	334-H11	11:44581346-44766989
A3 (CFA 18: 28.3-29.1Mb)	443-J18	18:28329968-28513580
	427-N06	18:28385269-28568184
	521-K12	18:28514174-28761057
	012-H11	18:28740202-28947811
	437-A10	18:28926939-29165678
B2 (CFA 13: 27.7-28.6Mb)	230-P12	13:27760461-27947289
	436-M07	13:27919770-28112778
	335-M01	13:28085282-28265714
	322-J03	13:28259484-28465596
	503-B01	13:28426744-28609016
B3 (CFA 14: 32.7-33.5Mb)	101-N14	14:32746871-32927900
	203-L15	14:32888876-33125590
	126-H12	14:33096787-33271891
	412-F03	14:33239518-33407145
	245-C04	14:33386835-33579230
B1 (CFA 31: 6.7-7.2Mb)	430-C22	31:6744970-6953060
	176-O08	31:6920084-7085628
	518-M15	31:7061066-7272016
C2 (CFA 6: 41.5-42.4Mb)	507-L04	6:41565280-41750903
	442-N16	6:41702499-41917747
	390-J08	6:4184225-42061613
	109-P13	6:41994977-42256794
	062-E09	6:42226005-42426757
C3 (CFA 37: 3.6-4.5Mb)	425-N05	37:3695351-3857779
	318-H21	37:3824921-4030805
	311-P03	37:3980381-4190953
	190-P20	37:4177704-4386749
	064-O20	37:4341431-4525759
C1 (CFA 38: 26.4-28.8Mb)	160-F08	38:26490012-26647053
	230-A04	38:26592080-26785007
	067-L21	38:26647059-26860061

Table 14. Demographic and clinical characteristics of the training set and the validation set

Clinical characteristic	Training set (n=121)	Validation set (n=39)	*P* value
Age (mean ± SD) (years)	7.7 ± 2.6	7.6 ± 2.8	0.748
Weight (mean ± SD) (Kg)	27.9 ± 12.7	32.4 ± 11.4	0.045
Gender			0.713
Male	53 (44.5%)	19 (48.7%)	
Female	66 (55.5%)	20 (51.3%)	
Histologic grade			0.542
High grade	84 (71%)	26 (68.4%)	
Intermediate grade	33 (28%)	11 (28.9%)	
Low grade	1 (1%)	1 (2.6%)	
DFI			
mean ± SD (days)	305 ± 298.2	255.7 ± 311	0.385
Median (days)	197	163	

Table 15. R square and P value of the regression analysis for the mean copy number of each of the nine loci and DFI

Locus tested	Training data set (n=121)		Validation data set (n=39)		Overall data set (n=160)	
	R^2	*P* value	R^2	*P* value	R^2	*P* value
A1	0.1387	<0.0001*	0.2342	0.0018*	0.1571	<0.0001*
A2	0.0072	0.3705	0.0029	0.7519	0.0038	0.4510
A3	0.0012	0.2400	0.0781	0.1213	0.0001	0.9159
B1	0.0035	0.5392	0.0029	0.7619	0.0016	0.6325
B2	0.0294	0.0832	0.0367	0.2778	0.0311	0.0392
B3	0.0172	0.1951	0.0226	0.4638	0.0030	0.5453
C1	0.0025	0.6019	0.0024	0.0850	0.0004	0.8141
C2	0.0583	0.0103	0.1151	0.0461	0.0553	0.0041*
C3	0.0054	0.4680	0.2904	0.0021*	0.0244	0.0760

* significant after Bonferroni correction

Table 16. Correlation matrix for nine genomic copy number aberrations based on 160 cases

	A1	**A2**	**A3**	**B1**	**B2**	**B3**	**C1**	**C2**	**C3**
A1	1.0000								
A2	0.2322	1.0000							
A3	0.0106	0.0146	1.0000						
B1	0.0010	0.1773	0.0400	1.0000					
B2	0.1181	0.0215	0.0690	0.1129	1.0000				
B3	0.1044	-0.0896	-0.0043	0.1406	0.0514	1.0000			
C1	0.1778	0.1011	0.3030*	0.0861	-0.0675	0.0599	1.0000		
C2	0.0879	-0.0485	0.2037	-0.0320	0.0391	0.2702	0.3992*	1.0000	
C3	0.1228	-0.0800	0.1539	-0.0622	-0.0184	0.0214	0.1205	0.3413*	1.0000

* Significant correlation after Bonferroni correction

Table 17. Correlation of copy number status of each aberration with tumor histologic grade

Locus of interest	Copy number status	Histologic grade		*P* value
		High grade (# of cases)	Intermediate grade (# of cases)	
A1	Gain[a]	27	19	0.061
	Normal[b]	14	6	
	Loss [c]	68	19	
A2	Gain	12	3	0.571
	Normal	46	21	
	Loss	48	16	
A3	Gain	11	1	0.134
	Normal	18	11	
	Loss	76	26	
B1	Gain	64	26	0.398
	Normal	24	11	
	Loss	13	2	
B2	Gain	33	12	0.995
	Normal	39	14	
	Loss	26	9	
B3	Gain	3	1	0.165
	Normal	22	13	
	Loss	66	17	
C1	Gain	7	8	0.024
	Normal	5	4	
	Loss	93	28	
C2	Gain	14	3	0.081
	Normal	18	13	
	Loss	73	22	
C3	Gain	16	8	0.053
	Normal	17	9	
	Loss	65	12	

[a]Gain was defined as mean copy number >2
[b]Normal was defined as mean copy number =2
[c]Loss was defined as mean copy number <2

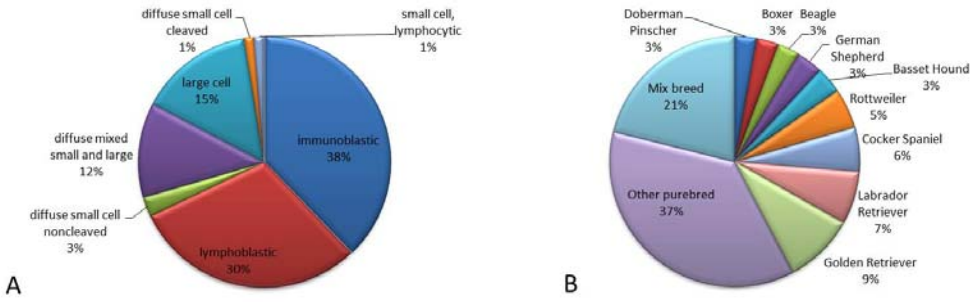

Figure 2. Distribution of canine lymphoma specimens (160 cases) by (A) histological subtype, and (B) breed. In chart B, 'other purebred' represents breeds with between one to four individuals.

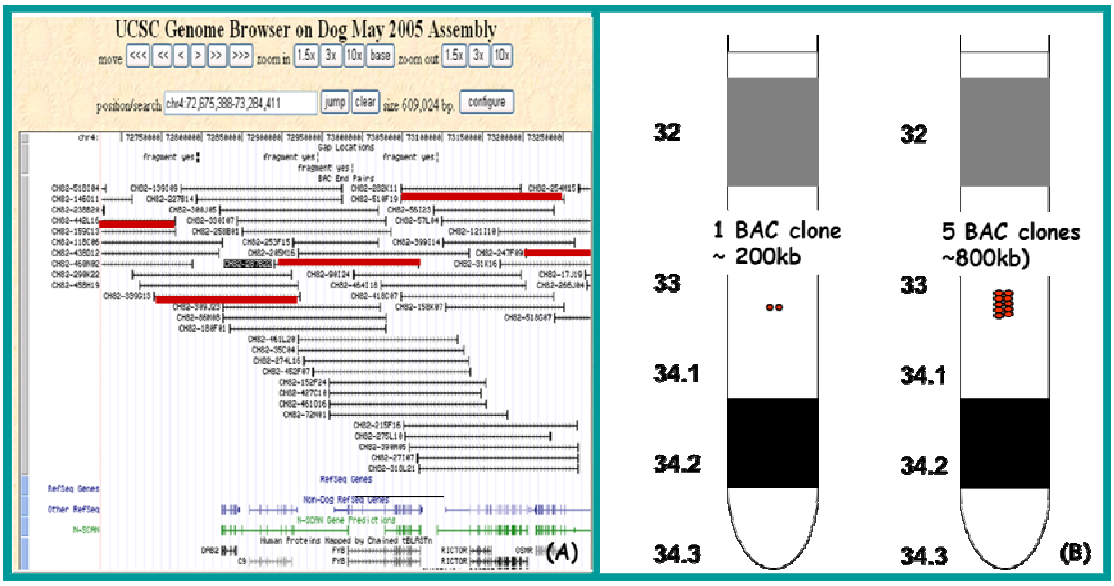

Figure 3. Probe design.
For each of the nine loci tested, up to five overlapping bacterial artificial chromosome (BAC) clones were selected. This resulted in an effective probe size of up to 800 kbp. (A) The overlapping BAC clones were selected from the UCSC dog genome browser. (B) Ideogram of a chromosome showing the probe design of contiguous BAC clones compared with a single BAC clone.

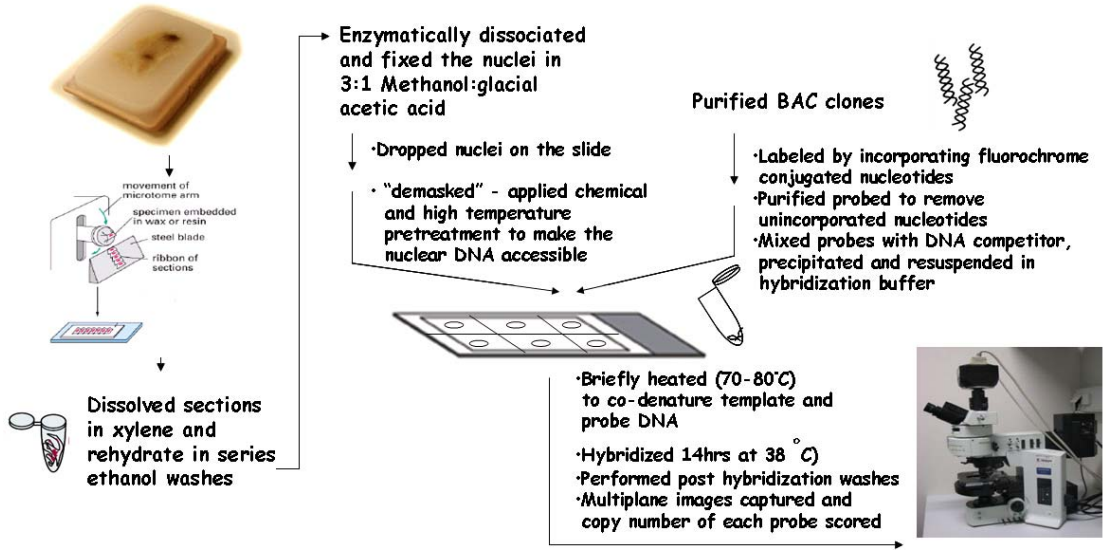

Figure 4. A outline showing the progression through the procedure for interphase fluorescence in situ hybridization (FISH) on cells isolated from paraffin embedded tissue biopsy specimens.
Single-cell suspensions were prepared by deparaffinizing 25 μm sections in microcentrifuge tubes in 1mL of xylene for 5x10 minutes. After deparaffinization and rehydration, the cells were resuspended in 0.5% of pepsin at 37°C for 30 minutes and then at 4°C overnight. Isolated nuclei were dropped on glass slides and followed by a "demasking" step in which the nuclei are subjected to high temperature (95°C, 10 minutes) and proteolytic (pepsin) digestion (38°C, 5 minutes) to make nuclear DNA more accessible to the probe. Purified, fluorochrome labeled probes were mixed, precipitated in the presence of competitor DNA and resuspended in hybridization buffer. The hybridization buffer was then added to the slides under a sealed coverslip and co-denatured at 80°C for 2 minutes. Hybridization was performed overnight at 38°C. After hybridization, the slides were washed in 2xSSC containing detergent (0.3% NP-40) at 75°C. Slides were counterstained using DAPI. Multiplane imaging of each probe was performed and data collated for each case.

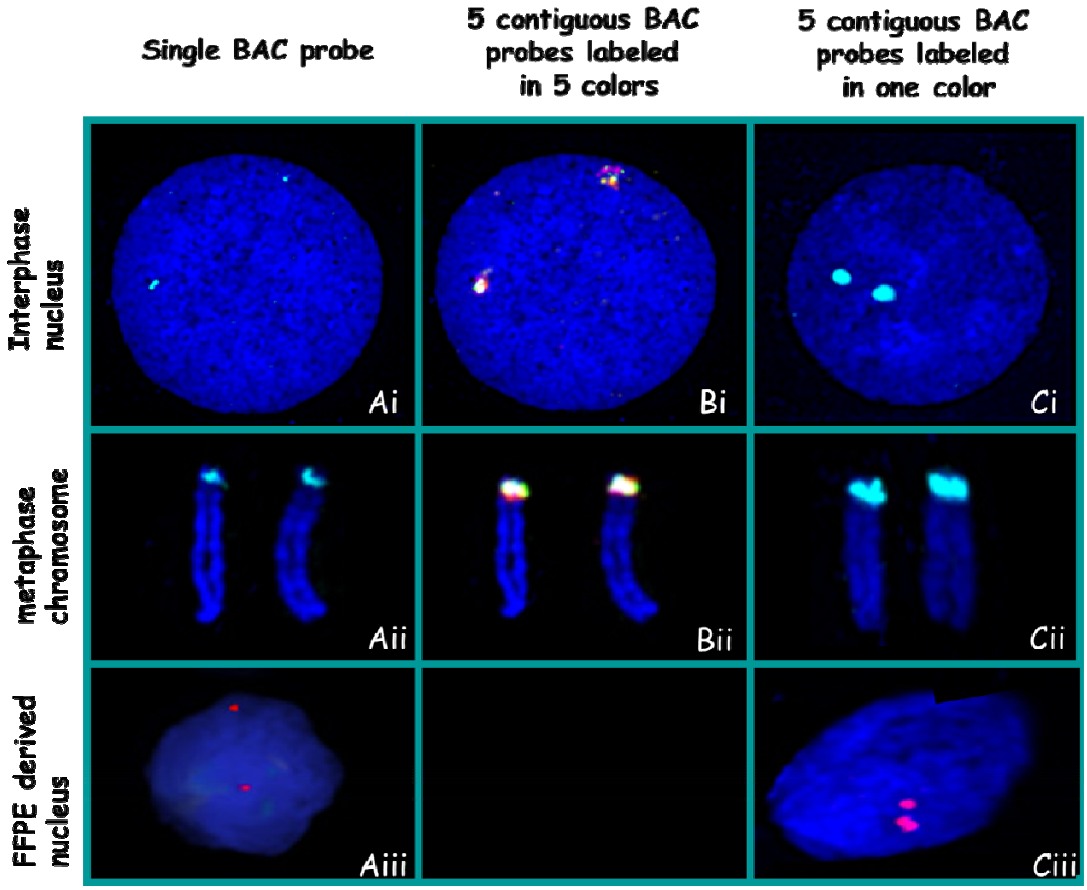

Figure 5. Examples of single 'vs' overlapping probe pools for FISH analysis.
Panels A, B and C show A) the hybridization of single BAC clone (200kb), B) 5 differentially labeled contiguous BAC clones (800kb) and C) 5 contiguous BAC clones labeled in the same color. Panels Ai, Bi and Ci show signals resulting from interphase nuclei derived from peripheral lymphocyte culture. Panels Aii, Bii and Cii show signals for metaphase chromosomes, while panels Aiii and Ciii show signals for paraffin embedded tissue derived nuclei.

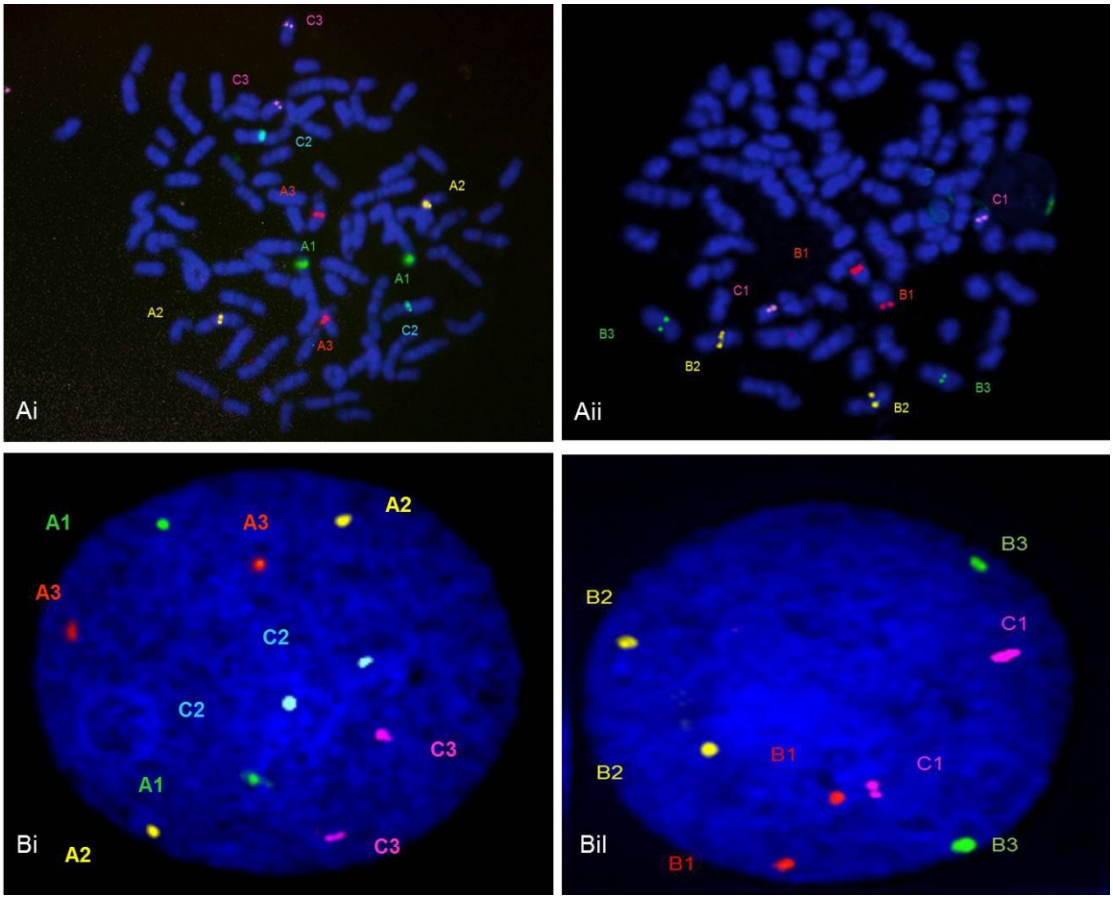

Figure 6. Probes specificity validation using fluorescence in situ hybridization analysis.
Five color FISH analysis of probes A1 (CFA 1), A2 (CFA 11), A3 (CFA 18), C2 (CFA6), C3 (CFA 37) (panel A) and B1 (CFA 31), B2 (CFA 13), B3 (CFA 14), C1 (CFA 38) (panel B) in metaphase chromosome spreads (Ai and Aii) and interphase nuclei (Bi and Bii) from a clinical normal dog. Analysis of the data reveals that all the probes represent normal copy number (n=2) and hybridized to the corresponding locations.

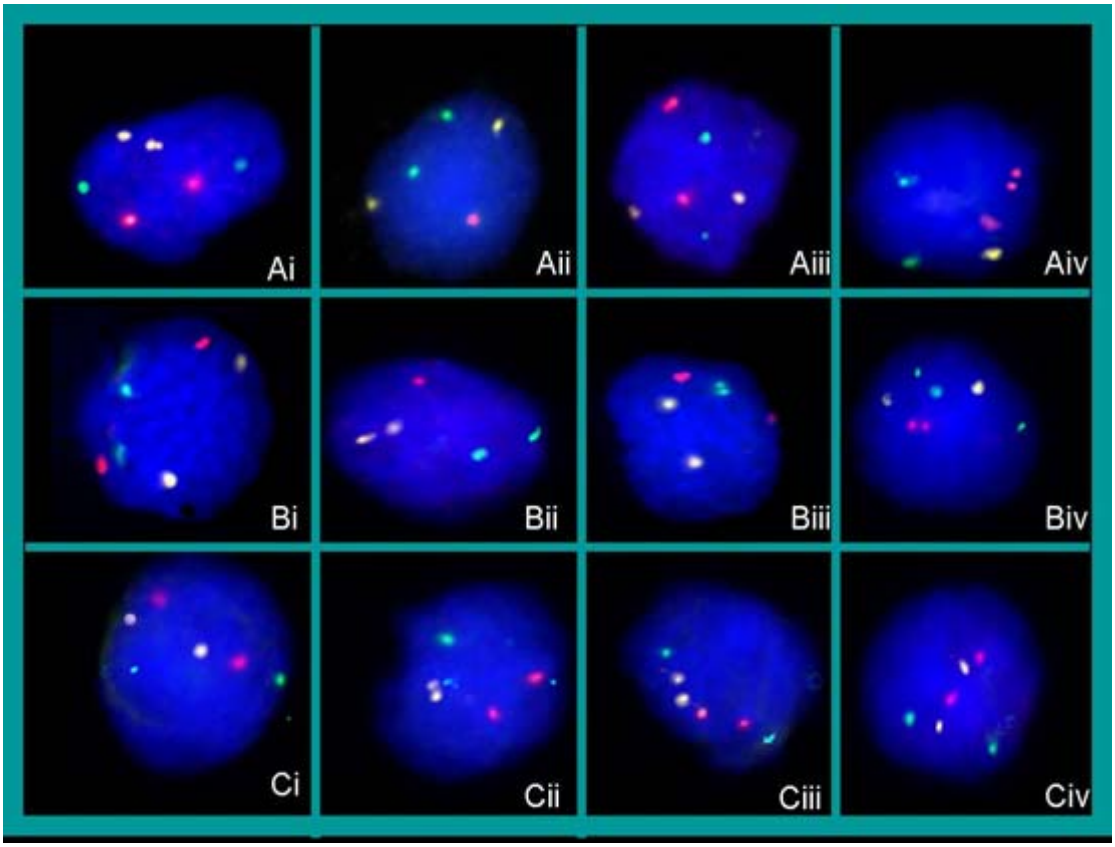

Figure 7. Examples of FISH data from paraffin embedded lymphoma specimens.
Panels A, B and C each show the co-hybridization of three differentially labeled BAC clones. Panel A shows signals resulting from clone pools specific for probes A1(red), A2 (yellow), and A3 (green), while panels B and C show signals for probes B1 (red), B2 (yellow), B3 (green), and probes C1 (red), C2 (yellow), and C3 (green), respectively. Panels Ai, Bi and Ci show hybridization of the BAC clones to nuclei derived from non-neoplastic paraffin embedded lymph node cells, revealing the expected copy number (n=2) of each locus per cell. Panels Aii-Aiv, Bii-Biv and Cii-Civ show the same clones hybridized to nuclei isolated from different canine lymphoma specimens; several abnormal hybridization signals were clearly observed.

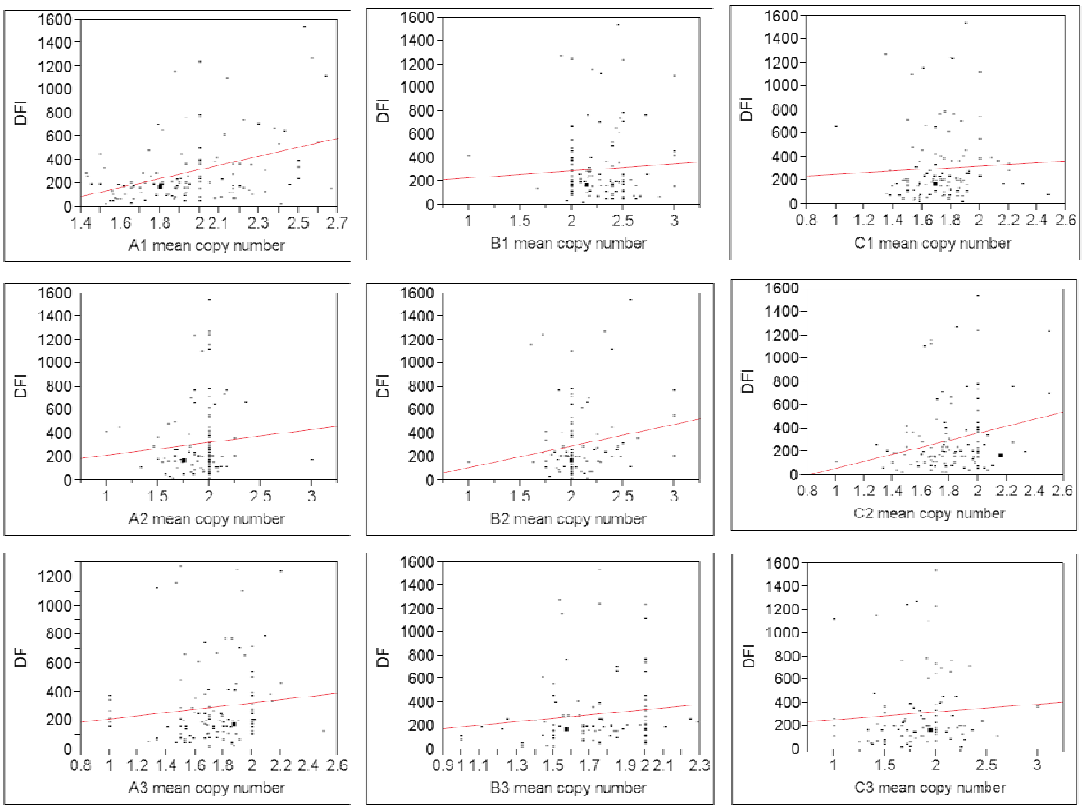

Figure 8. Scatter plots of the mean copy number of each of the nine loci versus DFI based on 121 lymphoma cases (the training set).
The red line indicates the linear fit.

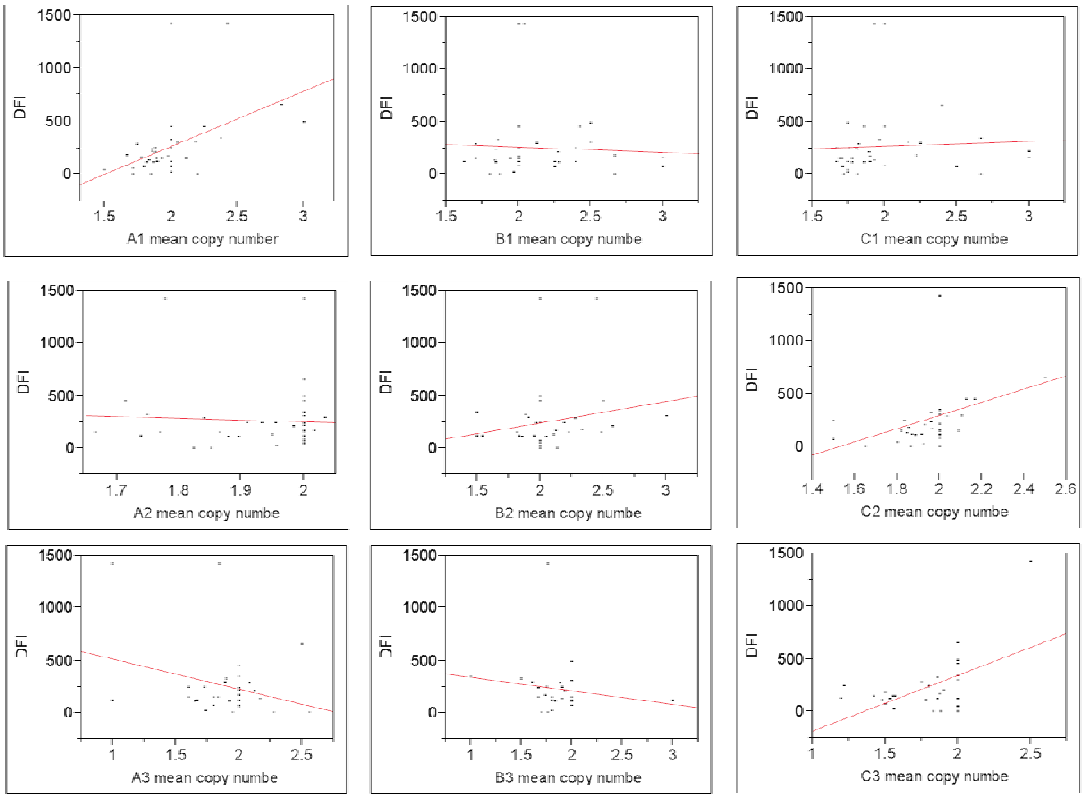

Figure 9. Scatter plots of the mean copy number of each of the nine loci versus DFI based on 39 lymphoma cases (the validation set).
The red line indicates the linear fit.

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CHAPTER 3: aCGH-guided Identification of Reliable Reference Gene for Real-Time Quantitative PCR Analysis in Commonly Occurring Canine Sarcomas

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Structured abstract

Objective- To identify suitable reference genes for real-time quantitative PCR analysis of commonly occurring canine tumors.

Sample Population- Malignant lymph-nodes (n=8), appendicular osteosarcomas (n=9) and histiocytic sarcomas (n=12) were analyzed in this study. Non-neoplastic, matched tissues were used as controls.

Procedures- aCGH data were used to guide the selection of nine new candidate reference genes. The stability of expression of these candidate genes, and four conventional reference genes, was evaluated in canine lymphoma, histiocytic sarcoma and osteosarcoma. The stability and suitability of each candidate reference genes was assessed by three programs; geNorm, NormFinder and BestKeeper.

Results- *LOC611555* was identified as the most stable reference gene across all three tumor types. Of the conventional reference genes, *HPRT* was stably expressed in histiocytic sarcoma, while *Ubi* and *RPL32* were relatively stable in osteosarcoma. A greater level of stability was evident in the proposed new reference genes than in the conventional reference genes.

Conclusions and Clinical Relevance- The rapidly advancing field of canine cancer gene expression profiling requires the use of stable, constitutively expressed reference genes. In the absence of reliable reference genes, accurate identification and quantification of both over- and under- expressed sequences in canine tumor tissues is at best difficult. The identification of *LOC611555* in our study provides a tissue-non-specific reference sequence to fulfill this requirement. Additionally we were able to recommend additional reference sequences for specific use either in canine lymphoma, osteosarcoma or histiocytic sarcoma, all of which are now intensively studied by the canine cancer research community.

Abbreviations

RT-qPCR- real-time quantitative PCR

aCGH- array-based comparative genomic hybridization

NF- normalization factor

C_T- cycle threshold

Introduction

Cancer is the primary of death overall in dogs [1]. Twenty-three percent of all dogs and 45% of dogs aged 10 years or older die of cancer [1,2]. Published cancer incidence rates for approximately all cancers ranged between 310 and 958 per 100,000 dogs [3-5]. Although numerous canine cancers have been proposed to be good models for human cancers, the exact molecular pathways underlying the formation and etiology of canine cancers are not yet well understood. With a complete high quality canine genome sequence assembly [6] and available tools for studying gene expression, there is increasing interest in examining canine tumors at the molecular level. Understanding gene expression profiles can provide insight into underlying regulatory interactions, and may assist in the identification of genes involved in tumorigenesis and cancer biology. At present, real-time quantitative PCR (RT-qPCR) provides quantitative information by the simultaneous measurement of the expression of select genes in different samples [7-9]. Although RT-qPCR has been recognized as the most accurate, sensitive, and reproducible method, compared with conventional quantification methods, the requirement for normalization is the same as for traditional methods of RNA quantification. Currently, reference genes are most frequently used to normalize expression results. Internal reference genes generally are housekeeping genes or constitutively expressed genes that are expressed at consistent and reproducible levels in tissues examined under different environmental conditions [10]. More recently, it has become clear that the commonly used reference genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta actin (*ACTB*) and beta-2-microglubulin (*B2M*) may be not expressed consistently across all tissue types and disease states [11,12]. Normalization of data using these reference genes without proper validation of their stability of expression can result in inaccurate conclusions in the study findings. Consequently, in order to increase the accuracy and sensitivity of RT-qPCR methods, the availability of an optimal set of reference genes for each experimental system has become increasingly important.

Array comparative genomic hybridization (aCGH) analysis is a key research tool for detecting genome-wide copy number aberrations of DNA segments/gene dosage in genomic DNA isolated from malignant cells [13-22]. Through our ongoing studies of canine lymphoma, osteosarcoma and histiocytic sarcoma, we have identified a series of recurrent DNA copy number imbalances present in each of these canine tumors. Simultaneously, for each of these cancers we have identified regions of the canine genome with predominantly balanced copy number and identified several that are balanced across different cancers. Within these shared regions of DNA copy number balance, the gene expression profiles may be more stable, thus providing a source of genes to be considered as new reference genes suitable for either a particular cancer type or across multiple types.

In this study we compared the aCGH profiles of 331 canine cancer cases representing approximately equal numbers of three different types of canine tumors that generally present with complex copy number profiles; lymphoma, osteosarcoma and histiocytic sarcoma. Based on these data, genomic regions that showed consistent copy number stability across all three tumor types were identified and used to select nine candidate reference genes. It has been reported recently that for canine tissues, the use of hypoxanthine-phosphoribosyl transferase (*HPRT*), ATP-synthase subunit 5B (*ATP5B*), ribosomal protein L32 (*RPL32*), and ubiquitin (*Ubi*) yield stable reference gene expression levels [23-25]. In order to relate the stability levels between aCGH directed candidate reference genes and commonly used reference genes, we also evaluated these four genes in all three tumor types. The relative stability of each reference gene in unaffected and malignant tissues was determined by RT-qPCR using three different validation software programs, geNorm [26], NormFinder [27] and BestKeeper [28]. The variability in expression of all 13 reference genes was studied across the three different tumor types, in order to assess their value as internal controls in studies of these tumors. The stability of the candidate reference genes were compared between tumor types.

Materials and Methods

Selection of reference gene candidates- Cytogenetic profiling data from canine lymphoma samples (n=122) [29], appendicular osteosarcoma (n=123), and histiocytic sarcoma (n=86) [30] were generated from aCGH analysis, using a 1Mb resolution [18], genome assembly-integrated microarray. Data for each tumor were compiled by calculating the frequency of copy number neutrality (n=2) for each of the 2,097 genomic loci represented on the array. To identify the most stable genomic regions across each of the tumor types, the prospective target regions were selected from genomic regions that show consistent copy number stability, defined as >90% of the observations shown as balanced. The candidate reference genes were then selected from those 'stable' genomic regions and the associated gene information reviewed to ensure no known involvement in lymphoma, osteosarcoma or histiocytic sarcoma. In this study, four genomic regions were identified from which nine new candidate reference genes were selected. The genomic location and the cytogenetic details of these stable regions and candidate reference genes are shown in Table 18.

Case materials- For the analysis of the new reference genes eight lymphoma, nine appendicular osteosarcoma and 12 histiocytic sarcoma cases were recruited from client-owned dogs admitted to the College of Veterinary Medicine at North Carolina State University. All specimens were acquired prior to initiation of chemotherapy or radiotherapy, under approved protocols and with informed client consent. Additionally, unaffected lymph node, spleen, lung and bone tissue had been obtained previously and as such, were routinely available. Immediately following surgical excision, half of the representative portion of each tumor specimen was fixed in 10% neutral buffered formalin, while the other half was snap-frozen in liquid nitrogen for subsequent RNA extraction.

Pathology – All tissue samples were submitted for evaluation by the NCSU CVM pathology service for routine Hematoxylin & Eosin (H&E) and immunohistocytochemistry, as appropriate, to confirm their diagnosis.

RNA extraction- RNA was isolated using a RNA isolation kit[a] according the manufacturer's instructions, supplemented with a genomic DNA digestion step[b]. The concentration of total RNA representing each sample was quantified first using a spectrophotometer[c] and the quality of each RNA sample was examined for RNA integrity using an Agilent Bioanalyzer 2100[d]. All RNA samples used in the study had a RIN>7.0.

RT-qPCR- Primer sequences were designed for each of the reference genes from available transcript sequences (NCBI) using the Primer 3 primer design software [31]. Reverse transcriptions (RT) were performed using a Quantiscript reverse transcription kit[e] according to the manufacturer's instructions. Briefly, 1μg of the isolated RNA was pre-incubated with genomic DNA wipeout buffer at 42^{o}C for 2 min. After the genomic DNA elimination step, RT primer mix, RT buffer and Quantiscript reverse transcriptase were added to each RNA sample and the samples then incubated at 42^{o}C for 15 min. Reverse transcriptase activity was inactivated by heating the samples at 95^{o}C for 3 min. All cDNAs were stored at -20^{o}C until use. The RT-qPCR assays were performed in triplicate using an iCycler[f] in a 96-well format using a SYBR Green PCR kit[g]. The reaction comprised 1x QuantiFast SYBR Green PCR Master Mix, 1 μM forward and reverse primer, 25 ng template cDNA, 0.5 μl Fluorescein Passive Reference Dye[h], and water in a final volume of 25 μl. The standard amplification conditions comprised 1 cycle at 95^{o}C for 5 min, 40 cycles of 95^{o}C for 10 s and 60^{o}C for 30 s. A melting curve was generated by heating the samples from 55 to 95^{o}C in 1^{o}C increments with a 15 sec hold at each temperature. No-template and no-RT controls were used for each assay to confirm for the absence of sample contamination and genomic contamination, respectively. None of the control reactions showed detectable products. The amplified RT-qPCR products were electrophoresed on 2% agarose gels and visualized using GelRedTM staining[i]. For determination of the RT-qPCR assay efficiency of each gene examined, RT-qPCR was performed in a 5-step, 10-fold dilution series of a pool of RNA samples isolated from non-neoplastic canine lymph node tissue [8]. A summary of the genes and corresponding primer sets used in the study is shown in Table 19.

Reference gene stability analysis- Expression levels of the candidate reference genes in all of the tissue samples were determined by the number of cycles needed for the amplification to reach a specific threshold fixed in the exponential phase of the PCR reaction. The expression data were exported into an Excel data sheet and three software applications. GeNorm [26]; NormFinder [27]; and BestKeeper [28]were used to analyze the expression stability of the candidate reference genes based on different mathematical algorithms.

GeNorm [26] is based on the principle that the expression ratio of two ideal reference genes always will be identical among samples, regardless of the experimental conditions. Therefore the program defines a stability measure (M) as the average pairwise variation of a particular reference gene compared with each of the other reference genes in one sample. Genes with lower M values indicate increased gene stability across samples. By stepwise removal of the least stable gene and recalculation of the M value, the most stable gene combinations with the lowest M values are identified. Therefore a normalization factors (NF) is calculated based on the geometric mean of the expression levels of the best performing reference genes. In addition, geNorm also allows estimation of the optimal number of control genes for normalization. The pair-wise variation $V_{n/n+1}$ was calculated out of two sequential normalization factors NF_n and NF_{n+1} to determine the benefit of adding extra reference genes for the normalization process.

The NormFinder [27] is a model-based approach and enables direct estimation not only of the overall variation of the candidate reference genes, but also of the variation between sample subgroups of the sample set. This distinguishing feature makes it more robust against the co-expressed genes. NormFinder generates a stability value where a lower value indicates increased stability in gene expression.

The BestKeeper [28] ranks the candidate reference genes according to the variation in the C_T values of each gene. Therefore, the standard deviation of C_T values (SD_{CT}) is used to describe reference gene stability. Where a lower SD_{CT} of candidate gene is observed, a more stable expression pattern can be assumed. Any studied gene with the SD_{CT} higher than 1 can be considered inconsistent.

Results

New candidate reference genes and RT-qPCR efficiency- The gene identities, putative functions, primer sequences, and RT-qPCR metrics of each candidate reference gene are shown in Table 19. To ensure comparability across RT-qPCR assays, the PCR efficiency of each individual gene was determined by a dilution method [8]. All PCR assays displayed efficiencies between 95% and 101% (Table 19). Furthermore, all RT-qPCR assays produced a single distinctive peak via melt-curve analysis and a single amplicon of the expected size via agarose gel electrophoresis.

RNA transcription levels of candidate reference genes- To evaluate the stability of expression of candidate reference genes, RT-qPCR was used to investigate the RNA transcription level of each gene in three different canine tumor types, as well as their corresponding unaffected tissues. The RNA transcription profiles of all 13 candidate reference genes are shown in Figure 10. The C_T values for different candidate reference genes were compared directly across all tissues. The expression data divided the genes into two groups. Group 1 comprised nine of the 13 genes, including all four of the commonly used reference genes (*RPL32*, *ATP5B*, *HPRT*, *Ubi)* and five new candidates *(L10E*, *SPPL3*, *TRIM37*, *LOC611555*, and *OASL*), all of which showed high expression levels (median C_T<30). Group 2 comprised four genes, all new candidates (*PPM1E*, *LAMA1*, *SMAD9*, and *HNF-1*) that showed lower expression levels (median C_T>30). For the transcription range, defined as the difference between the highest C_T and the lowest C_T in all tissues, it was observed that *HNF-1* (range=1.3), followed by *LAMA1* (range=1.6) and *LOC611555* (range=1.9) showed the lowest RNA transcription range.

Expression stability analysis in different tumors - Gene expression stability over different tumor types was assessed using three separate software tools, geNorm, NormFinder and BestKeeper. For canine lymphoma, geNorm ranked candidate genes according to their M value (Table 20). *LOC611555* was determined as one of the two most stable genes among

those examined. Similar to the findings from geNorm, *LOC611555* was the most stably expressed gene indicated by NormFinder (Table 21) and the 2nd according to BestKeeper (Table 22). Likewise, *PPM1E*, *SMAD9* and *TRIM37* were determined to be the least stably expressed genes by all three algorithms. The ranking order among the three algorithms was not consistent, although agreement generally was reached on the most and least stable genes. Table 6 summarizes the ranking order of the reference genes calculated by the three software programs implemented in geNorm, NormFinder and BestKeeper.

For canine histiocytic sarcoma, the software methods were not in agreement regarding the most stable gene (Table 20, 21 and 22). *LOC611555* was one of the two most stably expressed genes identified by geNorm, the 2nd by NormFinder, and the 3rd by BestKeeper. *HPRT* was the most stably expressed gene identified by NormFinder, but the 4th by both geNorm and BestKeeper. *OASL* was the most stably expressed gene indicated by BestKeeper and in the top two in geNorm, but was the 9th according to NormFinder. Based on the resulting mean rank order from those three methods (Table 23), the most stable reference genes for histiocytic sarcoma were *LOC611555*, followed by *HPRT* and *OASL*. *PPM1E*, *LAMA1* and *SMAD9* were the least stable reference genes.

For canine osteosarcoma, *LOC611555* was identified as the most stable gene by NormFinder (Table 21) and BestKeeper (Table 22), but only the 5th most stable gene by geNorm (Table 20). *Ubi* was one of the two most stable genes by geNorm, the 3rd most stable gene by NormFinder, and the 4th by BestKeeper. *RPL32* was the 2nd most stable gene by NormFinder and BestKeeper, and the 4th by geNorm. Overall, considering all three software programs, *LOC611555* emerged as the most stable gene, followed by *RPL32* and *Ubi* (Table 23). Conversely, *SMAD9, LAMA1*, *PPM1E*, and *HNF-1* were the least stably expressed genes.

Comparison of reference gene performance in different cancers- *LOC611555* consistently was identified as being one of the most stably expressed by geNorm and NormFinder or BestKeeper across three tumor types. (Table 23). *SMAD9* and *PPM1E* tended to be the least stable in all the tumor types evaluated. For the commonly used reference genes that have been evaluated previously in canine tissues, *HPRT* was stably expressed in

histiocytic sarcoma, both *Ubi* and *RPL32* were stable in osteosarcoma, but none of the three genes were identified consistently as the most stable reference gene by all methods across the three tumor types.

Number of reference genes for normalization- Apart from single gene of interest expression measurements, it is recommended that data be normalized against more than one reference gene and a NF be calculated [11,26-28]. In general, the variation in the average of multiple reference genes is smaller than the variation in a single reference gene. However, the number of the reference genes used is a trade-off between practical consideration and validity in the NF [26, 27]. To determine how many reference genes should be used for multiple gene normalization, geNorm calculates the degree of contribution for each gene to the variance of NF to measure the effects of adding further genes from the set of reference genes. A cut-off value of 0.15 is suggested, below which the inclusion of an additional reference gene is not required. In canine lymphoma, the V2/V3 value was <0.15 and so there was no need to include a 3rd gene in the NF. An optimal number of reference genes for normalization of lymphoma RT-qPCR data would therefore be two. By using the stability ranking established by the data (Table 20), the first two reference genes *LOC611555* and *HNF-1* were suggested as useful for calculating an NF in a canine lymphoma sample set. On the other hand, four genes are required for the normalization of canine histiocytic sarcoma and osteosarcoma sample sets. The summarized results are shown in Figure 11.

Discussion

A number of previous studies have evaluated the selection and effect of reference genes on normalized gene expression data, but most of the efforts have been concentrated on analysis of human samples. With the recent increase in canine studies, there is a need for validation of suitable reference genes in each experimental condition, especially among complex multiple tissues and treatment regimens. A number of different strategies have been applied to identify suitable reference genes for different experiments, such as evaluation of commonly used reference genes, or identification of new reference genes based on the transcriptional profiles

from microarray [23-25,32]. However, these strategies have not identified a new reference gene that shows consistent stability across multiple tissue types and disease situations. In this study, we compiled 1Mb resolution canine aCGH data generated from the evaluation of 331 canine cancer cases representing patients diagnosed with lymphoma, osteosarcoma and histiocytic sarcoma. From these data we identified just four regions of the genome that were considered to be highly stable in DNA copy number across these three cancers. From these regions we identified nine new candidate reference genes. The hypothesis for this study was that genes from relatively copy number stable regions of the genome would demonstrate greater expression stability across multiple tissue types.

In order to identify the optimal reference genes for RT-qPCR studies, we first evaluated the RNA transcription levels of 13 candidate reference genes (our nine new candidates and four traditionally used reference genes) across all three tumor tissues and their corresponding non-neoplastic tissues. We then calculated differences in RNA transcription among the selected genes. Our results show that C_T values for different reference genes were in highly variable quantity ranges. *SMAD9* had an unacceptably high transcriptional range, and in this study was the least stable in all tumor types evaluated. Genes found to have the lowest transcription range could be good candidate genes with invariable expression over all tissues. In this study *LAMA1*, *HNF-1* and *LOC611555* were observed to have the lowest range. *LAMA1* and *HNF-1* were in the low expression group and so the small variation might be caused by constitutively weak expression levels. The algorithmic analyses also demonstrated that both these genes were less stable in all tumor samples. *LOC611555* was in the high expression group and also showed a low variation in transcription across all tissues analyzed, suggestive of a good reference gene.

To gain insight into variations with each experimental system, we analyzed potential reference genes in three tumor types (lymphoma, osteosarcoma and histiocytic sarcoma) by the mathematical assessment of their expression levels. By ranking the tested candidate genes through three separate algorithms, *LOC611555* demonstrated the most stability and so appeared suitable for use in the normalization of gene expression in all three different tumor types examined. Although we identified a gene which is stably expressed in all tissues, a

proper validation of its use as a reference gene in other tissues or for other diseases is recommended, since no universal reference gene is optimal for all studies on the basis of available published evidence. *LOC611555* is an uncharacterized gene, conserved in human, chimpanzee, cow, mouse, rat, chicken, and zebrafish. The precise function of this gene is thus unknown, but since its expression appears to show stability across canine tissues types and disease status, we propose that this gene may be a useful candidate reference gene for other biological systems.

In order to relate the stability between the new reference genes and commonly used reference genes, four reference genes that have been demonstrated previously to be stable in canine tissues were also investigated. Limited information has been published on the validation of reference genes in canine tissues. Brinkhof et al. investigated nine reference genes in studies of tissues of prostate, kidney, mammary grand, left ventricle, and liver and found that *HPRT* was one of the most stably expressed genes [25]. Etschmann et al. examined 11 reference genes in canine mammary tissues including healthy and diseased ones [23]. *HPRT*, *RPL32*, *Ubi* and *ATP5B* were identified as the most stable genes [23]. Peters et al. investigated 11 potential reference genes and validated their use in bone marrow, colon, duodenum, heart, kidney, liver, lung, lymph nodes, skeletal muscle, pancreas, spleen and stomach [24]. *RPL32* was the most stably expressed gene in the majority of the tissues examined, and *HPRT* was also relatively stable in the bone marrow and lymph node investigated in our study [24]. These previous studies found that *HPRT* and ribosomal protein *RPL32* tend to have a stable expression profile in canine tissues. In our study, *HPRT* was stably expressed in histiocytic sarcoma tissues, *RPL32* and *Ubi* were relatively stable in osteosarcoma samples, but neither was stable in our canine lymphoma samples. Comparing the results between newly identified candidate genes and these previously used reference genes in multiple tumors, demonstrated the greater level of stability of the proposed new reference genes.

In this study, we used three different algorithms to rank reference gene stability in each experimental system. The program geNorm chooses the appropriate reference genes based on pairwise comparison approach which top ranks genes with the highest degree of similarity of their expression profile across the sample set [26]. Although GeNorm has a tendency to identify

co-regulated genes, revealing the similarity of expression profiles, use of NormFinder and BestKeeper avoids this problem [26, 27]. Since a number of new genes in this study have little annotated functional information, the assessment of stability using different algorithms provides us with a more robust result. In our study, the rank order determined from different methods was different, yet there were no major changes in the agreement of the most and least stable genes between the three methods. This supports the view that potential problems associated with co-regulation are unlikely to apply to our data set.

Normalization with multiple reference genes is becoming more commonplace in RT-qPCR analysis. In this study, we evaluated the expression stability of 13 genes, including four conventional reference genes in three different tumors and corresponding unaffected tissues. The evaluation, performed using geNorm, determined a series of reference genes for the multiple gene normalization of each tumor type. The NF was calculated based on the geometric mean of the best performing reference genes. In our study, two genes were suggested as the most appropriate to use for calculating a NF in a canine lymphoma sample set, while for histiocytic sarcoma and osteosarcoma, four genes are required for appropriate normalization.

In conclusion, we selected new reference genes based on assessment on copy number neutrality in a cohort of over 300 canine tumor samples. We identified multiple genes demonstrating greater stability than conventional reference genes in different tissues. *LOC611555* was the most stable gene in all tumor types and tissues analyzed, and we propose that this gene could be used to normalize expression levels of genes of interest within studies of canine lymphoma, histiocytic sarcoma or osteosarcoma. Analyses of the normalization factor for each tumor type revealed the best combination of the reference genes to be included for normalization. This study suggests that while canine researchers continue to use conventional reference genes for expression-based analyses, it is possible that such genes may not be the best option for all cancer types. This also raises concerns about prior data based on the assumption that such reference genes are indeed suitable.

Footnotes

a. RNeasy kit, Qiagen, Valencia, CA

b. Turbo DNA free kit, Ambion, Austin, TX

c. NanoDrop ND-1000 UV/Visible spectrophotometer, NanoDrop Technologies, Wilmington, DE

d. Agilent Bioanalyzer 2100, Agilent, Santa Clara, CA

e. QuantiTect Reverse Transcription Kit, Qiagen, Valencia, CA

f. iCycler, Bio-Rad, Hercules, CA

g. QuantiFast SYBR Green PCR kit, Qiagen, Valencia, CA

h. Fluorescein Passive Reference Dye, Affymetrix, Santa Clara, CA

i. GelRedTM nucleicm Acid Gel Stain, Biotium, Hayward, CA

Table 18. Four regions of the canine genome showed consistent copy number neutrality (>90%) among canine lymphoma (LSA), histiocytic sarcoma (HS) and osteosarcoma (OS) based on aCGH data using a 1Mb resolution canine BAC array. The CHORI-82 addresses of the four BAC clones assessed are shown along with their genome location in the Canfam2v2.0 genome assembly.

clone address	chromosome	position	neutrality % in LSA	neutrality % in HS	neutrality % in OSA	Genes in this region
326-J08	7	76,288,559-76,478,148	98.1	94.1	90.7	*LAMA1*
313-F22	9	36,694,888-36,863,023	98.1	91.0	92.6	*PPM1E*; *TRIM37*
315-P19	25	6,818,798-6,993,295	98.1	93.2	90.6	*SMAD9*
122-H22	26	19,737,413-19,909,922	98.8	90.8	96.7	*LE10*;*LOC611555*; *SPPL3*;*HNF-1*;*OASL*

Table 19. Identity, function, primer sequences, and RT-qPCR metrics of candidate reference genes considered in this study

Gene name	abbreviation	Accession No.	Gene function	Forward and reverse primers	Product length (bp)	R^2	PCR efficiency
laminin, α 1	*LAMA1*	XM_537324.2	Glycoproteins, cell differentiation and migration	F-GCACAACACCACGGGGGACC R-AGGTGGCAGGTGGGGCTGAA	141	0.99.3	100.4
protein phosphatase, Mg^{2+}/Mn^{2+} dependent, 1E	*PPM1E*	XM_848160	Protein phosphatase, CaM kinases inactivation	F-AAACAGATGGCACAGAAGGG R-TTTTTGATGGCATGGATTGA	160	0.998	95.8
tripartite motif-containing 37	*TRIM37*	XM_537697.2	Peroxisomal protein	F-CAGAGCTCCCTGACTTGGAC R-AATGCTCTCCACGCTCTGTT	156	1.000	95.8
SMAD family member 9	*SMAD9*	XM_852737.1	SMAD family polypeptide, cell signaling	F-GAGAGCCCCTATCAACACTCAGACT R-CGGGAGGATGCCTGGAACGTC	122	0.990	100.9
60S acidic ribosomal protein P0	*L10E/ RPLP0*	XM_846329.1	60S ribosomal protein	F-CTTCCCACTTGCTGAAAAGG R-TGTCCGATTCCAACTCTTCC	149	0.990	99.4
hypothetical protein LOC611555	*LOC611555*	XM_849238.1	Uncharacterized protein	F-GCCTGGGGCTTGGAGCAGTG R-TGGGCTCGGAATTCGGGGGT	158	0.999	96.8
signal peptide peptidase 3	*SPPL3*	XM_543427.2	Intra-membrane cleaving proteases	F-CGACCGTGGCATCCCGCATT R-GGCTCAGACCACATCCGCCG	127	0.999	100.5
HNF1 homeobox A	*HNF-1*	XM_543429	Transcriptional activator for liver-specific genes	F-GCCCAGAGCCCCTTCATGGC R-AAGACCTGCTTGGTGGGCGT	176	0.993	100.4
2'-5'-oligoadenylate synthetase-like	*OASL*	NM_001048093	Viral RNAs degradation	F-ACACCGCAGATCAATCATCA R-ACACCGCAGATCAATCATCA	188	0.997	99.2
ATP synthase, H^+ transporting, mitochondrial F1 complex, β polypeptide	*ATP5B*	NM_001686	Mitochondrial ATP synthase subunit, ATP synthesis	F-GCACGGAAAATACAGCGTTT R-TTGCCACAGCTTCTTCAATG	187	0.995	100.8
Hypoxanthine phosphoribosyltransferase 1	*HPRT*	NM_000194	Enzyme, purine metabolism.	F-TGCTCGAGATGTGATGAAGG R-TCCCCTGTTGACTGGTCATT	192	0.995	100.2
Polyubiquitin	*Ubi*	NM_001009202	Labeling proteins for proteasomal degradation	F-TCTTCGTGAAAACCCTGACC R-CCTTCACATTCTCGATGGTG	305	0.998	97.3
60S ribosomal protein L32	*RPL32*	XM_540107	Ribosomal protein	F-ATGCCCAACATTGGTTATGG R-CTCTTTCCACGATGGCTTTG	180	1.000	100.6

Table 20. Candidate reference genes ranked according to their expression stability estimated using geNorm algorithm

	Lymphoma	**Histiocytic sarcoma**	**Osteosarcoma**
Most stable[a]	*LOC611555-HNF-1*	*LOC611555-OASL*	*HPRT- Ubi*
↓	*LAMA1*	*SPPL3*	*L10E*
	OASL	*HPRT*	*RPL32*
	Ubi	*TRIM37*	*LOC611555*
	SPPL3	*L10E*	*OASL*
	HPRT	*RPL32*	*TRIM37*
	ATP5B	*ATP5B*	*SPPL3*
	RPL32	*SMAD9*	*ATP5B*
	L10E	*Ubi*	*SMAD9*
	SMAD9	*HNF-1*	*LAMA1*
	PPM1E	*PPM1E*	*HNF-1*
least stable	*TRIM37*	*LAMA1*	*PPM1E*

[a]The two most stably expressed reference genes cannot be ranked further due to the requirement for gene expression ratios for gene stability measurements.

Table 21. Candidate reference genes and their expression stability values calculated by NormFinder algorithm

	Lymphoma		Histiocytic Sarcoma		Osteosarcoma	
	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value
Most stable	*LOC611555*	0.231	*HPRT*	0.172	*LOC611555*	0.185
	RPL32	0.244	*LOC611555*	0.244	*RPL32*	0.324
	HNF-1	0.346	*Ubi*	0.281	*Ubi*	0.344
	LAMA1	0.357	*RPL32*	0.326	*HPRT*	0.361
	OASL	0.408	*SPPL3*	0.412	*OASL*	0.370
	HPRT	0.426	*TRIM37*	0.446	*SPPL3*	0.384
	TRIM37	0.495	*ATP5B*	0.499	*TRIM37*	0.391
	SPPL3	0.563	*L10E*	0.506	*L10E*	0.462
	L10E	0.573	*OASL*	0.530	*ATP5B*	0.507
	PPM1E	0.614	*PPM1E*	0.575	*SMAD9*	0.508
	ATP5B	0.630	*HNF-1*	0.675	*LAMA1*	0.577
	Ubi	0.658	*SMAD9*	0.848	*HNF-1*	0.617
Least stable	*SMAD9*	0.810	*LAMA1*	1.001	*PPM1E*	0.904

Table 22. Standard deviation of CT values of the tested candidate reference genes calculated with BestKeeper algorithm

	Lymphoma		Histiocytic Sarcoma		Osteosarcoma	
	Ranking	SD_{CT}	Ranking	SD_{CT}	Ranking	SD_{CT}
Most stable	*NHF-1*	0.26	*OASL*	0.53	*LOC611555*	0.59
	LOC611555	0.43	*HNF-1*	0.54	*RPL32*	0.65
	LAMA1	0.44	*LOC611555*	0.73	*PPM1E*	0.66
	OASL	0.76	*HPRT*	0.85	*Ubi*	0.72
	Ubi	0.87	*TRIM37*	0.91	*HNF-1*	0.80
	RPL32	0.98	*PPM1E*	0.96	*HPRT*	0.81
	L10E	1.00	*ATP5B*	0.98	*ATP5B*	0.82
	SPPL3	1.10	*Ubi*	0.99	*LAMA1*	0.82
	ATP5B	1.13	*SPPL3*	1.12	*L10E*	0.85
	HPRT	1.19	*RPL32*	1.14	*TRIM37*	0.86
	PPM1E	2.04	*L10E*	1.17	*SPPL3*	0.95
	TRIM37	2.15	*LAMA1*	1.25	*OASL*	0.99
Least stable	*SMAD9*	2.17	*SMAD9*	1.71	*SMAD9*	1.07

Table 23. Ranking order of 13 reference genes in three different tumors obtained by geNorm, NormFinder and BestKeeper.

	RPL32	*ATP5B*	*HPRT*	*Ubi*	*PPM1E*	*L10E*	*SPPL3*	*TRIM37*	*LAMA1*	*SMAD9*	*LOC611555*	*OASL*	*HNF-1*
Lymphoma													
GeNorm	9	8	7	5	12	10	6	13	3	11	1.5	4	1.5
NormFinder	2	11	6	12	10	9	8	7	4	13	1	5	3
BestKeeper	6	9	10	5	11	7	8	12	3	13	2	4	1
average rank	**5.7**	**9.3**	**7.7**	**7.3**	**11**	**8.7**	**7.3**	**10.7**	**3.3**	**12.3**	**1.5**	**4.3**	**1.8**
Histiocytic Sarcoma													
GeNorm	7	8	4	10	12	6	3	5	13	9	1.5	1.5	11
NormFinder	4	7	1	3	10	8	5	6	13	12	2	9	11
BestKeeper	10	7	4	8	6	11	9	5	12	13	3	1	2
average rank	**7**	**7.3**	**3**	**7**	**9.3**	**8.3**	**5.7**	**5.3**	**12.7**	**11.3**	**2.2**	**3.8**	**8**
Osteosarcoma													
GeNorm	4	9	1.5	1.5	13	3	8	7	11	10	5	6	12
NormFinder	2	9	4	3	13	8	6	7	11	10	1	5	12
BestKeeper	2	7	6	4	3	9	11	10	8	13	1	12	5
average rank	**2.7**	**8.3**	**3.8**	**2.8**	**9.7**	**6.7**	**8.3**	**8**	**10**	**11**	**2.3**	**7.7**	**9.7**

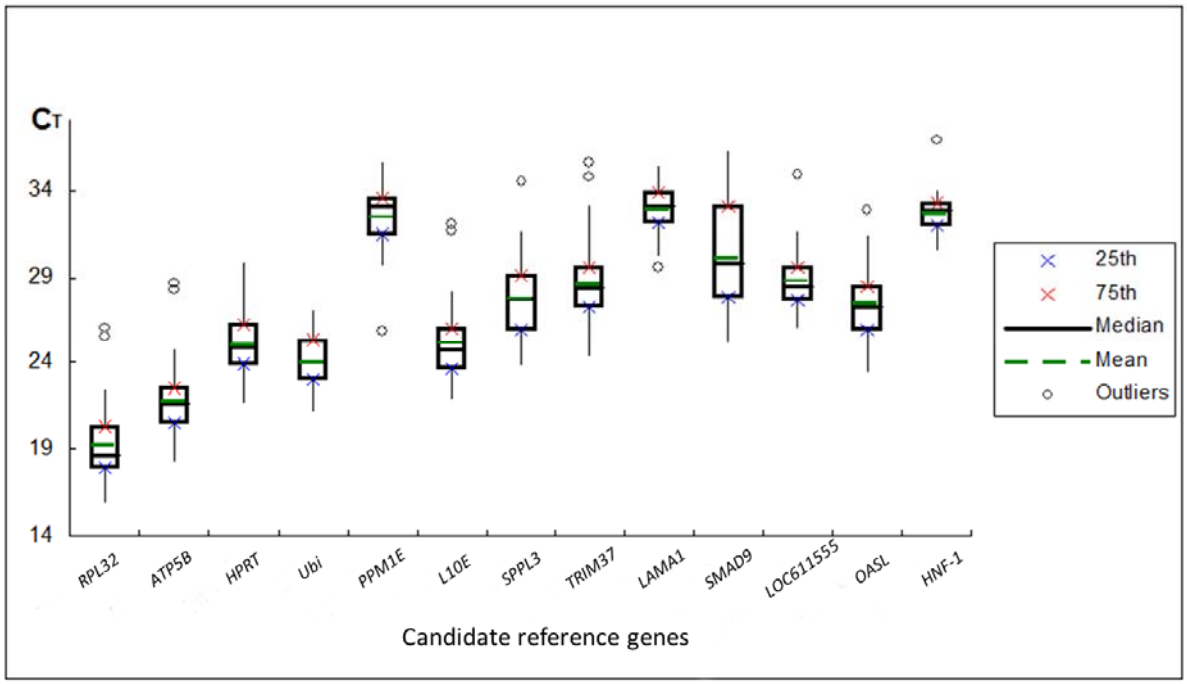

Figure 10. RNA transcription of the candidate reference genes, represented as CT values, across all tissues analyzed.
Boxplots indicate the 25th and 75th percentile (box boundaries), the median (bold lines), mean (lines), ranges (whiskers), and outliers (circles).

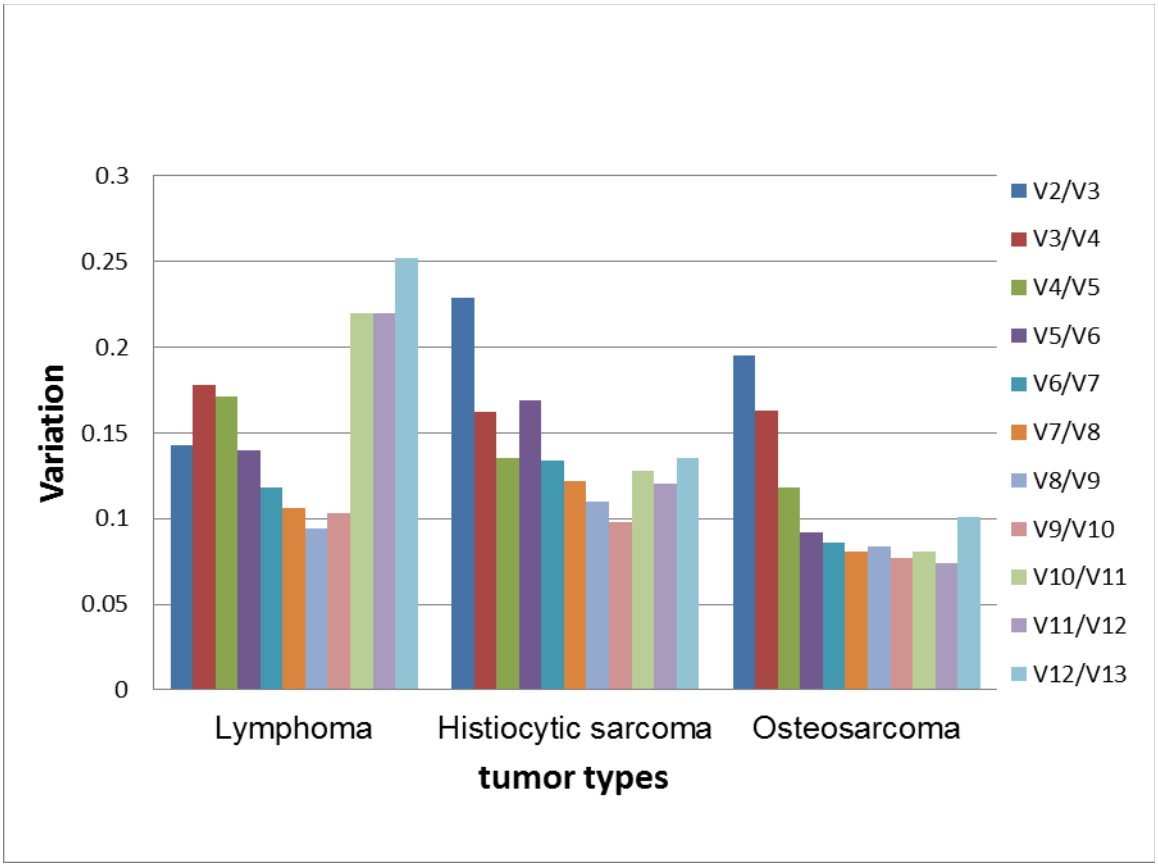

Figure 11. Determination of the optimal number of reference genes for normalization of each tumor.
The geNorm calculates the pairwise variation (Vn/n+1) between two sequential normalization factors (NFn and NFn+1). For example of lymphoma, the V2/V3 shows the variation below 0.15 which is a suggested cut-off value implying that there is no need for use of more than two reference genes.

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Figure legends

Figure 1 - RNA transcription of the candidate reference genes, represented as C_T values, across all tissues analyzed. Boxplots indicate the 25^{th} and 75^{th} percentile (box boundaries), the median (bold lines), mean (lines), ranges (whiskers), and outliers (circles).

Figure 2 - Determination of the optimal number of reference genes for normalization of each tumor. The geNorm calculates the pairwise variation ($V_{n/n+1}$) between two sequential normalization factors (NF_n and NF_{n+1}). For example of lymphoma, the V2/V3 shows the

variation below 0.15 which is a suggested cut-off value implying that there is no need for use of more than two reference genes.

CHAPTER 4: Use of Quantitative Real-Time PCR to Investigate the Molecular Alterations of Potentially Important Genes in Recurrently Aberrant Chromosomal Regions

Abstract

DNA copy number changes might lead to altered expression and function of oncogenes or tumor suppressors residing within affected chromosomal regions. In previous studies, we have identified several regions of the canine genome that are subject to recurrent copy number aberrations in lymphoma. To identify potential target genes located in these regions, 11 genes including *MYC*, *KIT*, *MDM2*, *PTEN*, *TSC2*, *H-Ras*, *MEOX-2*, *TLR5*, *POLD1* and *STAT1* were selected for further study based on their known tumor associated roles and history of deregulation in human lymphomas. Real-time quantitative PCR and molecular cytogenetic techniques were performed to detect the molecular alterations of these genes in nucleic acid samples isolated from 17 canine malignant lymph node specimens that were snap frozen at the time of biospy. Increased expression of *MYC* was detected in 9 (53%) cases; *MEOX-2* and *KIT* were downregulated and decreased expression of *MEOX-2* and *KIT* were detected in 17 (100%) and 13 (76%) of cases, respectively. Moreover, we observed that *KIT* was highly expressed in two T-cell lymphoma cases, which suggests that these cases may be potential targets for KIT-specific treatment using tyrosine kinase inhibitors. Furthermore, by combining gene expression data with cytogenetic changes, three genes demonstrated simultaneous change between mRNA expression and DNA copy number, *CDKN2A*, *TSC2* and *STAT1*. Deregulation of these genes may interfere with cell cycle control and signaling pathways that are involved in the pathogenesis of canine lymphoma.

Introduction

Lymphoma (LSA) is the most common life-threatening cancer in dogs, accounting for up to 24% of all canine neoplasia and over 80% of all canine hematopoetic cancers. Although the dog genome has been sequenced and displays a high degree of similarity to human genome (Kirkness et al 2003, Lander et al 2001, Lindblad-Toh et al 2005), the exact genetic events underlying the formation and development of canine lymphoma remain largely unknown. To

date, few aberrant genetic events have been reported in canine lymphomas, including *CDKN2A* (Fosmire et al 2007, Modiano et al 2007), *Rb* (Modiano et al 2007), *p53* (Nasir and Argyle 1999, Veldhoen et al 1998) and *N-ras* (Mayr et al 2003), *MYC* (Breen and Modiano 2008), and *FHIT* (Hiraoka et al 2009). Inactivation of CDKN2A and Rb has been associated with unfavorable outcome in canine lymphoma (Modiano et al 2007). Tumor associated genes have not been fully explored in canine lymphoma, as evidenced by a limited pool of available data.

In the past decade, a long list of characteristic chromosome aberrations have been identified in human lymphomas, and a proportion of them have served as molecular markers for various subtypes of human lymphomas. Such aberrant regions in the tumor genome are thought to harbor genes that may play an important role in tumor development and progression. Indeed, a number of the known tumor suppressor genes and oncogenes associated with lymphoma have been identified by fine mapping of these chromosomal aberrations, such as *BIM* (Tagawa et al 2005), *CDKN2A* (Dijkman et al 2006), *TP53* (Chen et al 2006, Kohlhammer et al 2004, Tagawa et al 2004), *FHIT* (Tagawa et al 2004), and *ATM* (Kohlhammer et al 2004). Chromosomal aberrations are also observed in canine lymphoma. In previous studies, we identified such recurrent aberrations using comparative genomic hybridization (CGH). Chromosomal aberrations that occur with high frequency include aberrations on CFA 1, 6, 11, 13, 14, 18, 31, 37, and 38 (Modiano et al 2005, Thomas et al 2003). A small homologous deletion involving *CDKN2A* locus (11q15dist-q16prox) was also identified during the process (Fosmire et al 2007). By examining these commonly affected genomic regions, we found that in addition to *CDKN2A*, these regions harbor many known tumor associated genes, including *MYC* (CFA13:28.23-28.24Mb), *KIT* (CFA13:50.04-50.12Mb), *MDM2* (CFA31:6.531-6.533 Mb), *PTEN* (CFA26: 40.92-40.98 Mb), *TSC2* (CFA6:41.90-41.94 Mb), *H-Ras (*CFA18: 28.632-28.634 Mb), *MEOX-2* (CFA14:33.20-33.27 Mb), *TLR5* (CFA38:26.65-26.66 Mb), *POLD1* (CFA1: 109.18-109.20 Mb) and *STAT1* (CFA37:4.52-4.54 Mb). These genes were selected based on their established roles in tumor development and history of altered gene expression in human NHLs (Aldinucci et al 2002, Badgwell et al 2004, Croce and Nowell 1985, Dave et al 2004, Goldman and McGuire 1992,

Goldsby et al 2002, Kang et al 2009, Kaplan et al 1998, Lesinski et al 2003, Li et al 2003, Moller et al 1999, Nakahara et al 1998, Pelicci et al 1986, Pinto et al 1994, Shankaran et al 2001, Song et al 2009, Turbin et al 2006) (see Table 24).

In this study, we explored the molecular alterations of these potentially important genes in canine lymphoma. As far as we know, there is no study that evaluates concomitantly the expression of the above tumor-associated genes in canine lymphoma and correlates their expression to cytogenetic features. Here, we used real-time quantitative PCR (RT-qPCR) and molecular cytogenetic techniques to investigate the molecular alterations of 11 potential target genes residing within the commonly aberrant genome regions in a cohort of 17 canine lymphoma samples. We combined the gene expression results with their corresponding cytogenetic data in order to identify genes with high correlation between gene expression and DNA copy number, which are more likely to be important for canine lymphoma development since primary events in lymphomagenesis generally include chromosomal abnormalities.

Materials and methods

Case materials for gene expression analysis

Canine lymphoma case samples (n=17; 12 B-cell and 5 T-cell) were recruited from client-owned dogs admitted to the College of Veterinary Medicine at North Carolina State University. All biopsies were acquired prior to initiation of chemotherapy or radiotherapy, under approved protocols (state the IACUC approval number) and with informed client consent. Representative portions of each tumor specimen were a) formalin-fixed/paraffin-embedded and b) snap-frozen in liquid nitrogen for subsequent RNA extraction. Non-neoplastic lymphoid tissues (n=2) were obtained at necropsy from non-diseased laboratory dogs through the College of Veterinary Medicine at North Carolina State University, approved by the Institutional Animal Care and Use Committees at North Carolina State University. All tissue samples were submitted for evaluation by the NCSU CVM pathology service for routine H&E and immunohistocytochemistry to confirm their diagnosis.

RNA extraction

For all tissue samples, total RNA was extracted from 25 mg of each sample using an RNeasy kit (Qiagen Inc., Crawley, UK) according the manufacturer's introductions. To remove residual genomic DNA, a genomic DNA digestion step was included (Ambion Inc., Austin, USA). The concentration of total RNA representing each sample was quantified using a NanoDrop ND-1000 UV/Visible spectrophotometer (NanoDrop Technologies Ltd., Utah, USA). Subsequently, samples were examined for RNA integrity in an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, USA). All RNA samples used in the study had a RIN>9.0.

Real-time quantitative PCR analysis

Gene expression levels of *MYC, CDNK2A, MDM2, PTEN, TSC2, KIT, H-Ras, MEOX-2, TLR5, POLD1* and *STAT1* genes were studied by real-time quantitative PCR (RT-qPCR). Primer sequences were designed for each gene from available transcript sequences (NCBI) using the Primer 3 primer design software. Reverse transcriptions (RT) were performed on 1 μg of isolated total RNA using the QuantiTect Reverse Transcription Kit (Qiagen Inc., Valencia, USA) according to the manufacturer's instructions. The RT-qPCR assays were performed in triplicate using an iCycler (Bio-Rad, Hercules, USA) in a 96-well format using the QuantiFast SYBR Green PCR kit (Qiagen, Valencia, USA), according to the manufacturer's instructions. No- template and no-RT controls were used for each assay. The standard amplification conditions consisted of 1 cycle at 95℃ for 5 min, followed by 40 cycles of 95℃ for 10 s and 60℃ for 30 s. To determine the RT-qPCR assay efficiency of each gene examined, RT-qPCR was performed in a 5-step, 10-fold dilution series of a pool of RNA samples isolated from non-neoplastic canine lymph node (Higuchi et al 1993). All PCR primers displayed efficiency between 95% and 101%. The relative gene expression was normalized against *LOC611555* expression. This gene was chosen as an internal calibrator after evaluating 13 genes in the canine model system using three separate mathematical algorithms (for details please see Chapter 3). Differential expression in each sample was determined by two-tailed unpaired t-test, implemented in the qGene application (Simon

2003). Group-wise comparison of expression results between malignant and healthy group was determined by REST algorithm (Pfaffl et al 2002). A summary of the genes and corresponding primer sets used in the study is shown in Table 25.

Cytogenetic analysis of canine lymphomas

Cytogenetic data of 17 canine lymphoma samples were obtained from array-based comparative genomic hybridization (aCGH) analysis, using a 1Mb resolution, genome assembly-integrate microarray (Thomas et al 2008, Thomas et al 2011). Log2 ratio values greater than 0.2 and less than -0.2 were considered as genomic gain and loss, respectively. Conventional fluorescence *in situ* hybridization (FISH) was performed on a representative subset of the lymphoma sample population to confirm the copy number changes revealed by aCGH data. FISH analysis was carried out as previously described (Chapter 2: Materials and methods; Conventional fluorescence in situ hybridization). DNA probes were hybridized first onto metaphase chromosome preparations from clinically healthy dogs to confirm the expected copy number (n=2) for the probe at the expected chromosomal location. Images were acquired from a minimum of 30 representative cells in each case.

Results

Gene expression profile analysis of potentially target genes

To investigate the molecular alterations of these potentially important genes, each of which is located in commonly aberrant regions of the genome in canine lymphoma, RT-qPCR analysis was performed to evaluate the RNA transcription level of each gene in lymphoma samples and in normal lymph node tissues. The statistical algorithm REST was used to determine whether there was a significant difference in transcriptional levels between lymphoma samples and non-neoplastic lymph node controls. The RNA transcription profiles of 11 potential target genes are shown in Figure 12. Analysis of the expression profiles reveals that *MYC* exhibited upregulation in lymphoma samples ($P = 0.014$). Increased expression of *MYC* mRNA was observed in 53% of the cases studied. In contrast, *MEOX-2* and *KIT* exhibited downregulation in lymphoma samples ($P = 0.001$ and 0.008, respectively).

Decreased expression of *MEOX-2* and *KIT* were observed in 100% and 76% of the cases studied, respectively. Other potential target genes did not show significant differences between groups of tumor samples and normal controls.

Correlation between expression data and cytogenetic results

To further access the possible roles of these potential target genes in canine lymphomas, expression results were compared with the corresponding cytogenetic data for each lymphoma case. A two-tailed unpaired t-test was used to determine differential expression in each sample. Table 26 summarizes the correlation between the copy number status determined by CGH analysis and the gene expression level detected by RT-qPCR for each individual gene. The level of correlation was defined as the percentage of cases showing consistent correlation between DNA copy number and mRNA expression level. Genes with better relationship between the RT-qPCR results and the cytogenetic profile include *STAT1*, *TSC2*, and *CDKN2A*, all of which showed correlation levels >80%. Copy number gain of the *STAT1* locus was seen in 3 of 17 cases. Within those three cases, two showed a simultaneously increased gene expression of *STAT1*. The other 14 cases with a normal copy number of the *STAT1* locus did not show any increased or diseased expression of *STAT1*. For *TSC2*, the three cases with gains of the *TSC2* locus showed a concomitant increase in mRNA expression level. However, the two cases with a hemizygous deletion of *TSC2* did not show any differences in expression levels. For *CDKN2A*, homozygous or hemizygous deletion was observed in 4 of 17 cases. The expression of *CDKN2A* was, as expected, almost undetectable in cases with an apparent homozygous deletion of the *CDKN2A* locus. However, one case where we detected a hemizygous deletion of *CDKN2A* did not show decreased expression of *CDKN2A*. The gene expression data and the corresponding cytogenetic results of each gene on 17 lymphoma cases are illustrated in Appendix Figure 20 to 30.

Discussion

Tumor progression is thought to be driven by the accumulation of genetic alterations and the consequent gene expression pattern changes. Abnormalities such as chromosomal

translocations or amplifications may lead to deregulated expression of oncogenes, which encode proteins important for cell proliferation, cell cycle progression, differentiation, or apoptosis regulation. However, deregulated expression of oncogenes may not be sufficient for malignant transformation and additional steps such as inactivation of tumor suppressor genes are required. Proteins encoded by tumor suppressor genes prevent malignant transformation by controlling cell cycle progression, protecting genomic DNA against damage, inducing apoptosis, or restraining cell migration and metastasis. Classic mechanisms for tumor suppressor gene inactivation include chromosomal loss, mutation, and methylation. The identification of oncogenes and tumor suppressor genes not only improves understanding of biological processes of tumorigenesis but also provides new targets for clinical treatment. In our previous efforts, we identified the most commonly encountered DNA copy number aberrations in canine lymphoma (Modiano et al 2005, Thomas et al 2003). In this study, RT-qPCR was used to investigate the expression level of a series of tumor associated genes residing within these commonly aberrant regions. By analyzing the expression profiles of each gene, we observed an upregulation of *MYC* and a downregulation of *KIT* and *MEOX-2* in canine lymphoma samples.

MYC is an oncogene and implicated in a variety of human cancers and animal neoplasias (Grisham 1997). In human NHLs, deregulation of *MYC* is primarily mediated by translocations that juxtapose *MYC* to the *IGH* enhancer sequences. Translocations involving *MYC* are typically associated with Burkitt's lymphoma (BL) (Taub et al 1982), but are also observed in diffuse large B-cell lymphoma (DLBCL), follicular lymphoma and, mantle cell lymphoma (Au et al 2004, Cigudosa et al 1999, Ladanyi et al 1992). In veterinary studies, *MYC-IGH*-like translocation has also been observed in canine BL and similarly leads to *MYC* overexpression (Breen and Modiano 2008). Furthermore, *MYC* overexpression has also been observed in canine DLBCL, a subtype that generally has chromosomal gain of *MYC* on CFA 13 (Breen and Modiano 2008, Thomas et al 2003). Within 17 canine lymphoma cases analyzed in this study, two of them were classified as BL and both showed overexpression of *MYC*. For other 15 non-BL cases, seven cases showed overexpression of *MYC*, but only three with a concomitant increase in gene dosage. These findings suggest that in addition to gene

translocation and amplification, other mechanisms may also contribute to the deregulation of *MYC* in canine lymphoma. It may be that while the copy number of *MYC* was balanced in numerous cases, one of the two copies was translocated or mutated resulting in overexpression. This is consistent with observations in human lymphoma (Stasik et al 2010), suggesting that multiple, complex mechanisms are involved in transcriptional regulation of *MYC* in both species.

KIT is a tyrosine kinase receptor expressed on the surface of hematopoetic progenitor cells and mature mast cells (Ashman et al 1987, Cambareri et al 1988). KIT binds to stem cell factor and plays an important role in hematopoiesis (Arber et al 1998, Ashman et al 1991, Natkunam and Rouse 2000). Aberrant expression of *KIT* has been described in several human hematopoietic malignancies, including T-cell lymphoblastic lymphoma, acute myeloid leukemia, Hodgkin's lymphoma, anaplastic large cell lymphoma, and in a small number of DLBCL (Aldinucci et al 2002, Brach et al 1992, Chen et al 2001, Pinto et al 1994, Sykora et al 1997). Lymphoma patients with high *KIT* expression are targets for applying target-specific therapy using tyrosine kinase inhibitors (Heinrich et al 2000, Smolich et al 2001). In our study, although *KIT* was down-regulated in a majority of lymphoma cases, we still observed 2/5 T-cell lymphoma cases with high *KIT* expression, including one lymphoblastic T-cell lymphoma and one peripheral T-cell lymphoma. This finding is consistent with previous observations on human lymphoma in which *KIT* overexpression was frequently detected in T-cell lymphoblastic lymphoma, but rarely been reported in B-cell ones (Ferrari et al 1993, Kiss et al 1993, Sperling et al 1997, Tomeczkowski et al 1998). Studies from Tomeczkowski et al. might provide an explanation for these observations. They report that KIT was involved in the growth regulation of thymocytes and appeared to play a role in the development of T-lineage leukemia and lymphoma (Sykora et al 1997, Tomeczkowski et al 1998). Additional studies with larger sample sizes are required to ascertain whether overexpression of *KIT* in canine lymphomas is restricted to T-cell lymphoma. Dog patients with high *KIT* expression may benefit from targeted therapeutic approach enrolling the tyrosine kinase inhibitor, such as toceranib phosphate.

Furthermore, in our study, the overall expression pattern of *KIT* was downregulated, although the expression of *KIT* in normal lymph nodes was also low. A previous study examined the distribution of KIT protein in a number of human lymphomas and normal lymphoid tissues (Pinto et al 1994). The expression of KIT was weak or absent in both normal lymphoid cells and 24 cases of NHLs other than anaplastic large cell lymphoma (Pinto et al 1994). Since we only investigated the mRNA expression level of KIT in this study, further proteomic studies are needed to confirm whether the KIT protein is actually downregulated in canine lymphoma samples.

The *MEOX-2* gene was dramatically downregulated in all lymphoma samples evaluated, with no genetic deletion detected. Homeobox gene *MEOX-2* is a transcription factor that controls many aspects of human vascular cellular processes such as proliferation, migration, and angiogenesis (Gorski et al 1993, Weir et al 1995). A recent study also reported that MEOX-2 is a direct regulator of *CDKN2A* transcription and regulates *CDKN2A*-mediated senescence pathway in human cells (Irelan et al 2009). However, in this study, we did not find any correlation between *CDKN2A* and *MEOX-2* expression. Furthermore, several studies have reported that *MEOX-2* was inactivated by promoter methylation in human lung cancer and human Wilms tumor (Cortese et al 2008, Ohshima et al 2009). Therefore, aberrant methylation of gene promoter might be a possible mechanism to inactivate *MEOX-2* expression in canine lymphoma. Further studies of how *MEOX-2* is deregulated in canine lymphoma and the potential influence of loss of *MEOX-2* expression in carcinogenesis will be discussed in Chapter 5.

In addition, by parallel analysis of expression profile and cytogenetic status, only three genes *CDKN2A*, *STAT1*, and *TSC2* demostrated a close relationship between mRNA expression level and DNA copy number. *CDKN2A* is a tumor suppressor gene that is frequently inactivated in a variety of human hematologic malignancies and canine lymphomas (Belaud-Rotureau et al 2008, Fosmire et al 2007, Krug et al 2002, Ruas and Peters 1998). In dog studies, the deletion or inactivation of *CDKN2A* is restricted to T-cell lymphoma (Fosmire et al 2007). A recent study further reports that dogs with inactivation of CDKN2A had significantly shorter survival (Modiano et al 2007, Thomas et al 2011). In our

study, we examined 17 canine lymphoma cases (12 B-cell tumors and 5 T-cell tumors). Four of five cases of T-cell lymphoma had loss of the *CDKN2A* locus; this abnormality was not seen in any of the B-cell lymphoma cases analyzed. These results support previous findings that *CDKN2A* deletion occurs exclusively in T-Cell canine lymphoma.

STAT1 is a transcription factor involved in regulating genes in response to signals by type I and II interferon (IFNs), which are key components of tumor surveillance (Dalton et al 1993, Sexl et al 2003, Street et al 2002). Therefore, it has been classified as a tumor suppressor gene, and *STAT1* knockout mice have been shown to develop solid tumors (Badgwell et al 2004, Kaplan et al 1998, Lesinski et al 2003, Shankaran et al 2001). However, according to a recent study, increased expression of STAT1 can also accelerate the development of hematopoietic tumors by up-regulation of MHC class I molecules, which is a general mechanism to escape tumor surveillance (Kovacic et al 2006). In our study, we observed *STAT1* overexpression in two T-cell lymphoma cases. Since the STAT1-induced effects on tumorigenesis seem to be biphasic; additional studies combined with clinical data are required to explain its roles in canine lymphoma.

TSC2 is another gene-dosage regulated candidate. It is a tumor suppressor gene; mutation of *TSC2* leads to tuberous sclerosis complex (TSC), which causes a non-malignant tumor in the brain and organs such as the kidney, skin, eyes, heart and lungs (Gomex et al 1999). TSC2 has been shown to act as a negative regulator of cell growth via the mammalian target of rapamycin (mTOR) signaling pathway (Astrinidis and Henske 2005) and G1-S transition in the cell cycle (Soucek et al 1997). It has been reported that *TSC2* was a direct target of *MYC* and that its transcription is negatively regulated by *MYC* (Ravitz et al 2007). However, another study indicated the opposite result: *TSC2* expression in peripheral lymphocytes was not affected by overexpression of *MYC* (Soucek et al 1997). In our study, *MYC* was overexpressed in 53% of the cases. However, we did not observe an inverse correlation between *MYC* and *TSC2*.

Additionally, among these tumor associated genes investigated in this study, *POLD1* (DNA polymerase δ catalytic subunit) is located within the most common region (MCR) of chromosomal aberration on CFA 1, whose mean copy number has been shown to be

associated with DFI in Chapter 2. POLD1 is a cell cycle-dependent protein with important roles in DNA replication, repair, and DNA methylation damage repair (Aboussekhra et al 1995, Halas et al 1997, Hindges and Hubscher 1997). Abnormal expression of *POLD1* has been observed in some human cancers (Kang et al 2009, Song et al 2009); POLD1 deficiency causes various cancers in mice, including lymphoma (Goldsby et al 2002). Regarding the gene structure of *POLD1*, human *POLD1* is adjacent to *SPIB* and shows 47% of gene identity with *SPIB*. A recent report indicated that these two genes share a common regulatory element(s) (Lenz et al 2008). As we mentioned previously in Chapter 2, *SPIB* is a candidate oncogene involved in activated B cell-like (ABC) diffuse large B-cell lymphoma (DLBCL), and its overexpression is associated with a frequent gain located in human chromosome (HSA) 19q (Lenz et al 2008). Since the region corresponding to the human *SPIB* locus lies within the MCR of aberration on CFA 1, we were interested in investigating the genetic and molecular alterations of *SPIB* in dog lymphoma. However, *SPIB* has not been annotated in the dog genome. Since *POLD1* shares sequence similarity and regulatory elements with *SPIB* and plays an important role in cell cycle and tumor development, we investigated *POLD1* instead. In this study, *POLD1* in dog lymphoma seems not to be expressed in a direct gene dosage dependent manner. Candidate target genes mapping to MCR of aberration on CFA 1 remain to be determined.

In conclusion, the present study characterized the RNA expression patterns of 11 tumor associated genes in canine lymphoma and identified three apparently gene-dosage regulated candidates**.** These findings provide new clues to the genetic basis of biological and clinical behavior of canine lymphoma and also provide potential targets for new therapy. Further studies with larger samples and protein level investigations are required to discover their functional relevance with respect to canine lymphoma.

Table 24. Candidate target genes considered in this study

Chromosomal location	Candidate genes	Gene function	Deregulation in tumorigenesis
CFA11:44,255,706-44,256,016	*CDKN2A*	Cell cycle kinase inhibitor	Human NHLs; canine T-cell lymphoma (Belaud-Rotureau et al 2008, Dijkman et al 2006, Fosmire et al 2007, Modiano et al 2007)
CFA13:28,238,008-28,242,545	*MYC*	Transcription factor	Human B-cell lymphomas; canine lymphoma (Au et al 2004, Cigudosa et al 1999, Croce and Nowell 1985, Ladanyi et al 1992, Pelicci et al 1986)
CFA13:50,040,826-50,122,327	*KIT*	Stem cell growth factor receptor	Human lymphoma and leukemia (Aldinucci et al 2002, Brach et al 1992, Chen et al 2001, Pinto et al 1994, Sykora et al 1997)
CFA18:28,632,560-28,634,065	*H-Ras*	Cell division	Human T-cell acute lymphoblastic leukemia (Goldman and McGuire 1992)
CFA26:40,921,802-40,981,821	*PTEN*	Cell cycle regulation	Human NHLs (Nakahara et al 1998)
CFA 31:6,531,719-6,533,725	*MDM2*	p53 inactivation	Human hematological malignancies (Moller et al 1999, Turbin et al 2006)
CFA1:109,184,543-109,200,788	*POLD1*	DNA replication	Human Cervical cancers; deficiency causes various cancers in mice, including lymphoma (Goldsby et al 2002, Kang et al 2009, Song et al 2009)
CFA14:33,208,198-33,273,050	*MEOX-2*	Regulator of vacular cells	Human lung cancer and Wilms tumor (Cortese et al 2008, Ohshima et al 2009)
CFA6:41,906,361-41,940,078	*TSC2*	Cell cycle regulation	Human Burkitt's lymphoma (Li et al 2003).
CFA38:26,650,008-26,662,100	*TLR-5*	Toll-like receptor	Human lymphomas (Adam et al 2008, Henault et al 2005, Wysocka et al 2004)
CFA 37:4,523,197-4,549,804	*STAT1*	Signal transduction	Human squamous cell carcinoma and breast cancer (Widschwendter et al 2002, Xi et al 2006).

Table 25. Genes and their primer sequences considered in this work.

Gene name	Forward and reverse primers (5'-3')
CDKN2A	F-TGATGATGGGCAGCACCCGC
	R-GCCGCGTCGTGCACAGGG
MYC	F-TCFCCTATTTGGGAAGACAC
	R-AAGCTGACGTTGAGAGGCAT
KIT	F-CGAAGATGTGTGAAGCAGGA
	R-GTGTCCGCTACCCTGGAATA
H-Ras	F-TGAAGGACTCTGACGACGTG
	R-TTGTGCTGTCGAATCTCTCG
PTEN	F-ACTTTGAGTTCCCTCAGCCA
	R-AGGTTTCCTCTGGTCCTGGT
MDM2	F-GGCAGGGGAGAGTGATACAG
	R-GCCAATTCTCACGAAGGGCCCAA
POLD1	F-TCCCACCTGAACGCCCTGGA
	R-GGGCCGAAGCGCCTCAGAAG
MEOX-2	F-AGACTGGCCGCTAGGGCTCC
	R-AGGCAGCCAAAGAGCGGGTG
TSC2	F-TCGTCGGACATCAACAACAT
	R-CCGCAGAGTCCGTGTTAGAT
TLR-5	F-GCATCCTGCTGCGTGGCTGA
	R-CGCTCCAGGAGGGGGAACGA
STAT1	F-GATGGGTAAGGGCCTTTGAT
	R-GACGGCCTGTAAGTGTCCAT

Table 26. Correlation between gene expression by RT-qPCR and copy number change by CGH

Gene examined	No. of patients studies	No. of tumor with CGH gain show simultaneous increased expression /No. of tumor with CGH gain	No. of tumor with CGH loss show simultaneous decreased expression /No. of tumor with CGH loss	No. of tumor with normal CGH show unchanged expression /No. of tumor with normal in CGH	% of cases show consistent correlation between expression and copy number change
MYC	17	3/5	0/1	6/11	53
CDKN2A	17	0/0	3/4	11/13	82
MDM2	17	0/0	0/0	14/17	71
PTEN	17	0/2	0/0	11/15	65
TSC2	17	3/3	0/2	12/12	88
KIT	17	2/4	0/0	2/13	23
H-Ras	17	0/1	1/4	12/12	76
MEOX-2	17	0/1	0/0	0/16	0
TLR5	17	0/0	0/4	5/13	29
POLD1	17	0/2	0/0	10/15	59
STAT1	17	2/3	0/0	14/14	94

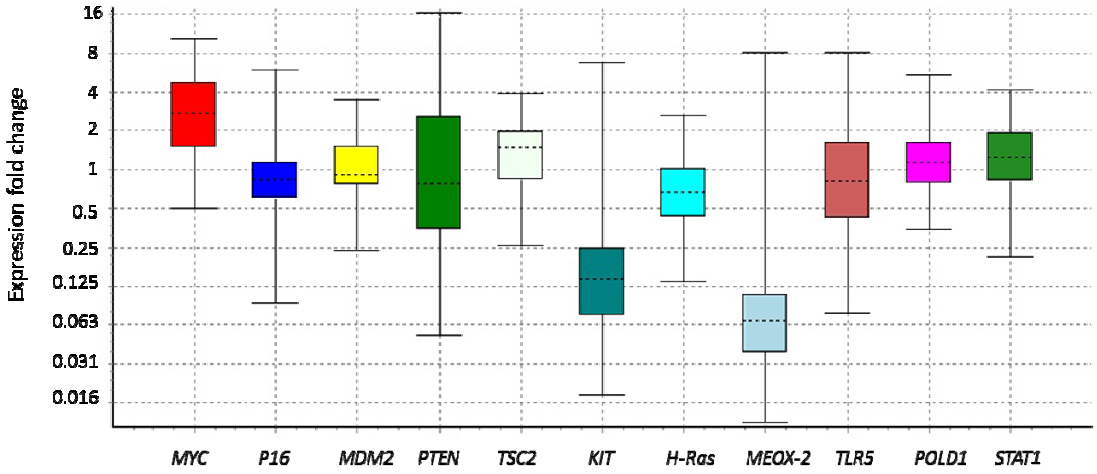

Figure 12. The RNA transcription of the tumor associated genes in expression fold changes over all tissues analyzed.
Boxplots indicate the 25 and 75 percentile (box boundaries), the medium (dot lines), and ranges (whiskers).

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CHAPTER 5: Aberrations of the Homeobox Gene *MEOX-2* in Canine Lymphoma

Abstract

The homeobox protein MEOX-2 is a transcription factor whose involvement in many aspects of vascular cellular processes has been recognized, in particular as a regulator controlling the angiogenic transition in vascular endothelial cells. Based on its global significance for development and cellular behavior, dysregulation of *MEOX-2* may potentially involve in oncogenesis. To investigate the possible role of *MEOX-2* in lymphoma, we used 14 lymphoma samples from canine lymphoma patients to examine the molecular alterations of *MEOX-2* that occur in association with lymphoma. Real-time quantitative PCR analysis showed that *MEOX-2* expression was silenced in all 14 lymphoma cases evaluated, with no corresponding genetic deletion detected by array comparative genomic hybridization (aCGH) and fluorescence *in situ* hybridization (FISH). Rather, dense promoter CpG island methylation of *MEOX-2* was detected in all lymphoma cases, but not in any non-neoplastic lymph nodes (n=8). These findings suggest that the expression of *MEOX-2* might be silenced by DNA hypermethylation. However, the functional roles of *MEOX-2* in the development of lymphocytes are still unknown. Additional studies are required to clarify the consequences of loss of *MEOX-2* expression in lymphoma. The tumor-specific methylation pattern of *MEOX-2* may have potential use as an epigenetic marker for molecular diagnosis of canine lymphoma.

Introduction

Homeobox genes are transcription factors containing a highly conserved DNA-binding domain termed the homeodomain, and play crucial roles in development as they control many different aspects of cellular differentiation, proliferation, migration, and apoptosis (Garcia-Fernandez and Holland 1996, Krumlauf 1994, McGinnis and Krumlauf 1992). During embryonic development, the earliest organ system to form is the cardiovascular system, which arises from mesoderm. Thus, it is not surprising that homeobox genes are also essential for regulating the development and functions of the vascular systems (reviewed in

(Douville and Wigle 2007). Recently, numerous examples of aberrant homeobox gene expression have been reported in a variety of malignancies, including lymphoma. Abate-Shen has proposed three categories for these deregulated homeobox genes in cancer (Abate-Shen 2002). The first category is those that are normally expressed during the development and/or in undifferentiated cells are re-expressed in cancer cells; the second is those that are not normally expressed in this tissue type but are expressed in cancerous tissues; the third is those that are normally expressed in differentiated and/or adult tissues and are epigenetically downregulated in cancerous tissues. Based on the significance of homeobox genes in developmental and tissue specific processes, expression analysis of homeobox genes is of interest for understanding the intimate relationship between differentiation processes and oncogenesis. Generally, homeobox genes that normally have tumor suppressive effects are silenced, while genes with an aberrant temporospatial expression pattern are associated with oncogenic effects (Shah and Sukumar 2010).

The diverged homeobox gene *MEOX-2* (also known as *MOX2* and *GAX)* is widely expressed in mesoderm and muscle precursor cells in the embryo (Candia and Wright 1996). In adults, its expression has been detected in cardiovascular system and mesoderm-derived tissues (Gorski et al 1993, Murthi et al 2007, Skopicki et al 1997). MEOX-2 has several characteristics suggestive of a key regulator controlling the angiogenic transition in vascular ECs. For example, *in vitro*, the expression of MEOX-2 is highest in quiescent vascular ECs, but is quickly reduced in response to mitogens and proangiogenic factors (Patel et al 2005). MEOX-2 expression inhibits vascular EC proliferation, migration, and VEGF-induced angiogenesis in both *in vitro* and *in vivo* models (Gorski and Leal 2003, Patel et al 2005). Moreover, studies of ECs revealed that proangiogenic factors, such as CXC chemokines, VEGF-C, and basic fibroblast growth factor (bFGF) are downregulated, whereas cyclin kinase inhibitors, such as $p19^{INK4D}$, $p57^{Kip2}$, and $p21^{WAF/CIP1}$ are upregulated in ECs that constitutively express MEOX-2 (Chan et al 1995, Smith et al 1997, Tsugu et al 2000). Two mechanisms through which MEOX-2 inhibits the activation of vascular ECs have been identified recently. First, MEOX-2 induces cell cycle arrest by upregulating $p21^{WAF/CIP1}$ through directly binding to its promoter and enhancer regions (Smith et al 1997). Second,

MEOX-2 inhibits the proangiogenic NF-κB activity in vascular ECs, resulting in the downregulation of downstream genes involved in vascular EC activation during angiogenesis (Patel et al 2005). In addition, in vascular smooth muscle cells (VSMC), consistent with its role in inhibiting cell growth and activation, MEOX-2 expression induces cell cycle arrest through direct upregulation of p21 expression and inhibits cellular migration through downregulation of integrin expression (Smith et al 1997, Witzenbichler et al 1999). MEOX-2 expression can also induce apoptosis through downregulation of the anti-apoptotic BCL-2 and upregulation of the pro-apoptotic BAX in VSMC (Perlman et al 1998). In fibroblasts, MEOX-2 expression induces p16INK4a-dependent senescence (Irelan et al 2009). These findings suggest that MEOX-2 is responsible for many aspects of cellular processes in various cell types and acts more like a tumor suppressor.

Recently, increasing evidence has shown that normal *MEOX-2* expression can be altered during a diseased state. For example, reduced *MEOX-2* expression has been described in human non-small cell lung cancer (NSCLC) and Wilms tumor (Cortese et al 2008, Ohshima et al 2009). Intriguingly, both dysregulations were mediated by aberrant promoter methylation. Although the current state of knowledge is insufficient to explain the significance of loss of *MEOX-2* in these tumor types, according to the categories defined by Abate-Shen, aberrant expression of *MEOX-2* falls into the third category in which tumor suppressive homeobox genes are epigenetically downregulated in tumorigenic cells (Abate-Shen 2002). These observations prompted interest in studies examining the expression pattern of *MEOX-2* in different tumor types. Unlike classic oncogenes or tumor suppressors that display broad specificities in many tumor types, homeobox genes show tissue-specific features and thus could be used as disease markers or therapeutic targets.

To our knowledge, no published study has examined the expression pattern of *MEOX-2* in lymphoma tissues. In the study herein, we focused on canine lymphoma, which is one of the most common cancers in dogs and accounts for up to 24% of all canine tumors, and 83% of all canine hematopoietic malignancies (MacEwen 1990, Moulton and Harvey 1990). Canine lymphoma shares biological and clinical features with human NHLs and has recently been used as a tumor model (Burnett et al 2003, Fournel-Fleury et al 1997, Greenlee et al 1990,

Hahn et al 1994, Hansen and Khanna 2004, Lana et al 2000). Unlike the traditional rodent model, canine lymphoma occurs spontaneously, and provides a compelling model to investigate heritable and sporadic factors that contribute to the pathogenesis of lymphoma. In this study, we first examined the mRNA expression of *MEOX-2* using a cohort of canine lymphoma samples and normal canine lymph nodes. We present evidence that *MEOX-2* was dramatically downregulated in all canine lymphoma cases. In exploring the possible mechanisms for the transcriptional inactivation of *MEOX-2* in canine lymphoma, we carried out methylation analyses and molecular cytogenetic evaluation to investigate the correlation between epigenetic and genetic alterations of *MEOX-2* and its expression.

Materials and Methods

Tumor samples

Canine lymphoma case samples (n=14; 9 B-cell and 5 T-cell) were recruited from client-owned dogs admitted to the College of Veterinary Medicine at North Carolina State University. All specimens were acquired prior to initiation of chemotherapy or radiotherapy, under approved protocols and with informed client consent. Representative portions of each tumor specimen were formalin-fixed/paraffin-embedded and also snap-frozen in liquid nitrogen for subsequent RNA/DNA extraction. Non-neoplastic lymphoid tissues (n=8) were obtained from non-diseased laboratory dogs through the College of Veterinary Medicine at North Carolina State University, approved by the Institutional Animal Care and Use Committees at North Carolina State University. All tissues were evaluated with CD3 and CD79a immunohistocytochemistry to verify the immunophenotype.

DNA and RNA preparation

DNA and RNA were isolated from snap-frozen tissue using Qiagen DNeasy tissue and RNeasy (Qiagen, Valencia, CA) according to the manufacturer's protocols, respectively. All RNA samples were treated with a DNase digestion step to remove residual genomic DNA (Ambion, Austin, TX). The RNA integrity was qualitatively assessed and quantified using

Bioanalyzer (Agilent Technologies, Palo Alto, CA). All RNA samples used in the study had a RIN>9.0.

Real-time quantitative PCR (RT-qPCR)

One μg of total RNA was used to generate cDNA using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Real-time quantitative PCR assays were performed in triplicate using the iCycler (Bio-Rad, Hercules, USA). Each reaction contained 1 μl of cDNA template, primers at a concentration of 1 μM, 1X SYBR master mix (Qiagen, Valencia, CA), and 0.5 μl of fluorescein (Ambion, Austin, TX). The primer sequences were as follows: MEOX-2 forward, 5'-CGGCTCGTCACAGCCTCTGC-3'; MEOX-2 reverse, 5'-CCTGGCGGCCGTAGTCTCCT-3'. The amplification conditions consisted of 1 cycle at 95^{o}C for 5 min, followed by 40 cycles at 95 ^{o}C for 10 s and 60 ^{o}C for 30 s. The relative gene expression was normalized against LOC611555 gene expression. This gene was selected as an internal reference by evaluating 13 genes in the canine model system using three separate mathematical algorithms (see Chapter 3). Group-wise comparison of expression results between the malignant and healthy group was determined by the REST algorithm (Pfaffl et al 2002).

Methylation-specific PCR (MS-PCR) analysis

The MS-PCR analysis used in this study was originally described by Herman et al.(Herman et al 1996). Bisulfite modification of genomic DNA was performed using the Qiagen Epitect bisulfite kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. The location of the CpG island of the MEOX-2 gene was determined using the canine CpG site map from the UCSC genome browser. It is located in an 1889-bp region encompassing the first exon of MEOX-2 (spanning from -746 to +1143 relative to the translation start site). Five sub-CpG islands (termed CGI 1 to 5) within the CpG island were determined by the Methprimer software (Li and Dahiya 2002). Bisulfite-treated DNA was used as a template

and amplified with primers specific for methylated and unmethylated sequences from those five sub-regions (see Figure 13). All primers were designed by Methyl Primer Express software ver.1.0 (Applied Biosystems). The primer sequences, locations, and expected product size are listed in Table 27. The PCR conditions consisted of 5 min at 95^{o}C for initial denaturation, followed by 35 cycles at 95^{o}C for 30 s, 57^{o}C for 30 s, and 72^{o}C for 30 s and a final extension of 10 min at 72^{o}C. For the preparation of positive control (100% methylated DNA), the genomic DNA obtained from pooled canine non-neoplastic lymphoid cells was treated with SssI methyltransferase to methylate cytosine residues in all CpG dinucleotides (New England Biolabs, Beverly, MA). The amplified PCR products were electrophoresed on 2% agarose gels and visualized using GelRed^{TM} staining (Biotium, Hayward, CA).

Combined bisulfite and restriction analysis (COBRA)

COBRA was originally described by Xiong and Laird (Xiong and Laird 1997). Bisulfite-treated DNA was used as a DNA template in a PCR reaction and amplified with the following primer: forward, 5'-GTTTTAATTTTTTAGTTTGGGTT-3'; and reverse, 5'-TATCCTTTTTTCTTAACCCTCTAA-3'. The PCR running conditions were as follows: initial denaturation for 5 min at 95^{o}C, followed by 35 cycles of denaturation for 30 s at 95^{o}C, annealing for 30 s at 55^{o}C, extension for 30 s at 72^{o}C, and a final extension of 10 min at 72^{o}C. The PCR product was purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA), then followed by restriction enzyme digestion using the enzymes: BstUI and HpyCh4IV (New England Biolabs, Beverly, MA). Ten microliters of PCR product were digested with 10 U BstUI for 4 h at 60^{o}C or 15 U HpyCh4IV for 4 h at 37^{o}C. Digested products were subjected to electrophoresis on 2% agarose gels, and visualized by GelRed^{TM} staining (Biotium, Hayward, CA).

Bisulfite sequencing

Three lymphoma cases and one normal dog lymph node tissues were amplified using the COBRA primer set and PCR conditions. The PCR products were purified and cloned into plasmids using the Promega pGEM-T easy TA cloning kit (Promega, Madison, WI). For

each case, three to four single colonies of transformed cells were selected and cultured in LB medium containing 50 μg/mL ampicillin at 37°C overnight. Individual plasmid DNA was extracted using the Qiagen REAL prep kit (Qiagen, Valencia, CA) and sequenced with universal primers using the ABI Prism® 3730 DNA Sequencer (Applied Biosystems, Foster city, CA).

Cytogenetic analysis of canine lymphomas

Cytogenetic profiling data of canine lymphoma samples were generated from an array-based comparative genomic hybridization (aCGH) analysis, using a 1Mb resolution (Thomas et al 2008), genome assembly-integrate microarray (Thomas et al 2011). Log2 ratio values greater than 0.2 and less than -0.2 were considered as genomic gain and loss, respectively. Conventional fluorescence *in situ* hybridization (FISH) was performed on a representative subset of the lymphoma sample population in order to confirm the copy number changes revealed by aCGH data. FISH analysis was carried out as previously described (Chapter 2: Materials and methods; Conventional fluorescence in situ hybridization). DNA probes were hybridized first onto metaphase chromosome preparations from clinically healthy dogs to confirm the expected copy number (n=2) for the probe at the expected chromosomal location. Images were acquired from a minimum of 30 representative cells in each case.

Results

MEOX-2 expression analysis in canine lymphoma samples and in normal lymph nodes

To investigate the expression pattern of *MEOX-2* in canine lymphoma, we assessed its mRNA expression in a collection of 14 primary canine lymphoma cases and eight non-neoplastic lymph node samples using RT-qPCR. As shown in Figure 14, *MEOX-2* was expressed in all non-neoplastic lymph node samples from healthy individuals, whereas the expression was dramatically reduced or barely detectable in all 14 lymphoma cases. *MEOX-2* exhibited significant downregulation in canine lymphoma samples compared with normal lymph nodes ($P = 0.001$). From the expression pattern of *MEOX-2* in canine lymphoma

samples, we conclude that *MEOX-2* expression is uniformly silenced in canine lymphoma cells, and the expression inactivation appears to be tumor-specific.

Methylation status of *MEOX-2* in canine lymphoma

As methylation of promoter CpG islands is a common mechanism for transcriptional silencing (Jones and Baylin 2002), we investigated the methylation status of *MEOX-2* CpG islands. By examination of the genomic context of the whole *MEOX-2* CpG island, we identified five sub-CpG islands around the *MEOX-2* Exon 1 (Figure 13). We examined the methylation status of these individual sub-regions using MS-PCR assays with five different primer sets (MSP 1 to MSP 5), corresponding to the five sub-CpG islands (Figure 13). The MS-PCR results are presented in Figure 15. For the MSP 1 primer set, DNA from normal lymph node samples was successfully amplified with the methylated primers as well as the unmethylated primers, while DNA from the lymphoma cases produced a strong and distinct PCR product with methylated primers, but a weaker or undetectable band with unmethylated primers. Similar results were observed in other primer sets. These findings suggested that the entire CpG island of *MEOX-2* was hypermethylated in canine lymphoma compared to healthy lymph node tissues.

To further confirm the MS-PCR results and gain a more detailed insight into the methylation status of *MEOX-2*, we performed COBRA and bisulfite sequencing analysis to examine the methylation status of the sub-CGI 4, which spans a putative promoter sequence predicted by the TSSW promoter prediction program (Solovyev and Salamov 1997) (Figure 13). The COBRA results are presented in Figure 16. The PCR amplicon (389 bp) contains five BstUI cut-sites and one HypCh4IV cut-site. The cleavage will occur only if the investigated CpG sites are methylated. The DNA from lymphoma cases showed extensive cutting by BstUI (100%; 14/14) leading to several small fragments because of multiple BstUI cut-sites, and at least partial cutting by HpyCh4IV (93%; 13/14) that produced the expected fragments of 298 bp and 91 bp. The DNA from normal lymph node samples was not cleavage by either BstUI (100%; 8/8) or HpyCh4IV (100%; 8/8). These results indicate that

the selected sub-CGI 4 was methylated in all lymphoma cases analyzed here, with no evidence to suggest methylation in any of the normal lymph node tissues.

The bisulfite sequencing results are presented in Figure 17. We conducted bisulfite sequencing for three cases of lymphoma (L7, 12 and 14) and one non-neoplastic lymph node (N4). Three to four clones of each sample were sequenced. As predicted by the COBRA results, CpG sites within this amplicon were extensively methylated in all three lymphoma cases, but only scattered methylated CpG sites were observed in non-neoplastic lymph node. Furthermore, for the convenience of comparing detailed results of COBRA and bisulfite sequencing, the locations of the restriction enzyme cut sites were marked in Figure 17. Based on the methylation pattern shown in Figure 17, each BstUI cut-site displayed high-frequency methylation in three evaluated lymphomas, which is consistent with the findings in COBRA that the PCR products were extensively digested by BstUI in lymphoma cases. Similarly, the HpyCH4IV cut-site displayed relatively lower frequency of methylation in lymphoma cases as suggested by COBRA results with partial digestion by HpyCH4IV in tumor DNAs. Overall, the bisulfite sequencing results were consistent with the COBRA findings, and confirmed the tumor-specific, dense methylation of the *MEOX-2* CpG island in canine lymphoma cases. These data strongly support our initial findings obtained from MS-PCR and thus revealed a possible mechanism for *MEOX-2* silencing in canine lymphoma.

Cytogenetic analysis of *MEOX-2* in canine lymphoma

The transcriptional repression of *MEOX-2* in canine lymphoma might also result from genetic deletion. We thus examined the copy number status of *MEOX-2* locus by our own aCGH data of canine lymphoma cases (Thomas et al 2011). Neither homozygous nor hemizygous deletion was apparent in any of the 14 lymphoma cases evaluated. To confirm the results obtained from aCGH data, FISH analysis was performed on a subset of lymphoma cases. The FISH results correlated well with the aCGH findings, with cells from all 14 cases presenting with two copies of the *MEOX-2* locus (data not shown). Therefore, the transcriptional repression of *MEOX-2* appears not to result from genetic deletion.

Discussion

The homeobox protein *MEOX-2* is a transcription factor that has important roles in development, regulating many aspects of cellular processes including cell proliferation, apoptosis, motility, and angiogenesis. Although a number of studies have addressed the expression and function of *MEOX-2*, most of these efforts have focused on vascular tissues under a normal physiological condition. Due to the high impact of *MEOX-2* in developmental and cellular processes, we became interested in investigating its expression signatures in tumors. In this study, we investigated *MEOX-2* expression in a series of canine lymphoma cases and normal lymph nodes. By comparing the expression patterns between the malignant and healthy group, we found that *MEOX-2* was significantly inactivated in all lymphoma cases.

Because epigenetic and genetic aberrations are commonly mechanisms leading to gene inactivation, we first focused on examining the methylation changes of the promoter CpG island of *MEOX-2* in canine lymphomas. MS-PCR was designed to screen the methylation status of the entire CpG island and the methylation pattern of lymphoma cases was compared with that of the normal counterpart, non-neoplastic lymph nodes. Our study showed that the entire CpG island of *MEOX-2* was heavily methylated in all lymphoma cases we evaluated and partially or only slightly methylated in normal lymph nodes. To confirm differential methylation of *MEOX-2* in lymphoma, COBRA and bisulfite sequencing were carried out to further investigate the methylation status of a selected region within the *MEOX-2* CpG island. The COBRA results demonstrate that the *MEOX-2* CpG island was methylated in all lymphoma cases analyzed. Moreover, the methylation is tumor-specific and appears not to be restricted to any particular subset of canine lymphomas. The bisulfite sequencing results provide greater detail about the methylation densities within malignant and non-malignant samples. These results support our initial findings and offer a possible mechanism for *MEOX-2* inactivation in canine lymphoma samples. As mentioned previously, *MEOX-2* has recently been described to be aberrantly methylated in human NSCLC and Wilms tumor, with a reduced mRNA expression concomitantly (Cortese et al 2008, Ohshima et al 2009). These findings suggest that aberrant methylation may be a common mechanism

for *MEOX-2* inactivation in tumors. However, further studies are still needed to determine whether aberrant methylation directly mediates *MEOX-2* silencing. Since methylation-mediated gene silencing is a reversible event, in future work, we are going to treat lymphoma cell lines with demethylating agents to see whether or not the expression of *MEOX-2* can be restored. If not, the expression of *MEOX-2* may be controlled by other intrinsic mechanisms.

Moreover, we also examined copy number status of *MEOX-2* in canine lymphoma cases. No homozygous or hemizygous deletion was detected in all 14 lymphoma cases. We further examined our high resolution aCGH data for 150 lymphoma cases (Thomas et al 2011), and found that only 1 of 150 cases (0.7%) was detected to have gene deletion in *MEOX-2* locus, indicating that *MEOX-2* tends to have a normal copy number. This cytogenetic investigation suggests that the loss of *MEOX-2* expression appears not to be the consequence of genetic deletion, but rather epigenetic modification.

Recently, a regulatory RNA miRNA-130a has been reported as a regulator of *MEOX-2* (Chen and Gorski 2008). This miRNA can directly downregulate *MEOX-2* expression through binding to the 3'UTR of *MEOX-2*, and has been shown to be responsible for the downregulation of *MEOX-2* in response to mitogens or proangiogenic factors. A more recent study reported that miRNA-130a is overexpressed in human NSCLC and associated with disease progression, metastasis, and prognosis (Wang et al). Given its role in regulating *MEOX-2* expression, overexpression of miRNA-130a in malignancies might contribute to the downregulation of *MEOX-2*. Although the role of miRNA-130a in lymphoma remains to be further investigated, we cannot entirely rule out the possibility that this miRNA is also involved in the downregulation of *MEOX-2*. Bear in mind that aberrant methylation of *MEOX-2* has also been observed in human NSCLC, suggesting that deregulation of *MEOX-2* in tumor might be attributable to multiple mechanisms.

In conclusion, this study represents the first report investigating the molecular changes of *MEOX-2* occurring in association with canine lymphoma. Our study showed the expression of *MEOX-2* was silenced in all studied lymphoma cases. This inactivation may result from its promoter methylation. Currently, the functional roles of *MEOX-2* in the development of lymphocytes are still unknown. Subsequent knockdown and overexpression approaches in

cell lines may allow us to elucidate downstream targets and cellular functions of *MEOX-2* in lymphocytes. To date, the evidence only hints at the mechanism underlying the inactivation. The significance of loss of *MEOX-2* expression in lymphoma remains to be clarified. Moreover, the dense, tumor-specific methylation pattern of *MEOX-2* could be used as an epigenetic marker for canine lymphoma.

Table 27. Primer sequence, locations, and expected product size for MS-PCR analysis in this study

Primer name	Primer sequence	CGI position	Product size (bp)
MSP 1-MF[a]	AGAAAATAGTTGCGAAAAGTTAGACGC	Sub-CGI-1	106
MSP 1-MR	AAAAAAAAAAAATTAATTTAACGCGCG	Sub-CGI-1	
MSP 1-UF[b]	AGAAAATAGTTGTGAAAAGTTAGATGT	Sub-CGI-1	106
MSP 1-UR	AAAAAAAAAAAATTAATTTAACACACA	Sub-CGI-1	
MSP 2-MF	AGAAAATAGTTGCGAAAAGTTAGACGC	Sub-CGI-2	210
MSP 2-MR	ACTTCTCTTATCACTACCACTCGAACG	Sub-CGI-2	
MSP 2-UF	AGAAAATAGTTGTGAAAAGTTAGATGT	Sub-CGI-2	210
MSP 2-UR	TCTCTTATCACTACCACTCAAACA	Sub-CGI-2	
MSP 3-MF	TTGAGACGTGTATGTTATGGAATATTC	Sub-CGI-3	184
MSP 3-MR	CTCGTTAAAATATCCCGCGA	Sub-CGI-3	
MSP 3-UF	TTTGAGATGTGTATGTTATGGAATATTT	Sub-CGI-3	188
MSP 3-UR	CCTCCTCATTAAAATATCCCACAA	Sub-CGI-3	
MSP 4-MF	AGGAGATTACGGTCGTTAGGC	Sub-CGI-4	190
MSP 4-MR	CCAAAAAAAACGAAACGCG	Sub-CGI-4	
MSP 4-UF	GTTAGGAGATTATGGTTGTTAGGT	Sub-CGI-4	190
MSP 4-UR	AACCAAAAAAAAACAAAACACA	Sub-CGI-4	
MSP 5-MF	TAGTACGTGAAATCGATTGTAAAGC	Sub-CGI-5	149
MSP 5-MR	AATCGAACCAATCGAAATCG	Sub-CGI-5	
MSP 5-UF	TGATAGTATGTGAAATTGATTGTAAAGT	Sub-CGI-5	149
MSP 5-UR	AAATCAAACCAATCAAAATCAA	Sub-CGI-5	

[a]MF and MR, forward and reverse primers for the methylated templates.

[b]UF and UR, forward and reverse primers for the unmethylated templates

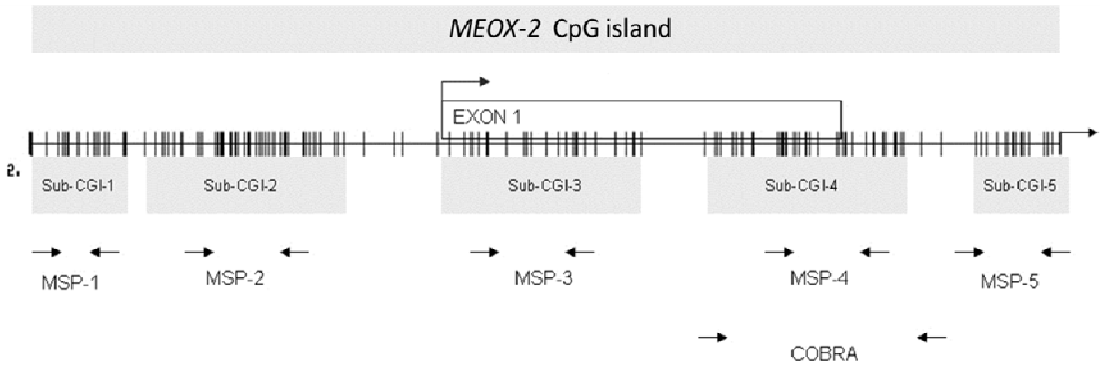

Figure 13. The schematic structure of the CpG island of MEOX-2 and the locations of primers used in MS-PCR and COBRA methods.
Open rectangle indicates exon 1. Short vertical lines represent the loci of the CpG dinucleotides. The top shaded rectangle indicates the range of MEOX-2 CpG island and the smaller shaded rectangles indicate the predicted sub-CpG island regions. Horizontal arrows indicate the locations of primers, and a bent arrow indicates the transcription start site.

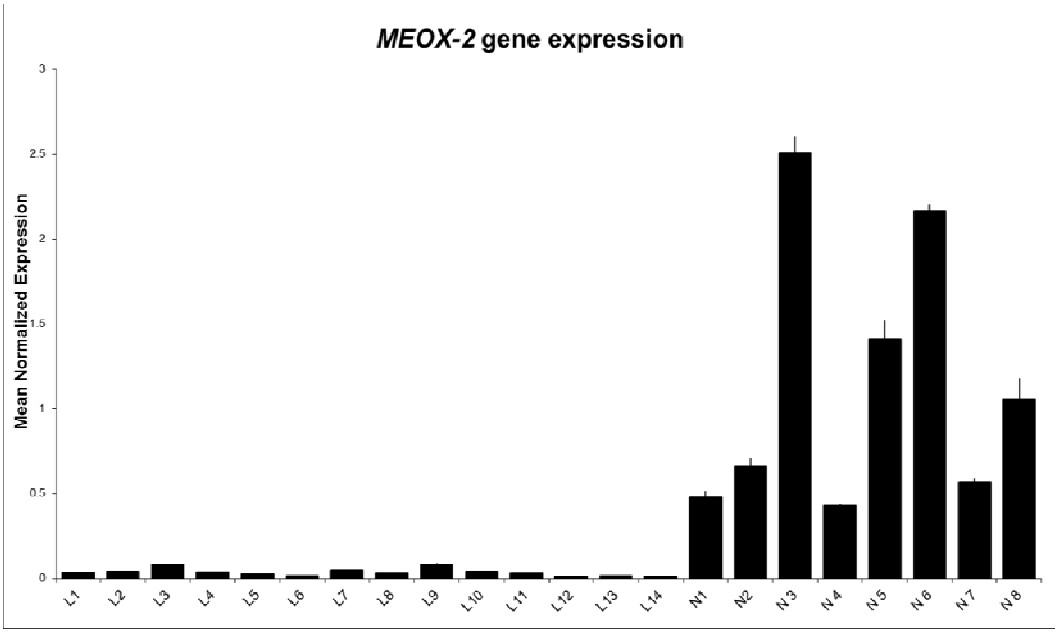

Figure 14. Relative MEOX-2 expression in canine lymphomas analyzed by real-time quantitative PCR. L1 to L14 represent canine lymphoma samples. For comparison, eight normal lymph node samples were included (N1 to N8). All values were normalized to LOC611555 expression for each sample. The displayed columns represent the mean value and the error bars indicate the standard error of triplicate determinations.

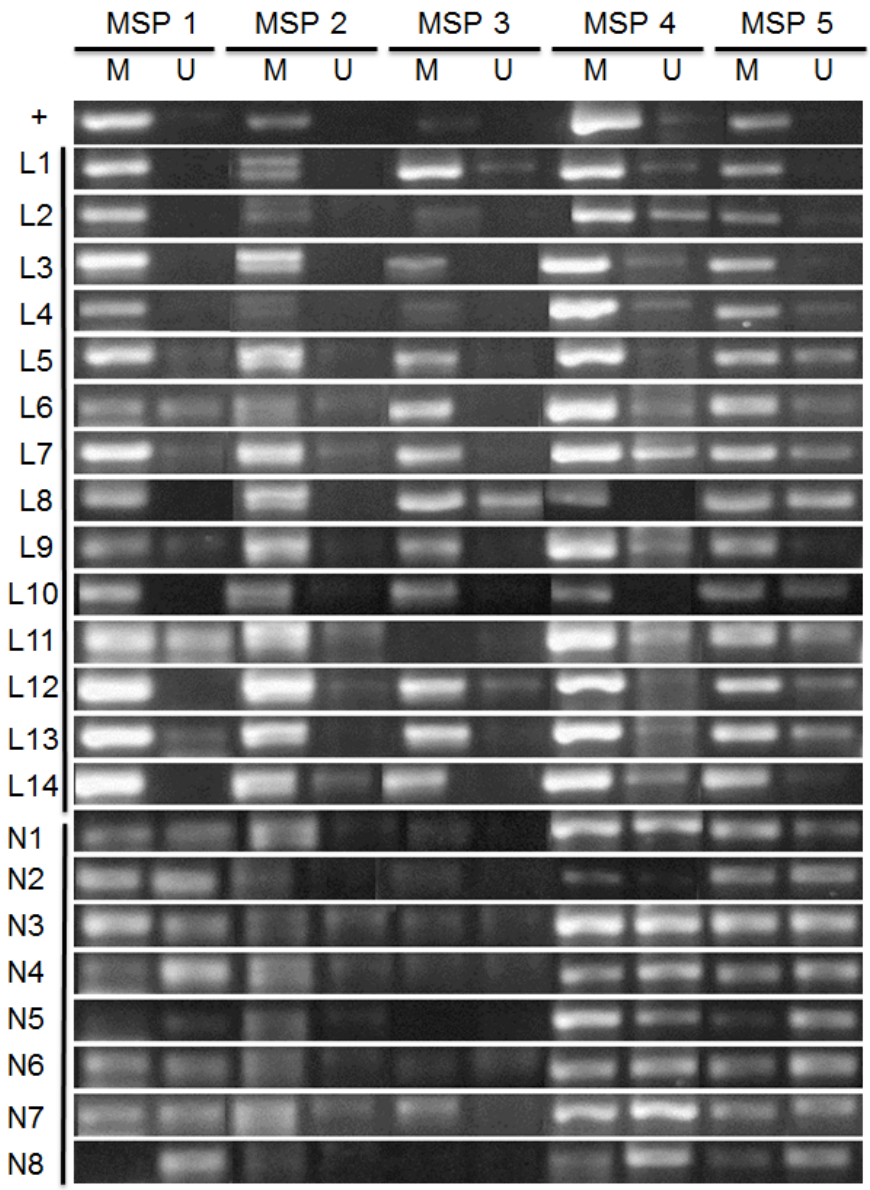

Figure 15. Methylation-specific PCR analysis for MEOX-2 gene in canine lymphoma cases.
DNA isolated from 14 lymphoma cases (L1 to L14) and 8 normal lymph node tissues (N1 to N8) from healthy dogs were subjected to bisulfite conversion followed by PCR amplification using five primer sets (MSP 1 to MSP 5) corresponding to five sub-CpG island regions. The presence of a PCR band under lanes M or U indicates methylated or unmethylated DNAs, respectively. In vitro methylated DNA is used as a positive control (+) for methylated DNA (top row).

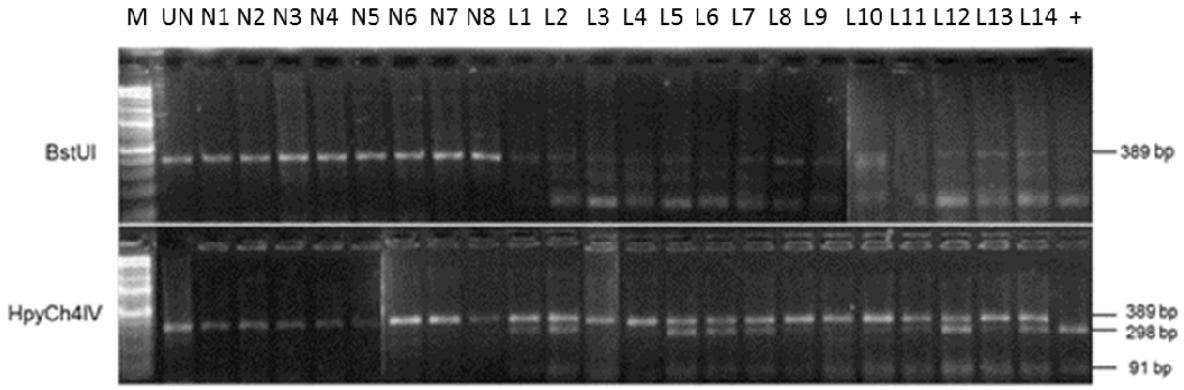

Figure 16. Figure 16. COBRA analysis of the sub-CGI 4 region of the MEOX-2 gene in normal lymph node tissues and lymphoma cases.
PCR products were digested with restriction enzyme BstUI (upper panel) and HpyCh4IV (lower panel). N1 to N8 represent normal lymph node samples and canine lymphoma samples are listed L1 to L14. Positive controls (+) are in the far right lane and the uncut negative controls are in the second lane (UN). Molecular markers (100 bp DNA ladder) are in the first lane (M).

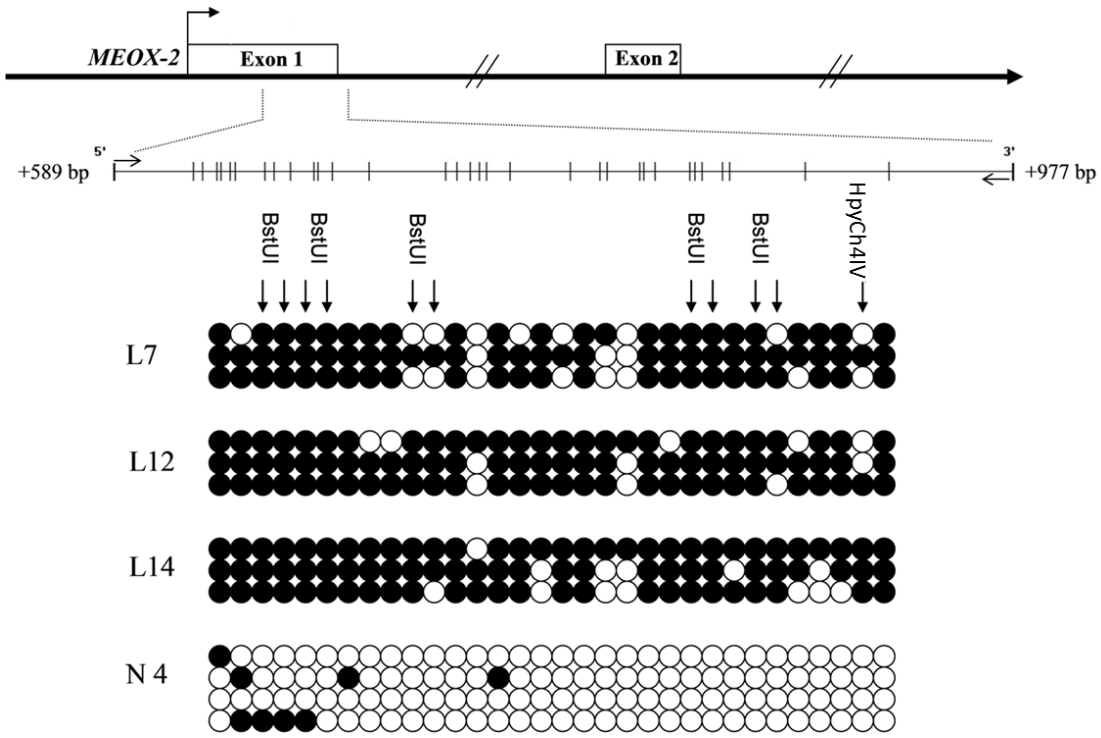

Figure 17. Schematic representation of bisulfite sequencing results for lymphoma cases and a normal lymph node control.
The upper panel shows a schematic of the region amplified by COBRA PCR relative to the MEOX-2 gene. Short vertical lines within the amplified region represent the loci of the CpG dinucleotides. The bisulfite sequencing results of three individual clones in each of three canine lymphoma cases (L7, L12 and L14) and four individual clones in a non-neoplastic lymph node sample (N4) are shown in the lower panel. Each circle represents a CpG dinucleotide. White and black circles represent unmethylated and methylated CpG dinucleotides, respectively. The vertical arrows indicate the CpG nucleotides within BstUI (CGCG) and HpyCh4IV(ACGT) restriction sites used in COBRA analysis.

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CHAPTER 6 : Dissertation Summary and Future Works

Lymphoma is one of the most common cancers in dogs. As is true for its human counterpart, non-Hodgkin's lymphoma (NHL), additional criteria are needed to develop improved and more refined schemes that can predict clinical outcomes. With the limited level of genetic heterogeneity within dog breeds, combined with the sophisticated genomic resources now available for the dog, the dog is now a powerful spontaneous model to identify heritable risk and genetic factors that are of pathological and clinical significance in lymphoma. Therefore, in order to improve the clinical management of canine lymphoma patients and provide opportunities for comparative studies, the works described in this dissertation focused on investigating genetic and molecular markers with potential clinical value in canine lymphoma.

It has been established that nonrandom chromosomal aberrations are key feature of a number of different human cancers. A proportion of such aberrations have been used as molecular markers for more comprehensive subclassification of tumor subtypes and for development of target-specific therapeutic protocols. In veterinary studies, nonrandom chromosomal aberrations also have been identified in canine lymphoma, and several aberrations have been linked to specific phenotypes of canine lymphoma (Modiano et al 2005, Thomas et al 2003b, Thomas et al 2011). However, investigation of the prognostic significance of these nonrandom aberrations has been hampered by the lack of large cohorts of patients with clinical follow-up data. Thus, the aim of the studies described in Chapter 2 was to determine the prognostic significance of nonrandom chromosomal aberrations in canine lymphoma. To achieve this, the study was designed to analyze the significance of nine recurring chromosome aberrations in a series of archival lymphoma specimens derived from dogs enrolled in a clinical trial. We examined 160 archival lymphoma specimens using multicolor interphase FISH with probes comprosing three to five contiguous BAC clones on each locus of interest. Based on the splitting-sample method, the results obtained from the training set (121 cases) using regression analysis showed that canine lymphomas with increased copy number of A1 (located on CFA 1) were associated with longer DFI. A

validation cohort of 39 cases was used to validate the results. These findings facilitate the definition of a subgroup of patients with more favorable outcome. Furthermore, because the canine lymphoma cases evaluated in the present study had been treated with single agent doxorubicin plus asparaginase, it will be interesting to evaluate whether copy number changes of A1 is similary associated with DFI in patients treated with multiagent, CHOP-based chemotherapy. Moreover, by using comparative genomic analysis, conserved chromosome segments of the human genome that shared with copy number aberration A1 could be defined. Further assessment of the presence of corresponding changes in human lymphoma samples may provide valuable data regarding the prognostic value of evolutionarily conserved DNA copy number aberrations shared between human and dog.

The rapidly advancing field of canine cancer gene expression profiling requires the use of stable, constitutively expressed reference genes. In the absence of reliable reference genes, accurate identification and quantification of both over- and under- expressed sequences in canine tumor tissues is at best difficult. Therefore, in order to provide reliable data in the following gene expression experiments, the study in Chapter 3 was designed to identify suitable reference genes for real-time quantitative PCR in canine experimental systems. In this study, we selected new reference genes based on assessment on copy number neutrality in a cohort of over 300 canine tumor samples representing three very different tumor types (Thomas et al 2011). The stability of these candidate genes, and four conventional reference genes, was evaluated in canine lymphoma, histiocytic sarcoma and osteosarcoma using three different algorithms. *LOC611555* was identified as the most stable reference gene across three tumor types. Of the conventional housekeeping genes, *HPRT* was stably expressed in histiocytic sarcoma, while *Ubi* and *RPL32* were relatively stable in osteosarcoma. A greater level of stability was evident in the proposed new reference genes than in the conventional housekeeping genes. This study also provided the most suitable combination of reference genes for multiple genes normalization for each type of tumor. The identification of *LOC611555* in this study provides a tissue-non-specific reference gene which showed greater stability across tissues types and disease status, and we thus propose that this gene may be a useful candidate reference gene for other biological systems. However, a proper validation of

its use in other tissues or diseases is recommended, since no universal reference gene is optimal for all studies on the basis of available published evidence.

To gain more insight into the molecular mechanisms underlying tumor development and disease progression in canine lymphoma, studies described in Chapter 4 were designed to further identify potential target genes residing within the recurrent chromosome aberrations identified in canine lymphoma. In this study, 11 genes were selected for further study based on their known tumor associated roles and history of deregulation in human NHLs. The gene expression profiling data showed that *MYC* was upregulated in lymphoma samples and increased expression of *MYC* was detected in 53% of cases; *MEOX-2* and *KIT* were downregulated and decreased expression of *MEOX-2* and *KIT* were detected in 100% and 76% of cases, respectively. *KIT* is located in CFA 13; gain of CFA 13 is the most frequently detected aberration in canine lymphoma (Thomas et al 2011). However, it is interesting that *KIT* expression was downregulated in a majority of lymphoma cases. Even so, we still observed two T-cell lymphoma cases with high *KIT* expression. This finding is consistent with previous observations on human lymphoma in which *KIT* overexpression was frequently detected in T-cell lymphoblastic lymphoma, but rarely been reported in B-cell ones (Ferrari et al 1993, Kiss et al 1993, Sperling et al 1997, Tomeczkowski et al 1998). Dog patients with high *KIT* expression may be potential targets for KIT-specific treatment using tyrosine kinase inhibitors. Additional studies in protein level with larger sample sizes are required to ascertain whether overexpression of KIT in canine lymphomas is restricted to T-cell lymphoma. Furthermore, by combining data of gene expression with cytogenetic changes, three gene-dosage regulated candidates were identified, including *CDKN2A*, *TSC2* and *STAT1*. Moreover, among these genes investigated in this study, *POLD1* is located in the MCR of aberration on CFA 1(harbored A1), which has been shown to be significantly associated with DFI in Chapter 2. However, *POLD1* in canine lymphoma was not expressed in a direct gene dosage dependent manner, suggesting that *POLD1* may not be the potential target gene on the A1 locus. Further studies of other candidate genes involving A1 locus such as *AKT2* and *BCL3* might be worthwhile to identify potential target genes on A1 locus. In addition, the candidate gene approach used in this study allows us to effectively highlight

potentially important genes in the regions with recurring copy number aberrations. However, this approach can only identify genes with previously established roles. Studies combining aCGH and gene expression microarray data from multiple canine lymphoma samples are underway in our lab. This integrated approach may allow us to discover novel tumor associated genes in canine lymphoma.

Furthermore, homoebox protein MEOX-2 is a transcription factor that controls many aspects of vascular cellular processes and angiogenesis. However, study of its expression and functional roles in cancer is still in its infancy. A preliminary study presented in Chapter 4 demonstrated that *MEOX-2* expression was uniformly silenced in all studied lymphoma cases. In Chapter 5, our aim was to elucidate further how the homeobox gene *MEOX-2* is deregulated in canine lymphoma. In this study, we presented evidence supporting our previous findings that *MEOX-2* was dramatically downregulated in all canine lymphoma cases, with no corresponding genetic deletion detected. Furthermore, we also demonstrated that the CpG island of *MEOX-2* was hypermethylated in all studied lymphoma cases, but not in normal lymph nodes. These findings suggest that *MEOX-2* expression might be silenced by DNA methylation. Further studies are needed to determine whether aberrant methylation directly mediates *MEOX-2* silencing. Additional studies are required to establish direct causal links between deregulation of *MEOX-2* and carcinogenesis of canine lymphoma. In addition, the tumor-specific methylation pattern of *MEOX-2* may have potential use as an epigenetic marker for molecular diagnosis of canine lymphoma.

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APPENDICES

Table 28. Cytogenetic data and corresponding DFIs of 160 lymphoma cases

CASE No[a]	DFI	A1	A2	A3	B1	B2	B3	C1	C2	C3
L1	196.00	1.77	1.92	1.77	2.00	2.40	1.68	1.74	2.00	1.93
L2	1246.00	2.00	2.00	1.90	2.00	1.71	1.75	1.80	2.00	1.71
L3	202.00	1.73	1.90	2.00	2.00	1.93	1.90	1.48	2.00	2.25
L4	766.00	1.93	1.86	1.81	2.16	1.87	1.57	1.82	1.93	2.15
L5	136.00	1.56	2.00	1.53	1.67	2.00	1.67	1.86	1.96	2.26
L6	402.00	2.00	2.00	1.88	2.19	1.90	1.51	1.73	1.77	2.04
L7	194.00	1.80	1.75	1.88	2.14	2.00	1.57	1.69	2.15	1.94
L8	243.00	1.90	1.81	1.78	2.20	1.95	1.77	1.79	1.86	2.46
L9	186.00	1.81	2.00	1.80	2.00	2.00	1.77	1.78	1.84	2.21
L10	203.00	1.81	2.03	2.02	2.27	1.96	1.97	1.68	1.96	2.23
L11	187.00	1.59	1.56	1.65	2.50	2.00	1.11	1.50	1.41	1.88
L12	156.00	1.70	2.00	1.50	2.33	2.00	2.00	1.58	1.84	2.35
L13	169.00	1.67	2.00	2.00	2.50	1.87	1.88	2.30	2.15	
L14	413.00		1.00	1.83	1.00	1.75	1.64	1.89	2.00	2.06
L15	154.00	2.00	2.04	1.85	2.00	1.63	1.73	1.73	1.97	1.79
L16	28.00	1.65	1.61	1.86	1.95	1.78	1.47	1.54	1.75	1.75
L17	155.00	1.70	1.55	1.41	2.00	2.00	1.67	1.50	2.00	1.80
L18	147.00	1.88	1.74	1.73	2.20	2.29	1.84	1.39	1.66	2.10
L19	1100.00	2.14	1.93	1.93	3.00	2.00		1.53	1.63	1.93
L20	261.00	1.43	1.67	1.73	2.07	2.22	1.76	1.63	1.93	1.88
L21	111.00	1.56	1.33	1.83	2.43	2.00	2.00	1.69	1.98	1.67
L22	203.00	2.05	2.25	2.00	2.50	3.00	1.67	1.50	1.36	1.67
L23	1158.00	1.88	2.00	1.47	2.20	1.60	1.55	1.61	1.67	1.42
L24	450.00	1.50	1.13	1.89	3.00		2.00	1.60	1.76	2.07
L25	1274.00	2.57	2.00	1.50	1.89	2.32	1.54	1.35	1.85	1.81
L26	610.00	2.13	2.00	1.63	2.45	2.10	1.44	1.79	1.81	1.93
L27	167.00	1.89	1.71	1.69	2.00	2.00	2.00	1.58	1.73	1.64
L28	79.00	2.00	2.00	1.70	2.40	2.00	1.00	1.65	1.82	1.50
L29	286.00	1.71	1.45	1.70	2.00	2.44	1.64	1.66	1.77	2.00
L30	478.00	1.74	1.61	1.50	2.00	2.00		1.53	1.71	1.40
L31	700.00	1.79	1.82	1.91	2.27	2.17	1.85	1.88	2.50	2.06
L32	18.00	1.53	1.65	1.70	2.11	2.15	1.50	1.58	1.47	1.67
L33	196.00	1.50	1.67	1.90	2.43	2.05	1.71	1.70	2.33	2.29
L34	281.00	1.78	1.81	1.58	2.18	1.92	1.58	1.38	1.61	1.86
L35		1.55	1.56	1.71	2.43	2.08	1.67	1.56	1.68	2.18
L36	171.00	1.62	3.00	1.67		2.00	1.22	1.42	1.48	1.33
L37	197.00	1.45	2.12	1.68	2.00	2.48	1.54	1.46	1.62	1.59
L38	656.00	1.81	1.85	1.52			2.00	1.00	2.00	
L39	229.00	1.89	1.85	1.79	2.00	1.71	1.45	1.72	1.67	1.68
L40	285.00	1.43	2.00	1.50	2.14	2.12	1.56	2.20	2.00	2.17

Table 28 Continued

L41	357.00	2.26	2.25	1.60	2.46	2.65	1.71	1.58	1.77	1.78
L42	158.00	1.88	2.00	1.00	2.63		1.50	1.50	1.89	
L43	162.00	1.65	1.67	1.53	2.13	2.26	1.70	1.50	1.69	1.71
L44	385.00	2.00	2.00	2.13	2.00	2.00		1.75	1.91	1.75
L45	203.00	1.90	2.00	2.00	2.00	2.00	1.50	1.73	1.75	1.50
L46	181.00	2.17	1.50	2.00	2.40		2.00	1.58	1.60	2.13
L47	259.00	1.75		1.00	2.50	2.00	1.75	1.91	2.05	1.70
L48	364.00	1.93	1.50	2.00				1.81	1.55	1.50
L49	256.00	1.94	1.69	1.59	2.73	2.44	2.25	1.73	1.76	1.89
L50	262.00	2.00	2.00	2.00	2.27	2.00	1.25	1.92	1.29	1.00
L51	78.00	2.11	2.00	1.50	2.00	2.00	1.67	2.47	1.67	1.50
L52	188.00	2.45	2.00	1.00	2.08	2.13	2.11	1.67	1.43	2.00
L53	120.00	2.15		2.50	2.00		1.50	1.80	1.69	1.67
L54	70.00	2.08	2.00	1.59	2.67	2.20	1.67	1.75	1.70	1.43
L55	77.00	1.88	1.86	1.75			1.00	1.59	2.11	2.29
L56		2.17	1.00	1.42	2.00	2.50	2.00	1.80	1.91	2.00
L57	244.00	1.90	2.00	1.63	2.50	2.33	2.00	1.68	1.78	1.69
L58	171.00	2.20	2.00	2.00	2.00			2.17	1.60	2.00
L59	42.00	1.92	1.85	1.47	2.40	2.00	1.75	1.58	1.94	1.71
L60	122.00	2.33	1.83	2.00	2.50			1.80	1.67	2.00
L61	63.00	1.67	2.00	1.33	2.50	2.00	1.75	1.58	1.33	1.25
L62	69.00	1.57	2.08	1.50	2.25	2.00	2.00	1.45	1.78	
L63	150.00	2.17	2.00	2.00	2.40	1.00	1.50	1.78	1.70	2.00
L64	70.00	2.17		1.67	2.17	2.18		1.38	1.58	1.33
L65	231.00	2.12	1.86	1.70	2.15	2.00	2.29	2.00	2.00	2.00
L66	161.00	1.94	2.00	1.73	2.00	1.75	2.00	1.63	1.91	1.33
L67	314.00	2.33	2.00	2.00	2.17	2.50		1.56	1.80	1.50
L68	1233.00	2.00	1.86	2.20	2.50		2.00	1.81	2.50	2.00
L69	63.00	1.87	1.82	1.53	2.25	1.88	2.00	1.84	2.05	1.50
L70	161.00	1.92	1.88	1.82	2.00	2.14	1.75	1.58	1.67	1.47
L71	244.00	2.00		2.00	2.00	1.83	2.00	1.62	1.50	
L72	213.00	1.86	1.86	1.50	2.08	1.80	2.00	1.83	1.80	1.43
L73	156.00	2.22	1.60	1.80	2.29	2.00	2.00	1.91	1.75	2.00
L74	105.00	1.84	2.09	1.69	2.33	2.05	1.81	1.84	1.63	1.88
L75	99.00	1.83	1.69	1.69	2.14	1.86	1.85	1.75	1.76	1.80
L76	667.00	2.38	2.36	1.75	2.00	2.15	1.85	1.63	1.81	2.14
L77	154.00	2.67			3.00	2.00	1.83	1.57	2.00	2.00
L78	206.00	1.66	1.67	1.70	2.38	1.90	1.82	1.75	1.88	1.59
L79	20.00	2.41	2.00	1.88	2.10	2.00	1.33	1.88	1.78	2.25
L80	193.00	1.80	2.00	1.86	2.13	1.96	1.82	1.85	1.84	1.90
L81	204.00	1.93	2.00	1.83	2.57	2.00	2.00	1.87	2.00	1.00

Table 28 Continued

L82	242.00	2.50	2.00	1.67	2.17		1.40	2.00	1.75	
L83	196.00	1.94	1.89	1.52	2.38	2.08	1.71	1.88	1.77	1.92
L84	188.00	1.80	2.00	1.80	2.45	2.00	1.67	1.88	2.00	
L85	272.00	2.17	2.00	2.00	2.17	2.25	2.00	1.93	2.25	
L86	116.00	2.17	2.11	1.75	2.18	1.92	1.44	1.54	1.57	1.75
L87	216.00	2.00	2.00	1.00		2.00				
L88	497.00	2.00	1.67	2.00	2.40	2.00				
L89	98.00	1.92	1.92	2.00	2.50	2.00				
L90	101.00	1.78	2.00	2.00	2.00					
L91	141.00	2.25	2.00							
L92	114.00	1.71	2.00	1.80				1.50	1.00	1.00
L93	46.00	1.55	1.75	1.57	2.50	2.00	1.33	1.55	1.43	1.38
L94	742.00	2.22	2.14	1.67	2.47	2.00	2.00	2.00	2.00	2.00
L95	365.00	2.17	2.00	1.00	2.33	2.00			1.50	
L96	45.00	1.56	1.57	1.53	2.33	2.00	2.00	1.47	1.67	1.38
L97	53.00	1.72	2.00	1.27	2.50	2.13		1.63	2.00	1.36
L98	103.00	1.62	2.06	1.50	2.00	2.00	1.00	1.50	1.40	
L99	331.00	1.52	2.00	2.14	2.40		2.00	1.69	1.59	1.85
L100	415.00	2.09	2.00	1.69	3.00	2.00	2.00	1.69	1.50	
L101	1120.00	2.64	2.00	1.33	2.29	2.40	2.00	2.00	1.67	1.00
L102	63.00	1.60	1.94	1.91	2.67	2.00	1.67	1.74	1.90	1.75
L103	784.00	2.00	2.00	2.09	2.50	2.00	2.00	1.76	2.00	1.91
L104	63.00	1.58	2.00	1.80	2.86	2.00	1.75	1.88	2.05	1.92
L105	41.00	2.00	2.00	1.86	2.63	2.00	2.00	1.78	2.07	1.50
L106	393.00	2.50	1.80	2.00	2.00	2.25	1.75	2.08	2.00	1.75
L107	763.00	2.00	2.17	1.86	2.71	3.00	2.00	1.72	2.25	1.67
L108	714.00	2.30	2.00	2.00	2.50	2.00		1.50	1.75	2.33
L109	116.00	1.93	2.17	1.76	2.50	2.57	2.00	1.80	1.71	1.86
L110	1540.00	2.53	2.00		2.44	2.57	1.75	1.90	2.00	2.00
L111	69.00	2.18	1.94	1.33	2.60	2.09	1.71	1.79	2.00	2.00
L112	89.00	1.86	1.94	1.57	2.00	1.88	1.50	1.35	1.53	2.14
L113	652.00	2.43	2.05	1.95	2.43	2.00	2.00	1.70	1.71	2.00
L114	532.00	2.40	2.00	2.00	2.40	2.00				
L115	103.00	2.27	2.00	1.89	2.00	1.75	2.00	1.91	1.83	2.60
L116	336.00	2.50		1.00	2.00	1.75	2.00	2.20	2.07	2.00
L117	385.00	2.08	2.00	1.67		2.00		2.00	2.00	2.00
L118	549.00	2.60	2.00		2.00	3.00	1.50	2.00	2.00	
L119	367.00	2.20	2.00	2.00	2.00	2.00		1.86	2.00	3.00
L120	363.00	2.00	2.00	2.00			2.00	2.13		
L121	456.00	1.91	2.00	2.20	2.00	2.40	1.50	1.90	2.00	2.20
L122	248.00	2.00	1.95	1.60	2.40	2.19	1.91	1.80	1.83	1.80

Table 28 Continued

L123	145.00	1.93	1.77	1.83	2.00	2.23	1.80	1.70	1.82	1.42
L124	203.00	1.89	1.98	2.13	2.28	2.58	1.92	1.89	1.93	1.91
L125	297.00	2.05	2.03	1.89	2.13	1.91	1.60	2.25	2.11	2.00
L126	105.00	1.82	1.90	1.65	1.85	1.86	1.83	1.86	2.00	1.78
L127	171.00	1.98	2.02	2.00	2.00	2.13	1.73	1.90	1.96	1.88
L128	18.00	2.00	1.96	1.74	1.96	2.00	1.80	1.75	1.92	1.56
L129	119.00	1.89	2.00	2.00	2.40	1.55	2.00	1.75	1.91	1.86
L130	119.00	2.00	2.00	1.00	1.63	1.83	1.80	1.90	1.87	2.00
L131	289.00	1.75	1.84	2.08	1.71	2.27	1.89	1.82	2.04	1.75
L132	126.00	1.83	1.95	2.17	1.83	2.11	1.91	1.93	1.85	1.20
L133	454.00	2.00	1.71	2.00	2.00	2.50	.	1.86	2.17	.
L134	1427.00	2.00	1.78	1.84	2.04	2.45	1.76	1.93	2.00	2.50
L135	147.00	1.89	1.67	1.80	1.71	2.48	1.67	1.69	2.00	1.57
L136	112.00	1.87	1.88	1.93	2.28	2.05	2.00	1.78	1.89	1.48
L137	245.00	1.88	1.91	1.73	2.00	1.96	1.75	1.76	1.83	1.22
L138	121.00	1.89	1.74	1.67	2.25	1.95	2.00	1.67	2.00	1.53
L139	0.00	1.85	1.82	2.27	1.88	2.00	1.70	1.81	1.87	1.83
L140	0.00	2.20	1.85	1.95	1.80	2.13	1.76	1.72	1.65	1.88
L141	239.00	1.86	2.00	2.00	1.85	1.97	1.67	1.81	1.96	1.80
L142	153.00	2.12	1.87	1.60	1.94	1.82	2.00	1.78	2.09	1.56
L143	327.00	2.00	1.75	1.90	1.86	1.88	1.50	1.96	1.96	1.86
L144	249.00	2.00	1.93	1.60	2.00	2.00		1.67	1.50	
L145	216.00	1.86	2.00	2.00				3.00	2.00	
L146	303.00	2.19	2.00		2.50	3.00	2.00	2.17	2.00	
L147	163.00	1.78	2.00		3.00			3.00	2.00	
L148	0.00	1.71		2.56	2.67	2.00		2.67	2.00	2.00
L149	176.00	1.67	2.00	2.00	2.67	2.33		2.22	1.86	1.50
L150	69.00	2.00		1.80	2.25	2.00	2.00	1.71	1.50	1.50
L151	490.00	3.00	2.00		2.50	2.00	2.00	1.75		2.00
L152	453.00	2.25	2.00		2.43	2.00		2.00	2.13	2.00
L153	42.00	1.50	2.00					1.75	1.80	2.00
L154	84.00	1.67	2.00		2.00			2.00	2.00	
L155	1422.00	2.43	2.00	1.00	2.00	2.00		2.00	2.00	
L156	75.00	1.80	2.00	2.00	3.00	2.00		2.50		
L157	652.00	2.83	2.00	2.50				2.40	2.50	2.00
L158	49.00	1.71	2.00	2.00		2.00				2.00
L159	345.00	2.38	2.00	2.00		1.50	1.00	2.67	2.00	2.00
L160	119.00	1.90	2.00		2.00	1.50	3.00			

[a]L1 to L121: training data set
L122 to L160 : validation dataset

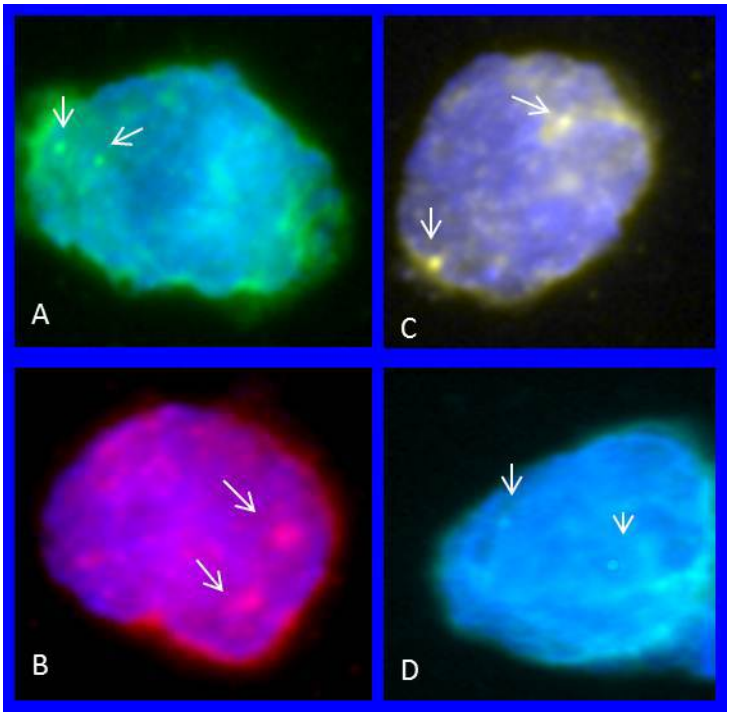
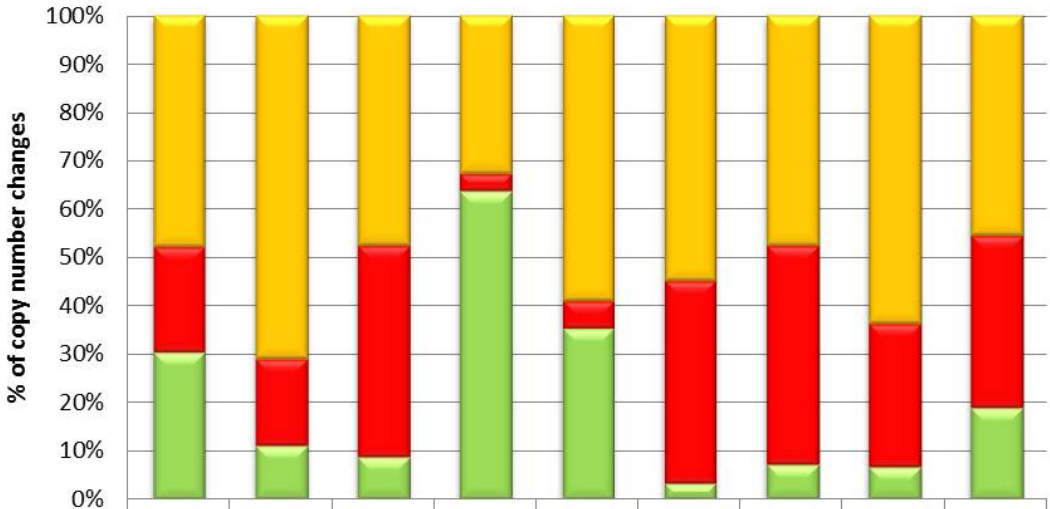


Figure 18. Examples of hybridization signals on paraffin embedded specimens in our initial hybridization tests. Arrows indicate the hybridization signals.



	A1	A2	A3	B1	B2	B3	C1	C2	C3
Normal	47.8	71.1	47.7	32.9	59.0	54.8	47.7	63.8	45.5
Loss	22.0	17.8	43.6	3.4	5.8	42.1	45.0	29.5	35.6
Gain	30.2	11.2	8.7	63.7	35.3	3.2	7.3	6.7	18.9

Figure 19. Frequency of the nine copy number aberrations in 160 cases of canine lymphoma, determined by FISH analysis.
Copy number gain was defined as a mean copy number greater than 2.25 (which means more than 25% of counted nuclei with copy number greater than 2), while copy number loss was defined as a mean copy number less than 1.75 (which means more than 25% of counted nuclei with copy number less than 2. Mean copy number between 1.75 and 2.25 was defined as normal.

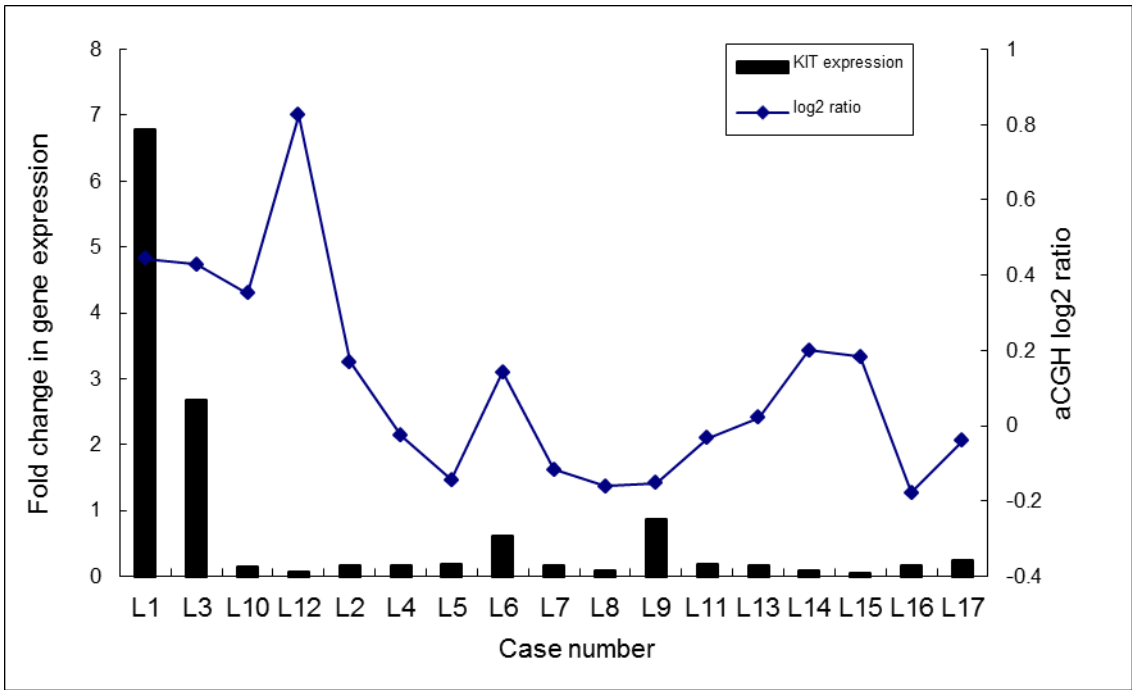


Figure 20. The gene expression data and the corresponding cytogenetic results of KIT in canine lymphoma cases.

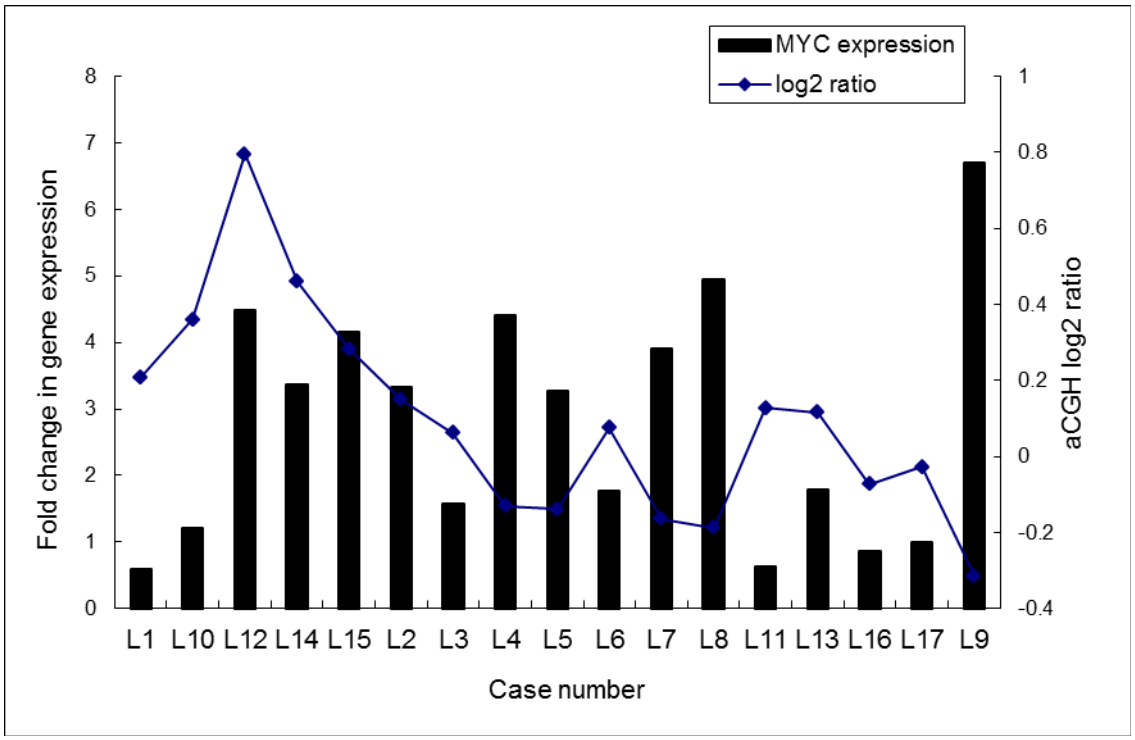


Figure 21. The gene expression data and the corresponding cytogenetic results of MYC in canine lymphoma cases.

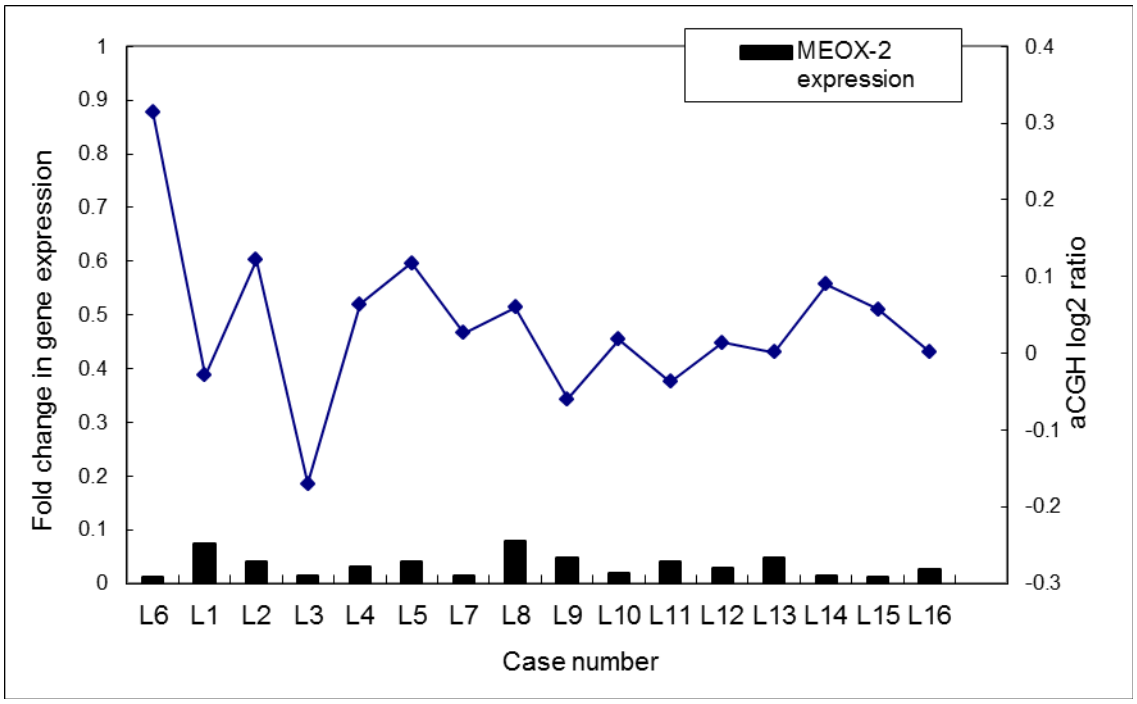


Figure 22. The gene expression data and the corresponding cytogenetic results of MEOX-2 in canine lymphoma cases.

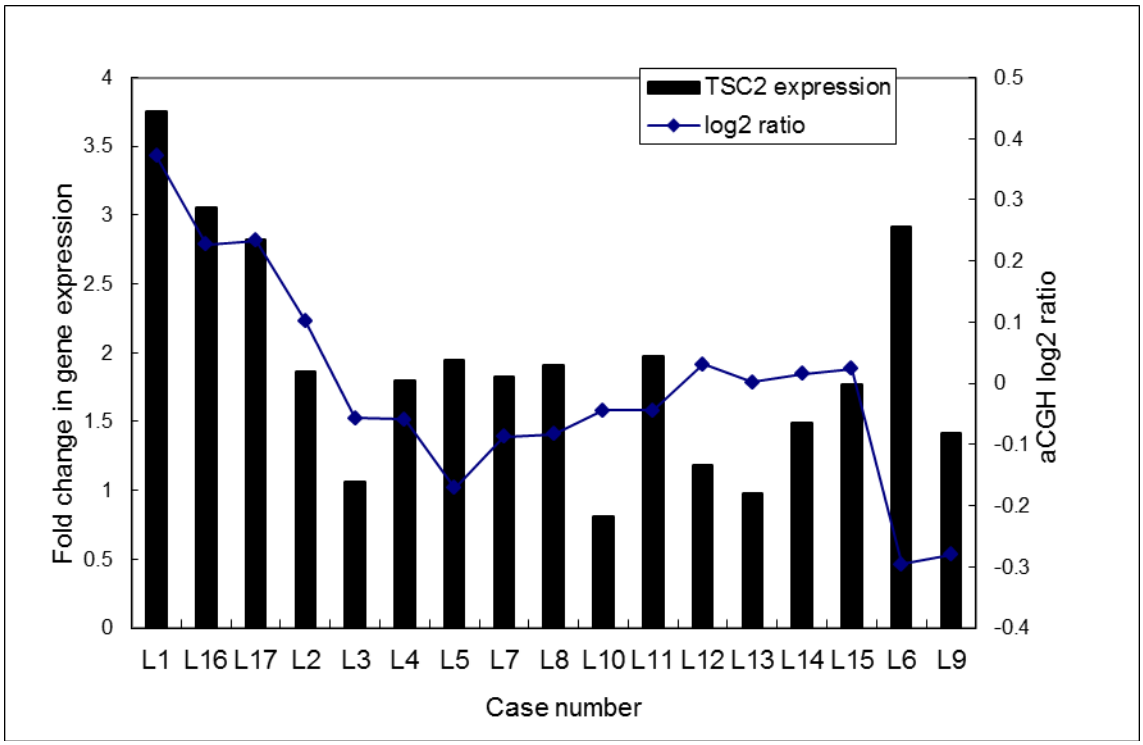


Figure 23. The gene expression data and the corresponding cytogenetic results of TSC2 in canine lymphoma cases.

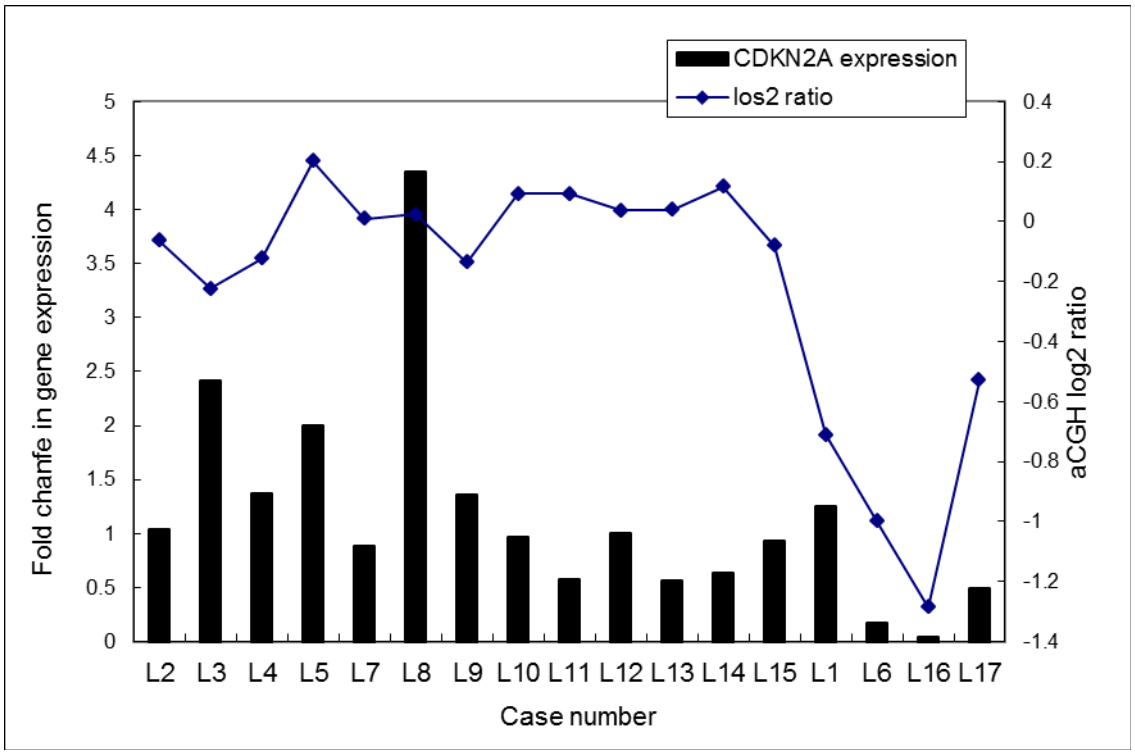


Figure 24. The gene expression data and the corresponding cytogenetic results of CDKN2A in canine lymphoma cases

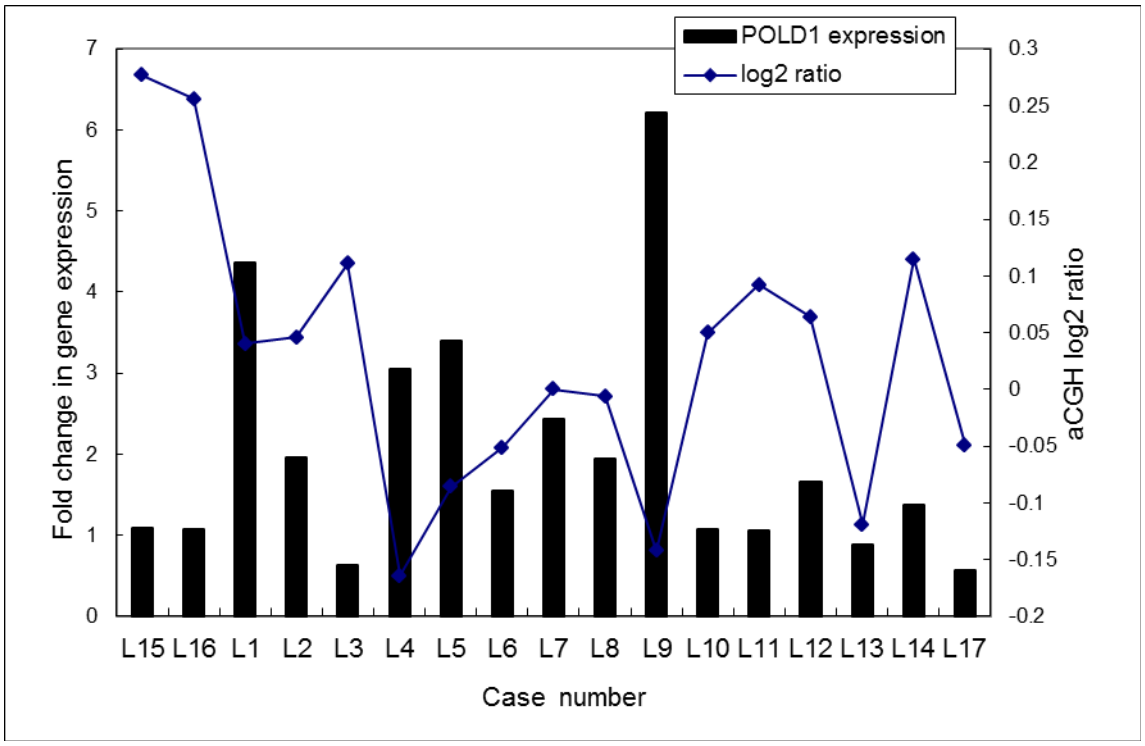


Figure 25. The gene expression data and the corresponding cytogenetic results of POLD1 in canine lymphoma cases.

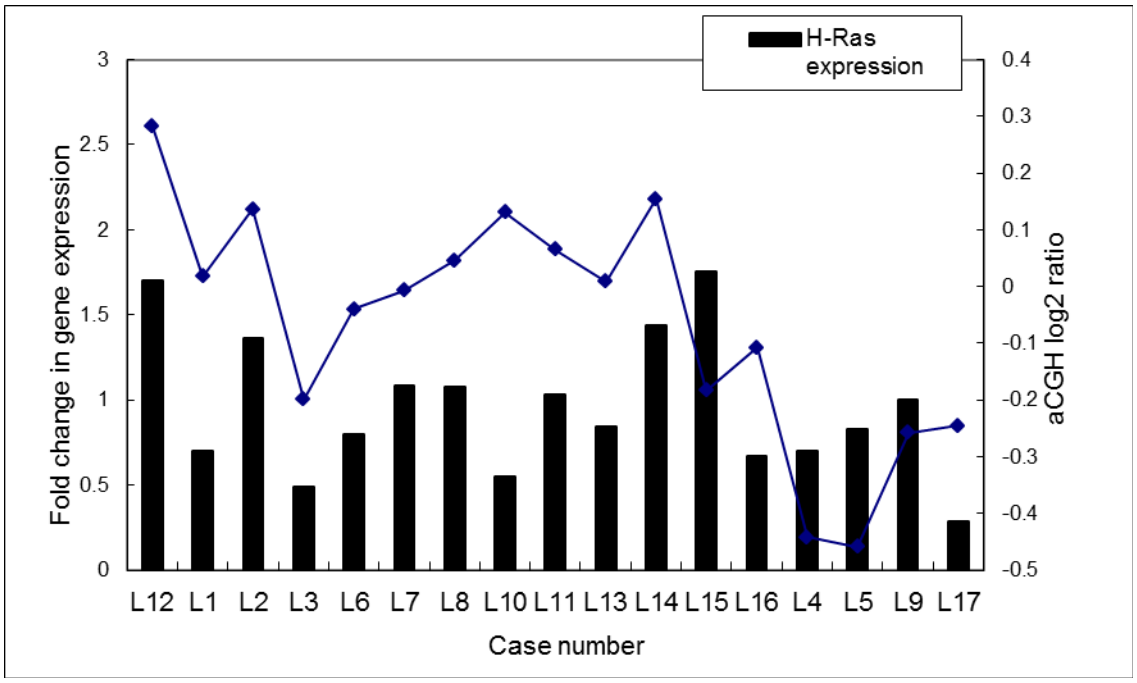


Figure 26. The gene expression data and the corresponding cytogenetic results of H-Ras in canine lymphoma cases.

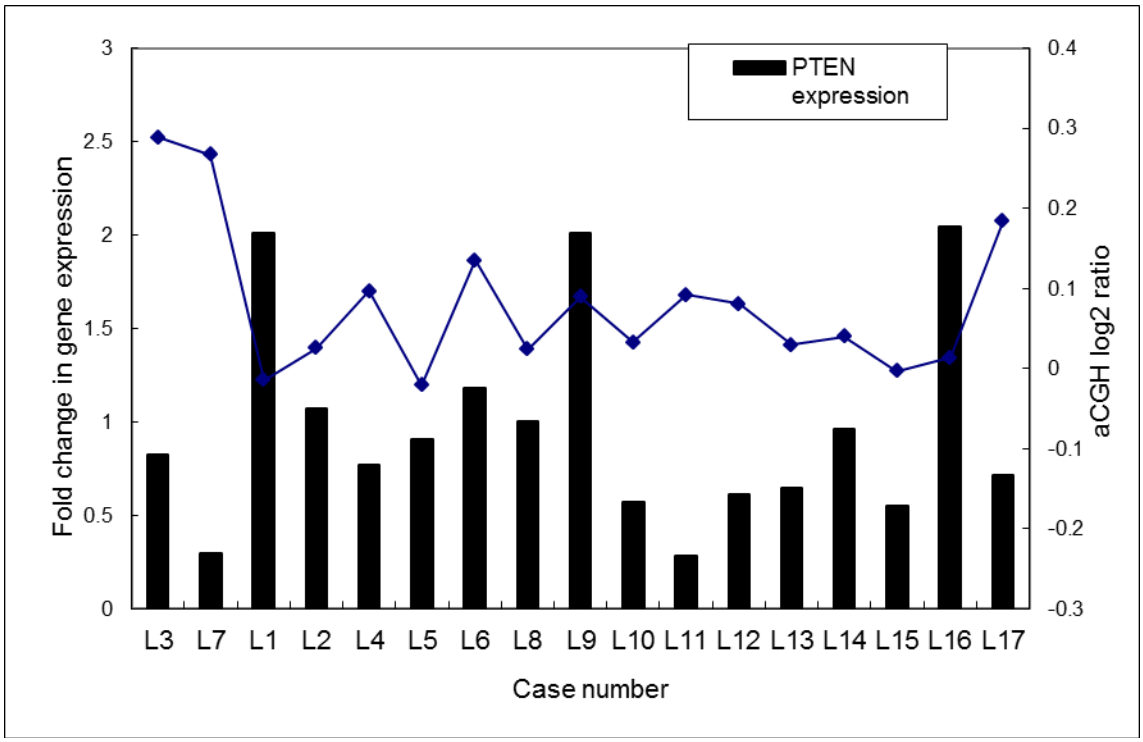


Figure 27. The gene expression data and the corresponding cytogenetic results of PTEN in canine lymphoma cases.

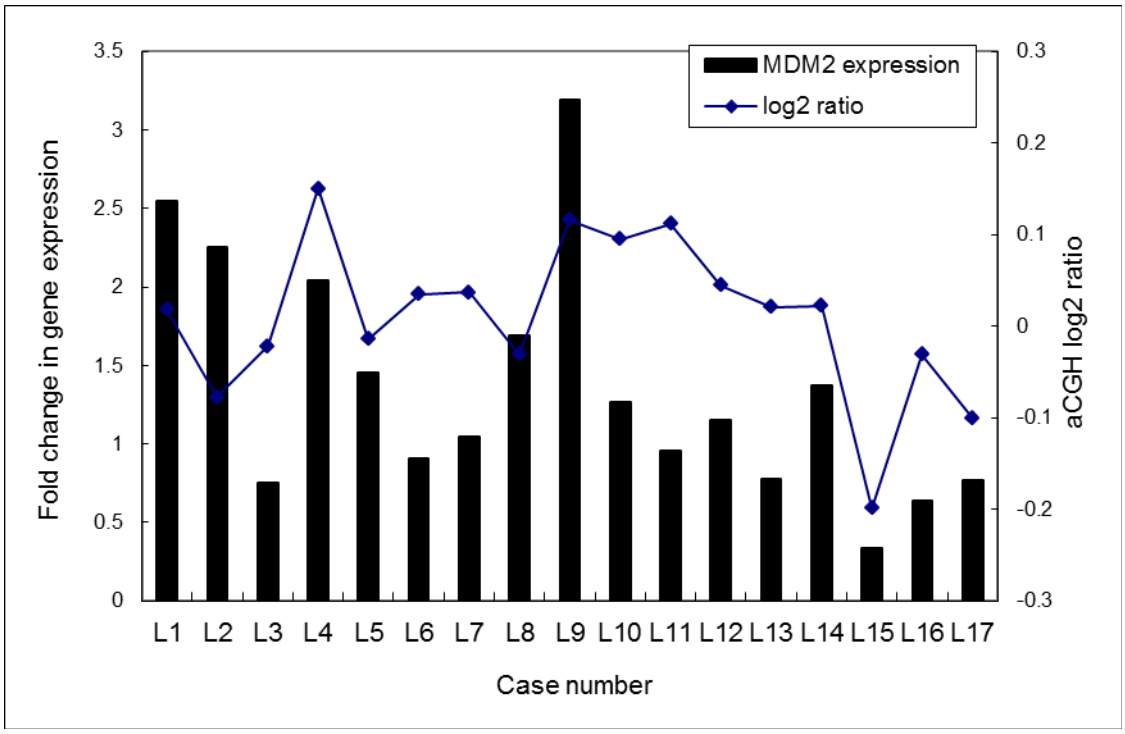


Figure 28. The gene expression data and the corresponding cytogenetic results of MDM2 in canine lymphoma cases.

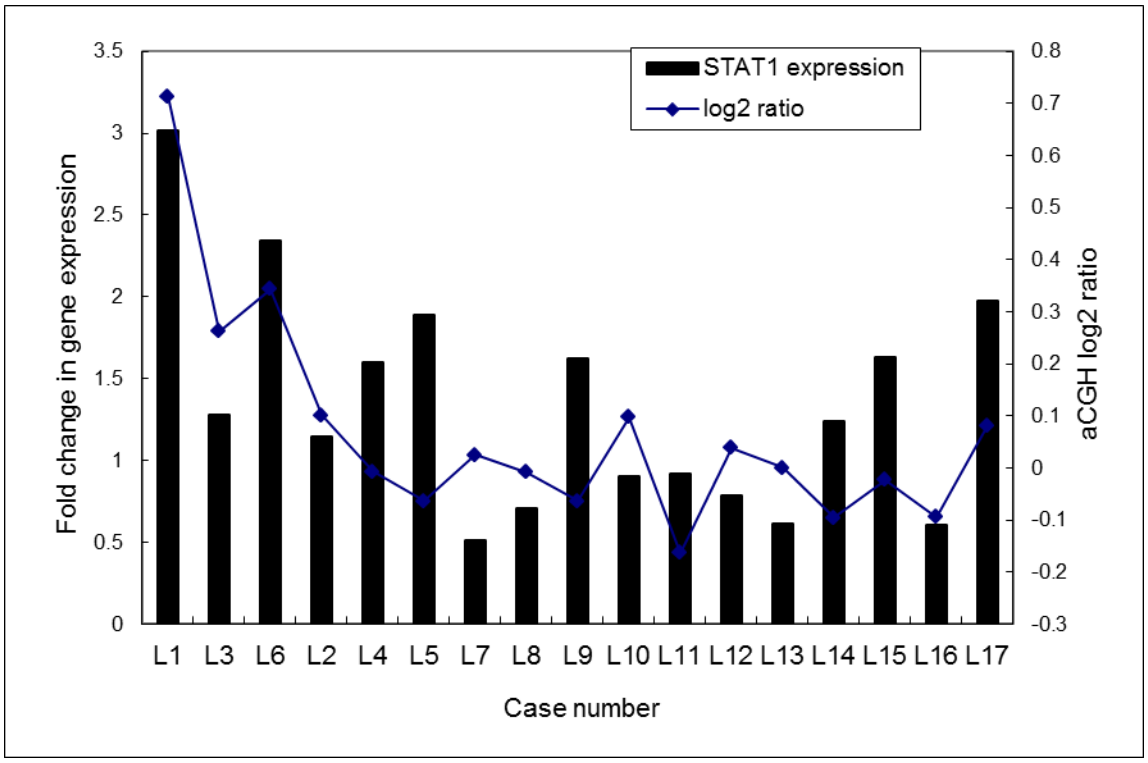


Figure 29. The gene expression data and the corresponding cytogenetic results of STAT1 in canine lymphoma cases.

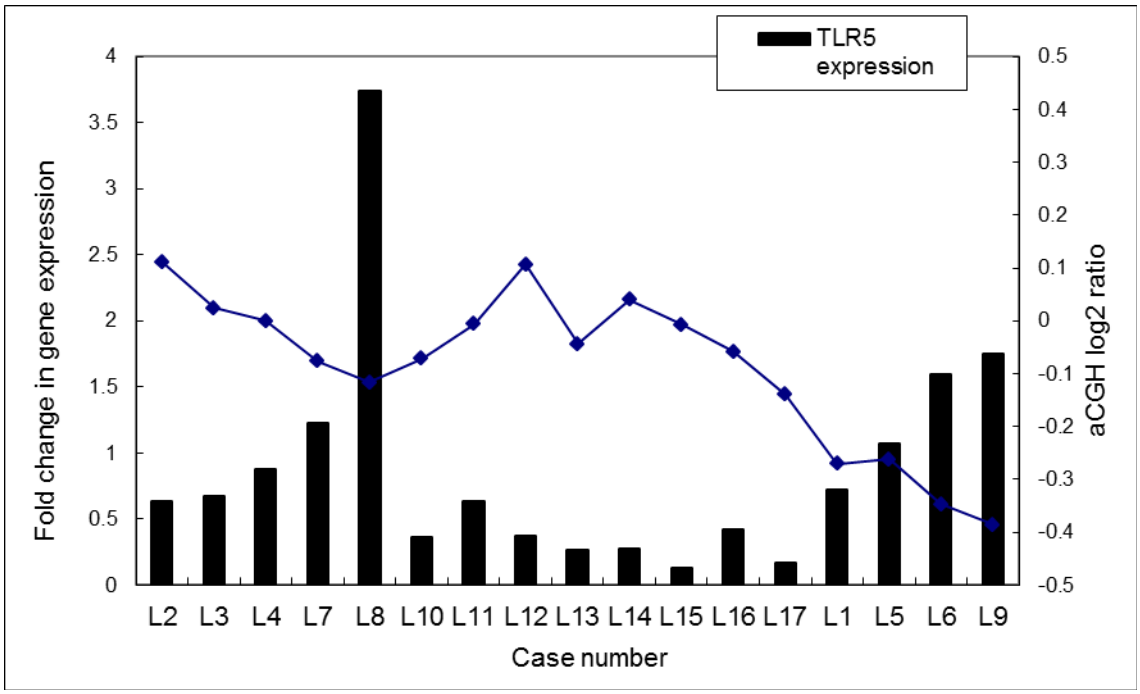


Figure 30. The gene expression data and the corresponding cytogenetic results of TLR5 in canine lymphoma cases.