

Evolutionarily conserved cytogenetic changes in hematological malignancies of dogs and humans – man and his best friend share more than companionship

Matthew Breen^{1,2*} & Jaime F. Modiano^{3,4}

¹Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606, USA; Tel: +1-919-513-1467; Fax: +1-919-513-7301; E-mail: Matthew_Breen@ncsu.edu; ²Center for Comparative Medicine and Translational Research, North Carolina State University, Raleigh, NC, USA; ³Integrated Department of Immunology and Cancer Center, School of Medicine, University of Colorado at Denver and Health Sciences Center, Denver, CO, USA; ⁴Present address: Department of Veterinary Clinical Sciences – College of Veterinary Medicine, and Cancer Center, University of Minnesota, St Paul, MN 55108, USA

*Correspondence

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Abstract

The pathophysiological similarities shared by many forms of human and canine disease, combined with the sophisticated genomic resources now available for the dog, have placed ‘man’s best friend’ in a position of high visibility as a model system for a variety of biomedical concerns, including cancer. The importance of nonrandom cytogenetic abnormalities in human leukemia and lymphoma was recognized over 40 years ago, but the mechanisms of genome reorganization remain incompletely understood. The development of molecular cytogenetics, using fluorescence *in situ* hybridization (FISH) technology, has played a significant role in our understanding of cancer biology by providing a means for ‘interrogating’ tumor cells for a variety of gross genetic changes in the form of either numerical or structural chromosome aberrations. Here, we have identified cytogenetic abnormalities in naturally occurring canine hematopoietic tumors that are evolutionarily conserved compared with those that are considered characteristic of the corresponding human condition. These data suggest that humans and dogs share an ancestrally retained pathogenetic basis for cancer and that cytogenetic evaluation of canine tumors may provide greater insight into the biology of tumorigenesis.

Abbreviations

ALCL	anaplastic large-cell lymphoma	CLL	chronic lymphocytic leukemia
BAC	bacterial artificial chromosome	CML	chronic myelogenous leukemia
BCR	breakpoint cluster region	FISH	fluorescence <i>in situ</i> hybridization
BL	Burkitt’s lymphoma	HSA	<i>Homo sapiens</i>
CFA	<i>Canis familiaris</i>	MZL	marginal zone lymphoma
cDLBCL	canine diffuse large B-cell lymphoma	NHL	non-Hodgkin lymphoma
CHORI	Children’s Hospital Oakland Research Institute	PBL	peripheral blood lymphocytes
		Ph	Philadelphia chromosome
		RCPI	Roswell Park Cancer Institute
		SLP	single-locus probe

Introduction

The demographic history of humans and dogs has progressed in parallel. As hunter-gatherers, humans and dogs co-existed in a relationship built on mutual benefit. Dogs were selected for their ability to aid humans in their survival; they served a primarily functional role, be it in hunting, retrieving or guarding our livestock and ourselves. As humans began to develop permanent settlements, we invited our dogs into our homes. The relationship continued and dogs became more than working animals; they became our companions and the unique bond we share was established. The subsequent emergence of many modern purebred dogs has resulted from the selection of individuals that possessed physical and behavioral traits that we perceived to be desirable characteristics, rather than those that afford a functional role. As such, many breeds have been developed in the past few hundred years under conditions of intensive artificial selection of a restricted gene pool in genetic isolation. The huge diversity in phenotypic variation across the hundreds of dog breeds we see today is a reflection of the genetic plasticity of the canine genome when under such stringent selective pressure. While this process is necessary for the development of a population of individuals that share the collection of characteristics required of the 'breed', if allowed to go unchecked this process will ultimately also generate serious health concerns within the resulting population. This is evident in the fact that, unlike dogs of mixed ancestry, many purebred dogs in the 21st century are affected by susceptibility to a wide variety of genetic disease and cancers, many of which seem to affect just one breed or a few closely related breeds (Parker *et al.* 2004, Sutter *et al.* 2004, Ostrander 2007). This is an indicator that during the development of breeds the deliberate selection of particular desirable traits has brought with it the unintentional selection of deleterious genes.

As our pet, the dog is exposed to our daily environment and the pathophysiological similarities shared between humans and dogs are such that both species develop many of the same kinds of disease and are susceptible to the same range of spontaneously occurring tumors (Withrow & Vail 2006). The recent completion of genome sequences for humans and dogs (Lander *et al.* 2001, Kirkness *et al.* 2003, Lindblad-Toh *et al.* 2005) has demonstrated the high

degree of similarity between our two genomes. In combination, these factors indicate that we are faced with a unique opportunity to advance our understanding of evolutionarily conserved diseases using comparative genomics between man and his best friend.

For more than 40 years the importance of nonrandom cytogenetic abnormalities in human leukemia and lymphoma has been recognized. We hypothesized that the close pathogenetic relationship between human and dog would extend to the presence of evolutionarily conserved cytogenetic changes in comparable cancers. In this study we used comparative genomics to select canine bacterial artificial chromosome (BAC) clones in regions of the canine genome that were evolutionarily related to regions of genome reassembly in three different human cancers. Molecular cytogenetic analysis of these clones in tumor cells derived from a series of canine cancer patients revealed the retention of ancestral chromosomal aberrations in comparable cancers of human and dog. These findings add to the wealth of data supporting the dog as a highly suited biomedical model system for cancer research.

Materials and methods

Three well-characterized blood cancers that are morphologically similar in both species were selected to test our hypothesis: chronic myelogenous leukemia (CML), sporadic Burkitt lymphoma (BL) and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL).

Case materials

Samples were selected from archival cases of leukemia and lymphoma collected through our ongoing studies of canine cancer genetics. Some cases have been reported previously (Dickerson *et al.* 2002, Fernandes *et al.* 2002, Burnett *et al.* 2003). The rest were collected from affected or healthy family-owned dogs, with informed consent from the owners, using procedures that were reviewed and approved by the appropriate Institutional Review Board and Institutional Animal Care and Use Committee. Diagnoses were made using complete blood counts, cytopathology, histopathology and immunophenotyping to

classify leukemias and lymphomas according to the REAL/WHO scheme (Valli *et al.* 2002). Samples from healthy dogs with known karyotype status were used as controls.

Selection of BAC clones as single-locus probes (SLPs) for FISH analysis

Comparative cytogenetic/genomic data (Breen *et al.* 1999, 2004, Yang *et al.* 1999) and the dog genome browser [http://genome.ucsc.edu/index.html?org = Dog](http://genome.ucsc.edu/index.html?org=Dog) were used to identify regions of the canine karyotype that are evolutionarily related to regions of the human karyotype that are known to be involved in cytogenetic aberrations in hematopoietic tumors. The browser was also used to define bacterial artificial chromosome clones within the CHORI-82 canine BAC library (<http://bacpac.chori.org/library.php?id=253>) that contained the orthologues of genes of interest for use as SLPs. DNA from each clone was isolated using a Qiagen RealPrep kit according to the manufacturer's instructions.

FISH

Cryopreserved suspensions of lymph node biopsies and bone marrow aspirates were thawed and used to establish short-term cultures. Chromosome preparations and interphase nuclei were prepared according to our routine protocols (Breen *et al.* 2001). Labeling of BAC clone DNA for FISH, template preparation, and all FISH and imaging procedures were as described previously (Breen *et al.* 2004). In all cases the clones selected for FISH were used first to probe chromosome preparations and interphase nuclei from a clinically normal dog to verify that the signals were each located where expected. For each case, multiplane images of no fewer than 50 cells were acquired and processed using a multicolor FISH workstation driven by dedicated software (SmartCapture 2.3, Digital Scientific, Cambridge, UK).

Immunoprecipitation and immunoblotting

ABL immunoreactive proteins were immunoprecipitated from cryopreserved cells using a monoclonal antibody (24-11) directed against the ABL C-terminal domain (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoprecipitates were immunoblotted with antibodies directed against the

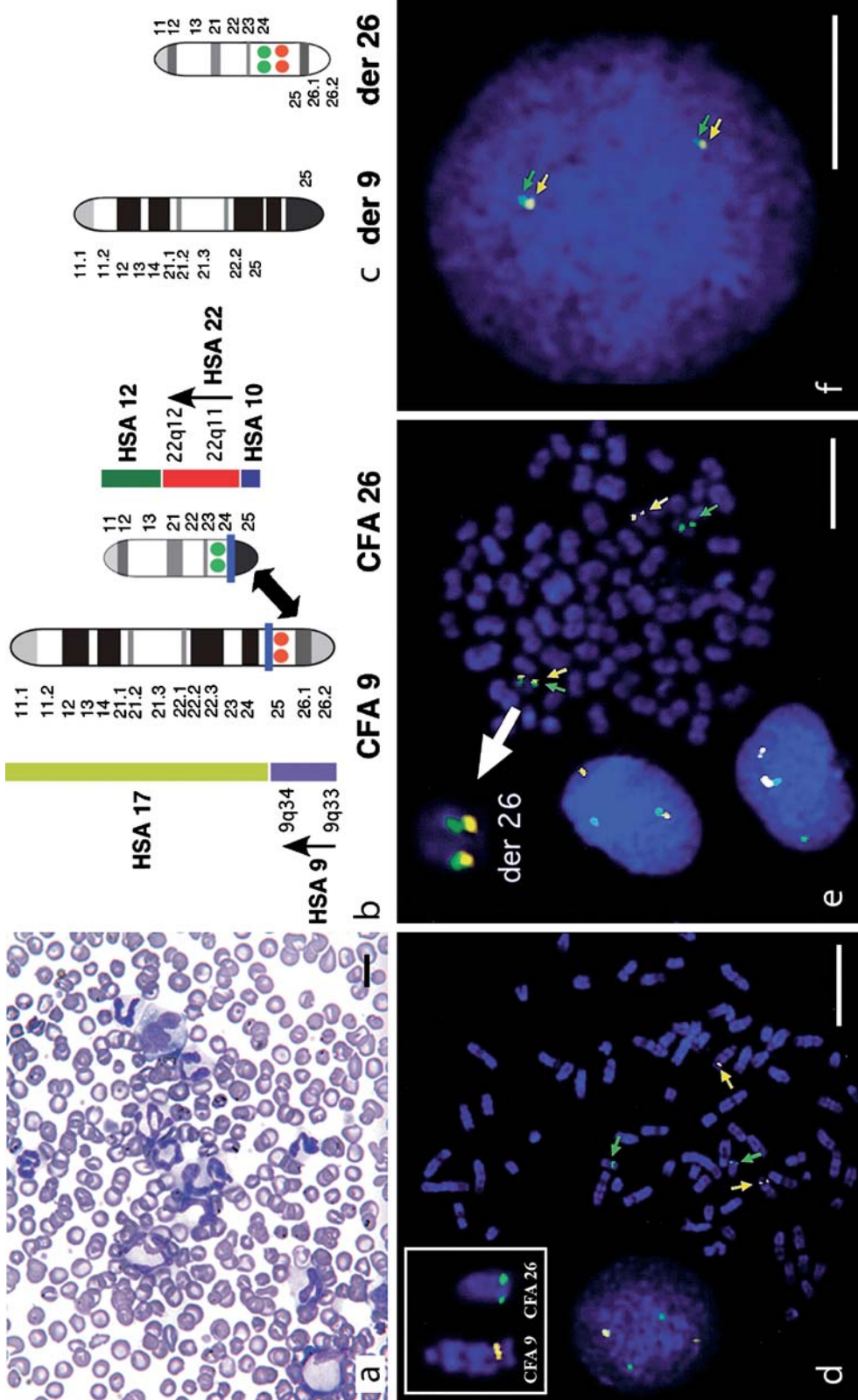
C terminus (24-11) or the kinase domain (K-12, Santa Cruz) of ABL, or against the N terminus of BCR (N-20, Santa Cruz). Expression of c-MYC and RB in CLL samples was examined using monoclonal antibodies N-262 (Santa Cruz) and G3-245 (BD PharMingen, San Diego, CA, USA), respectively. Expression of β -actin was used as a loading control (Ritt *et al.* 2000). The antibodies used are broadly cross-reactive against the target proteins in mammals; they recognize highly conserved epitopes that show no or minimal variability in humans, dogs, and rodents.

Results

Characterization of a rearrangement homologous to human t(9;22) in canine CML

Human CML is characterized by the presence of an aberrant *BCR-ABL* fusion transcript. In the majority of cases, the fusion arises from a reciprocal translocation between the *c-abl* oncogene located in 9q34 (*ABL* locus) and the breakpoint cluster region (BCR) located in 22q11. The rearrangement, described as t(9;22)(q34;q11) results in the classical Philadelphia (Ph) chromosome (Rowley 1973) and is considered a hallmark of CML (Kurzrock *et al.* 2003). Various lines of evidence support the causal association of t(9;22) translocations with CML. First, this group of aberrations is seen in as many as 95% of adult CML patients (Kurzrock *et al.* 2003). Second, elevated tyrosine kinase activity of chimeric BCR-ABL proteins is crucial to their oncogenic potential, as demonstrated by the ability to induce and maintain remission of CML patients using imatinib mesylate (Gleevec Novartis Pharmaceuticals Corp, East Hanover, NJ), a BCR-ABL kinase antagonist (Kurzrock *et al.* 2003). Third, adoptive transfer of hematopoietic stem cells carrying the BCR-ABL fusion product into lethally irradiated mice recapitulates the disease (Wertheim *et al.* 2002). Given the clinical and morphological similarities between human and canine CML, we surmised that a homologous translocation might be detectable in dogs with naturally occurring CML.

For this study we evaluated five cases of canine CML, all of which had similar cytological appearance (Figure 1a). Reciprocal chromosome painting of dog and human has shown previously that the human chromosome (HSA) regions 9q33-q34 and 22q211



are evolutionarily related to dog chromosome (CFA) regions 9q25–26.1 and 26q23–24, respectively (Breen *et al.* 1999, 2004, Yang *et al.* 1999) (Figure 1b). In cytogenetic terms, the presence of a canine equivalent to the Philadelphia chromosome would be a derivative CFA 26 and the translocation would be described as t(9;26)(q25dist-q26.1;q24) (Figure 1c). The dog genome browser ([http://genome.ucsc.edu/index.html?org = Dog](http://genome.ucsc.edu/index.html?org=Dog)) indicates that the canine orthologue of ABL is located on CFA 9 at ~56 Mb and that of BCR is located on CFA 26 at ~31 Mb. BAC clones (CHORI-82) containing segments of ABL (326f17) and BCR (486k17) have been isolated and shown to localize to the expected regions on CFA 9 and 26 (at 9q25 and 26q24) when hybridized to chromosome preparations of a healthy dog (Figure 1d). Further, analysis of over 50 interphase nuclei revealed the consistent presence of two copies for each locus with no evidence of coincidental physical proximity or association. We hybridized the clones representing ABL and BCR to metaphase preparations and interphase nuclei prepared from all five cases of canine CML. In one case we were able to generate chromosome preparations, analysis of which indicated that there

was clear co-localization of the two target BAC clones on a single derivative chromosome and also in interphase nuclei (Figure 1e). We observed this t(9;26) translocation in 26% (13/50) of cells analyzed in this case, suggesting that the progenitor cell giving rise to malignant progeny had not effaced normal hematopoietic elements. Predictably, when present the translocation event was heterozygous in the majority (11/13) of cells, as indicated in Figure 1e. Homozygous translocations occurred in the remaining two cells harboring this abnormality (Figure 1f). Similar results were found in our analysis of interphase nuclei from four additional cases of canine CML with the percentage of affected cells ranging from 11% to 34%.

We used antibodies directed against conserved regions of ABL and BCR to verify the generation of

← **Figure 1.** Conserved cytogenetic rearrangement in canine CML. (a) Photomicrograph of a peripheral blood smear from a dog with CML (Diff-Quik stain; original magnification, $\times 600$). Scale bar = 10 μm . (b) Comparative ideograms showing corresponding regions for HSA 9q34 and HSA 22q11 on canine chromosomes 9 and 26. Orange and green spots indicate the location of canine ABL and BCR. A horizontal blue line on each ideogram identifies the location of the predicted breakpoints on CFA 9 and CFA 26, below which the two regions would be exchanged to form the aberrant derivative chromosomes. (c) Schematic representation of the predicted derivative CFA 9 and derivative CFA 26 that would be produced by such a reciprocal translocation, with the derivative 26 showing co-localized signals from ABL and BCR. (d) FISH analysis of metaphase preparations and interphase nuclei in normal canine leukocytes using canine BAC clones representing canine ABL and BCR on CFA 9 (orange spots) and CFA 26 (green spots), respectively. Inset shows enlarged, single metaphase chromosomes for CFA 9 and 26 correctly oriented. (e) Hybridization of the same two BAC probes to metaphase chromosomes and interphase nuclei of a canine CML. Heterozygous co-localization of these two BAC clones to the derivative chromosome 26 is evident in the metaphase preparation and co-localization of one green and one yellow spot is also evident in the two interphase nuclei. Inset shows the derivative CFA 26 enlarged and correctly oriented (compare to predicted der26 in (c)). (f) Interphase nucleus from the same case of CML showing the presence of an apparent homozygous 9/26 translocation event, with both yellow and green spots showing close association. Scale bars = 8 μm .

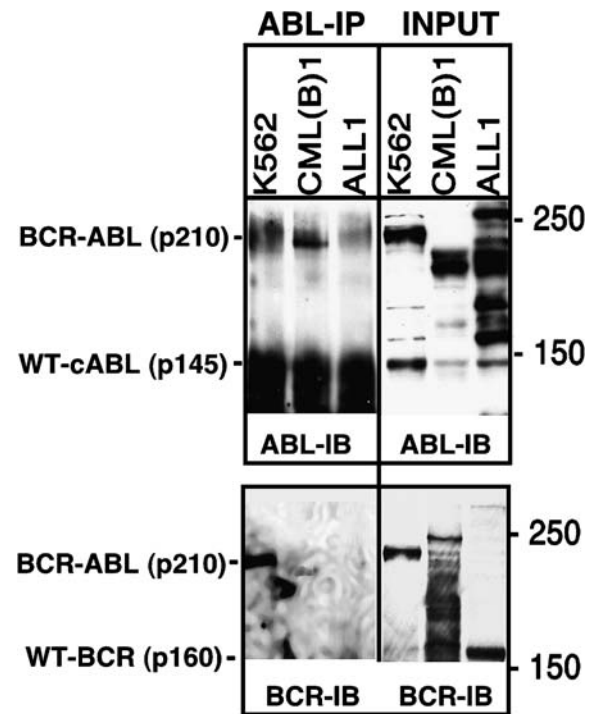


Figure 2. Demonstration of canine BCR-ABL fusion protein. ABL protein was immunoprecipitated (ABL-IP) from whole-cell lysates of K562 human erythroleukemia cells, a dog with CML in blast crisis (CML(B)1), and a dog with acute lymphoblastic leukemia (ALL1). *Left panel:* Immunoprecipitates were probed using antibodies against the C-terminal end of ABL (top) or the N-terminal end of BCR (bottom) to identify the presence of BCR-ABL fusion proteins. *Right panel:* Whole-cell lysates used as input (INPUT) for the immunoprecipitates were probed with the same antibodies to document ABL and BCR immunoreactive proteins. The prototypical BCR-ABL fusion protein found in K562 (p210) and wild-type ABL (p145) and BCR (p160) are identified.

a BCR-ABL fusion protein. Figure 2 shows that a high-molecular-weight BCR-ABL fusion protein was present in cells from a dog with CML in blast transformation. This protein was comparable to the common ~190 kDa form seen in human CML and slightly smaller than the ~210 kDa form present in Ph⁺ human K562 erythroleukemia cells. We also identified the presence of t(9;26) in a case of canine acute lymphoblastic leukemia (ALL). Despite the

presence of multiple ABL-immunoreactive bands in cells from this dog with ALL, none was consistent with a BCR-ABL fusion protein, suggesting a distinct variant form of the translocation event (Haigh & Cuthbert 2004).

Characterization of a rearrangement homologous to human t(8;14) in canine BL

In humans the BL/ALL-L3 complex is characterized by translocations that involve rearrangement of the *MYC* gene on HSA 8 (Ferry 2006). In approximately 80% of B cell lymphomas, the translocation of *MYC* from HSA 8q24 to HSA 14q32 places *MYC* under the control of the immunoglobulin heavy-chain enhancer (*IGH*). The pathogenesis of BL seems to require temporally regulated overexpression of *MYC* driven by various mechanisms (Taub *et al.* 1984, Hecht & Aster 2000). Among numerous canine lymphomas examined, we identified three cases with morphological features of high-grade sporadic BL (Valli *et al.* 2002, Jubala *et al.* 2005). Cells from a representative case are shown in Figure 3a. To look for *MYC*-*IgH* translocations, canine BAC clones were selected that represent the canine *MYC* gene (RPCI-81 396j05), which maps to CFA 13q12 (Thomas *et al.* 2003b), and a BAC clone from CFA 8q33 (corresponding to HSA 14q32). (CHORI-82 clone 293n12 was selected to contain *IgH* but did not produce a reliable signal. A clone known to map 8q33 was thus selected as a proxy). Although we were unable to generate chromosome preparations from these cases, hybridization to interphase nuclei of these cases revealed co-localization of these two clones in 10–26% of the cells, substantiating the presence of a *MYC*-*IGH*-like translocation (Figure 3b). To verify its biological significance, we compared expression of *Myc* in

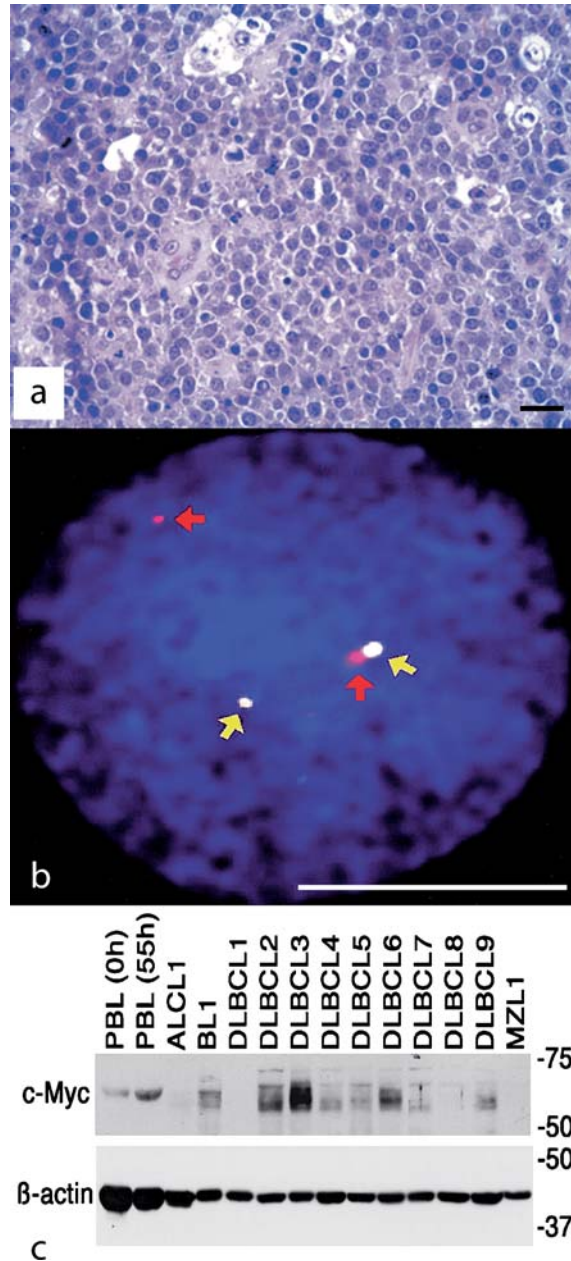


Figure 3. Conserved cytogenetic rearrangement in canine Burkitt's lymphoma. (a) Photomicrograph of a lymph node section from a dog with Burkitt lymphoma (H&E stain; original magnification, $\times 400$) Scale bar = 25 μm . (b) Interphase cell from a dog with Burkitt's lymphoma showing heterozygous co-localization of a canine BAC clone containing the *MYC* gene (yellow spots indicated by yellow arrows) and a BAC clone that maps to the same cytogenetic band as the *IgH* locus (red spots, identified by red arrows) Scale bar = 5 μm . (c) *Myc* expression in nonstimulated normal canine PBLs (0 h), mitogen-stimulated PBLs (55 h), or lymph node cells from dogs with anaplastic large cell lymphoma (ALCL1), Burkitt lymphoma (BL1), diffuse large B cell lymphoma (DLBCL1–DLBCL9) or nodal marginal zone lymphoma (MZL1). β -Actin was used as a loading control.

normal peripheral blood lymphocytes (PBLs) and non-Hodgkin lymphoma (NHL). Figure 3c shows that, as is true for human PBL, Myc was inducible in normal canine PBL after 55 h of mitogenic stimulation. Among the cases of canine NHL, Myc was expressed constitutively in seven of nine cases of canine diffuse large B cell lymphoma (cDLBCL), a disease we have shown previously to show amplifications of CFA 13 (Thomas *et al.* 2003c). More importantly, among the NHL subtypes that do not have CFA 13 amplification (anaplastic large-cell lymphoma or ALCL, BL, and marginal zone lymphoma, or MZL) Myc expression was only seen with BL.

Deletion of the *RB-1* locus in canine CLL

CLL frequently presents with a hemizygous deletion of the q14 region of HSA 13 that includes the gene *RB1* (Dohner *et al.* 2000). If deletion of 13q14 were

evolutionarily conserved in the dog, it would manifest as a deletion within CFA 22q11.2 (Thomas *et al.* 2003a) (Figure 4a). To explore this, we initially performed multicolor FISH on metaphase preparations from a case of canine CLL (Figure 4b), using a selection of seven BAC clones that spanned the full length of CFA 22 (Breen *et al.* 2004) including a clone containing the *RB1* locus (RPCI-81 312-b24) (Thomas *et al.* 2003a). Differential spectral labeling of these BACs revealed clearly a hemizygous deletion of the *RB1* locus (Figure 4c). In addition, the retained *RB1* allele, which is normally localized to CFA 22q11.2, was distal to this location at 22q12.1, suggesting an inversion involving this locus (compare Figures 4a and 4c). Five of seven additional CLL cases examined showed hemizygous or homozygous deletion of *RB1* in >25% of cells, and RB protein was reduced or absent in eight of nine cases examined by immunoblotting (Figure 4d), indicating that loss of this region of CFA 22 was a

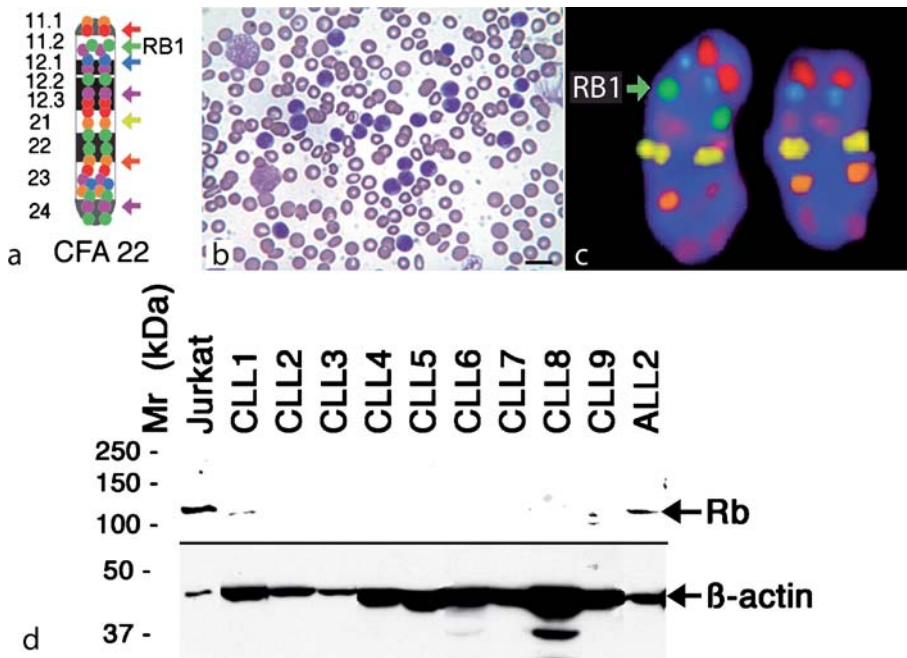


Figure 4. Conserved cytogenetic rearrangement in canine CLL. (a) Schematic assignment of 21 canine BAC clones across the length of CFA 22, derived by multicolor FISH analysis. The location of the BAC clone containing *RB1* in 22q11.2 is indicated. (b) Photomicrograph of a peripheral blood smear from a dog with CLL (Diff-Quik stain; original magnification, $\times 600$, scale bar = 15 μm). (c) Co-localization of seven differentially labeled canine BAC clones (arrowed in (a)) to the homologues of CFA 22 in a case of canine CLL. The clone labeled in green contains the canine *RB1* gene. The homologue on the left appears normal, with the exception of a small inversion involving the *RB1* locus; the homologue on the right also lacks the green signal, indicating a deletion of the *RB1* locus, in addition to the inversion. (d) Reduced expression of Rb (p110) in cells from nine dogs with CLL (CLL1–CLL9). Rb expression was evident in a case of ALL (ALL2), and human Jurkat cells were also assessed to confirm the similar electrophoretic mobility of Rb in human and dog cells. β -Actin was used as a loading control.

functionally significant event. Yet, even though both species seem to harbor RB1 deletions in CLL, it was shown recently in humans that the significance of the 13q14 deletion in CLL may trace to regulation of two microRNAs (miR-15a and miR-16-1) that control Bcl2 (Cimmino *et al.* 2005, Calin & Croce 2006). Whether the same is true for canine CLL remains to be investigated. Nevertheless, the observation that a comparable deletion exists in this indolent disease despite the exclusive B-cell phenotype in humans (Schlegelberger *et al.* 1994) and the prevalent T-cell phenotype in dogs (Burnett *et al.* 2003) suggests that these micro-RNAs, and/or other genes in this region are essential factors in the pathogenesis of the clinical syndrome of chronic lymphocytic leukemia regardless of the potential of these cells to progress along either immunophenotypic lineage.

Discussion

As mammals, humans and dogs are essentially differential arrangements of the same collection of ancestrally related genes that began to diverge over 50 million years ago from a common ancestor. Their genomes have since been modified through the ongoing process of genome evolution/speciation and natural selection. In contrast to the gradual selective pressures placed upon the human genome, numerous purebred dog breeds have been subjected to intense selection pressure over just the past few centuries. This high level of selective pressure has exploited the enormous plasticity of the domestic dog, enabling it to be 'molded' relatively quickly to generate the diverse phenotypic endpoints considered desirable by mankind. Despite the huge diversity in morphology of the domestic dog, the gross genome architecture has remained apparently constant. Using molecular cytogenetic evaluation of three different naturally occurring canine hematopoietic malignancies, our studies revealed the presence of evolutionarily conserved chromosome aberrations shared with the corresponding condition in humans. We propose that two major conclusions can be drawn from our data. First, despite tens of millions of years of divergence, the evolving mammalian genome seems to have retained an underlying common mechanism for genome reassembly associated with cancer, leading

to the presence of evolutionarily conserved recurrent chromosomal aberrations shared between naturally occurring blood cancers of humans and dogs. This supports the idea of a fundamental association between cytogenetic abnormalities and tumor phenotype. Second, these conserved aberrations have similar consequences in both species, i.e., the over-expression or silencing of homologous proteins or the generation of fusion proteins, suggesting these are pathogenetically significant evolutionarily conserved features of genome reassembly. Recent studies evaluating the comparative cytogenetics of numerous mammals have demonstrated that many karyotypes have retained large blocks of shared evolutionarily conserved chromosome segments (e.g. O'Brien *et al.* 1999, Murphy *et al.* 2005). More detailed investigation of the genome organization in mammals revealed that almost 20% of chromosome breakpoints were reused during chromosome evolution, and that there was a close association between the location of breakpoints associated with chromosome evolution and breakpoints associated with cancers (Murphy *et al.* 2005). Our study provides further support for this concept by demonstrating that divergent mammalian genomes undergoing comparable spontaneous malignancy have retained evolutionarily conserved regions of genome reassembly. Furthermore our data support the idea that, as one would expect, the biological consequences of such restructuring are also conserved, resulting in the presentation of pathologically comparable malignancies. These data suggest that such sites of genomic reorganization may be inherently fragile and so may serve as evolutionarily retained 'hot spots' for chromosomal reassembly associated with tumorigenesis. It may be also the case that such sites are the result of powerful somatic selection for the survival of rare events of mammalian genome reassembly that ultimately favor survival, regardless of the millions of years of divergent evolution that now separate the species in which they occur.

The predisposition of dog breeds to selectively develop types of cancer (Modiano *et al.* 2005) suggests that, through the process of breed development, dogs have acquired genomes that render specific cells distinctly susceptible to malignant transformation. The presence of evolutionarily conserved chromosome aberrations in naturally occurring cancers will

thus allow us to determine whether the apparent structural instability in these regions is indeed a conserved feature of the ancestral genome. Furthermore, a thorough investigation of recurrent breakpoint regions in canine tumor genomes, especially those that have high breed specificity, offers the potential to define regions that contain genes that have so far not been associated with tumorigenesis in humans or other species. This is especially true for abnormalities that are identifiable in canine tumor genomes but that may remain undetectable in corresponding human tumors using currently available technologies. Comparative genomics is providing strong evidence that man and his best friend share more than companionship.

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