

## The *MTAP-CDKN2A* Locus Confers Susceptibility to a Naturally Occurring Canine Cancer

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### Abstract

**Background:** Advantages offered by canine population substructure, combined with clinical presentations similar to human disorders, makes the dog an attractive system for studies of cancer genetics. Cancers that have been difficult to study in human families or populations are of particular interest. Histiocytic sarcoma is a rare and poorly understood neoplasm in humans that occurs in 15% to 25% of Bernese Mountain Dogs (BMD).

**Methods:** Genomic DNA was collected from affected and unaffected BMD in North America and Europe. Both independent and combined genome-wide association studies (GWAS) were used to identify cancer-associated loci. Fine mapping and sequencing narrowed the primary locus to a single gene region.

**Results:** Both populations shared the same primary locus, which features a single haplotype spanning *MTAP* and part of *CDKN2A* and is present in 96% of affected BMD. The haplotype is within the region homologous to human chromosome 9p21, which has been implicated in several types of cancer.

**Conclusions:** We present the first GWAS for histiocytic sarcoma in any species. The data identify an associated haplotype in the highly cited tumor suppressor locus near *CDKN2A*. These data show the power of studying distinctive malignancies in highly predisposed dog breeds.

**Impact:** Here, we establish a naturally occurring model of cancer susceptibility due to *CDKN2* dysregulation, thus providing insight about this cancer-associated, complex, and poorly understood genomic region. *Cancer Epidemiol Biomarkers Prev*; 21(7); 1019–27. ©2012 AACR.

### Introduction

Although many genes have been associated with rare, high-penetrance cancer syndromes in humans, such

syndromes account for only a fraction of familial cancer risk (1). A recent explosion of genome-wide association studies (GWAS) has identified several putative cancer-associated risk alleles, many of which are located near known cancer genes, although not within classic exonic boundaries (reviewed in ref. 2). These noncoding, low-penetrance, cancer-susceptibility alleles likely contribute to quantitative changes in gene expression and, as such, are difficult to find.

Dogs are particularly well suited to studies of malignancy (3) as cancer is the most frequent cause of disease-associated death in dogs, and naturally occurring cancers are well described in several breeds (4, 5). The high incidence of breed-specific cancers offers opportunities to identify sequence variants leading to disease susceptibility that have been difficult to find in humans. Application of the canine system is particularly efficacious when multiple closely related breeds or pure breeding populations of the same breed exist, each with predisposition to the same disease and as such, are likely to segregate the same founder mutation (6, 7, 8).

Histiocytic sarcoma is a highly aggressive and lethal dendritic cell neoplasm that occurs in 15% to 25% of Bernese Mountain Dogs (BMD; refs. 9–12). Localized histiocytic sarcoma most commonly develops in the skin

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**Note:** Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

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doi: 10.1158/1055-9965.EPI-12-0190-T

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or subcutis of an extremity. The tumor is locally invasive with metastasis to lymph nodes and/or blood vessels. Disseminated histiocytic sarcoma is a multisystem disease with tumors appearing in numerous organs, including the spleen, liver, and lungs. Progression to death is rapid (12). Almost no information is known about the genetic underpinnings histiocytic sarcoma in humans or animals (13), largely because of the lack of a well-characterized biologic system for study. In this article, we summarize findings from 2 independent histiocytic sarcoma GWAS in BMDs, offering insights into this poorly understood class of neoplasms as well as establishing a foundation for future studies of histiocytoses in humans.

## Materials and Methods

### Sample collection

All dog owners provided informed consent consistent with Animal Care and Use Committees at their collecting institution. DNA was isolated from 475 BMD blood samples. Two hundred forty, 95, and 140 were provided from North America, France, and The Netherlands, respectively. All dogs with available pedigree data were unrelated at the grandparent level. For detailed collection information see Supplemental Material S1. Whole blood was collected with either EDTA or ACD anticoagulant. In North America and The Netherlands, genomic DNA was isolated using a standard phenol–chloroform protocol (14). In France the Nucleon BACC Genomic DNA Extraction Kit was used (GE Healthcare). All samples were stripped of identifiers, numerically coded, and aliquoted for long-term storage at  $-80^{\circ}\text{C}$ .

### Genotyping and PCA

Samples were genotyped using the Canine SNP20 Bead-Chip panel (Illumina), which included approximately 22,000 single nucleotide polymorphisms (SNP). After removing SNPs with a minor allele frequency  $<0.01$  and genotyping rate  $<80\%$ , 17,218 SNPs remained. The final data set included 114 and 120 case and control dogs, respectively, from North America, and 128 and 112, respectively, from Europe. More than 96% of European dogs came from France and The Netherlands. Two rounds of principal components analysis (PCA) were carried out on the data set using EIGENSTRAT (15). The first removed genetic outliers and the second determined the amount of stratification within the data set. Eight dogs were removed because they were  $>6$  SDs from the average across the top 10 PCs. The remaining 466 dogs (228 North American and 238 European) were clustered according to the top 10 PCs. The process was repeated in the North American and the European samples independently, and 2 additional outliers were removed.  $F_{st}$  values and inbreeding coefficients were calculated by continents, countries, and case/control status. The complete data set of genotypes and phenotypes has been submitted to Gene Expression Omnibus (GEO) under accession number GSE38011.

### GWAS analyses

In the first of 2 GWAS, 111 cases and 117 controls BMD from North America were analyzed using PLINK v1.07 (16). Standard  $\chi^2$  values of association were calculated (17). Spurious missing data was imputed using BEAGLE and the analysis repeated, correcting for multiple testing with 10,000 permutations using PRESTO (18, 19). The data were analyzed using EMMA to correct for stratification and cryptic relatedness (20).

In the second GWAS, 125 European cases and 111 controls were analyzed using the same methods. The 2 North American and European GWAS results were compared and the data sets combined. Association was calculated without correction in PLINK, stratified by continent, and permuted 10,000 times in PRESTO, and corrected for population structure using an additive kinship matrix implemented by EMMA. Loci that were significantly associated with disease in both populations were considered further.

### Fine mapping

A custom SNPlex genotyping assay (Applied Biosystems) was run on 175 cases and 162 control BMDs from North America (213) and Europe (124). Selected SNPs spanned 9.7 Mb (chr11:39,072,460–48,846,456) and surrounded highly associated markers from the combined GWAS. After removing failed and uninformative SNPs, 229 remained (Supplementary Table S2).

Genotypes from SNPlex were imputed on all 466 dogs from the original GWAS using BEAGLE (18, 21). The data sets were divided by continent before imputation to account for differences in population structure, an additional 212 SNPs were added to the original GWAS data and association was calculated, as previously described. Phasing and association was carried out using BEAGLE. Finally, the data sets were combined and association calculated with correction for population structure across the genome, including and excluding chromosome 11. Frequency of the associated haplotype was calculated in cases and controls from each population.

### Sequencing

Amplicons were sequenced within a 300-kb region (chr11:44,001,369–44,331,631) that included the 198 kb (chr11:44,133,881–44,331,630) associated haplotype and all predicted exons of *CDKN2A*, *CDKN2B*, and *MTAP*. Primers were designed using Primer3 v0.4.0 (ref. 22; Supplementary Table S2). Segments were amplified from 24 case and 20 control North American BMD using standard protocols and sequenced using BigDye Terminator v3.1 on an ABI 3730xl DNA Analyzer (Applied Biosystems). Sequencing 306 amplicons revealed 133 SNPs. The complete sequence of the *INK4A* transcript and the genomic sequence of *CDKN2A* exon1a and promoter have been submitted to National Center for Biotechnology Information (NCBI) GenBank (accession numbers JN086563 and JN086564, respectively).

Sequences were analyzed using Phred/Phrap/Consed (23–25) with SNPs identified by Polyphred (26). BEAGLE was used to estimate haplotypes, impute genotypes, and calculate Fisher exact association for SNPs and haplotypes after 10,000 permutations (21, 27). The sequenced SNPs were imputed on the North American SNPlex data set to calculate association. Markers with >25% missing data were removed before imputing. Pairwise LD and haplotype block analysis was done using Haploview v4.1 (28).

To confirm the relative strength of SNP associations from imputed data, 9 SNPs were genotyped in an additional 109 cases and 89 controls (Supplementary Table S3). Also, 10 dogs from 7 non-histiocytic sarcoma breeds were genotyped with the same SNPs.

### Quantitative PCR in dendritic cells from healthy dogs

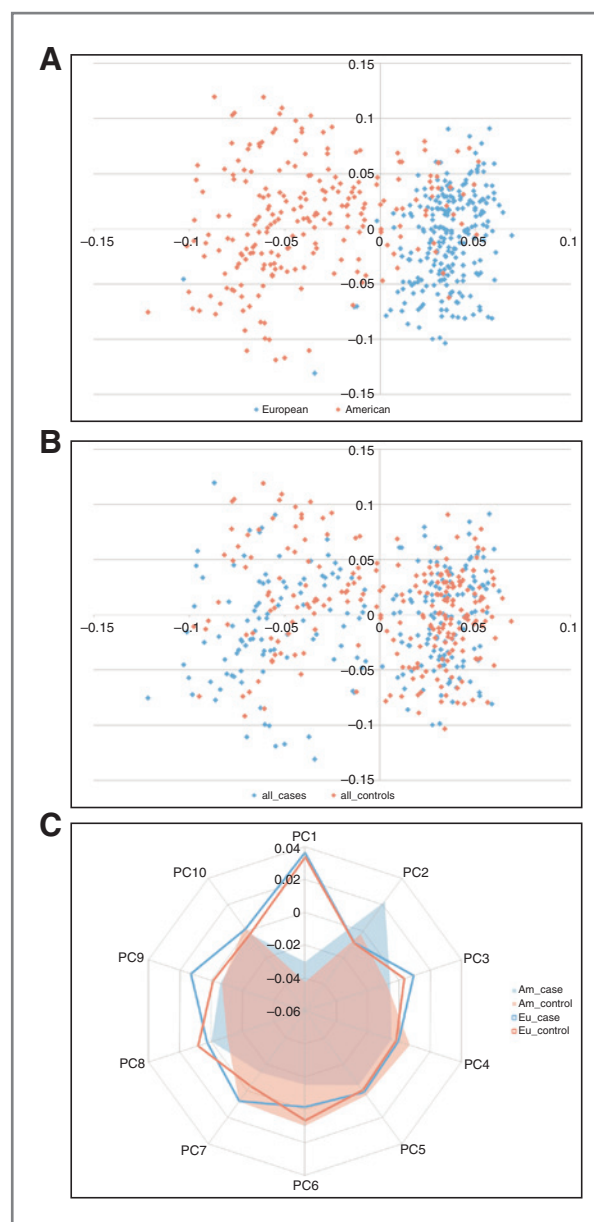
Four mL blood samples were obtained from 53 healthy BMD, randomly selected from approximately 500. Peripheral mononuclear cells were isolated, uniformly plated, and allowed to expand in the presence of interleukin-4 (50 ng/mL) and granulocyte macrophage colony-stimulating factor (33 ng/mL) to select for dendritic precursor cells (29, 30). At 19 days, the cells were harvested and DNA/RNA was extracted using standard methods.

Samples were assigned haplotypes based on their genotypes at chr11:44,201,923 and 44,215,162. Pre-designed TaqMan assays were obtained for the *B2M* (endogenous control) and *MTAP* genes, whereas primers and probe were designed for *CDKN2A* and *CDKN2B* using Primer Express (Applied Biosystems). Real-time PCR was carried out on 24 ng of cDNA for each assay using standard protocols. Each sample was run in triplicate and  $C_T$  values averaged. Relative quantities of the transcripts and average fold change were calculated using the  $\Delta C_T$  method compared with an endogenous control and a reference tissue (testis) and then corrected for amplification efficiency (31, 32). Data were collected for 6 dogs homozygous for the CA haplotype, 6 heterozygous dogs, and 5 dogs lacking the CA haplotype.  $P$  values were calculated for the differences in distributions of transcript quantities using both the 2-tailed Student  $t$  test and the nonparametric Wilcoxon rank sum test.

## Results

### Principal component analyses

PCA of the entire data set of 474 dogs and 17,218 SNPs revealed significant stratification among the populations of BMD from North America and Europe (Fig. 1C). Plots of PCs 1 and 2 show separation of North American and European populations (Fig. 1A) although cases and controls are fully integrated (Fig. 1B). Calculations of  $F_{st}$  averaged over all loci showed that divergence between cases and controls is an order of magnitude lower than between geographic localities



**Figure 1.** PCA of BMD populations from Europe and North America. PCs were calculated from whole genome SNP data in cases and controls from North America and Europe. A, two distinct yet overlapping populations are identified when comparing North American (light red) and European (light blue) populations ( $F_{st} = 0.01$ ). B, cases and controls are distributed evenly throughout both populations ( $F_{st} = 0.001$ ). C, one of the top 10 principal components differentiates cases from controls, all others divided the samples along continental lines. North American cases = red fill, North America controls = blue fill, European cases = red line, European controls = blue line. EU, Europe; NA, North America.

(average  $F_{st} = 0.001$  and 0.015, respectively). Overall, North American dogs showed a higher level of inbreeding than either of the European populations. However, none of the case groups were significantly more inbred than the controls (Supplementary Table S4).

### Genome-wide association study

A GWAS was conducted using 111 affected (cases) and 117 unaffected (controls) BMD from North America revealing >20 markers within a single peak of association on CFA11 spanning approximately 9 Mb from 38.5 to 47.1 Mb ( $P_{\text{raw}} = 1.41 \times 10^{-9}$ ,  $P_{\text{emp}} < 1 \times 10^{-4}$ , 10,000 permutations; Fig. 2A). After correcting for population stratification and cryptic relatedness, the most associated marker was CFA11:47,179,346 ( $P_{\text{corrected}} = 5.6 \times 10^{-6}$ ).

A second GWAS, carried out using 125 cases and 111 control European BMDs, revealed histiocytic sarcoma loci on CFA11 at 47.1 Mb ( $P_{\text{raw}} = 1.5 \times 10^{-6}$ ,  $P_{\text{emp}} = 0.0064$ ) and CFA14 from 10.9 to 14.0 Mb ( $P_{\text{raw}} = 9.8 \times 10^{-8}$ ,  $P_{\text{emp}} = 0.0003$ ). After correction with EMMA, both loci remained significant ( $P_{\text{corrected}} = 1.50 \times 10^{-7}$  and  $P_{\text{corrected}} = 6.59 \times 10^{-6}$ , respectively; Fig. 2B).

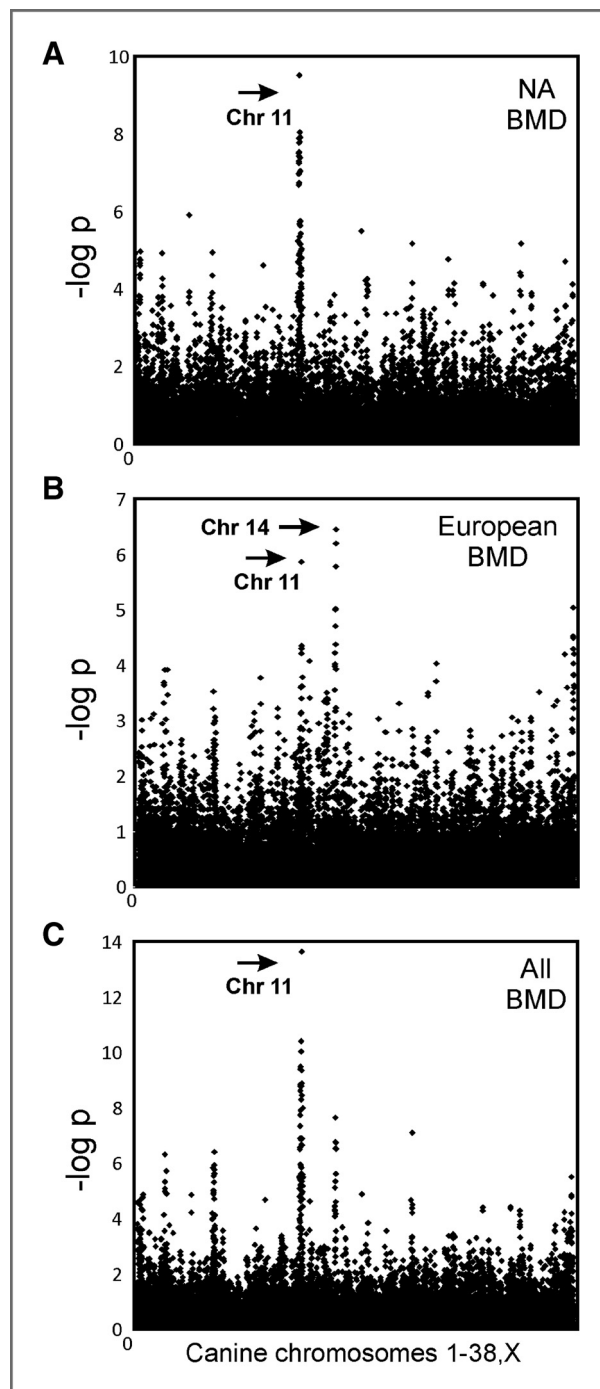
The data sets were combined and association analysis with correction for population structure revealed the same 2 loci as above (Fig. 2C); however, only the CFA11 locus was associated in both the individual and combined GWAS. The SNP at CFA11:47,179,346 had the strongest association with disease susceptibility by all methods with  $P_{\text{raw}} = 1.11 \times 10^{-11}$ ,  $P_{\text{emp}} < 1.00 \times 10^{-4}$ , and  $P_{\text{corrected}} = 1.76 \times 10^{-8}$ . Quantile–quantile plots, showing the distribution of  $P$  values before and after population correction, are shown in Supplementary Fig. S5. The top 10 associated SNPs from each data set and analysis method are listed in Supplementary Table S6, followed by a list of possible candidate genes from the locus on CFA14 for future studies (Supplementary Table S7).

### Fine mapping the CFA11 locus

To refine the locus on CFA11, we genotyped an additional 229 SNPs spanning 9.7 Mb (Supplementary Table S2) in 327 dogs from the combined BMD data set and imputed the genotypes using all 468 dogs. In all populations 2 markers in complete LD with one another showed the highest association with  $P_{\text{corrected}} = 4.15 \times 10^{-12}$ ,  $3.15 \times 10^{-8}$ , and  $9.90 \times 10^{-21}$ , in North American, European, and the combined set, respectively (Supplementary Fig. S8). These markers were located at 44,191,398 and 44,215,162 in the CanFam2 assembly.

Genotypes across the region were phased and multi-marker association computed. All dogs carrying the case-associated allele at position 44,191,398 carried an identical 3 SNP haplotype at positions 44,191,398, 44,215,162, and 44,254,083. The haplotype was common in BMD, however, and comprised 80% of case haplotypes in all populations, but ranged from 49% to 64% in controls (Table 1). Strikingly, 65% of cases were homozygous for the CA haplotype within each population, with >95% of cases carrying the CA haplotype on at least one chromosome. By comparison, only 18% to 39% of the controls were homozygous for the CA haplotype (Table 1).

These 3 SNPs define a 198-kb region (44,133,881–44,331,630) that spans methylthioadenosine phosphorylase gene (*MTAP*) and the cyclin-dependent kinase inhibitors 2A (*CDKN2A*) and 2B (*CDKN2B*).



**Figure 2.** Genome-wide analyses of histiocytic sarcoma in 2 populations of BMD identify an association on CFA11. The Y-axis indicates the negative log of the uncorrected  $P$  value. The X-axis shows marker position from the top of CFA1 through CFAX. A, a total of 240 U.S. BMD with maximum association at CFA11 bp 41,359,032,  $P_{\text{raw}} = 1.41 \times 10^{-9}$ . B, a total of 234 European BMD with 2 peaks of association, CFA11 bp 47,179,346,  $P_{\text{raw}} = 1.50 \times 10^{-6}$  and CFA14,  $P_{\text{raw}} = 9.80 \times 10^{-8}$ . C, U.S. and European cohorts combined for a total of 474 dogs with maximum association at CFA11 bp 47,179,346,  $P_{\text{raw}} = 1.11 \times 10^{-11}$ . Additional peaks found on chromosomes 2, 5, and 20 are reduced to background levels after correcting for population structure.

**Table 1.** Allele frequency of the haplotype associated with histiocytic sarcoma in the BMD

|          | CA allele frequency <sup>a</sup> | % carrying CA allele | Homo | Hetero | Absent |
|----------|----------------------------------|----------------------|------|--------|--------|
| All      |                                  |                      |      |        |        |
| Cases    | 80                               | 96                   | 65   | 31     | 4      |
| Controls | 54                               | 84                   | 24   | 60     | 16     |
| American |                                  |                      |      |        |        |
| Cases    | 80                               | 95                   | 65   | 30     | 5      |
| Controls | 49                               | 80                   | 18   | 62     | 20     |
| European |                                  |                      |      |        |        |
| Cases    | 81                               | 96                   | 65   | 31     | 4      |
| Controls | 59                               | 88                   | 30   | 58     | 12     |
| French   |                                  |                      |      |        |        |
| Cases    | 81                               | 97                   | 65   | 33     | 2      |
| Controls | 64                               | 90                   | 39   | 51     | 10     |
| Dutch    |                                  |                      |      |        |        |
| Cases    | 81                               | 96                   | 66   | 30     | 4      |
| Controls | 56                               | 85                   | 26   | 60     | 14     |

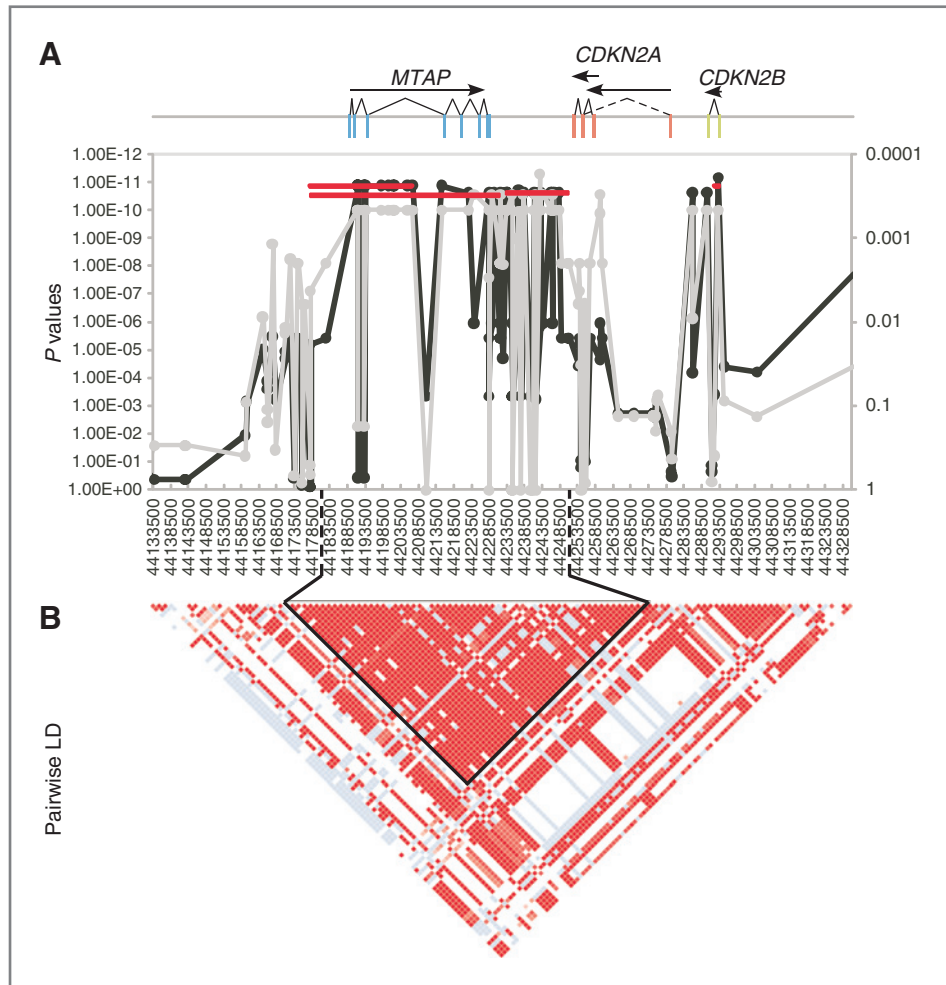
<sup>a</sup>CA = case associated.

**Sequencing in North American BMD**

We identified 139 informative SNPs by sequencing, including 115 within the 198-kb haplotype (Supplementary Table S2). Two coding mutations were found in *CDKN2A*; a silent mutation in exon 1a and a mutation in exon 2 that is silent in p14<sup>ARF</sup> but changes an asparagine to a histidine in p16<sup>INK4a</sup>. The altered amino acid is not conserved across species and the SNP does not segregate with the disease (Fishers exact  $P = 0.09893$ ). The SNP 88 bases upstream of exon 1a that is likely within the 5'-untranslated region showed an association with histiocytic sarcoma (Fisher exact  $P = 1.09 \times 10^{-6}$ ,  $P_{emp} = 0.00029$ ). However, this SNP alone is unlikely to be causative as the associated allele was found in 8 of 10 dogs from breeds in which histiocytic sarcoma is rare (Supplementary Table S3).

Thirty SNPs spanning positions 44,191,314 to 44,293,447 were in complete LD with the 2 most highly associated SNPs from the combined GWAS (Fig. 3B). The associated haplotype was reduced to 75,920 bases (44,177,956–44,253,875) including SNPs at positions 44,177,978 to 44,251,174 surrounding the *MTAP* gene and ending within intron 2 of *CDKN2A*. This haplotype is broken by a single SNP at position 44,232,491 that seems to have arisen

**Figure 3.** A 75-kb region spanning the *MTAP* gene and continuing through the last exon of *CDKN2A* is highly associated with histiocytic sarcoma. A 195-kb region between CFA11 44,133,500 and 44,328,500 is shown. The X-axes for all plots list the SNPs in order from centromere to telomere. A, positions of 3 genes are shown at the top of the graph. Exons are indicated as colored rectangles, introns are the connecting lines. Transcripts are indicated as arrows below gene names. Fisher exact association of allele frequency with histiocytic sarcoma is plotted along the Y-axis for each SNP. The gray line shows association in the discovery set of 24 cases and 20 controls with *P* values on the right Y-axis. The black line shows association in the full data set after imputation, with *P* values on the left Y-axis. The red lines show association of the haplotypes across the region with the *P* values in the left Y-axis. B, pairwise LD plot was calculated using Haploview. Solid red blocks indicate  $D' = 1$  with a LOD score of 2. The haplotype block containing 28 of 30 equally associated SNPs is outlined in black. Another 2 SNPs form a short 3.4-kb haplotype in the *CDKN2B* region in perfect LD with the larger 75-kb haplotype. Differences in *P* values between the haplotypes are the result of a single cross-over in a control dog.



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on the CA haplotype. Haplotypes on either side of this SNP are nearly identical in frequency with only one dog of 228 being a possible recombinant.

More than 65 kb of the 75 kb haplotypes have been sequenced in the discovery set. The remaining 10 kb is divided among 25 loci ranging from <10 to nearly 2,000 bps and is largely composed of repetitive elements. Thus far, no single marker or combination of markers within the 75.9-kb haplotype conveyed significantly more risk than any other (Fig. 3A). LD in dog breeds can be expansive, extending more than 1 Mb at some loci (33, 34). Because of the near-perfect LD found within this disease-associated region and the lack of coding mutations, finding the causative mutation remains outside the scope of this article. However, functional approaches can be applied to determine the most probable effect of the elusive mutation(s).

### Correlation of haplotype with candidate gene expression

The disease-associated haplotype lies across *MTAP* and continues through the last exon of *CDKN2A*. We carried out quantitative real-time PCR across the region to determine whether there were changes in transcript levels that correlated with the CA haplotype. Expression was measured on total RNA from histiocytes cultured from whole blood samples of healthy BMDs carrying 0, 1, or 2 copies of the CA haplotype. No significant changes in *MTAP* expression were observed. However, individuals with 2 copies of the CA haplotype produced significantly higher amounts of both *CDKN2A* and *CDKN2B* transcripts, averaging 16 ( $P = .0173$ , Wilcoxon rank sum) and 4 times ( $P = 0.00866$ ) higher, respectively, compared with those lacking a CA haplotype (Table 2). Heterozygotes had approximately half the homozygote level of transcript, but the differences were not significant given the small sample sizes tested. These data suggested that there are variants within the CA haplotype that affect the expression of the *CDKN2A* and *CDKN2B* in histiocytic sarcoma-susceptible dogs.

### Discussion

Dissecting the genetic underpinnings of dendritic cell neoplasms presents unique challenges to canine and human researchers alike, because of confusion about the origin of these immune cell tumors. Although human disorders, such as Langerhans cell histiocytosis, have been well characterized clinically, etiologies remain elusive. We hypothesized that identification of susceptibility loci in the BMD would likely reveal genes of interest for both canine and human disorders, thus leading to a better understanding of the genetic underpinnings of this complex family of neoplasms.

Our data set consisted of dogs from 1 breed but 2 major geographic areas. Average  $F_{st}$  values show that these BMD populations differ at a level similar to human populations from unique European countries (35). This is an order of magnitude lower than differences found between

**Table 2.** Expression levels of 3 genes surrounding the case-associated haplotype in healthy dogs showing all 3 genotypes

|        | Genotype <sup>a</sup> | Amount <sup>b</sup> | Increase <sup>c</sup> | P <sup>d</sup> |
|--------|-----------------------|---------------------|-----------------------|----------------|
| CDKN2A | 2                     | 0.002577            | 16.8988               | 0.0173         |
|        | 1                     | 0.001334            | 8.7482                | >0.05          |
|        | 0                     | 0.000153            | 1                     |                |
| CDKN2B | 2                     | 0.000697            | 3.976                 | 0.0087         |
|        | 1                     | 0.000518            | 2.9567                | >0.05          |
|        | 0                     | 0.000175            | 1                     |                |
| MTAP   | 2                     | 0.009758            | 1.2412                | >0.05          |
|        | 1                     | 0.009028            | 1.1484                | >0.05          |
|        | 0                     | 0.007862            | 1                     |                |

<sup>a</sup>The genotype is the number of copies of the case associated (CA) haplotype with 2 = homozygous for the CA haplotype, 1 = heterozygous, and 0 = no copies of the CA haplotype.

<sup>b</sup>The amount of transcript is relative to the amount of B2M transcript and normalized by comparison with expression in testes.

<sup>c</sup>Increase in expression of each gene in individuals carrying the CA haplotype compared with those without.

<sup>d</sup>P values were calculated by Wilcoxon rank sum on the distribution of relative expression levels within each genotype group.

breeds, yet significant to find global alterations in haplotype and allele frequencies (8, 36).

There is slightly higher population-wide heterozygosity in the North American BMD compared with European BMD; however, individuals are characterized by reduced heterozygosity. The effect of such population differences is apparent in a complex disorder such as histiocytic sarcoma. In the North American population, only one locus segregates with the disease. However, the European population shows at least 2. The second European locus may not be important in the North American population, or the underlying mutation may be present at such a high frequency that it is approaching fixation. A brief examination of markers across the region shows that all North American dogs share allele frequencies similar to the cases from Europe, supporting the latter alternative.

This GWAS study unambiguously localized the major histiocytic sarcoma locus to a 9.7-Mb region on CFA11. The advantages of genetic mapping in dogs, in which loci are quickly identified with small numbers of samples, can be offset by the potentially difficult transition from disease-associated haplotype to causative mutation (37). We compared mapping results from 2 populations of the same breed to reduce LD. The European population showed overlapping association with the North American population in a relatively small region of <200 kb, compared with the approximately 10 Mb identified in the original GWAS. Extensive

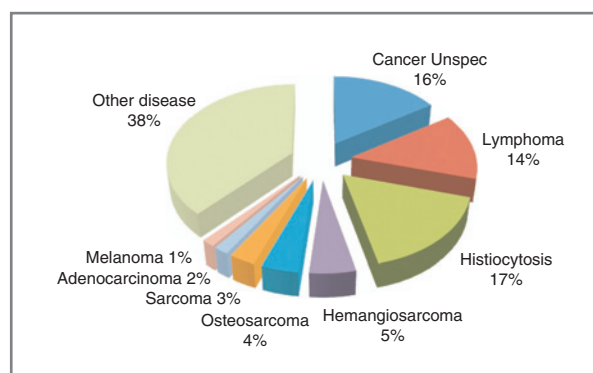
sequencing revealed a single 75-kb disease-associated haplotype.

More than 85% of the CA haplotype has been sequenced. The majority of the unsequenced segments are within the large third intron of *MTAP*. Expression levels of the *CDKN2* genes show a much greater range in cells from dogs carrying the CA haplotype ( $SD = 0.0052$ ) compared with those with control haplotypes ( $SD = 8.5 \times 10^{-5}$ ). It is formally possible that this is a consequence of small sample size; more likely it indicates that the causal variant is present on only a subset of CA haplotypes and has yet to be discovered. The second explanation also accounts for the relatively high incidence of risk-associated haplotypes in unaffected dogs.

Expression analysis suggests that histiocytic sarcoma is caused by a regulatory mutation(s). Unfortunately, little is known about regulatory elements in the dog. However, we can compare the canine locus to the corresponding human region and predict regulatory potential. For example, based on ENCODE chromatin state predictions from human ChIP-seq data (build NCBI36/hg18; refs. 38–40), there is a strong enhancer immediately downstream of *MTAP*. The homologous region in the dog contains at least one of the 28 highly associated histiocytic sarcoma SNPs and a region containing a series of SINE elements and repeats that may be amenable to deletion, insertion, or rearrangement in addition to base pair changes, providing an attractive site for further investigation. Another of the highly associated SNPs, at position 44,215,162, lies within a second predicted enhancer region.

Our data suggest that variants on a risk-associated haplotype surrounding *MTAP* and continuing through exon 3 of *CDKN2A* affect the expression of both *CDKN2A* and *CDKN2B*. All 3 of the proteins transcribed from the *CDKN2* genes; p16<sup>INK4A</sup>, p14<sup>ARF</sup>, and p15<sup>INK4B</sup> have unique promoters, but share regulatory elements (reviewed in ref. 41). Loss of the *CDKN2A-CDKN2B* region through mutation, deletion, or silencing is among the most frequent alterations found in human cancers, including histiocytic sarcoma (42, 43). In addition, CGH analysis shows that a region of at least 1 Mb centered on the *CDKN2* locus is lost in approximately 60% of histiocytic sarcoma tumors in BMD (44). Although unexpected, increased expression of p16<sup>INK4a</sup> and p14<sup>ARF</sup> has been noted in multiple cancers, including prostate, ovarian, cervical, and mammary (45–47), and is typically associated with poor prognosis (48–50). Studies have suggested that, in these neoplastic cells, p16 inhibits apoptosis, particularly in response to DNA damage. Further investigation of *CDKN2* gene regulation in BMD with and without histiocytic sarcoma may better illuminate the roles of these common cancer-associated genes.

*MTAP* is important for the salvage of methionine and adenine, encoding an enzyme that plays a role in polyamine metabolism (51). Recently it has been suggested that *MTAP* may also be a tumor suppressor (52). In our study, variants within or near the *MTAP* gene are asso-



**Figure 4.** Diagnoses for 3,785 BMD who have died since 1995 collected by the Berner Garde Foundation. Frequency was determined by dividing individual cancers by the total number of BMD deaths. "Histiocytosis" includes histiocytic sarcomas, malignant histiocytosis, and reactive histiocytoses.

ciated with altered expression of *CDKN2A/CDKN2B*, but not changes in *MTAP* expression. Thus, our data offer a new perspective on role of *MTAP* in cancer. Specifically, mutations within *MTAP* likely lead to dysregulation of *CDKN2A/B*.

The established importance of the *MTAP/CDKN2A/CDKN2B* locus in multiple cancer types, in combination with our finding that naturally occurring sequence variants in BMDs are associated with expression changes in these genes, suggests that the CA haplotype could be relevant for susceptibility to multiple cancers. Some 16.9% of U.S. BMDs reportedly die of histiocytic sarcoma-related causes (Fig. 4). Because 38% of a random sample of U.S. BMDs ( $n = 53$ ) was homozygous for the CA haplotype (Supplementary Table S9), we hypothesize that multiple types of BMD cancer may be related to variants within the *MTAP-CDKN2A* region. This concept mimics what has been observed at human chromosome region 9p21, which is associated with susceptibility to several types of human cancer as well as other complex disorders (53).

Here we present the first GWAS of histiocytic sarcoma in any species. Using a population-guided mapping approach followed by sequencing, we have identified a 75.9-kb haplotype found in 96% of all histiocytic sarcoma affected dogs. This haplotype contains features that affect expression of the *CDKN2A* and *CDKN2B* genes, which may be a primary contributor to histiocytic sarcoma susceptibility in the BMD. The CA haplotype overlies the *MTAP* gene and likely contains one or more variants that alter the expression of *INK4A/ARF/INK4B* but do not affect *MTAP* expression. It is plausible that numerous cancers developed by BMD are associated with sequence variants in this region. These findings lead us to hypothesize that BMDs are an excellent system for the study of cancer susceptibility due to *INK4A/ARF/INK4B* dysregulation, allowing for systematic studies about the role of naturally occurring sequence variants in this increasingly important locus.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

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## Acknowledgments

The authors thank the Berner Garde Foundation, Bernese Mountain Dog Club of America, French Association for Swiss dogs, and the Swiss, Italian, and Belgium Bernese Mountain Dog Associations for providing data and distributing information; the many breeders, owners, and clinicians who collected data and samples; and Dr. James Rocco for supplying biologic reagents and Dr. Erik Teske for cytology review.

## Grant Support

This work was supported by the Intramural Program of the National Human Genome Research Institute at NIH. Additional support was received from the AKC-Canine Health Foundation grants 2667 and 760 (M. Breen), 336 and 935 (E.A. Ostrander and C. André); the CNRS and French Association for Swiss dogs (C. André); NIH NCI R01 CA69069, NIH U01 AI07033, and the Harvard Breast Cancer SPORE P50 CA89393 (E. V. Schmidt); the Alberto Vittoni Award (B. Hedan, E. Cadieu, and G.R. Rutteman); the Committee of Preventive Health Care of the Netherlands Royal Society of Veterinary Medicine and the breed societies for Bernese mountain dogs in the Netherlands, Belgium, Germany and Austria (G.R. Rutteman).

Received February 16, 2012; revised April 9, 2012; accepted April 27, 2012; published OnlineFirst May 31, 2012.



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