Comparative Genomics Reveals Shared Mutational Landscape in Canine Hemangiosarcoma and Human Angiosarcoma

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Abstract

Angiosarcoma is a highly aggressive cancer of blood vessel-forming cells with few effective treatment options and high patient mortality. It is both rare and heterogeneous, making large, well-powered genomic studies nearly impossible. Dogs commonly suffer from a similar cancer, called hemangiosarcoma, with breeds like the golden retriever carrying heritable genetic factors that put them at high risk. If the clinical similarity of canine hemangiosarcoma and human angiosarcoma reflects shared genomic etiology, dogs could be a critically needed model for advancing angiosarcoma research. We assessed the genomic landscape of canine hemangiosarcoma via whole-exome sequencing (47 golden retriever hemangiosarcomas) and RNA sequencing (74 hemangiosarcomas from multiple breeds). Somatic coding mutations occurred most frequently in the tumor suppressor TP53 (59.6% of cases) as well as in two genes in the PI3K pathway: the oncogene PIK3CA (29.8%) and its regulatory subunit PIK3R1 (8.5%). The predominant mutational signature was the age-associated deamination of cytosine to thymine. As reported in human angiosarcoma, CDKN2A/B was recurrently deleted and VEGFA, KDR, and KIT recurrently gained. We compared the canine data to human data recently released by The Angiosarcoma Project, and found many of the same genes and pathways significantly enriched for somatic mutations, particularly in breast and visceral angiosarcomas. Canine hemangiosarcoma closely models the genomic landscape of human angiosarcoma of the breast and viscera, and is a powerful tool for investigating the pathogenesis of this devastating disease.

Implications: We characterize the genomic landscape of canine hemangiosarcoma and demonstrate its similarity to human angiosarcoma.

Introduction

Angiosarcoma is an aggressive cancer of blood vessel-forming cells, associated with poor survival times (1–3). There is an unmet need for new diagnostics and therapies for these patients. However, the rarity of this cancer (approximately 0.01% of all cancers; refs. 4, 5) has limited large-scale genomic studies so far. Canine hemangiosarcoma is a relevant clinical model for understanding the pathophysiology of human angiosarcoma. The human and canine diseases share many clinical similarities, and hemangiosarcoma is common in dogs, occurring in some breeds (notably the golden retriever) with a frequency up to 20% (6). This means that using canine hemangiosarcoma as a model for human disease would yield sample cohorts of a magnitude inaccessible using human data alone. However, for dogs to be an effective...
model of this disease in the era of precision medicine, detailed genomic characterization of canine hemangiosarcoma must be undertaken, and the results directly compared with existing and emerging genomic data from human angiosarcoma.

Angiosarcoma can form anywhere in the vasculature. In human patients, it most commonly occurs in the skin of the head, neck, and scalp, the breast, the extremities, and less frequently in the liver, right auricle of the heart, bone, and spleen (7). Prognosis is poor, with metastatic disease occurring in approximately 50% of cases (8), and a median overall survival time of approximately 50 months for local disease and 10 months in metastatic cases (9). Treatment involves surgical resection with wide margins, plus or minus radiotherapy, as well as adjuvant chemotherapy in the metastatic disease setting (3). Many angiosarcomas are initially sensitive to doxorubicin, paclitaxel, or targeted agents, but resistance to these therapies is virtually inevitable (3).

While most cases of angiosarcoma in humans occur without known cause, there are several known risk factors. These tumors can arise secondary to radiotherapy for other cancers or chronic lymphedema (3). Other known risk factors include UV irradiation, given the typical locations of cutaneous angiosarcomas on the head and neck (10–12), as well as occupational exposure to vinyl chloride (13), arsenic exposure (14), and use of anabolic steroids (15). Genetically, angiosarcoma is associated with familial syndromes including Li-Fraumeni syndrome (TP53 mutations; ref. 16) and Klippel–Trenaunay syndrome (PIK3CA mutations; ref. 17). However, these syndromes do not solely cause angiosarcoma (7), and angiosarcomas are not present in the majority of cases.

Canine hemangiosarcoma is the histopathologic equivalent of human angiosarcoma (18), and follows a similar, aggressive clinical course. In dogs, the most common tumor locations are the spleen, right auricle of the heart, liver, and skin or subcutaneous tissue (19, 20). The different anatomic distribution seen in the human and canine diseases is likely due, at least in part, to the lack of secondary cases in dogs. Treatment protocols for dogs with hemangiosarcoma similarly involve wide surgical resection, followed by adjuvant chemotherapy. Survival times are short—a median of 4–6 months after surgical resection with adjuvant chemotherapy, with a 1-year survival rate of approximately 10% (21–23). Biological risk factors have not yet been identified in dogs. Genetically, dog breeds display differential predisposition to specific cancers, indicating that there are heritable risk factors that have become common as a result of inbreeding based on selection or drift. In a previous genome-wide association study, we identified several loci significantly associated with the risk of hemangiosarcoma in the golden retriever (24).

Recent targeted next-generation sequencing of human angiosarcomas has begun to reveal the somatic mutational spectrum of this disease. It has so far proven to be fairly heterogeneous—no pathognomonic mutations or copy number aberrations occur in all cases, and tumors from different primary locations or with different underlying etiologies have genomic differences. TP53 and genes in the MAPK pathway are frequently mutated (25), and mutations in P16CG1 and PTEN are common, particularly in secondary angiosarcomas (25, 26). Although the PI3K pathway is activated in some human angiosarcomas (27, 28), PIK3CA mutations have so far not been commonly reported in human angiosarcoma studies (26, 29). Genes in the VEGF pathway are frequently gained or amplified, including VEGFA and KDR (25, 28). The tumor suppressor CDKN2A is frequently deleted, while the MYC oncogene is frequently amplified, most commonly in radiation-associated tumors (25, 28).

In dogs, a recent whole-exome sequencing (WES) study of a small cohort of 20 hemangiosarcomas showed that the top recurrently mutated genes were PIK3CA and TP53 (30). Earlier candidate gene studies of canine hemangiosarcomas reported mutations in TP53 (31), PTEN (32), and PDGFR and PDGFRB (33). An analysis of somatic copy number aberrations in visceral hemangiosarcomas from five breeds found that VEGFA was recurrently gained, while CDKN2A was frequently deleted (34). MYC copy number gain was infrequent, likely reflective of the fact that secondary hemangiosarcomas are not seen in canine patients (34). While these earlier studies provide clues as to the genetic features of canine hemangiosarcoma, there is very little of the genome-wide data needed for comprehensive comparison with human angiosarcoma.

To assess the potential utility of hemangiosarcoma as a model for angiosarcoma at the molecular level, we performed the largest exome-sequencing study of hemangiosarcoma to date, and complemented our exome data with oligonucleotide array comparative genomic hybridization (oaCGH) copy number data and RNA-sequencing (RNA-seq) data in partially overlapping cohorts of hemangiosarcoma cases. We then performed comparative analyses of our results with those released by The Angiosarcoma Project direct-to-patient initiative. In this way, we have created a detailed genomic profile of this cancer in the golden retriever breed, and begun vetting this canine cancer as a comparative model for human angiosarcoma, and potentially other tumors.

Materials and Methods

Additional Materials and Methods can be found in the Supplementary Materials and Methods, and a workflow in Supplementary Fig. S1.

Canine hemangiosarcoma sample collection for exome sequencing

Samples were obtained as part of necessary diagnostic procedures with owner consent. DNA from tumor tissue and whole blood was collected from 47 golden retrievers with visceral hemangiosarcoma. Samples were collected from dogs referred for treatment at the University of Minnesota (UMN) Veterinary Medical Center and samples submitted to the Modiano laboratory for diagnostic assessment and/or use in research (n = 34), from cases seen at the North Carolina State University (NCSU) Veterinary Hospital (n = 8), or from diagnostic samples sent to Antech Diagnostics (n = 5). Cases where date of diagnosis was known (n = 36), were diagnosed between 2000 and 2014. Tumor samples were either frozen (n = 17), or formalin-fixed, paraffin-embedded (n = 30; Supplementary Table S1). Procedures involving animal use were approved by the Institutional Animal Care and Use Committees at the Broad Institute (Cambridge, MA), UMN (Minneapolis, MN), or NCSU (Raleigh, NC).

Sample preparation

DNA was extracted and sequencing libraries prepared using the Kapa Hyper Prep Kit (Supplementary Table S2). For 66 samples, additional cycles of PCR were required to obtain sufficient DNA for exome capture, while libraries from 28 samples were
reconstructed from source DNA using the standard protocol (see Supplementary Information).

**Exome capture**

The Roche Nimblegen SeqCap-EZ capture canine exome (120705_CE3_Uppsala_Broad_EZ_HX1) was used for hybrid exome capture, following the manufacturer’s protocol.

**Sequencing and read alignments**

The barcoded exome-captured libraries were multiplexed in pools of eight, and sequenced on the Illumina HiSeq 2500 to a target depth of 60 × in the tumor and 30 × in the normal, reaching a mean depth of 78 × in the tumor and 63 × in the normal. Reads were aligned to the CanFam3.1 reference genome using BWA. PCR duplicate reads were flagged using the Picard tool MarkDuplicates. Following the Genome Analysis Toolkit (GATK) Best Practices, we then performed Base Quality Score Recalibration using a set of approximately 19 million known canine germline variant locations.

**Somatic variant calling**

Somatic mutations were called using MuTect2, using the default settings with the addition of the dontUseSoftClippedBases option, which was added to avoid calling a large number of artifactual indels in our FFPE-processed samples (see Supplementary Methods). To further refine our set of somatic variant calls, and to avoid artifacts, we also called variants using the GATK4 version of Mutect2, and kept only the consensus of calls which passed in both the GATK3 and GATK4 versions. Both sets of calls were filtered using a "panel of normals" created using all 47 normal (germline) samples, as well as germline samples from previous studies of canine lymphoma, osteosarcoma, and melanoma. Calls were further filtered to exclude oxidation artifacts, and were removed if they overlapped locations with known germline variants. Variants were further filtered if the position had low coverage (defined as a read depth less than 20 in the normal, less than 40 in the tumor, or less than four alternate allele reads in the tumor) or had excessive read depth (greater or equal to mean read depth + 5 × SD), to filter out potential alignment errors. Finally, variants with a median read position < 10 were filtered to remove potential artifacts.

**Variant annotation**

Variants were annotated using the variant effect prediction program Snpeff v4.2. Where multiple effects were predicted for a single variant, the most damaging predicted effect was selected. Coding variants were analyzed using the smg function in GenomeMuSiC 0.4 to determine which genes were significantly mutated above the background rate. The merge-concurrent-muts option was applied to count multiple mutations in the same gene within a sample as a single mutation, and a FDR threshold of 0.1 using the convolution test (CT) was applied.

**Canine mutational signature discovery**

Mutational signatures, considering point mutations and their genomic context, were extracted using a Bayesian nonnegative matrix factorization (NMF) algorithm. The discovered signatures were compared with known signatures in COSMIC (35), as well as those reported in the literature for human angiosarcoma (36). The overall landscape of mutations was plotted for different groupings of samples using the SomaticSignatures package.

**RNA sequencing of canine hemangiosarcomas**

Seventy-four snap-frozen samples were obtained from 73 dogs with hemangiosarcoma (41 golden retrievers and 32 dogs from 13 other breeds or from mixed breeding; Kim and colleagues, manuscript in preparation). RNA-seq data from 51 of the hemangiosarcoma tissues had been published previously (37, 38). RNA-seq libraries were generated as described previously (37).

**Somatic copy number detection in canine hemangiosarcomas**

Analysis of somatic copy number aberrations (SCNA) in our canine WES data was limited by the inclusion of both frozen and formalin-fixed samples. The FFPE samples appeared to have an increased number of amplifications and deletions when compared with the frozen samples, possibly as a result of DNA fragmentation during the fixation process (39). While we were able to partially account for this using correction for guanine and cytosine content (40), we were not able to completely remove the bias between the two groups (Supplementary Fig. S6). Hence, we instead used oaCGH data to interrogate copy number changes in a cohort of 69 golden retrievers with hemangiosarcoma tumors, of which 28 were also included in the exome sequencing cohort. SCNAs were called from oligonucleotide array comparative genomic hybridization (oaCGH), as described previously (34), using an approximately 180,000-feature microarray (Agilent Technologies) with approximately 26 kb resolution throughout the dog genome.

**Accessing human data from The Angiosarcoma Project for comparative analysis**

We compared the somatic mutations and SCNAs present in our cohort of canine hemangiosarcomas to the results reported by The Angiosarcoma Project, a direct-to-patient sequencing project run by the Count Me In initiative (JoinCountMeIn.org). Somatic mutations and SCNAs derived from WES data from 48 samples from 36 patients were downloaded from cBioPortal, and somatic mutations classified as nonsense, missense, splice site, or splice region were kept for analysis. For our analysis, we selected only the annotated primary tumor site from each patient, including 30 tumors—17 breast, five visceral (one each of right atrium, spleen, lung, abdomen, and bladder wall), and eight head, face, neck, and scalp (HFNS) tumors.

**Pathway analysis in canine and human data**

Pathway analysis was performed on the canine and human mutational data using the DAVID Functional Annotation Tool. For the overall enrichment analyses, all genes with nonsynonymous splice site, or splice region somatic mutations were used. Functional annotation charts were created for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the default options, using the Benjamini–Hochberg method to control the FDR. Analysis was performed independently for the canine data and the human data, mapping genes from both species to *Homo sapiens* reference to avoid confounding due to differences in gene annotation between the species. Canine Ensembl gene IDs were mapped to their 1:1 orthologs in the human GRCh37 reference genome annotation using Ensembl’s BioMart (41).

**Data availability**

The WES data, as well as the RNA-seq data that has not been previously published, has been submitted to the NCBI Sequence Read Archive. WES data: BioProject PRJNA552034, BioSamples SAMN12173468 - SAMN12173561. RNA-seq data:
Results

Simple somatic mutations

**TP53 and PIK3CA are commonly mutated in canine hemangiosarcomas.** The seven significantly mutated genes (SMGs) in the canine hemangiosarcomas contained well-known cancer genes, including TP53, as well as two genes in the PI3K pathway (Table 1; Fig. 1). Tumor suppressor TP53 was most frequently mutated (28/47 cases, 59.6%), with all 28 cases carrying at least one mutation affecting the DNA-binding domain (Fig. 2). Oncogene PIK3CA (14/47, 29.8%) and its regulatory subunit PIK3R1 (4/47, 8.5%) were both mutated. Ten of the 14 cases with PIK3CA mutations had a mutation at amino acid position 1047, a hotspot frequently mutated in many types of human cancers (ref. 42; Fig. 2). The remaining four SMGs were ORC1 (4/47, 8.5%), RASA1 (4/47, 8.5%), ARPC1A (3/47, 6.4%), and ENSCAG00000017407 (2/47, 4.3%), a “one-to-many” ortholog of human ATP5PD with 27 paralogs in the canine genome. Overall, 59 genes that were mutated at least once in the canine dataset are annotated as having likely causal somatic mutations in the COSMIC Cancer Gene Census (43), including TP53, PIK3CA, and PIK3R1 (Supplementary Table S3).

**Variant allele fraction and effect predictions.** For further insight into whether the four SMGs not annotated in COSMIC are likely to be driver mutations, we investigated the variant allele fraction (VAF) of each and whether missense mutations were predicted to be deleterious or tolerated using the SIFT (44) score via Ensembl’s Variant Effect Predictor (45). The median VAF for the four

Table 1. Significantly mutated genes in golden retriever hemangiosarcoma tumors

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Indels</th>
<th>SNVs</th>
<th>Total mutations</th>
<th>Covd Bps</th>
<th>Muts pMbp</th>
<th>P</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>Tumor protein p53</td>
<td>3</td>
<td>25</td>
<td>28</td>
<td>72,582</td>
<td>386</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha</td>
<td>0</td>
<td>14</td>
<td>14</td>
<td>156,415</td>
<td>90</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PIK3R1</td>
<td>Phosphoinositide-3-kinase regulatory subunit 1</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>124,857</td>
<td>32</td>
<td>7.3 \times 10^{-6}</td>
<td>5.9 \times 10^{-4}</td>
</tr>
<tr>
<td>ORC1</td>
<td>Origin recognition complex subunit 1</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>133,219</td>
<td>30</td>
<td>1.3 \times 10^{-7}</td>
<td>8.2 \times 10^{-4}</td>
</tr>
<tr>
<td>RASA1</td>
<td>RAS p21 protein activator 1</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>156,359</td>
<td>29</td>
<td>3.2 \times 10^{-6}</td>
<td>1.6 \times 10^{-2}</td>
</tr>
<tr>
<td>ARPC1A</td>
<td>Actin-related protein 2/3 complex subunit 1A</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>72,351</td>
<td>41</td>
<td>1.3 \times 10^{-5}</td>
<td>5.1 \times 10^{-2}</td>
</tr>
<tr>
<td>ENSCAG00000017407</td>
<td>1-to-many ortholog of human ATP synthase peripheral stalk subunit d (ATPSPD)</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>22,074</td>
<td>91</td>
<td>1.7 \times 10^{-5}</td>
<td>5.9 \times 10^{-2}</td>
</tr>
</tbody>
</table>

NOTE: Significantly mutated genes with an FDR < 0.1, calculated using Genome MuSiC. Abbreviations: Covd Bps, number of basepairs with adequate coverage in gene; SNV, single nucleotide variants; Muts pMbp, mutations per megabase.
mutations was 0.12 (range 0.08–0.23). Three mutations were nonsense mutations, with the fourth a missense mutation predicted to be deleterious (SIFT score 0), supporting a causal role for these mutations. Similarly, the median VAF of the three ARPC1A mutations was 0.16 (range 0.12–0.17), with one nonsense mutation, and two missense mutations at the same position, predicted to be deleterious (SIFT score 0). The median VAF for the four ORC1 mutations (all at the same position) was lower, at 0.08, and the change was predicted to be tolerated (SIFT score 0.06). The two mutations in ENSCAFG00000017407 also had a low median VAF (0.05), and were predicted to be tolerated (SIFT score 1).

Comparison with top mutated genes in human angiosarcoma. The most commonly mutated genes in the human data were TP53 (8/30 tumors, 29%), followed by KDR, LRP2, RYR2, and ABCA13 with mutations in 7 of 30 tumors each, and PIK3CA, FG3, ASXL3, MYH14, and UNCI13C, mutated in 6 of 30 tumors each. However, the distribution of these mutations varied by tumor location. In the human data, breast tumors were the only location to carry PIK3CA mutations (n = 6 patients), and were the most common location for KDR mutations. TP53 mutations occurred in all locations, but were more common in HFNS and visceral tumors. KDR was much less frequently mutated in the canine cohort, with only one case having a nonsynonymous mutation, predicted to be tolerated. Two human tumors also had mutations in the canine SMG RASA1. One was a nonsense mutation, the other predicted to be deleterious.

Differential patterns of mutations found in some tumor locations. The pattern of mutations in the PI3K gene family varied by tumor location in the canine data, as well. In the canine cases, a slightly higher proportion of heart tumors (11/16, 68.8%) compared with splenic tumors (9/22, 40.9%) had PI3K alterations, although this
Table 2. KEGG pathways enriched in hemangiosarcoma, visceral and breast angiosarcoma, and HFNS angiosarcoma

<table>
<thead>
<tr>
<th>Term</th>
<th>Angiosarcoma - visceral and breast</th>
<th>Hemangiosarcoma</th>
<th>Angiosarcoma - HFNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa04020:Calcium signaling pathway</td>
<td>5.4E-03</td>
<td>3.6E-04</td>
<td>8.6E-03</td>
</tr>
<tr>
<td>hsa04730:Long-term depression</td>
<td>7.1E-03</td>
<td>3.8E-04</td>
<td>1.9E-02</td>
</tr>
<tr>
<td>hsa04015:Rap1 signaling pathway</td>
<td>9.0E-03</td>
<td>5.9E-04</td>
<td>9.6E-04</td>
</tr>
<tr>
<td>hsa04530:Focal adhesion</td>
<td>2.2E-02</td>
<td>6.7E-04</td>
<td>11E-03</td>
</tr>
<tr>
<td>hsa04720:Long-term potentiation</td>
<td>2.2E-02</td>
<td>1.4E-03</td>
<td>1.0E-03</td>
</tr>
<tr>
<td>hsa04102:MAPK signaling pathway</td>
<td>2.3E-02</td>
<td>1.4E-03</td>
<td>3.8E-03</td>
</tr>
<tr>
<td>hsa05230:Central carbon metabolism in cancer</td>
<td>2.3E-02</td>
<td>1.5E-03</td>
<td>8.1E-03</td>
</tr>
<tr>
<td>hsa05200:Pathways in cancer</td>
<td>4.8E-03</td>
<td>1.5E-03</td>
<td>8.1E-03</td>
</tr>
</tbody>
</table>

NOTE: Blue, pathways enriched in both visceral and breast angiosarcoma; yellow, pathways enriched in both HFNS angiosarcoma and hemangiosarcoma; green, pathways enriched in all three groups.
difference was not significant ($P^2 = 0.09$). Liver tumors had slightly fewer overall alterations in the PIKK gene family (3/8, 37.5%), and were less likely to have PIK3CA mutations (1/8, 12.5%), although this was not statistically significant.

**Comparison of mutational burden by tumor location in canine and human data.** We found fewer nonsynonymous and splice site/region mutations in the canine tumors than reported in the human tumors overall, while the number of mutations per sample varied significantly by tumor location in human cases. In the canine cohort, there were a median of 23 nonsynonymous and splice site/region mutations in the canine tumors than reported in the human breast sample varied significantly by tumor location. In the dogs, there was a small difference in the total number of nonsynonymous and splice site/region mutations removed) and splenic (n = 22, median = 17.5) tumors ($P_{\text{TukeyHSD}} = 0.04$). In the human data, there was a significant difference in the median number of mutations by tumor location, with head, face, neck, and scalp (HFNS, n = 8) tumors having a higher mutational burden (median = 596.5) than breast or visceral tumors ($P_{\text{ANOVA}} = 3.6 \times 10^{-3}$; ref. 46).

**Comparative pathway analysis highlights similarity between canine and human tumors.** We compared the gene functional annotations enriched in hemangiosarcoma to those enriched in breast and visceral angiosarcoma and those enriched in HFNS angiosarcoma (Table 2). KEGG pathways enriched in canine hemangiosarcoma overlapped with KEGG pathways enriched in both subtypes of angiosarcoma. Pathways enriched in hemangiosarcoma included a larger fraction of those enriched in visceral and breast angiosarcoma (75%, vs. 48% of pathways enriched in HFNS angiosarcoma); however, the total number of pathways shared was greater between hemangiosarcoma and HFNS angiosarcoma (10 pathways). Pathways shared between hemangiosarcoma and visceral and breast angiosarcoma included pathways in cancer ($P_{\text{cancer}} = 3.6 \times 10^{-4}$), Rap1 signaling pathway ($P_{\text{cancer}} = 5.8 \times 10^{-3}$), central carbon metabolism in cancer ($P_{\text{cancer}} = 3.0 \times 10^{-3}$), and long-term depression ($P_{\text{cancer}} = 2.3 \times 10^{-3}$). Pathways shared between hemangiosarcoma, visceral and breast angiosarcoma, and HFNS angiosarcoma included focal adhesion pathway ($P_{\text{cancer}} = 3.6 \times 10^{-4}$), PI3K-Akt signaling ($P_{\text{cancer}} = 3.0 \times 10^{-3}$), and Ras signaling ($P_{\text{cancer}} = 2.3 \times 10^{-3}$). In addition, functional annotation enrichment analysis of the genes mutated in hemangiosarcoma, visceral and breast angiosarcoma, and HFNS angiosarcoma found that they were enriched in many of the same protein domains. Shared domains include eNOS, NO synthase, and p38MAPK. These pathways were enriched in both canine and human angiosarcoma, and shared between hemangiosarcoma and HFNS angiosarcoma.
Pathways and gene families previously reported in the angiosarcoma literature. Several pathways and gene families have been previously reported to be affected in the human angiosarcoma literature. Mutations in both PLCG1 and PTPRB have been reported to be recurrent (26), particularly in secondary angiosarcoma. In the human data, 12 patients had mutations in eight phospholipase C genes, including PLCG1 (n = 5, Table 3). Seven canine cases (15%) had mutations in PLC genes, including one in PLCG1. Twenty protein tyrosine phosphatases were mutated in 12 samples in human, including 2 patients with PTPRB mutations. While none of the canine tumors had somatic mutations in PTPRB, 8 cases (17%) harbored a mutation in one of nine other protein tyrosine phosphatase genes (Table 3).

The MAPK pathway has been reported to be frequently affected by mutations in angiosarcoma (25), and we found the visceral and breast angiosarcomas to be enriched in mutations in MAPK pathway genes (P = 0.02, Table 2). We noted mutations in 25 genes in the MAPK pathway in the canine cohort, including the SMGs TP53 and RASA1. In the human data, 67 genes in the MAPK pathway were mutated. Thirteen of these genes were mutated in both the canine and human data (Table 3). Although we found mutations in genes involved in histone methyltransferase/demethylase activity in both species, consistent with earlier human studies (47), we saw no significant enrichment on a pathway level in either the canine or human cohorts (Table 3). Tyrosine protein kinases were enriched in both hemangiosarcoma and angiosarcoma. In the human data, 55 genes in the GO Protein Tyrosine Kinase Activity pathway were mutated, while 22 genes in this pathway were mutated in the canine data, with 12 genes mutated in both human and canine samples (Table 3). Low-density lipoprotein receptors were enriched for mutations in the canine (P = 0.03) and human HFNS tumors (P = 0.05), and LRPS2 was one of the most frequently mutated genes in the human dataset (mutated in 73/30 tumors). While mutations in the LDL receptor–related protein family have not been widely reported in human angiosarcoma, they have recently been shown to play a role in a variety of cancers (48). We found that four LDL receptor–related protein genes were mutated in both hemangiosarcoma and angiosarcoma, with an additional gene mutated in angiosarcoma only (Table 3).

Mutational signature of aging is present across all canine tumors. Analysis of mutational signatures, which capture the mutational landscape of tumors and are shaped by both genetic factors and environmental exposure, revealed similarities between canine hemangiosarcoma and human angiosarcoma, as reported by Thibodeau and colleagues (36). We analyzed the signatures of trinucleotide mutational frequencies present in golden retriever hemangiosarcomas, and found a strong signature of mutations arising through spontaneous deamination of methylated cytosines in CpG islands, corresponding to COSMIC signature SBS1 and generally associated with aging (ref. 35; Fig. 3; Supplementary Figs. S3–S5). In addition, we see a faint signature not described in COSMIC (Fig. 3). The overall mutational landscape was consistent across all canine hemangiosarcoma tumors, regardless of tumor location or tissue preservation method (Supplementary Figs. S2–S4).

Canine RNA-seq analysis validates exome mutations and extends findings to additional breeds. We validated our exome-sequencing somatic mutation calls using RNA-seq data from a partially overlapping set of dogs (n = 74 tumor samples from 14 breeds. We validated our exome-seq data from a partially overlapping set of dogs (n = 74 tumor samples from 14 breeds, as well as mixed-breed dogs; Supplementary Table S4). Thirteen golden retrievers from the exome cohort with mutations in SMGs had the same tumor location included in the RNA-seq data, with a total of 25 SMG mutations among them. We excluded two frameshift deletion variants from the validation, as they showed evidence of deletion in the pileup data, but also unexpected alternate alleles. Of the remaining 23 variants, we validated 15 (65%) mutations. Of the eight sites which were not validated, one was a nonsense mutation, which may have been subject to nonsense-mediated decay, five had a variant allele fraction (VAF) less than 0.1, and three variants came from samples with tumor purity estimate (≤ 35%) on histologic examination, suggesting that tumor heterogeneity and stromal contamination may be a contributing factor to variants not being replicated (Supplementary Table S5). In the RNA-seq data, four tumor samples from three dogs with variants in the SMGs were from a different tumor site than was included in the exome discovery cohort. In this subset, we were able to detect the mutation discovered in the same individual, but a different tumor location in 4 of 8 (50%) cases. Overall, the group of variants, which we were unable to validate, came from tumor samples with a significantly lower estimated tumor purity on histology (Pval = 0.04), but this difference was not significant based on tumor purity estimates from the program ESTIMATE (Pval = 0.4; Supplementary Table S5).

The RNA-seq cohort also confirmed that the TP53 and PI3K pathway mutations were not breed-specific, but were present across different breeds. Excluding the 19 golden retrievers who
were also included in the exome cohort, we looked to see whether somatic mutations corresponding to those observed in the exome cohort could be found in other cases, which included Golden Retrievers (n = 22), Portuguese water dogs (PWD, n = 6), German Shepherd dogs (GSD, n = 6), mixed breed dogs (n = 6), boxers (n = 2), Labrador retrievers (Labs, n = 2), Keeshonds (n = 2), and other breeds (n = 8, one each of American Staffordshire Terrier, Bernese Mountain Dog, Bichon Frise, Briard, Bullmastiff, Gordon setter, Parson’s Russell Terrier, and Saluki). We found mutations at the same sites as discovered in the exome cohort in TP53 (n = 14, 26%, 5 breeds), PIK3CA (n = 11, 20%, 6 breeds), and PIK3R1 (n = 4, 7%, 4 breeds; Supplementary Table S6).

SCNAs
SCNAs in canine hemangiosarcoma recurrently affect known cancer genes. We surveyed SCNAs in genes known to be involved in hemangiosarcoma. The genes most recurrently affected by DNA copy number aberrations in the oaCGH data were VEGFA, with copy number gain in 19% of cases, KDR, gained in 22%, KIT, gained in 17%, and the tumor suppressor CDKN2A/B, deleted in 22% (Supplementary Table S8). The MYC oncogene had copy number gain in 9% of cases. Copy number aberrations in the top significantly mutated genes were relatively rare (Fig. 1A; Supplementary Table S8).

Copy number gains in KDR and losses in AXIN1 are common in both dogs and humans. Comparison of SCNAs in the human data and canine oaCGH data revealed recurrent copy number aberrations in known cancer genes KDR and AXIN1 in both species. Copy number gains in KDR occurred in approximately 27% of human samples, and 22% of canine samples. AXIN1 was lost in 20% of human and 22% of canine samples. In addition, the genes PTK6, ARFRP1, and RTEL1 showed copy number loss in 17% of human and canine samples; however, these genes are close together, and also showed copy number gains in a number of canine samples (PTK6, 8.7%; ARFRP1, 13%; RTEL1, 13%; Supplementary Table S9).

SCNA profiles differ among cases with and without PIK3CA mutations. We examined the SCNA profiles of hemangiosarcoma cases with and without TP53 and PIK3CA mutations in the 28 cases with both exome sequencing and oaCGH data. There were no significant differences in the relative frequency of any given CNA between the CNA profiles of cases with and without TP53 mutations. However, significant differences were detected between cases with PIK3CA mutations and those without (Fig. 4), using a two-tailed Fisher exact test and a minimum differential threshold of 25% between the two groups. A region on chromosome 11 at approximately 22.6 Mb, near the UBE2B (ubiquitin-conjugating enzyme E2B) gene, was deleted in 4 of 11 cases with PIK3CA mutations, and 0 of 17 without (P < 0.016). Similarly, the CDRN2B gene, located distally on chromosome 11 at 41.2 Mb, was deleted in 4 of 11 cases with PIK3CA mutations, and 0 of 17 cases without (P < 0.016). A region on chromosome 24 at 21.2 Mb was gained in 7 of 17 cases without PIK3CA mutation and 0 of 11 with PIK3CA mutation (P < 0.023). This region overlaps the antiapoptotic BCL2L1 gene. Broad copy number gains along the length of chromosome 31 were more frequent in cases without PIK3CA mutations compared with those with PIK3CA mutations.

Discussion
Detailed molecular profiling of canine hemangiosarcoma has revealed both similarities and differences in the genetic landscape between dogs and humans. In particular, visceral canine hemangiosarcoma showed strong similarities with human angiosarcoma of the viscera and breast. Our findings have important implications for comparative oncology, as the study of canine hemangiosarcoma has the potential to improve our understanding of the pathophysiology of both canine hemangiosarcoma and human angiosarcoma, and to improve treatment and outcomes in both species. Tumor suppressor TP53 was the top significantly mutated gene in the canine data. The majority of mutations occurred in the DNA-binding domain, likely causing loss of function (Fig. 2). TP53 was also the only significantly mutated gene in the human Angiosarcoma Project data, and it has been frequently reported as mutated in targeted sequencing studies of angiosarcoma (25).

We found that the PI3K pathway was commonly mutated in canine hemangiosarcoma. A total of 23 canine tumors (48.9%) had at least one somatic mutation affecting this gene family (Fig. 1A). Tumors with a mutation in the PI3K family tended to have only one mutation in this family. The PI3K pathway is one of the most commonly altered pathways in cancer, playing an important role in signal transduction leading to cell proliferation, survival, differentiation, and regulation of metabolism and immunity (49, 50). PIK3CA is an oncogene (51) that has been shown to be mutated in human glioblastoma, breast, gastric, colorectal, lung, and endometrial cancers (52). Ten of the 14 PIK3CA mutations in our canine cohort occurred at amino acid position 1047, a mutational hotspot in many human cancers (42). Mutations within this domain have been shown to increase catalytic activity (53).

Of the less frequently mutated SMGs, PIK3R1 is annotated as a likely driver gene by COSMIC. In addition, variant allele fraction and SIFT scores support a potential role for RASAI and ARPC1A as driver genes. RASAI is a negative regulator of the Ras and MAPK pathways, and plays an important role...
in vascular formation (54, 55). Germline RASA1 mutations
can cause capillary malformation—arteriovenous malformation
syndrome (56). Somatic mutations in this gene have been found
in a subset of human basal cell carcinomas, and expression
has been correlated with survival in invasive ductal breast carci-
nomas and hepatocellular carcinomas (57, 58). ARPC1A plays
an important role in regulating the actin cytoskeleton, which func-
tions in the migration and invasiveness of pancreatic carcinoma
cells (59). There is less support for a causal role for ORC1 and
ENSCAFG000000017407; however, it is important to note that the
SIFT score does not annotate activating mutations; for example,
the hotspot mutations in PIK3CA (known to be drivers in many
human cancers) are also predicted to be tolerated.

There were also potentially important differences in somatic
mutations between the two species. In the human data, mutations
in TP53 and PIK3CA tended to be mutually exclusive, while we did
not see this pattern in the canine tumors. In addition, PIK3CA
mutations were exclusively found in breast tumors in the small
human dataset, while we found them to be common in cardiac
and splenic tumors in dogs. Within the canine visceral tumors, we
found fewer mutations in PIK3CA in liver tumors. These differ-
ences in distribution of PIK3CA mutations by tumor location may
due to genetic heterogeneity of the cancer, with tumors in
different locations activating the PI3K pathway at different
points, or relying on alterations in different pathways to affect an essential
protein downstream. It is also possible that this difference is due
to the small number of human visceral angiosarcomas currently
sequenced, and the small number of liver tumors in the canine
cohort.

Another potentially important difference between the two
species is that, while copy number gains in KDR are common
in both species, somatic mutations in this gene were seen
in over 20% of human tumors, but in only one canine tumor.
As the KDR receptor is upstream of the PI3K pathway, it
is possible that mutations in either may lead to a similar
phenotype. We also saw more copy number gains of VEGFA
in our canine cohort (19% vs. 0 in the human data), and as
VEGFA is upstream of KDR, this copy number gain may serve
a similar role to KDR mutations in the canine tumors. In addi-
tion, whole genome sequencing will be necessary to determine
whether there are common regulatory mutations affecting this
gene in canine hemangiosarcoma.

In the human data, mutation rates were significantly different
between different tumor locations, with HFN5 angiosarcomas
having a much higher mutational burden, as reported by Painter
and colleagues (46). It is possible that the higher mutational
burden in these tumors is due to UV exposure. It would be
interesting to compare these findings to canine cutaneous and
subcutaneous hemangiosarcomas, which were not included in
this study. In the canine visceral hemangiosarcoma, a slight
difference was found between mutational burden in heart and
splenic tumors. It is possible that the golden retrievers have a
lower overall somatic mutation burden than humans, as they
likely have a higher germline risk burden, given the high incidence
of hemangiosarcoma in the breed. Further studies in dogs will be
necessary to determine whether the lower mutation rate is cor-
related with breed risk, or if in this case it is an artifact of how
mutations were called.

Tumors in both dogs and humans were enriched for mutations
in protein tyrosine kinases, which are important regulators of
cellular growth and division signals and are commonly mutated
in cancers. There were also recurrent mutations in the protein
tyrosine phosphatase gene family in both species. This may
suggest an alternate mechanism of tyrosine kinase overactivation,
as protein tyrosine phosphatases deactivate tyrosine kinase sig-
naling by dephosphorylating proteins in opposition to kinase
phosphorylation (60). In addition, phospholipase C proteins
play a crucial role in cellular signaling pathways by hydrolyzing
phosphatidylinositol 4,5-bisphosphate (PIP2) into the second
messengers DAG and IP3, passing on signals from receptor
tyrosine kinases (61). The canine hemangiosarcomas were
enriched for phospholipase C genes, including PLCG1, and
PLCG1 mutations were recurrent in the human tumors.

The shared enriched pathways between canine hemangio-
sarcomas and human angiosarcomas provide insight into
disease pathogenesis for both species. Tyrosine kinase inhibi-
tors have been effective against angiosarcoma in the clinic, but
tumor heterogeneity and the development of resistance have
limited their long-term utility. They have also shown promise
against hemangiosarcoma in vitro (62), but so far have been
less promising in the veterinary clinic (63). Future investiga-
tion of the interaction between the many affected pathways
will help to determine the potential for combination therapy
targeting multiple of these pathways or a common down-
stream effector to combat resistance.

A recent study in 13 radiation-induced and three spontaneous
breast angiosarcomas detected the irradiation signature, as well as
the aging signature, and a unique C>T signature (36). The median
age of patients in this human cohort was 74.5 years (36). Canine
hemangiosarcomas looked very similar, in that we primarily saw
the aging signature and low levels of a novel C>T signature. This
novel signature bore some resemblance to the signature reported
in the human angiosarcomas, including higher levels of C>T
mutations at C nucleotides flanked by A-A or A-T; however, there
were also differences, such as a high number of mutations flanked
by T-G in the dogs, and a low number of these in the human
cohort. A larger study will be needed to decipher whether this is a
novel angiosarcoma-related signature or whether it represents
noise. The lack of the irradiation signature was anticipated as
our canine data did not include any tumors secondary to
radiotherapy.

We examined copy number changes in canine hemangio-
sarcoma in genes previously reported to be affected in human
angiosarcoma and angiosarcoma. Most common were copy number
gain of VEGFA and KDR, and loss of CDK24A/B. The MYC
oncogene, which has been reported amplified in human angio-
sarcoma and canine hemangiosarcoma, was only rarely gained
in our dataset and showed no evidence of high-level amplification.
This makes sense, given that it is more common in radiation-
induced tumors, which were not present in the canine cohort.

Our data suggest that visceral canine hemangiosarcoma
could be developed as a model for primary human angiosar-
coma. Detailed molecular characterization of canine hemangio-
sarcoma revealed many similarities, but also some impor-
tant differences, between canine hemangiosarcoma and human
angiosarcoma. Future work should include analysis of larger
cohorts, including recalling all currently available human
angiosarcoma data using the same methods, to decipher poten-
tial molecular subtypes and to facilitate a more complete
comparison between tumors in different locations. An inte-
grated understanding of the interaction between mutations in
the many enriched signaling pathways may be useful for
determining treatment strategy; for example, the feasibility of combinations of targeted inhibitors or the prevention of convergent resistance. Our data suggest that clinical trials evaluating therapeutic approaches in dogs with this disease might also inform human medicine.

Disclosure of Potential Conflicts of Interest
J.F. Modiano is a partner (paid consultant) for Veterinary Research Associates, reports receiving a commercial research grant from Boston Scientific, and has ownership interest (including patents) in Half Moon Bay Biotechnology, LLC. No potential conflict of interests were disclosed by the other authors.

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Acknowledgments
The authors would like to thank all of the dogs and owners who participated in our research, as well as the veterinarians who collected samples. The results included here include the use of data from the Angiosarcoma Project [https://angiosarcoma.project/], a project of Count Me In [https://joincountmein.org/], downloaded March 2019. We would also like to thank Dr. Scott Moroff, Vice-President and Chief Scientific Officer of Antech Diagnostics, for contributing tumor and paired blood samples to our research. This work was funded by American Kennel Club (AKC) Canine Health Foundation (CHF) grants #422 (to J.F. Modiano), 1131 (to J.F. Modiano), and 1889-G (to J.F. Modiano, M. Breen, K. Lindblad-Toh, E.K. Karlsson). NIH grants R03CA191713 (to J.F. Modiano), P30CA07598 (NIH Comprehensive Cancer Center Support Grant to the Masonic Cancer Center, University of Minnesota), R37CA218570 (to E.K. Karlsson, C. Painter), and R24OD018250 (to E.K. Karlsson). Cancerfonden (K. Lindblad-Toh). National Canine Cancer Foundation (NCCF) grants DM05CCO-003 (to J.F. Modiano) and JHK15SMN-004 (to J.-H. Kim), and Morris Animal Foundation grant D10CA-501 (to J.F. Modiano, M. Breen, K. Lindblad-Toh). K. Lindblad-Toh is the recipient of a Distinguished Professor award from the Swedish Research Council. I. Elvers is supported by a postdoctoral fellowship from the Swedish Medical Research Council, SSMF. J.F. Modiano is supported by the Alvin and June Perlman Chair in Animal Oncology at the University of Minnesota. M. Breen is supported in part by the Oscar J. Fletcher Distinguished Professorship in Comparative Oncology Genetics at NC State University. A.L. Sarver is supported by NCI R50 CA211249.

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Received February 22, 2019; revised July 12, 2019; accepted September 25, 2019; published first September 30, 2019.

References
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doi:10.1158/1541-7786.MCR-19-0221

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