Chromatin run-on sequencing analysis finds that ECM remodeling plays an important role in canine hemangiosarcoma pathogenesis

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Abstract
Background Canine visceral hemangiosarcoma (HSA) is a highly aggressive cancer of endothelial origin that closely resembles human visceral angiosarcoma, both clinically and histopathologically. Currently there is an unmet need for new diagnostics and therapies for both forms of this disease. The goal of this study was to utilize ChRO-seq and immunohistochemistry (IHC) to identify gene and protein expression signatures that may be important drivers of HSA progression.

Methods Chromatin run-on sequencing (ChRO-seq) was performed on tissue isolated from 17 HSA samples and 4 normal splenic samples. Computational analysis was then used to identify differentially expressed genes and these factors were subjected to gene ontology analysis. Next, RT-PCR was performed on a subset of candidate genes to validate the ChRO-seq data. We then performed Masson’s trichrome, H&E, and IHC staining on these tissues to investigate the morphological features of HSA tumor tissue as well as the expression patterns of several proteins identified in our ChRO-seq analysis.

Results ChRO-seq analysis revealed over a thousand differentially expressed genes in HSA tissue compared with normal splenic tissue (FDR <0.005). Interestingly the majority of genes overexpressed in HSA tumor tissue were associated with extracellular matrix (ECM) remodeling. This observation correlated well with our histological analysis, which found that HSA tumors contain a rich and complex collagen network. Additionally, we characterized the protein expression patterns of two highly overexpressed molecules identified in ChRO-seq analysis, podoplanin (PDPN) and laminin alpha 4 (LAMA4). We found that the expression of these two ECM-associated factors appeared to be largely limited to transformed endothelial cells within the HSA lesions.

Conclusion Outcomes from this study suggest that ECM remodeling has an important role in HSA progression. Additionally, our study identified two potential novel biomarkers of HSA, PDPN and LAMA4. Interestingly, given that function-blocking anti-PDPN have shown anti-tumor effects in mouse models of canine melanoma, our studies raise the possibility that these types of therapeutic strategies could potentially be developed for treating canine HSA.

Background (introduction)
Angiosarcomas (AS) are highly aggressive malignant tumors originating from endothelial cells. They account for approximately 2% of all soft tissue sarcomas in humans with the number of cases increasing significantly over the past 30 years [1-5]. For patients presenting with non-metastatic AS, the reported five-year survival rate is ~ 35%. These patients also have a 75% chance of local recurrence within 24 months and a 50% likelihood of metastases developing despite local treatment[4][6]. When metastases are already present at the initial presentation, the five-year survival rate becomes negligible, with a median survival time of just 3 months[4]. Although published research on this rare tumor is increasing, we still know very little about the pathogenesis of this disease.

Canine hemangiosarcoma (HSA) is histopathologically similar to angiosarcoma, with both forms of this disease following a similar clinical course [7]. Canine HSA is most commonly observed in the spleen, but also occurs in other organs such as the heart, liver, and the dermis with the later often associated with ultraviolet radiation associated oncogenesis. The prognosis for the visceral forms of HSA is poor, with most dogs dying from this disease within months of their diagnosis[8][9]. For dogs diagnosed with splenic HSA, surgical removal of the spleen can increase the life expectancy up to 6 months, and combined with chemotherapy, can prolong their life for up to 12 months [8] [9].

While all breeds are susceptible to hemangiosarcoma, German Shepherds, Golden Retrievers, and Labrador Retrievers, are particularly predisposed to developing this disease [10], with the estimated lifetime risk of Golden Retrievers developing HSA being 20%. This breed predisposition suggests an inherited component to HSA [11]. Genome wide association studies have been carried out to identify risk loci [12][13]. SNP array analysis on genomic DNA from HSA Golden Retriever tumor samples identified a risk locus on chromosome 5 that was shared by ~ 20% of the cases. In another study, microarray analysis on genomic DNA revealed gained copy number aberrations on several genes such as PDGFRA, KDR and VEGFA. A recent whole exome sequencing study revealed somatic mutations in tumor suppressor genes, including TP53, and two genes (PIK3CA and PIK3R1) in the PI3K pathway. These mutations were not breed-specific but were present across many different breeds. Comparison of somatic copy number aberration profiles in human angiosarcoma and canine hemangiosarcoma
identified recurrent copy number gains in KDR (31% in human, 22% in canine) and KIT (17% in both) [14]. These genome-wide association studies revealed that while specific genetic aberrations are associated with HSA in some populations, these alterations are not sufficient to explain the majority of HSA cases, thus further supporting the heterogeneity of HSA pathogenesis.

Transcriptome analysis has been previously performed on both HSA cell lines and tumor tissues to identify molecular features that define canine HSA. Gene expression profiling of HSA cell lines and non-malignant proliferating endothelial cells revealed that HSA cell lines appeared to overexpress genes associated with inflammation, angiogenesis, adhesion, invasion, metabolism, cell cycle progression, and patterning [15]. Microarray and RNA-seq analysis of visceral HSA tumors identified three distinct molecular subtypes; angiogenesis, inflammation and lipogenesis. These molecular subtypes did not appear to be associated with a specific breed or tumor morphologic subtype [16].

A variety of analytic tools exist for assaying the transcriptome and molecular alterations in tissue. One of these tools, ChRO-seq, uses RNA polymerase activity to measure transcription and, as such, provides for a highly-sensitive, base-pair level readout of gene expression [17][18]. Given the lack of clarity regarding the molecular underpinnings of HSA, the major goal of this study was to utilize chromatin run-on sequencing (ChRO-seq) to document changes in gene expression between normal splenic tissue and HSA tumor tissue. Our results show that the majority of genes that are upregulated in HSA tumor tissue appear to be associated with extracellular matrix (ECM) remodeling. Additionally, we further characterize two ECM-associated molecules that we found to be highly overexpressed in HSA tumor tissue, podoplanin (PDPN) and laminin alpha 4 (LAMA4), and show by immunohistochemistry (IHC) that the expression of these two cancer-associated factors appears to be primarily limited to HSA tumor cells.

Materials And Methods

Samples

Canine tissue samples were obtained from the Canine Comparative Oncology & Genomics Consortium and Cornell University Hospital for Animals. Pathology was independently confirmed and patient demographics are described in the supplemental material (Table S1). ChRO-seq libraries were made
from splenic hemangiosarcoma (n = 20) and normal splenic tissues from HSA dogs (n = 4).

**Precision Run On sequencing**

Chromatin was extracted from each tissue sample and chromatin run-on was performed as described previously[17][18][19]. ChRO-seq library preparations were executed according to the Illumina protocol and were sequenced using Illumina NextSeq500 sequencing. Raw sequence FASTQ files were assessed by FastQC for quality control[20]. Single-read sequencing data were preprocessed and mapped to the canine genome assembly by proseq 2.0 pipeline (https://github.com/Danko-Lab/proseq2.0)[21]. Briefly, Single-read sequencing data were preprocessed to remove the adapter sequences and trim based on base quality, and deduplicating the reads based on unique molecular identifiers in RNA adapters. Sequencing reads were mapped into a canine genome assembly (CanFam 3.1) using the Burrows-Wheeler Aligner software package. Aligned BAM files were converted into bigWig format, which were used to create the matrix of read counts mapping to each annotated gene (CanFam3 ensGene). 7007 genes (> 20 rpkm) were used to calculate the Spearman’s rank correlation coefficients to create the matrix and dendrogram by GENE-E (https://software.broadinstitute.org/GENE-E/). The list of differentially expressed genes and an MA-plot were produced by running DESeq2 (false discovery rate < 0.01)[22]. Upregulated genes (369 genes, FDR < 0.0005) and down regulated genes (358 genes, FDR < 0.005) in HSA were subjected to PANTHER (Protein Analysis Through Evolutionary Relationships) overrepresentation gene ontogeny analysis (GO) on Reactome pathway dataset (version 65, released 2018-06-12)[23].

**Immunohistochemistry and histology staining**

Prior to all staining protocols, paraffin section slides were rehydrated in xylene followed by sequential washes in 100, 90, 80, and 70% EtOH. H&E and Masson’s trichrome staining were performed on 10 HSA samples and 4 normal spleen sections. Gill’s hematoxylin and Eosin Y solutions were used for H&E. Masson’s trichrome staining was performed following the manufacture’s protocol (Masson’s Trichrome Stain Kit, Polysciences, Inc #25088-100). Immunohistochemistry (IHC) experiments using anti-canine PDPN (clone PMab-38 [24], now commercially available at FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; 017-27091, 1:200) and anti-LAMA4 (Sigma-Aldrich Corp. St. Louis, MO,
HPA015693, Human Atlas antibodies for IHC, Rabbit polyclonal, epitope homology 85%, 1:300), were performed on 8 HSA samples and 2 normal spleens following a standard protocol[25]. Briefly, slides were boiled for 40 minutes in 0.01 M sodium citrate to retrieve antigens. Slides were cooled and washed in running tap water for 20 minutes, then incubated for 15 minutes in 3% hydrogen peroxide to quench endogenous peroxidases. Slides were blocked in 2.5% normal goat serum for 30 minutes at room temperature. Primary antibodies were diluted in 1% BSA/TBST, and incubated overnight at 4C. Slides were washed three times with TBS-T, then incubated with a secondary antibody for 1 hour at room temperature (Goat anti-mouse-HRP, Vector laboratories, Inc., Burlingame, CA; #MP7452 or Goat anti-rabbit-HRP, Vector laboratories, Inc., #MP7451). ImmPACT NovaRED peroxidase substrate (Vector laboratories, Inc., SK-4805) with hematoxylin counterstain was used for signal detection. Slides were dehydrated through 80, 95 and 100% EtOH to Xylene and coverslipped.

RT-PCR
Snap frozen tissue was pulverized in tissue crasher and total RNA was extracted with Trizol reagent. 1× g of RNA was reverse-transcribed to cDNA using Applied Biosystems High-capacity cDNA reverse transcription kit following the manufacture’s protocol. All cDNA reactions were diluted 5-fold with water prior to use in PCR. 1× l of diluted cDNA was used for PCR using Go-Taq master mix (Promega Corporation) with canine specific primers (supplement table 2), and amplified products were analyzed on agarose gel electrophoresis.

Discussion
In this study, we first utilized precision-run-on sequencing to identify RNA polymerase activity in hemangiosarcoma tumor tissue and normal splenic tissue. Analysis of the correlation matrix for our ChRO-seq dataset finds that 14 of the HSA tumor samples clustered together, while 3 of the HSA samples appeared to be more similar to normal splenic tissue (Fig. 1). While speculative, it is possible that these 3 samples represent a different subtype of HSA and, as such, displayed a gene expression profile that is more similar to normal tissue. Alternatively, it is also possible that, these 3 presumptive HSA tissues were actually tumor-adjacent normal tissue or a mix of normal and tumor tissue. While grading of canine hemangiosarcoma is not often utilized due to the aggressive nature of the
neoplasm, a grading scheme does exist and differences in gene expression may correlate to progression and eventual outcome[26].

Previous HSA transcriptomic profiling studies have implicated a number of signaling pathways in HSA pathogenesis. Tamburini et al, for example, found HSA cells exhibited a several different distinct gene expression profiles, including signatures for angiogenesis and inflammation[15]. In another study, Gorden et al performed microarray and RNA-seq analyses on visceral HSA tumor tissues and identified three distinct tumor subtypes that were associated with either angiogenesis, inflammation, and adipogenesis. Our study differs from these previous reports in that we found ECM remodeling to be the most significantly upregulated biological process in HSA tumor tissue. Precisely what accounts for the discrepancy between our study and previous studies is not clear.

Regarding the specific types of ECM factors that were upregulated in our dataset (Table 1), we identified genes encoding fibrous components of the ECM, including 12 collagen genes, 3 laminin genes, and fibronectin. Several ECM binding proteins were also identified in this dataset including, Lumican and Biglycan, which are small leucine-rich proteoglycans that regulate collagen fibril and matrix assembly [27–30]. Integrin alpha 2 is directly associated with fibril-forming collagens (1,2,3,5,6,14, 18)[31] while DDR2 functions as a cell surface receptor for fibrillar collagen and regulates cell differentiation by remodeling ECM[32, 33]. Additionally, Integrin alpha 5 binds directly to fibronectin while integrin alpha 6 binds to laminin [34][35]. Several ECM related enzymes were also found in this dataset including P4HA2 and PLOD1 which catalyze collagen biosynthesis [36, 37] along with ADAMTS14, ADAMTS4, ADAM12 and TLL1 which process procollagen to collagen by cleaving N-propeptide and C-propeptide [38, 39]. Lastly, two molecules directly involved in ECM turnover, tissue inhibitor of metalloproteinase 1 (TIMP1) and matrix metalloproteinase 14 (MMP14), were also found in this dataset [40, 41][42].

The extent to which ECM genes were upregulated in HSA tumors in our study is highlighted by our GO analysis which found that 9 of the top 10 biological process categories were ECM-related (Fig. 2). Interestingly, 5 of these 9 categories related to collagen function. We more directly tested for the abundance of collagen fibers in the tumor samples using Masson’s trichrome stain and found
extensive collagen deposition throughout the tumor tissue (Fig. 3). Collagen deposition was observed both in the more solid areas of the tumor and, in particular, in the tumor regions filled with malformed vascular channels. In these vascular regions, neoplastic endothelial cells are often found to encircle the collagen bundles forming “gumball”-shaped structures that look like inverted blood vessels. While stromal fibroblasts are presumably primarily responsible for the synthesis of these collagen fibers, it is also possible that the tumor cells may also be partly responsible for synthesis of these collagen fibers.

In addition to collagen, we also found that PDPN and LAMA4 were highly overexpressed in HSA tumor tissue when compared to normal tissue. These molecules were of particular interest to us given their close association with cancer progression in other types of cancer. Podoplanin is a mucin-type protein that contains an extracellular region, transmembrane domain, and intracellular tail. It is widely known as a marker for lymphatic endothelial cells and also to play a critical role in heart and lung development and in development of the lymphatic endothelial system [43–45]. PDPN appears to play several roles in cancer progression. A number of studies have shown that PDPN expression in cancer cells promotes tumor cell proliferation and invasion [46–48]. In addition to cell-intrinsic effects, PDPN is also believed to promote tumor metastasis by interacting with its receptor, CLEC2, on the platelets. This interaction then promotes the coating of tumor cells by platelets, thereby protecting tumor cells from the immune system [49]. In addition to its role in human cancers, PDPN is also overexpressed in canine squamous cell carcinomas and melanomas [50] and PDPN mAbs were recently found to have potent anti-tumor activity in mouse xenograft models of canine melanoma [51]. Interestingly, overexpression of PDPN in mice leads to disseminated intravascular coagulation [52], a condition that is strikingly similar to that seen in many dogs with HSA [53]. Our ChRO-seq data demonstrated that PDPN expression varies significantly between HSA samples (Fig. 1, 4). IHC analysis found that PDPN was robustly expressed in transformed endothelial cells in certain HSA tumor samples. However, similar to our ChRO-seq finding, we did find that PDPN appears to only be expressed in a subset of HSA tumors. In future studies, we plan to test whether PDPN expression in HSA tumors correlates with disease severity.
Laminins form large heterotrimeric $\alpha\beta\gamma$ protein complexes and are a prominent component of basement membranes. LAMA4 is distinct from other laminin isoforms in that it lacks a polymerization domain with the loss of this domain potentially facilitating tumor cell migration [54]. Interestingly, LAMA4 was recently described as an “oncolaminin” due to its strong association with cancer cell migration and tumor progression in a range of cancers [55]. These links to cancer include recent studies which found that LAMA4 and MCAM (melanoma cell adhesion molecule) are highly enriched in tumor blood vessels in renal cell carcinoma and colorectal carcinoma. Additionally another study found that expression of these molecules is enhanced in locally invasive and metastatic clear cell renal cell carcinoma [56]. Further, antisense oligonucleotides against laminin-8 (LAMA4 and LAMB1) were found to block the invasion of glioma cells and neovascularization in vitro [57]. Taken together, these published studies indicate that LAMA4 plays a key role for vascular development, tumor progression and metastasis. In our study, deseq2 analysis found that LAMA4 is highly transcribed in HSA tumor tissue. This observation was supported by PCR analysis of mRNA isolated from HSA tumor tissue (Fig. 4). Additionally, our IHC analysis found that LAMA4 protein expression appears to be primarily limited to malignant endothelial cells (Fig. 5). Given the previously defined roles for LAMA4 in cancer progression and tumor metastasis, our findings raise the possibility that LAMA4 is an important mediator of canine HSA pathobiology.

Conclusions

Outcomes from our studies found that the majority of upregulated genes in HSA tumor tissue appear to be associated with ECM remodeling. This finding was supported by IHC analysis which found a robust collagen network throughout HSA tumor tissue. Additionally, we further characterized two ECM-associated factors, PDPN and LAMA4, found that their expression was largely limited to tumor cells in HSA tissue. Both of these molecules have been previously identified as potential biomarkers in other types of cancer. Given the recent previous preclinical mouse studies showing that anti-canine PDPN antibodies can block the growth of melanomas, our findings raise the possibility that similar types of therapies may have utility for treating canine HSA.

Declarations
Ethics approval and consent to participate

Not applicable. All samples in this study were purchased from CCOGC and Cornell Veterinary Biobank.

Consent to publish

Not applicable.

Availability of data and material

Fastq files will be submitted to GEO upon the manuscript accepted.

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

CM, CD and SAC were involved in conception and design of this study. CM, ER, ZW, LC, SPC, BAM, CD, SAC prepared the library and analyzed bioinfomatic data. CM, EC, LA, KS, EK, YK, AM, SAC acquired and interpreted histology data. CM and SAC wrote the manuscript. All authors have been involved in revising the manuscript and approved the manuscript.

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Tables
Due to technical limitations, tables are only available as a download in the supplemental files section.

Figures
Figure 1

Correlation matrix and MA-plot from ChRO-seq analysis. (a) Spearman's rank correlation of
17 HSA (red bar) and 4 Normal samples (green bar). The sample order is based on single-linked hierarchical clustering of the matrix, shown by the dendrogram. 7007 genes (>20 rpkm) were used to calculate the correlation coefficients by GENE-E. The light green box identifies a cluster containing the normal samples plus 3 HSA samples. Note that these HSA samples also have similarities with other HSA samples. The pink box highlights a large cluster which contains all HSA samples. (b) MA-plot of DESeq2 analysis. Results show that ECM-associated gene expression tends to be upregulated in HSA while genes involved in normal splenic function tend to be downregulated. X-axis represents average expression over all samples. Y-axis represents log2 fold change between HSA and normal. Genes with an adjusted p-value below 0.01 are shown in red.
Figure 2

Gene ontology analysis of differentially expressed genes. Upper panel: Top annotation categories identified in upregulated HSA gene set. Lower panel: Top annotation categories
identified in downregulated HSA gene set. X axis represents fold enrichment, FDR values are labelled under each bar.

Figure 3

Histology of H&E (top) and Masson's trichrome (bottom) stained HSA tumor sections. H&E
staining shows that collagen bundles are surrounded by neoplastic endothelial cells and appear to form "inside out" blood vessels (a and b). Trichrome-stained HSA sections (c and d) show collagen bundles stained as blue in vascular regions of the tumor that are surrounded by endothelial cells. (a and c) : low magnification, (b and d) : high magnification. Scale bars represent 50 µm in a-d, and 10 µm in insets in b and d.
Figure 4

PDPN and LAMA4 transcription and mRNA expression in normal and HSA samples. (a)

Genome browser views (UCSC browser) of the ChRO-seq dataset showing the PDPN and
LAMA4 gene loci. ChRO-seq reads on sense and anti-sense strands are shown in red and blue, respectively. Red lines indicate the encoding gene in the Ensemble database. (b) RT-PCR analysis documenting PDPN and LAMA4 transcript expression in normal (3) and HSA samples (3). GAPDH primers were used as a positive control. Negative control (NC) lacks cDNA template. Results show that PDPN and LAMA4 mRNA do not appear to be expressed in normal spleen. LAMA4 transcripts were observed in all three HSA samples while PDPN showed strong expression in one of the three HSA samples.
Figure 5

Immunohistochemical localization of PDPN protein in normal and HSA tumor tissue. (a) PDPN detection in normal splenic tissue was low to absent. (b) PDPN staining appears weak in the cytoplasm of endothelial cells of blood vessels that are adjacent to tumor masses. (c and d) PDPN staining is strong in cells within the tumor’s vascular regions. In these regions the PDPN-positive tumor cells were seen to surround what appears to be collagen bundles. PDPN was also detected in tumor cells from the more avascular regions of the tumor (data not shown). c=low magnification d= high magnification. Scale bar represents 50 μm.
Figure 6
Immunohistochemical localization of LAMA4 protein in normal and HSA tumor tissue. (a) LAMA4 detection was low to absent in normal splenic tissue. (b) LAMA4 detection appears weak in the cytoplasm of endothelial cells of blood vessels that are adjacent to tumor masses. (c and d) LAMA4 staining is strong in the cytoplasm of tumor cells within the tumor’s vascular regions. In these regions the LAMA4-positive tumor cells were seen to surround what appears to be collagen bundles. LAMA4 was also detected in tumor cells from the more avascular regions of the tumor (data not shown). c=low magnification d= high magnification. Scale bar represents 50 μm.

Supplementary Files
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