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Genomic profiling reveals extensive heterogeneity in somatic DNA copy number aberrations of canine hemangiosarcoma

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Abstract

Canine hemangiosarcoma is a highly aggressive vascular neoplasm associated with extensive clinical and anatomical heterogeneity and a grave prognosis. Comprehensive molecular characterization of hemangiosarcoma may identify novel therapeutic targets and advanced clinical management strategies, but there are no published reports of tumor-associated genome instability and disrupted gene dosage in this cancer. We performed genome-wide microarray-based somatic DNA copy number profiling of 75 primary intra-abdominal hemangiosarcomas from five popular dog breeds that are highly predisposed to this disease. The cohort exhibited limited global

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ETHICAL STANDARDS

Experiments described in this manuscript comply with the current laws of the country in which they were performed (USA). All institutional and national guidelines for the care and use of laboratory animals were followed.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

genomic instability, compared to other canine sarcomas studied to date, and DNA copy number aberrations (CNAs) were predominantly of low amplitude. Recurrent imbalances of several key cancer-associated genes were evident; however the global penetrance of any single CNA was low and no distinct hallmark aberrations were evident. Copy number gains of dog chromosomes 13, 24 and 31, and loss of chromosome 16, were the most recurrent CNAs involving large chromosome regions, but their relative distribution within and between cases suggests they most likely represent passenger aberrations. CNAs involving *CDKN2A*, *VEGFA* and the *SKI* oncogene were identified as potential driver aberrations of hemangiosarcoma development, highlighting potential targets for therapeutic modulation. CNA profiles were broadly conserved between the five breeds, although subregional variation was evident, including a near two-fold lower incidence of V*EGFA* gain in Golden Retrievers versus other breeds (22% versus 40%). These observations support prior transcriptional studies suggesting that the clinical heterogeneity of this cancer may reflect the existence of multiple, molecularly-distinct subtypes of canine hemangiosarcoma.

Keywords

canine; chromosome; hemangiosarcoma; comparative genomic hybridization (CGH)

INTRODUCTION

Canine hemangiosarcoma is a highly aggressive malignant neoplasm arising from cells involved in blood vessel formation, characterized by extensive clinical and anatomical heterogeneity, local invasiveness and a high incidence of local recurrence and metastatic disease. Hemangiosarcoma represents up to 7% of all malignant tumors of the domestic dog, translating to more than 50,000 new diagnoses in the US each year (Vail and MacEwen 2000; Tamburini et al. 2009), most commonly arising in the spleen, right atrium, liver and subcutis. While cutaneous hemangiosarcoma has a relatively favorable prognosis, intra-abdominal masses frequently remain undetected until presentation as acute, late-stage disease with extensive necrosis, coagulopathies and untreatable internal hemorrhage secondary to rupture of the tumor. In the most common splenic form, median survival is typically shorter than six months with standard-of-care surgical resection and intensive chemotherapy (reviewed in Bergman 2010).

Improved strategies for early detection and effective clinical management are an acutely high priority for those breeds that are highly predisposed to visceral hemangiosarcoma. Represented among these are several of the most popular family owned pure breeds, including the Golden Retriever, Labrador Retriever and German Shepherd Dog (Schultheiss 2004; Thamm 2007; Bergman 2010; American Kennel Club 2012). These breeds also are among the ten most common contributors to the genetic ancestry of mixed-breed dogs, which themselves account for 53% of all family owned pet dogs within the US (Mars Veterinary 2011). Consequently, advances in clinical management of hemangiosarcoma may have tremendous impact across the full breadth of the pet dog population. The strong association between genetic background and hemangiosarcoma susceptibility suggests the presence of breed-associated heritable risk factors and molecular signatures reflecting biologically significant characteristics of their underlying pathogenesis. The lifetime risk for

hemangiosarcoma in the Golden Retriever (GR) is remarkably high, with estimates of greater than one in five (Glickman et al. 2000; Schultheiss 2004; Shearin and Ostrander 2010). The genetic history of this breed is well documented (American Kennel Club 2006), and for over 30 years the GR has constituted one of the five most popular family-owned breeds in the US, yielding an estimated 50,000 new American Kennel Club registrations each year (American Kennel Club 2012). The GR therefore offers a comprehensive resource of naturally occurring clinical specimens and epidemiologic data in a population with restricted heterogeneity and well-defined ancestry, conferring vast potential for identifying tumor-associated risk factors and therapeutic targets for hemangiosarcoma.

Prior studies have described tumor-associated disruption of global transcriptional profiles in canine hemangiosarcoma, and gene-inactivating sequence mutations of key pathways associated with angiogenesis (Dickerson et al. 2005; Tamburini et al. 2009; Tamburini et al. 2010). Germline risk factors are now being recognized that may explain the elevated susceptibility of the purebred GR population to this disease (Tonomura et al. submitted). To date, however, there remains no published knowledge of global somatic genome instability in the form of gene dosage imbalance in this cancer as a potential mechanism for transcriptional dysregulation. We used oligonucleotide array comparative genomic hybridization (oaCGH) analysis to develop the first description of the landscape of non-random tumor-associated DNA copy number aberrations (CNAs) within primary visceral hemangiosarcoma. We explored the diversity of CNA profiles within and between genetically distinct populations through parallel evaluation of 40 GR tumors and 35 tumors from four additional breeds that exhibit an elevated incidence of this disease (Australian Shepherd Dog [ASD], Bernese Mountain Dog [BMD], Flat Coated Retriever [FCR] and German Shepherd Dog [GSD]). In combination these findings identify the subset of somatic hemangiosarcoma-associated CNAs that are shared across breeds, defining genomic intervals and genes that may play a fundamental role in disease pathogenesis and/or represent effective therapeutic targets. Additionally, we highlight breed-associated signatures consistent with differential gene dosage effects involving signaling pathways that are intimately involved in angiogenesis. Finally we draw comparisons with recently reported profiles of chromosomal instability in angiosarcoma (Italiano et al. 2012b), a rare and understudied human counterpart for which advances in clinical management may be aided greatly by the availability of a pertinent canine model.

MATERIALS AND METHODS

Case recruitment and histological evaluation

Clinical specimens of primary visceral hemangiosarcoma were acquired from family-owned dogs between 2003 and 2012 from community or institutional veterinary practices distributed broadly across the continental USA, during routine diagnostic procedures, under approved institutional protocols and with informed client consent. Representative biopsies were formalin-fixed and paraffin-embedded (FFPE) for histological evaluation. A diagnosis of hemangiosarcoma was confirmed by examination of hematoxylin and eosin (H&E)-stained sections. All histological specimens were reviewed independently by two or more board-certified veterinary pathologists, one of whom (LB) was common to all cases.

Consecutive 25μm sections from each hemangiosarcoma tissue block were macrodissected to eliminate gross regions of healthy tissue by reference to the corresponding H&E slide, using dedicated equipment for each case to prevent cross-contamination. Tumor DNA was isolated from the fixed tissue sections using a QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA) for use in oaCGH analysis. FFPE tissue processing (based on the recommendations of van Essen and Ylstra (2012)) included an extended treatment with Qiagen Proteinase K (four days at 56°C) and a one hour incubation in a guanidine hydrochloride -based lysis buffer (Qiagen Buffer ATL) at 90°C. These procedures aid removal of DNA-protein cross-links created during formalin fixation, yielding DNA templates suitable for high-resolution, dual-channel Agilent oaCGH platforms without need for amplification or further fragmentation (Krijgsman et al. 2012; supplementary figure 1).

Identification of tumor-associated DNA copy number aberrations

oaCGH analysis was performed as described previously (Thomas et al. 2011) using a ~180,000-feature microarray (Agilent Technologies, Santa Clara, CA) comprising repeat-masked ~60-mer oligonucleotides distributed at approximately 13kb intervals throughout the dog genome sequence assembly (canFam version 2.0, May 2005, Lindblad-Toh et al. 2005). To maximize continuity and aid data integration, tumor DNA from each case was hybridized against common reference DNA samples comprising equimolar quantities of constitutional DNA from 10 clinical healthy GRs of the same gender as the patient. Array image files were processed using Feature Extraction version 10.10 and Genomic Workbench version 7 (Agilent Technologies, Santa Clara, CA) and were then imported into Nexus Copy Number version 7 (Biodiscovery, El Segundo, CA). Raw data were filtered to exclude probes exhibiting non-uniform hybridization or signal saturation. Recurrent CNAs within each tumor were defined using the FASST2 segmentation algorithm in Nexus Copy Number, based on a minimum of three consecutive probes with $\log_2$ tumor:reference values ≥ 0.201 (copy number gain) or ≤ −0.234 (copy number loss), resulting in an effective resolution of ~26kb (two intervals of ~13kb). High amplitude gains and losses within individual tumors were defined using default $\log_2$ tumor: reference values of ≥1.14 and ≤−1.1, respectively.

oaCGH data for all cases were compiled using a threshold of 20% penetrance of the cohort to define the broadest regions of recurrent genomic imbalance. Genes and uncharacterized coding sequences within recurrent CNAs were defined using the UCSC canine genome sequence browser (http://genome.ucsc.edu/) and the Gene database at www.ncbi.nlm.gov/gene. These data were filtered to flag regions of known natural copy number polymorphism defined by prior studies (Chen et al. 2009; Nicholas et al. 2009). Significant differences between pairwise comparisons of discrete subpopulations were defined as regions exhibiting ≥20% relative difference in CNA frequency with p-values < 0.05 based on a two-tailed Fisher's Exact test. The 'comparisons' tool of Nexus was used to identify breed-associated aberrations by sequential comparison of genome-wide CNA penetrance in each breed against the mean CNA penetrance for all other breeds. The GISTIC algorithm (Beroukhim et al. 2007) was then used to identify genomic regions for which the frequency and magnitude of genomic gain and loss was significantly increased relative to the 'background' level, indicating CNAs that are unlikely to occur by chance alone (G score < 0.05). Benjamini-Hochberg false discovery rate (FDR) correction for multiple testing was applied (Benjamini

and Hochberg 1995), yielding a peak region of highest significance with minimum Q-bound value, flanked by a broader region with lower significance.

Discrete genomic regions are herein denoted according to their cytogenetic location and then their Mb position on that chromosome, based on the canFam v2.0 (Lindblad-Toh et al. 2005) dog genome sequence assembly. The absence of data for the dog Y chromosome in the female dog genome sequence assembly precluded the inclusion of data analysis for this chromosome.

Targeted fluorescence *in situ* hybridization analysis

Cases exhibiting discrete high level amplifications of the vascular endothelial growth factor A gene (*VEGFA*) in oaCGH were evaluated further by targeted fluorescence *in situ* hybridization (FISH) analysis. FISH analysis was performed using bacterial artificial chromosome (BAC) clones from the CHORI-82 canine BAC library (http://bacpac.chori.org, BACPAC Resources, Children's Hospital Oakland Research Institute, Oakland, CA), containing the full coding sequence of the *VEGFA* gene (clone 152L05) and associated receptor (*VEGFR2/KDR*, clone 34E11). FISH probes for *VEGFA* and *VEGFR2/KDR* were generated by incorporation of fluorescent-conjugated nucleotides into BAC DNA using nick translation, and their unique chromosomal location was verified by conventional FISH analysis of chromosome preparations from clinically healthy dogs (Breen et al. 2004). Thin (5 micron) sections from FFPE tissue blocks were floated onto positively charged slides and deparaffinised with xylene. Specimens were then exposed to one hour enzymatic treatments at 37°C with hyaluronidase type VIII (45 U/μl in 250mM TRIS-buffered saline, pH7.4, Sigma-Aldrich, St. Louis, MO) followed by collagenase (236U/μl each of collagenases I and II, and 0.4U/μl of collagenase III [Invitrogen/Life Technologies. Carlsbad, CA] in Hank's Balanced Salt Solution supplemented with 2.4μM $CaCl_2$). Specimens were then exposed to Abbott VP2000 Pre-Treatment Reagent (Abbott Laboratories, Chicago, IL) for one hour at 80°C, and then subjected to further digestion for one hour with Abbott Protease II Solution at 37°C. Labeled probes (100ng each) were combined with 25μg of sonicated dog total genomic DNA and applied to processed FFPE sections and denatured *in situ* at 80°C for five minutes. Hybridization was performed for 16 hours at 38°C, followed by a two minute wash in 0.4× saline-sodium citrate buffer (SSC)/0.3% IGEPAL (pH7) at 73°C and a one minute wash in 2 × SSC/0.1% IGEPAL (pH7) at ambient temperature. Image analysis was performed as described previously (Breen et al. 2004), using incremental capture (Smart Capture 3, Digital Scientific, Cambridge UK) of successive vertical planes to permit visualization of probe signals throughout the three-dimensional depth of each cell.

RESULTS

Genome-wide profiles of recurrent CNAs in canine hemangiosarcoma

The cohort of 75 hemangiosarcomas exhibited limited global DNA copy number instability, ranging from low amplitude imbalances of large chromosomal regions and entire chromosomes to infrequent, high amplitude aberrations of focal subchromosomal regions suggestive of structural rearrangement. A genome-wide penetrance plot of the frequency and relative distribution of CNAs within the cohort indicated that the global incidence of any

single CNA was low (Figure 1a). Of 317 discrete CNAs that were classed as recurrent (shared by ≥ 20% of cases), none were common to more than 45% of the cohort, and only ten were evident in ≥ 25% of cases (Supplementary Table 1). These 317 regions (164 gains and 153 losses) ranged in length from 4.5kb to 19.6Mb, but were heavily skewed toward small intervals (mean 654kb, median 241kb), with the vast majority (88%, 278/317 regions) smaller than 1Mb in size. Of the 317 recurrent CNAs, 125 overlap with natural copy number variants (CNVs) identified in prior surveys of healthy dogs (Chen et al. 2009; Nicholas et al. 2009), including all ten CNAs that were common to more than 25% of hemangiosarcoma cases. No recurrent high-level copy number amplifications or apparent homozygous deletions were detected within the cohort of 75 cases.

The most physical extensive CNAs present in ≥ 20% of the cohort were copy number gain along the full length of dog chromosomes 13 (CFA13), 24 and 31, and loss of CFA16 (Figure 1a). These events resulted in concomitant copy number imbalance of several cancer-associated genes with distinct biological relevance to hemangiosarcoma. Three such genes lie consecutively within a 754kb region of CFA13, namely the platelet-derived growth factor receptor gene *PDGFRA* (CFA13q21.3:49.7Mb, gained in 20% of cases), the *KIT* oncogene (CFA13q21.3:50.1Mb, gained in 27% of cases) and the *KDR* receptor tyrosine kinase gene (also known as *FLK1/VEGFR2*, CFA13q21.3:50.3Mb, gained in 28% of cases). The angiopoetin 1 gene *ANGPT1,* located proximally at CFA13q12.1:11.2Mb, was gained in 20% of cases. Additional members of the angiopoetin family were also contained within recurrent CNAs, specifically angiopoetin 2 (*ANGPT2*, CFA16q25.2:61.4Mb, 20% loss) and angiopoetin 4 (*ANGPT4*, CFA24q21:23.0Mb, 31% gain), and the angiopoetin-like 6 gene (*ANGPTL6*, CFA20q16:54Mb, 24% gain). A distinct peak of recurrent gain was evident on CFA12q13, corresponding to increased copy number of the vascular endothelial growth factor gene *VEGFA* (CFA12q13:15.2Mb) in 29% of cases, of which four cases (5% of the cohort) were consistent with a high level amplification. oaCGH detected deletion of the *CDKN2A/B* tumor suppressor gene region (CFA11q16:44.3Mb) in 28% of cases, while the *CDKN2A* interacting protein gene *CDKN2AIP* (CFA16q24:49.9) was deleted in 20% of cases. CNAs of other well-described tumor associated genes including *PDGFRB* (CFA4q32), *TP53* (CFA5q22), *MYC* (CFA13q13) and *PTEN* (CFA26q25) were not recurrent, occurring with < 20% penetrance within the cohort.

These data were interrogated further using GISTIC analysis to distinguish CNAs containing candidate genes as potential drivers of tumor pathogenesis from the background of generalized ‘passenger’ aberrations occurring at random. GISTIC identified 89 discrete CNAs whose amplitude and relative distribution indicated they were significantly unlikely to occur by chance alone (30 gains and 59 losses, G-score > 1.0, Q-bound value < 0.05, Figure 1b and Supplementary table 2). The broad genomic distribution of these regions suggests that the extensive recurrent gains of CFA13, 24 and 31, and losses of CFA16, are more consistent with passenger rather than driver aberrations. Of the 16 regions identified by GISTIC that share no apparent overlap with known natural CNVs, the most significant was a region on CFA12q13:15.1Mb that flanks the *VEGFA* gene (CFA12q13:15.2Mb, G-score = 13.9, Q-bound score = 5.83×10^{-7}). The second most significant (G-score = 13.6, Q-bound score = 2.44×10^{-5}) was deletion of a region on CFA11q16:44,3Mb that contains the *MTAP*, *CDKN2A* and *CDKN2B* loci, with the peak of significance localized at the *CDKN2A* locus.

Also of note was a significant peak of gain on CFA5q32:60Mb (G-score = 10.9, Q-bound score = 1.48×10^{-5}), detected in 21% of cases, which coincides with the *SKI* gene (v-ski sarcoma viral oncogene homolog). The combination of their penetrance in the population, accompanied by their significantly non-random relative distribution and amplitude and their biological function, supports the intimate association of disrupted dosage of *VEGFA, CDKN2A and SKI* genes with the disease phenotype.

FISH analysis of cases exhibiting high level amplification of *VEGFA* showed extensive heterogeneity in the number and distribution of *VEGFA* and *VEGFR2/KDR* probe signals within histological confirmed regions of tumor. The *VEGFA* probe frequently showed clusters of intense fluorescent signal suggestive of tandem duplication, while the *VEGFR2/KDR* probe showed only modest increase in copy number in cases showing gain of this region in oaCGH (Figure 2). Aberrant cells were typically interspersed with pockets of cells with grossly normal copy number, indicative of a highly heterogeneous cell population.

Comparison of CNA profiles in GRs with other breeds

oaCGH data were subcategorized by breed as either ‘GR’ (n = 40) or ‘other’ (n = 35) for initial assessment of evidence for breed-associated CNAs. This analysis demonstrated that global CNA profiles were broadly conserved in both groups as generalized characteristics of hemangiosarcoma, including gains of CFA13, 24 and 31, and loss of CFA16 (Figure 3a). The relative distribution of these CNAs showed subregional variation; for example, GRs exhibited recurrent deletion along the full length of CFA16, while in other breeds this was restricted primarily to the distal 75% of this chromosome. Similarly, while GR cases showed broad gain along both CFA20 and 31, in other breeds this was restricted to the distal 25% of these chromosomes. The peak in incidence of gain on CFA24 was proximal in GR cases (located at ~16Mb) versus distal in other breeds (~39Mb). Furthermore, the peak of recurrent gain on CFA12 around the *VEGFA* gene was diminished by almost half in GR cases (22% of cases) compared to those of other breeds (40%). Annotated below the CNA penetrance plots in figure 3a are the 188 discrete regions of significant difference between the two categories (Supplementary Table 3). Of these 188 regions, 50 coincide with known natural canine copy number variants (CNVs, and none overlap with cancer-associated genes shown to be involved in recurrent CNAs within the full cohort of 75 cases. The majority of these regions represented small genomic intervals (mean = 221kb, with 181/188 regions <1Mb in size and the remaining seven regions <2.4Mb in size) and were distributed widely throughout the genome. Figure 3a shows, however, that the incidence of CFA31 gain was significantly elevated in GRs within the region spanning CFA31q11-q15.1 (4.3–28.4Mb), reaching a maximum of 45% of GRs versus 20% of other breeds. An additional cluster of significant regions reflected the increased incidence of deletion on CFA16qprox in GRs versus other breeds.

Assessment of breed-associated CNA profiles

Similar principles were then applied to genome-wide CNA profiles of the ‘other breeds’ category, by examination of the oaCGH data contributed by each of the four component breeds (Figure 3b). The most remarkable differences were evident from comparison of ASD

tumors against all other cases. Hemangiosarcoma cases of this breed showed significantly elevated gain of both CFA24 (including *ANGPT4*) and 35, and loss of CFA33 and 37. Partial deletion of the distal half of CFA16 (including the *CDKN2A*-interacting protein *CDKN2AIP*), and gain of CFA31qdist (including the transcription factor gene *RUNX1*) and subregions of CFA13 (including *KIT*, *KDR* and *PDGFRA*), were also significantly elevated in this breed. Interestingly ASD cases exhibited significantly increased incidence of gain both of the platelet-derived growth factor gene *PDGFA* (CFA6q15, 30% of cases) and of the receptor tyrosine kinase gene *PDGFRA* (CFA13q21.3, 50% of cases), relative to other breeds. The incidence of *PDGFA* gain was also significantly elevated in the GSD, along with subregional gain along CFA6qprox and CFA20qdist, and partial deletions of CFA7 and 38. FCR cases exhibited a significant peak of gain flanking *VEGFA* on CFA12q13. While BMD cases also showed significantly elevated CFA12qprox gain, this region was located ~4Mb proximal to the *VEGFA* locus; however these cases showed a significant association with deletion of CFA5qprox and gain of CFA36qdist. Figure 4 summarizes the CNA frequencies for genes discussed in the text both within the full cohort and in each breed, and additional details are provided in Supplementary Table 4.

DISCUSSION

Genome-wide profiles of copy number imbalance in canine hemangiosarcoma

Somatic DNA copy number profiling of 75 canine hemangiosarcomas revealed no highly recurrent CNA signatures that might be considered hallmarks of this cancer. Rather, hemangiosarcoma exhibits a relatively low level of global CNA compared to the extensive aneuploidy evident in other canine sarcomas evaluated using similar techniques (Angstadt et al. 2011; Hedan et al. 2011; Thomas et al. 2011), with remarkably few aberrations exceeding 20% penetrance. This is likely a consequence of the high degree of cellular heterogeneity associated with hemangiosarcoma, which was evident from FISH analysis in the form of discrete pockets of tumor interspersed with extensive regions of grossly normal stromal tissue. Targeted FISH analysis of high-level amplification events also demonstrated substantial cell-to-cell variation in both probe copy number and distribution within individual cases, suggestive of variable, complex structural rearrangement through multiple rounds of chromosome breakage and fusion, as is typical of solid tumors (for example, Thompson and Compton 2011).

Despite macrodissection of fixed tissue specimens to enrich for malignant cell populations it is highly likely that residual microscopic heterogeneity would result in attenuation of CNA penetrance due to contaminating stromal DNA. The variable degree of aneuploidy apparent between individual cases may be associated in part with differential progression rates and stromal involvement; however, the extensive range in the number and distribution of CNAs evident within the cohort, despite their histomorphologic characteristics, supports prior transcriptional studies suggesting the existence of several related but molecularly distinct forms of hemangiosarcoma (Dickerson et al. 2005; Tamburini et al. 2009; Tamburini et al. 2010).

Recurrent imbalance of extensive genomic regions in hemangiosarcoma was restricted primarily to gain of CFA13, 24 and 31, and loss of CFA16. Increased copy number of

CFA13 is recurrent in a diverse range of canine cancers, including non-Hodgkin's lymphoma, appendicular osteosarcoma, leukemia, glioma and histiocytic sarcoma (Thomas et al. 2009; Angstadt et al. 2011;Becker et al. 2011;Hedan et al. 2011; Thomas et al. 2011). Similarly, the incidence of CFA31 gain in hemangiosarcoma was comparable to that of a broad range of canine cancers studied to date, including appendicular osteosarcoma (20–35% of cases) (Angstadt et al. 2012; Karlsson et al. in press), glioma (35% of cases) (Thomas et al. 2009) and B-cell non-Hodgkins lymphoma (~20% of cases) (Thomas et al. 2011). The present data therefore add to evidence that gains of CFA31, and of CFA13 in particular, both represent generalized CNAs rather than hallmarks of specific tumor subtypes. CFA24 gain is less widely evident in prior reports of canine cancers, but occurs in hemangiosarcoma at similar frequency to that of appendicular osteosarcoma (30–45% of cases, Angstadt et al. 2012; Karlsson et al. in press), histiocytic sarcoma (20–30% of cases, Hedan et al. 2011) and glioma (~30% of cases, Thomas et al. 2009). In contrast, among published studies to date, recurrent loss of CFA16 has been reported only in histiocytic sarcoma (~70% of cases, Hedan et al. 2011), T-cell non-Hodgkins lymphoma (~20% of cases, Thomas et al. 2011) and appendicular osteosarcoma (50–70% of cases, Angstadt et al. 2012; Karlsson et al. in press), suggesting that this CNA may be a characteristic of the broader category of solid tumors defined as sarcomas.

GISTIC analysis revealed a significant peak of deletion in hemangiosarcoma on CFA11q16 encompassing *CDKN2A/B* and *MTAP*, with maximum significance coinciding with the *CDKN2A* gene, highlighting this locus as a potential driver of tumor development. Several additional dog cancers with recurrent CFA11 deletion also show a focal peak in penetrance at CFA11q16, similarly indicative of a fundamental role for this locus in tumor pathogenesis (for example, T-cell non-Hodgkins lymphoma (Thomas et al. 2011), appendicular osteosarcoma (Angstadt et al. 2012; Karlsson et al. in press) and histiocytic sarcoma (Hedan et al. 2011). Like hemangiosarcoma, canine histiocytic sarcoma is a highly aggressive and locally invasive cancer with rapidly fatal clinical course, frequently manifesting in a disseminated form with multiple organ involvement, and with a strong breed predilection. Interestingly the *MTAP and CDKN2A/B* region at CFA11q16 lies within the major germline risk haplotype for histiocytic sarcoma in the BMD (Shearin et al. 2012). Furthermore, a germline risk haplotype for appendicular osteosarcoma has now been mapped to the same region in the Greyhound (Karlsson et al. in press). This direct association between germline risk and somatic aberration provides strong support for the fundamental involvement of *MTAP* and/or *CDKN2A/B* dysregulation as a driver of these disease phenotypes. Interestingly, however, despite recurrent deletion of the CFA11q16 locus in hemangiosarcoma (28% of all cases, and 30% of all GRs), a recent genome-wide association study of germline risk factors in the GR (Tonomura et al. submitted) shows no significant overlap with somatic CNA. Although 21/75 hemangiosarcoma cases exhibited deletion of the *CDKN2A/B* region, only two cases were consistent with homozygous deletion. While this may be a consequence of stromal contamination, the relative peak in CNA penetrance at this site relative to its flanking regions was less distinct in hemangiosarcoma than in osteosarcoma, histiocytic sarcoma or T-cell lymphoma, in which homozygous deletion is frequent (Angstadt et al. 2012;Hedan et al. 2011; Thomas et al. 2011). This suggests that CFA11 may harbor other major gene targets for hemangiosarcoma pathogenesis. The

extension of these genome-wide association studies to additional purebred dog populations will reveal whether these risk haplotypes are more closely linked to the disease phenotype or to genetic ancestry. Moreover, many recurrent CNAs overlap with CNVs identified in prior studies of clinically healthy dogs (for example Chen et al. 2009; Nicholas et al. 2009). There is growing evidence for the involvement of these natural polymorphisms in a variety of human diseases, including cancer (for example, Fanciulli et al. 2010; Shlien and Malkin 2010). CNVs are less well characterized in dogs, however, and thus at present there is insufficient evidence to permit any association between specific natural polymorphisms and the disease phenotype.

GISTIC also identified *VEGFA* as a likely target for the recurrent subregional gain on CFA12q13, consistent with prior transcriptional studies supporting the role of this growth factor and associated receptors in malignant proliferation of hemangiosarcoma (Yonemaru et al. 2007;Tamburini et al. 2009;Tamburini et al. 2010). A previous study (Yonemaru et al. 2006) demonstrated elevated mRNA and protein expression of *VEGFA* and its receptors *VEGFR1/Flt-1* and *VEGFR2/Flk-1/KDR* in malignant cells of hemangiosarcoma, compared to benign hemangioma. In the present study genomic gain of *VEGFR2/Flk-1/KDR* occurred in 28% of all cases, in contrast to *VEGFR1/Flt-1* gain in only 8% of cases. This is consistent with the generalized description of the former as the major mediator of the angiogenic effects of *VEGFA*, although the distinction may be somewhat more complex (Tamburini et al. 2009). Aberrant upregulation of *VEGFA* in hemangiosarcoma has also been associated with the presence of inactivating point mutations and deletions within the *PTEN* tumor suppressor gene on CFA26q25, resulting in unregulated endothelial cell growth and angiogenesis (Dickerson et al. 2005). While *PTEN* deletion is highly recurrent in other dog sarcomas (Angstadt et al. 2011; Hedan et al. 2011; Thomas et al. 2011), only 5% of hemangiosarcoma cases (4/75) showed copy number loss of this locus, suggesting that genomic deletion does not constitute an alternative mechanism for loss of *PTEN* function in this disease. GISTIC analysis identified the *SKI* oncogene as a strong candidate for the target of the CFA5q32 gain in 21% of hemangiosarcoma cases. Upregulation of *SKI,* a negative regulator of the TGFβ signaling pathway, has been reported in a diverse range of human cancers, promoting tumor growth and induction of angiogenesis and conferring a negative prognosis (reviewed in Bonnon and Atanasoski 2012). Moreover, elevated *SKI* expression has been identified in human pediatric hemangiomas, with the strongest expression evident in the most actively proliferating of these benign endothelial cell tumors (O et al. 2009). Transcriptional regulation of the human *SKI* gene is not yet well understood, and the mechanism by which *SKI* dysregulation occurs in malignant cells is also unclear (Bonnon and Atanasoski 2012). To our knowledge there are no prior studies of the *SKI* gene in canine tumors, and definition of its potential relevance will therefore require focused attention in future investigations.

Assessment of differential CNA profiles in GR hemangiosarcoma

Subclassification of the cohort by breed permits the first insight into the relationship between somatic DNA copy number aberrations and genetic background in canine hemangiosarcoma. Comparison of all GR cases against those of the four other selected breeds showed that CNA profiles are broadly conserved between these subcategories, but

revealed that GRs exhibit significantly more frequent gain of CFA31 and subregional loss on CFA16qprox, as well as a contrasting profile of copy number increase on CFA24. Of particular note was the reduced incidence of *VEGFA* gain in GRs (22% of GRs versus 40% of cases from the four other breeds). Prior studies have indicated that transcriptional profiles of hemangiosarcoma in the GR are distinct from those of other breeds, highlighting differential involvement of members of the *VEGF* pathway. Tamburini et al. (2009) reported elevated mRNA and protein expression of *VEGFR1/Flt-1* in GR hemangiosarcoma, not as a consequence of gross elevated gene expression but rather through enrichment of *VEGFR1/Flt-1* in conjunction with other functionally related signaling molecules. Targeted inhibition of *VEGFR1/Flt-1* increased tumor proliferation in GR hemangiosarcoma, but was ineffective in other breeds. Conversely, immunohistochemical staining of hemangiosarcoma cell lines from non-GRs showed elevated *VEGFR2/Flk-1/KDR* expression compared to GR cells (Tamburini et al. 2009). These observations were not reinforced by significant differences in the DNA copy number profiles of either *VEGF* receptor when comparing GR tumors with other breeds in the present study. The identification of *VEGFA* as a potential driver of tumor development, coupled with the almost two-fold reduction in *VEGFA* gain in GR tumors and the highly variable incidence of both *VEGFA* and *VEGFR2/Flk-1/KDR* gain in other breeds, however, continues to highlight the fundamental relevance of this pathway. In recent years, critical advances in human medicine have yielded several monoclonal antibodies and tyrosine kinase inhibitors of the VEGF pathway, with others showing promise in clinical trials (Saharinen et al. 2011). Of particular note, a recent phase II trial showed bevacizumab, a recombinant humanized antibody against VEGF, to be safe and effective for the treatment of angiosarcoma (Agulnik et al. 2012). The increasing volume of evidence for an intimate association between hemangiosarcoma pathogenesis and the VEGF pathway reinforces the urgency for further investigation of therapeutic options in veterinary medicine, and raises the possibility for breed-associated strategies for clinical management.

Emerging evidence for breed-associated CNA profiles in hemangiosarcoma

The inclusion of cases from four non-GR breeds enables the first glimpse into the relationship between genetic ancestry and somatic DNA copy number imbalance in hemangiosarcoma. FCR cases showed the most compelling evidence for intimate association between *VEGFA* copy number gain and tumor pathogenesis, but were otherwise globally consistent with observations across the entire cohort. Significantly elevated CFA20qdist gain was revealed in GSD cases, while BMD cases exhibited elevated CFA5 imbalance. ASD cases demonstrated the most complex aberration profiles, with a series of marked deviations away from the global penetrance profiles of the full cohort. The global recurrence of CFA16 deletions and gains of CFA24 shown in figure 1a are strongly driven by the ASD, which in turn exhibits significantly elevated incidence of several genes that are fundamental to tumor pathogenesis (Figure 4). Among these are members of the angiopoietin gene family, which are central to the regulation of angiogenesis, endothelial cell survival, proliferation and migration, and which have been implicated in angiosarcoma development in human patients (Brown et al. 2000), The products of the *ANGPT1* (CFA13q12.1) and *ANGPT4* (CFA24q21) genes act as ligands for the angiopoetin tyrosine kinase receptor Tie2, which is expressed on vascular endothelium, promoting endothelial cell survival and proliferation and blood vessel stability. Conversely, the *ANGPT2* gene product (CFA16q25.2) generally acts

as an antagonist, promoting endothelial cell death and regression of blood vessels (Cascone and Heymach 2012). It is interesting to note, therefore, that the pro-angiogenic factors *ANGPT1* and 4 each lie on chromosomes exhibiting recurrent genomic gain in hemangiosarcoma (CFA13 and 24), with a significantly increased incidence of *ANGPT4* in the ASD (70% of ASD cases versus a mean of 31% in other breeds). In contrast, the typically anti-angiogenic *ANGPT2* ligand lies on CFA16, the chromosome with highest incidence of global deletion, but showed a low incidence of copy number loss across the cohort (20% of all cases). Promising results are now emerging using various inhibitory factors for regulation of angiopoetins in a wide range of human cancers (Cascone and Heymach 2012). The results of the present study suggest that these principles may be applicable to hemangiosarcoma, potentially with breed-associated efficacy. Despite global conservation in their CNA profiles there is, therefore, emerging evidence also for additional breed-associated CNAs in hemangiosarcoma outwith the GR.

Canine hemangiosarcoma as a potential model for human angiosarcoma

Several reports have proposed hemangiosarcoma as a naturally occurring model of human angiosarcoma, principally based on commonalities in their clinicopathologic features (for example, Tamburini et al. 2010;Andersen et al. 2013;Dickerson et al. 2013). Angiosarcoma, a highly vascular endothelial neoplasm, commonly arises *de novo*, but may also manifest as a secondary tumor subsequent to radiation therapy or in association with chronic lymphedema (for example Roy et al. 2004). Both forms share grossly comparable clinical behavior and histomorphology (Billings et al. 2004). As with canine hemangiosarcoma, options for effective clinical management of angiosarcoma are limited, and the prognosis is typically poor (~30% overall five-year survival (Fury et al. 2005). The genomic basis of angiosarcoma remains poorly understood, in large part due to its relative rarity, comprising just 1–2% of the ~10,500 new soft-tissue sarcoma cases reported each year in the US (Baumhoer et al. 2005; Antonescu et al. 2009; Jemal et al. 2010; Lahat et al. 2010). The relative rarity of angiosarcoma will inevitably restrict resources for evaluating the efficacy of novel therapeutic agents in human subjects and for clinically predictive patient stratification. The extensive repository of clinical materials and data associated with the high incidence of hemangiosarcoma in defined purebred dog populations could represent an opportunity to address limitations resulting from the infrequency of this cancer in human populations. This concept has been supported by several studies that demonstrate related tumor-associated transcriptional profiles and gene-inactivating sequence mutations of key pathways associated with angiogenesis in angiosarcoma and hemangiosarcoma, and commonalities in the potential involvement of specific micro-RNA elements (Dickerson et al. 2005; Dews et al. 2006; Yonemaru et al. 2006; Yonemaru et al. 2007; Antonescu et al. 2009; Tamburini et al. 2009; Sarver et al. 2010; Tamburini et al. 2010; Guo et al. 2011; Taylor et al. 2011; Italiano et al. 2012a; Italiano et al. 2012b). The findings of the present study afford the first opportunities to establish whether the similarities between angiosarcoma and hemangiosarcoma extend to their CNA profiles. Comparison of these canine data with 18 angiosarcoma profiles generated using an oaCGH platform with comparable resolution (Italiano et al. 2012b) demonstrates that both tumors present with a relatively low degree of aneuploidy, with few individual CNAs exhibiting >20% penetrance. High-level amplification of the *MYC* locus on human chromosome 8q24.21:128.8Mb occurs in 50–60% of

angiosarcoma cases, representing one of few consensus chromosomal aberrations to be identified in this cancer. This CNA is associated with concomitant transcriptional upregulation of the *MYC* oncogene, resulting in downstream inhibition of angiogenesis (Dews et al. 2006; Manner et al. 2010; Guo et al. 2011; Italiano et al. 2012b). In the present study, increased copy number of *MYC* occurred in only 17% of canine hemangiosarcoma cases, and exceeded 20% penetrance in only two breeds, the ASD (30%) and BMD (33%). This event was associated almost exclusively with gain of a broad interval on CFA13, and only a single case exhibited high level focal *MYC* amplification. This may in part reflect the nature of the canine cohort as primary tumors, since *MYC* amplification has historically been regarded as a hallmark of secondary angiosarcoma (Manner et al. 2010; Guo et al. 2011). A more recent, high-resolution oaCGH study has since demonstrated that *MYC* amplification is also recurrent in *de novo* human angiosarcoma, and revealed significant conservation of genomic profiles in primary and secondary cases, blurring their distinction at a molecular level (Italiano et al. 2012b). Aside from lack of conservation in *MYC* copy number status, global comparison of data from the present study with human angiosarcoma data from (Italiano et al. 2012b) indicates negligible overlap in their CNA profiles. It seems likely that the apparent contrast in the molecular characteristics of hemangiosarcoma and angiosarcoma may be driven by the relative anatomical distribution of tumors in these populations. Angiosarcoma arises in a variety of skin, soft tissue or visceral locations but occurs most commonly as a cutaneous lesion (Lucas 2009) while the majority of canine cases manifest in the abdominal or thoracic cavities (Bergman 2010). The 18 angiosarcoma cases evaluated by Italiano et al. (2012b) represent a combination of both cutaneous and soft-tissue tumors from a broad range of anatomical locations, which precludes the ability to perform more focused comparisons with canine genomic data. Comparisons of histopathologic morphology showed that the visceral hemangiosarcoma specimens in the present study were grossly indistinguishable from human anaplastic angiosarcoma arising from non-cutaneous sites, while angiosarcoma arising from the skin or breast parenchyma typically exhibited more well-differentiated features (Italiano et al. 2012b). It is therefore possible that greater cross-species conservation in CNA profiles will be evident through comparison of canine cutaneous hemangiosarcoma with human angiosarcoma. Cutaneous hemangiosarcoma lesions, while globally infrequent, are prevalent in several dog breeds with short hair and limited or variable skin pigmentation, such as the Whippet and Dalmation (Hargis et al. 1992; Schultheiss 2004). The extension of cytogenomic studies to cases from these breeds will clarify the influence of genetic background on molecular profiles in hemangiosarcoma. In the meantime, the results of the present study indicate the need for more extensive assessment of the global validity of the canine system as a relevant model for human angiosarcoma biology.

Conclusion

Visceral hemangiosarcoma constitutes a tremendous challenge for veterinary practitioners due to its aggressive and rapid clinical course, limited treatment options and heterogeneity in anatomical location and intratumoral histomorphology. Through genomic profiling of 75 primary cases we demonstrate that the clinicopathologic heterogeneity of canine hemangiosarcoma is recapitulated by extensive variation in the distribution of non-random somatic DNA copy number aberrations, both within and between individuals and distinct

genetic populations. These data reveal recurrent gene dosage imbalances with direct biological relevance to tumor pathogenesis that offer potential for therapeutic modulation of discrete pathways involved in tumor development. This in turn provides the first opportunity for comparative assessment of genome-wide somatic DNA copy number aberrations in canine hemangiosarcoma and clinicopathologically similar human angiosarcoma. Both present with limited aneuploidy but there is little conservation in their genomic profiles, indicating the need for further assessment of the relevance of the dog as a model for the human disease. Our findings provide an insight into the relationship between somatic DNA copy number profiles and genetic ancestry through parallel evaluation of cases from five dog breeds with elevated predisposition to hemangiosarcoma. The high degree of intra- and intertumoral heterogeneity in CNA profiles indicates a need for extension to larger cohorts and additional breeds for refining evidence for the existence of discrete molecular subtypes.

These findings contribute toward an expanding catalogue of complementary genomic resources directed towards improved strategies for early detection and effective clinical management of canine hemangiosarcoma, which will also aid comprehension of its underlying pathogenesis and validity as a model for human angiosarcoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ASD	Australian Shepherd Dog
BAC	bacterial artificial chromosome
BMD	Bernese Mountain Dog
CFA	*Canis familiaris*
CNA	copy number aberration
CNV	copy number variant

FCR	Flat-Coated Retriever
FFPE	formalin-fixed, paraffin-embedded
FISH	fluorescence *in situ* hybridization
GR	Golden Retriever
GSD	German Shepherd Dog
H&E	hematoxylin and eosin
oaCGH	oligonucleotide array comparative genomic hybridization
SSC	saline-sodium citrate

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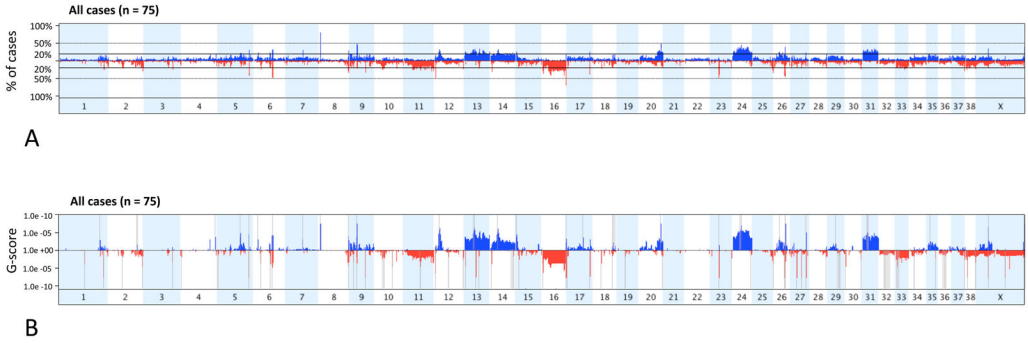

Figure 1.

A) Penetrance plot of recurrent CNAs identified within the cohort of 75 hemangiosarcoma cases. Genomic locations are plotted along the x-axis. The y-axis indicates the percentage of the corresponding cohort that demonstrated either copy number gain (shown in blue above the midline) or loss (shown in red below the midline) of the corresponding chromosome region. The horizontal bars immediately above and below the midline indicate the 20% cutoff used to define recurrent CNAs. B) GISTIC analysis of CGH profiles from all 75 cases identified 89 discrete CNAs whose amplitude and relative distribution was significantly increased above background levels (G-score > 1.0, Q-bound > 0.05). The peak region of significance is highlighted in dark grey, flanked by a broader region of reduced significance shown in pale grey. Genomic locations are plotted along the x-axis, and G-scores for statistical significance are shown on the y-axis (larger bars indicate higher significance).

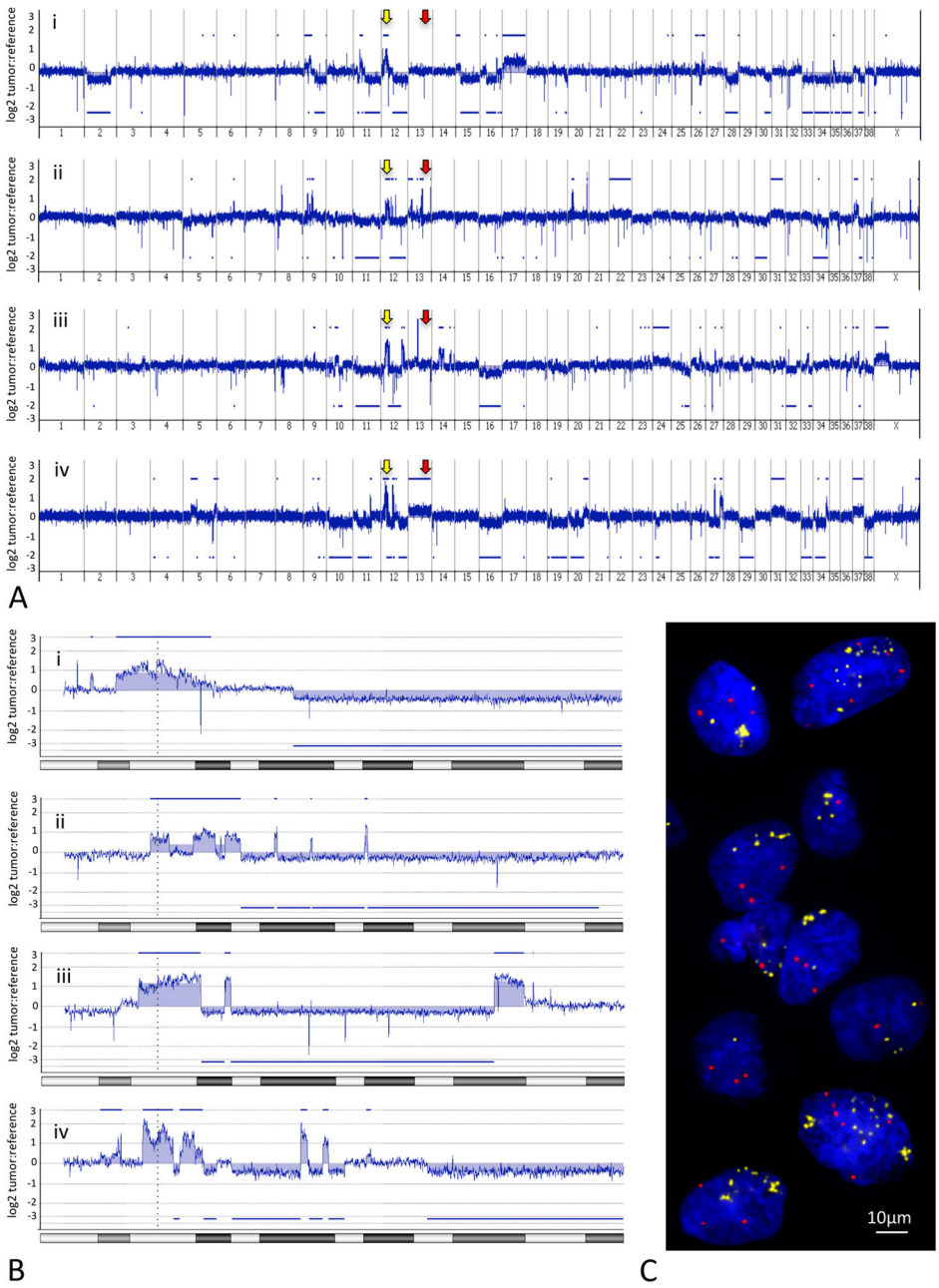

Figure 2.

Hemangiosarcoma cases exhibited *VEGFA* amplification detected by oaCGH and FISH. A) Genome-wide oaCGH profiles for each of four cases with *VEGFA* amplification (denoted i through iv) demonstrating the extensive variation in the number, size and distribution of CNAs between cases, which are indicated by horizontal lines above (gain) or below (loss) the midline. The chromosomal locations of the *VEGFA* and *VEGFR2/KDR* genes are indicated by yellow and red arrows, respectively. All four cases show a discrete amplification at the *VEGFA* locus on CFA12q13. The *VEGFR2/KDR* locus is balanced in all cases except case [iv], which shows a low-amplitude copy number gain. Case [iii]

exhibited the sole example of *MYC* amplification, evident as the spike of high amplitude copy number increase on CFA13q13, proximal to the *VEGFR2/KDR* locus. B) Enlarged view of the CGH profile of CFA12 in each case, indicating *VEGFA* amplification (dotted line). Additional disruption in copy number along the length of the chromosome is suggestive of multiple structural rearrangements. C) FISH analysis revealed clusters of probe signal consistent with tandem duplication of the *VEGFA* locus (yellow signal). The number and distribution of *VEGFA* probe signals varied even within the same field of view, indicative of extensive heterogeneity in cell populations and stromal contamination. FISH analysis of *VEGFR2/KDR* (red signal) was grossly normal in all but case [iv, pictured], which showed a minor increase in copy number consistent with oaCGH analysis.

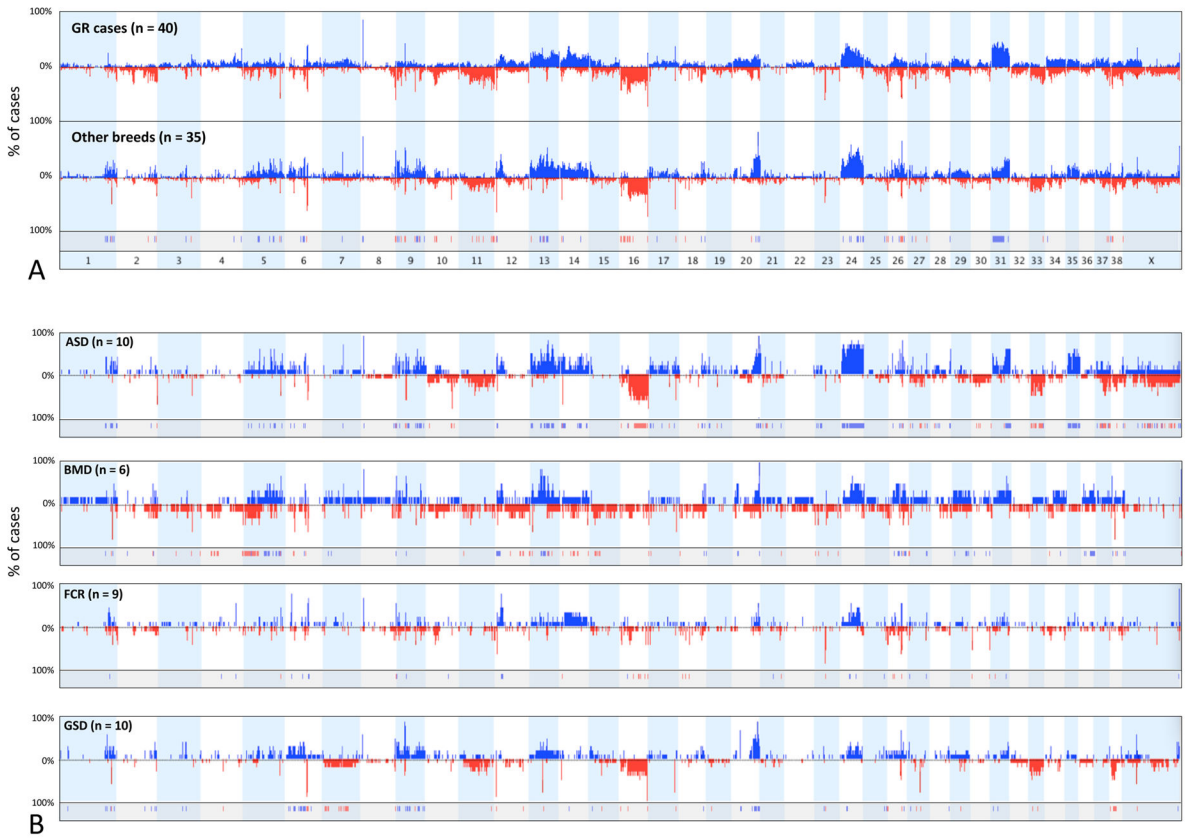

Figure 3.

A) Penetrance plot of recurrent CNAs in GR hemangiosarcoma (n = 40) compared with 'other breeds' (n = 35). Comparison of these data identifies discrete regions of significant difference in their CNA profiles. These regions are denoted by red or blue bars below the upper panel that shows the relative difference in CNA frequency between the two groups. B) CNA penetrance plots of each non-GR breed group, annotated to show regions of significantly different CNA identified within each breed when compared sequentially against the mean penetrance within all other cases. The highly penetrant gain on CFA8 coincides with the dog T cell receptor alpha chain immunoglobulin domain, and is evident as an apparent CNA due to the rearrangement of this region in the blood-derived reference DNA.

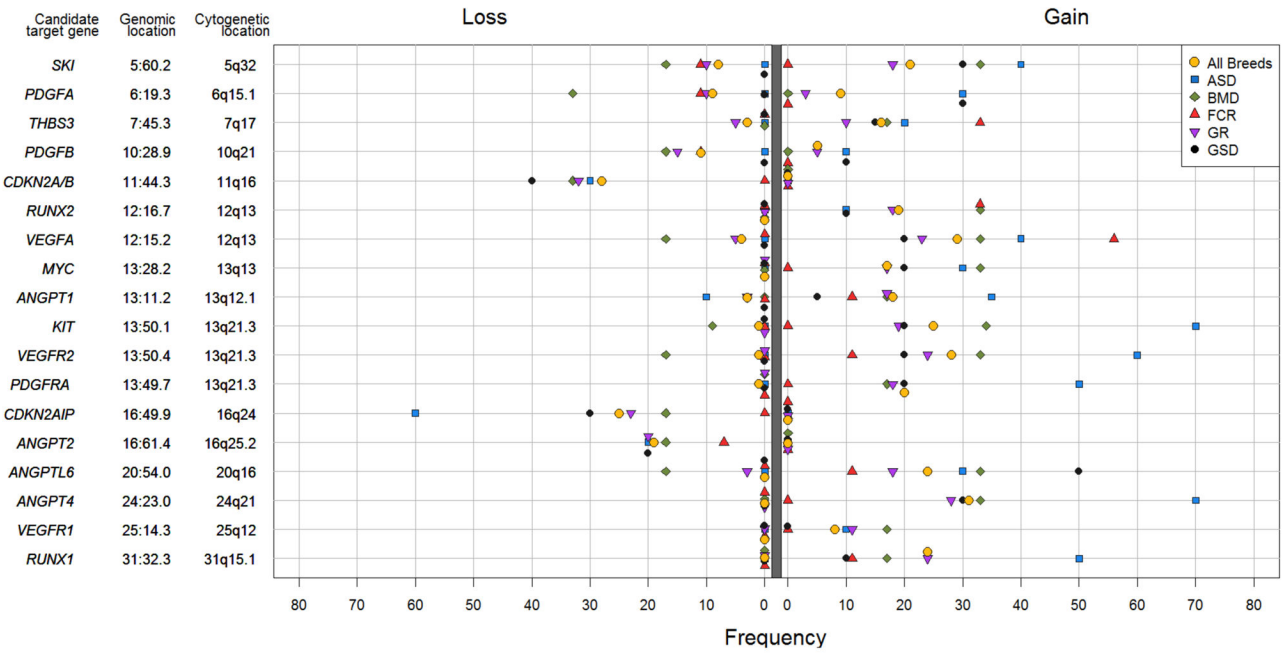

Figure 4.

Forest plot summarizing penetrance data for genes involved in recurrent CNAs identified within the cohort of 75 hemangiosarcoma cases, and within each breed. Genes described in the text are ordered on the vertical axis according to their physical location in the dog genome. The frequency of gain and loss of each locus in each breed is shown on the horizontal axis as a percentage of that population, in addition to the frequency in the entire cohort. Asterisks indicate CNA penetrance values that deviate significantly in one breed compared to all other breeds (Fishers Exact test, $p < 0.05$).