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Novelty and Impact :

Due to its rarity, histiocytic sarcoma (HS) is not well known and treatment remains a challenge. Using the canine sponteanous model, we highlighted the association of *PTPN11* somatic mutations to a disseminated subtype of HS in humans and dogs. Thanks to in-house canine HS cell lines, we tested MAP kinase inhibitors as a proof of concept of the interest of this model for the development and selection of efficient therapies.

List of abbreviations

HS: Histiocytic Sarcoma

MH: Malignant Histiocytosis

BMD: Bernese Mountain Dogs

LOH: Loss Of Heterozygozity

ctDNA: cell free circulating tumor DNA

Abstract

In humans, histiocytic sarcoma (HS) is an aggressive cancer involving histiocytes. Its rarity and heterogeneity explain that treatment remains a challenge. Sharing high clinical and histopathological similarities with human HS, the canine HS is conversely frequent in specific breeds and thus constitutes a unique spontaneous model for human HS to decipher the genetic bases and to explore therapeutic options. We identified sequence alterations in the MAPK pathway in at least 63.9% (71/111) of HS cases with mutually exclusive *BRAF* (0.9%; 1/111), *KRAS (*7.2%; 8/111) and *PTPN11* (56.75%; 63/111) mutations concentrated at hotspots common to human cancers. Recurrent

PTPN11 mutations are associated to visceral disseminated HS subtype in dogs, the most aggressive clinical presentation. We then identified *PTPN11* mutations in 3/19 (15.7%) human HS patients. Thus, we propose *PTPN11* mutations as key events for a specific subset of human and canine HS: the visceral disseminated form. Finally, by testing drugs targeting the MAPK pathway in eight canine HS cell lines, we identified a better anti-proliferation activity of MEK inhibitors than PTPN11 inhibitors in canine HS neoplastic cells. In combination, these results illustrate the relevance of naturally affected dogs in deciphering genetic mechanisms and selecting efficient targeted therapies for such rare and aggressive cancers in humans.

Introduction

Histiocytic sarcomas (HS), also referred as Malignant Histiocytosis (MH), are rare tumors characterized by proliferation of cells with the phenotype of mature tissue histiocytes[1]. Half of the cases occur in the context of a previous lymphoid or myeloid neoplasm, while the other half are considered as primitive proliferation of histiocytes[1]. This aggressive tumor leads to a high mortality and currently there is neither consensus on prognostic factors, nor on standard treatment. Recent publications showed that the MAPK pathway, with *BRAF* alterations, plays a crucial role in the development of HS[2–6]. Drugs targeting this pathway such as vemurafenib or sorafenib have shown clinical responses in HS cases[7,8]. The rarity and heterogenous nature of this cancer poses significant challenges to advancing knowledge, limiting opportunities to identify recurrent alterations with driver roles and aiding selection of targeted therapies.

Interestingly, this tumor type, occurring naturally and well-characterized in the domestic dog, is especially prevalent (up to 20%) in some popular predisposed breeds, including Bernese mountain dogs (BMD), rottweilers and retrievers[9–11]. Genetic predispositions in these breeds have led to the availability of many naturally affected cases with homogeneous clinical presentation in a specific canine breed[9,10]. Indeed, BMDs more frequently display disseminated HS with simultaneous involvement of

multiple internal organs at time of diagnosis, whereas retrievers more often develop localized HS with tumor initially restricted to joint, muscle or subcutaneous tissue. Using this unique resource to characterize HS, we identified recurrent gain-of-function mutations in the MAPK pathway in canine and then in human HS. The *PTPN11* mutations - the main recurrent alterations in canine HS (56.75%; 63/111)- are associated with the visceral disseminated canine HS subtype. We thus proposed that *PTPN11* mutations are also characteristic of a visceral disseminated form of human HS. By developing seven canine HS in-house cell lines, we tested drugs targeting directly *PTPN11* or the MAPK pathway and confirmed the antitumor activity of MEK inhibitors in canine HS neoplastic cells, illustrating the potential of the dog model to screen drugs to improve therapies of this rare and aggressive cancer.

Material & Method

Sample collection

Blood and tissue biopsy samples from dogs were collected by veterinarians through the Cani-DNA BRC (http://dog-genetics.genouest.org) and DNA/RNA was extracted as previously described[12]. Blood was also collected on Cell-free DNA BCT (Streck, La Vista, Nebraska, USA) and cell-free circulating DNA was extracted with NucleoSnap® DNA Plasma (Macherey Nagel, Düren, Germany) according to the manufacturer protocol. The canine HS cases were diagnosed by histopathological analysis (JA) with adequate immunostaining (CD204 SRA-E5 Abcam MAB1710).

Seventeen human DNA samples were collected through the French Histiocytosis Registry, approved by the Comité de Protection des Personnes Ile de France III (#2011-A00447-34) over a fifteen-year period (2003-2018). Diagnosis of primary HS was established according to the classification of histiocytosis[1] and cases were independently reviewed by experienced human pathologists. DNA was extracted either from FFPE tissues or from snap frozen tissue biopsies samples, part of them by the Department of Pathology (APHP University Hospital Ambroise Paré, Boulogne, France) thanks to Jean-Francois Emile. Two additional cases were obtained from the literature[13](Table 1).

Sequencing

RNA-Seq and Sanger sequencing were performed on canine samples as previously described[12]. For all mutations, their somatic status was assessed with the corresponding constitutional DNAs. Human DNA libraries for *PTPN11* sequencing were prepared by targeted enrichment and amplicon tagging (Access array barcode - Fluidigm, San Francisco, USA). When high quality and quantity DNA samples were available, *BRAF* and *KRAS* hot spots were also amplified. The libraries were sequenced by MiSeq following

Illumina's protocol (sequencing platform GEH, Biosit, Rennes). Primers are available in Supplementary Table 1.

Cell lines

The 8 cell lines include one commercial cell line (DH82 (ATTC, CRL-10389 ; RRID:CVCL_2018) and seven cell lines developed in the team from HS affected dog fresh tissues (supplementary table 2). These seven cell lines are available on request. Cells were cultivated in a complete RPMI medium containing RMPI 1640 GlutaMAX supplemented medium (Gibco. life technologies) with 10% of Fetal Bovine Serum (HyClone, GE Healthecare, Life ScienceS, Logan, USA) and 0,025% of Primocin (InvivoGen, Toulouse France) at 37 °C in a humidified 5% CO2 incubator. All cell lines were tested for mycoplasma with MycoAlert™ Plus kit (Lonza; Rockland; ME; USA) and were mycoplasma-free cells.

Drug tests on canine HS cell lines

We tested four drugs targeting the MAPK pathway: U0126 (Promega Co., Madison, USA), GS-493, kindly provided by Dr Birchmeier and Dr Land, Sorafenib (Santa Cruz Biotechnology Inc., Dallas, USA) and Trametinib (Active Biochemicals Co., Hong Kong). Stock solutions of drugs were made in DMSO. The cells were cultured for 24h in 96-well plates and the drug or the DMSO alone was then added for additional 72H. Six biological replicates were used for each drug concentration tested, and 12 replicates for the DMSO controls. Cell proliferation was evaluated by methylene blue assays. A Dixon test was performed for each replicate and the proliferation rate was normalized with the DMSO control for each cell line. IC25s were fitted by non-linear regression (Weibull equation, maximum 1, minimum estimated) using the GraphPad Prism version 6.00 (GraphPad Software, La Jolla California USA).

Statistical analyses

Statistical analyses were performed using the R software (Vienna, Austria). Fisher's Exact tests were used to evaluate the association between *PTPN11* mutations and clinical parameters, Wilcoxon Mann Whitney test was used to compare IC25 distribution between homozygous and heterozygous *PTPN11* mutated cell lines. Values of $p < 0.05$ were considered statistically significant.

Data availability

RNA-seq data are available at European Nucleotide Archive (accession number: PRJEB36828). Other data will be made available upon reasonable request.

Material & Method for the primer design, the Western-blots, the Cytogenetic and LOH analyses are available in Supplementary Material & Method.

Results

To characterize somatic alterations in histiocytic sarcomas, we performed RNA-Seq on three canine HS cases and the commercial cell line DH82 and we identified two somatic mutations in *PTPN11*. These two mutations are homologous to the human *PTPN11* E76K and G503V mutations occurring in hotspot regions of this gene in human cancers (COSMIC database)[14]. To estimate the frequency and analyze the relevance of *PTPN11* mutations, and more widely of the MAPK pathway involvement in HS oncogenic events, we searched for the presence and recurrence of somatic mutations in *PTPN11*, *KRAS*, *NRAS*, *HRAS*, *BRAF* and *CBL* in all 111 canine HS cases. All tumors except one were found wild-type for *BRAF*. This *BRAF*–mutated case presented two mutations orthologous to human S465T and G469A *BRAF* mutations. We found *PTPN11* and *KRAS* somatic mutations in 56,7% (63/111) and 7.2% (8/111) of cases respectively, indicating alterations of the MAPK pathway in 63.9% of canine HS (Figure 1 and Supplementary Table 2). These mutations were mutually exclusive in all but one case, a composite heterozygous case. We found that *PTPN11* mutations were enriched, but not significantly, in the BMD breed (58.8% vs 47.6% p-value=0.46, Fisher's Exact test), in

agreement with the recent identification of the *PTPN11* mutations in canine HS associated with BMD[15]. In our large canine cohort, we identified an association between *PTPN11* somatic mutations and the internal localization of tumors or HS disseminated forms (p-value=$1.27x10^{-4}$, and p-value=$1.6x10^{-6}$ respectively, Fisher's Exact test). These results indicate that *PTPN11* mutations are associated with the aggressive form of disseminated HS rather than the localized HS forms in dogs.

These mutations in the *PTPN11* protein in canine HS are distributed at two hotspots, identical to those observed in human cancers (Figure 1 B) and inducing over-activation of PTPN11 in juvenile myelo-monocytic leukemia[16]. As expected with the strong sequence identity (99%) between the human and canine *PTPN11* proteins, these gain-of-function mutations in dogs are also associated with MAPK pathway activation[15]. By Western Blot analyses, we confirmed the constitutive activation of the MAPK pathway (pERK) in 2 HS cell lines harboring *PTPN11* mutations (Supplementary Figure 1).

Moreover, with the high incidence of this tumor in dogs, this natural model allows to study the life history of HS and the timing of *PTPN11* mutations in disseminated HS. Using CGH and Loss of Heterozygozity (LOH) analyses of 13 tumor samples from five dogs presenting with several histiocytic masses, we showed that 4/5 cases had a common origin with a clonal linear evolution, demonstrating the metastatic origin of disseminated HS (Figure 2, Supplementary Figure 2). For the one case presenting different CGH profiles between lung tumor and spleen/lymph node tumors, the LOH analysis is in favor of a common origin of disseminated HS ($P_{chromosome}$ = 0.0058 and P_{marker}= 0.0022) with subsequent genetic divergence (Supplementary Table 4). This case suggests that *PTPN11* mutations probably occur in late stages of the tumor.

Considering the high frequency of the *PTPN11* gain-of-function mutations in disseminated canine HS, we hypothesize that *PTPN11* mutations can also be a key event in a subset of human HS. We then explored the frequency of *PTPTN11* mutations in 19

human MH cases with visceral masses. Using high throughput sequencing, we identified *PTPN11* mutations in three cases (Table 1). Interestingly, the recent report of Shanmugman et al. (2019), identifying two *PTPN11* mutations in two patients with internal and multifocal tumors, confirmed that *PTPN11* mutations are enriched in visceral disseminated cases (p-value = 0.0476 one sided Fisher's Exact Test)[5]. Moreover, Egan et al. (2019) recently showed the existence of a distinct subtype of primary HS characterized by *PTPN11* alterations enriched in gastrointestinal tract localizations[17]. Altogether, these results strongly suggest that *PTPN11* mutations drive the growth of histiocytic neoplasms in humans as in dogs. Moreover, the canine data highlight that the key *PTPN11* mutations are associated with visceral dissemination and aggressiveness of HS. For four samples with enough available DNA, we also explored *BRAF* and *KRAS* hotspots, previously found exclusively mutated with *PTPN11* in canine HS. Interestingly, in WT-*PTPN11* human patients, we identified two *KRAS* mutations (G12D and Q61H) and one *BRAF* mutation (G464V) (Table 1). While the number of cases is limited, our results and the literature confirmed our hypotheses that *PTPN11* mutations, as *BRAF* and *KRAS* mutations are a key mechanism of the MAPK pathway activation, playing a crucial role in the physiopathology of HS in humans and dogs.

The identification of driver events is a key step in proposing targeted therapies, as shown by Diamond *et al.* who demonstrated the clinical efficacy of MEK and RAF inhibitors on *MAP2K1*- and *ARAF*-mutated Histiocytic Neoplasms[7]. We thus proposed the spontaneous disseminated form of canine HS as a relevant model to evaluate candidate drugs for treating human *PTPN11*-mutated HS patients. Thus, we developed seven canine in-house HS cell lines. These cell lines as well as the commercial cell line DH82 were all mutated for *PTPN11* (homozygous or heterozygous state); one cell line was also heterozygous for the *KRAS* Q61H mutation (Supplementary Table 3). We studied their sensitivity, together with the DH82 cell line, to three drugs targeting the MAPK pathway. Based on the development of personalized medicine and interest of targeting PTPN11 in human cancers[18], we tested a PTPN11 inhibitor, GS-493, expected

to inhibit the phosphatase activity in PTPN11 gain of fonction mutant[19]. We compared it with Trametinib and Sorafenib, two FDA-approved drugs with clinical efficacy in human histiocytic neoplasms[7]. The three inhibitors decreased the cell density for all cell lines (Figure 3). Homozygous or compound heterozygous *PTPN11* mutated cell lines were more sensitive to the PTPN11 inhibitor GS-493 than heterozygous cell lines (p-value= 0.02857 One Sided Wilcoxon Mann Whitney test). However, the highest sensitivity to inhibitors was observed with Trametinib (IC25 = $3{,}01.10^{-3}$ µM; $\pm 5{,}10.10^{-3}$ µM) (Supplementary Table 5).

Based on the *PTPN11* and *KRAS* mutations detected in the *BRAF* wild type canine HS, we confirmed that targeting the RAF/RAS/MEK pathway with MEK inhibitors such as Trametinib is a promising therapeutic option for patients with histiocytic sarcoma especially disseminated cases, harboring *PTPN11* mutations. These results emphasize the relevance of the canine spontaneous model for the selection of more efficient therapies.

Discussion

Comparative oncology across species appears useful to identify driver molecular alterations in similar nosological entities, and may guide research for both species, which could prove extremely useful for rare human cancers. The present complementary approach is based on large cohorts and identifies *PTPN11* mutations both in frequent canine and rare human aggressive histiocytic sarcoma. Our results demonstrated the association of *PTPN11* mutations with disseminated canine HS, the most aggressive subtype, and highlighted that human patients harboring *PTPN11* mutations also share a similar disseminated clinical presentation. Our results expand the current understanding of the pathogenesis of histiocytic neoplasms and offer new opportunities for the comprehension of the genetic mechanisms. They also provide clues to new therapeutic approaches for the mutual benefit of human and veterinary medicine. Last, this approach, using naturally affected dogs, in a non-experimental

frame, is respectful of the ethics and of the 3R rule, by replacing experimental animals by pet dogs in the frame of their health care.

Through a unique series of 111 canine HS cases, we showed the high prevalence of *PTPN11* mutations at the two key hot spots (56.75%) and that these alterations are linked to an aggressive HS subtype. This work confirmed the recent identification of *PTPN11* mutations in canine HS associated to the BMD breed[15]. In our cohort, we detected a more significant association with the visceral disseminated HS forms than with the breed, due to the fact that BMD is mainly affected by disseminated HS form[10]. The link between *PTPN11* mutations and an aggressive clinical presentation of this histiocytic neoplasm is supported by the identification of three *PTPN11* mutations in human cases with internal tumors as well as cases from the literature: except for one case with "unspecified lymph node" localization, all published human cases to date (10 cases), with *PTPN11* somatic alterations presented internal masses, including kidney, spleen or brain localizations[4,5,17,20–22]. Moreover the mutated patients presented multifocal masses with dramatic evolution similar to canine disseminated HS[4,5,20–22]. Finally, the recent work of Egan *et al*. clearly showed that human HS cases with *PTPN11* alterations form a distinct subtype of primary HS with predilection to gastrointestinal tract[17], these recent results reinforcing our work. Altogether, our data refined our comprehension of the genetic bases of HS in dogs and humans, by associating *PTPN11* mutations to an aggressive clinical presentation associated to internal/visceral masses.
In this study, we also identified mutations exclusive to *PTPN11* in the same hotspots of *KRAS* and *BRAF* in canine and human HS cases, confirming the driving role of these *KRAS* and *BRAF* mutations in histiocytic neoplasms as previously shown[4,5,17,20–23]. Noticeably, the *BRAF* mutations identified in canine and human HS cases were found in the GSGSFG phosphate binding loop (P-loop) at residues 464–469 and constitute novel relevant mutations since this region, with the V600 region, contain the majority of *BRAF* mutations in human cancers [24].

The attractiveness of the spontaneous canine model, is based on the higher HS frequency due to a strong breed predisposition[10,11] and clinical homogeneity, recapitulating the clinical presentations of primary human HS. This model thus offers a great potential to decipher the genetic mechanisms involved in rare human clinical subtypes and also to screen for targeted therapies. Previous studies orientated treatments towards MEK inhibition in canine and in human HS[7,20,25,26]. In the context of *PTPN11* alterations, we tested a specific *PTPN11* inhibitor, which surprisingly was, *in-vitro*, less efficient than MEK inhibitors. Interestingly, one cell line (Dog-HS-13281) is clearly more sensitive to the tested inhibitors probably due to the presence of *KRAS* complementary mutation (Supplementary table 4). Future studies are needed to explore the targeted therapies, especially in case of partial[20] or refractory responses[8]. The mechanisms of resistance, only explored to date in one patient[5], are expected especially if the MAPK pathway activation with *PTPN11* mutations occurs at a late stage of tumoral development as suggested by our results. We hypothesize that this late event leads to HS dissemination but further studies are needed to exclude that *PTPN11* mutated HS could represent a different nosological entity with a specific origin of histiocytes than *PTPN11* wild-type HS. Last, we succeeded in detecting *PTPN11* mutations in cell free circulating tumor DNA (ctDNA) of canine cases (Supplementary Table 2). Thus the spontaneous canine HS model, with the availability of numerous cases and the possibility of therapeutic response follow-up with the ctDNA is a unique opportunity to explore therapeutic options and resistance mechanisms through *in vitro* screening in canine HS cell lines and *in vivo* pre-clinical trials in pet dogs.

In conclusion, through access to a large cohort of canine HS, we identified MAPK pathway-activating *PTPN11* mutations, which point out a specific aggressive disseminated HS subgroup in dogs and in humans. We propose that the key *PTPN11* mutations occur in late stage of tumorigenesis with tumoral dissemination. Our findings strongly advocate the rationale for MAPK inhibition in *BRAF*-wild-type HS cases and we propose to take advantage of the unique resource of canine cases to screen inhibitor

efficiency as has already been done in integrated approaches with pet dogs clinical trials[27]. Finally, this work confirms the value of naturally occurring canine cancers as models for the study of rare human cancers from the discovery of mutations to the development and the screening of targeted therapies.

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Disclosure of Conflicts of Interest

No conflicts of interest

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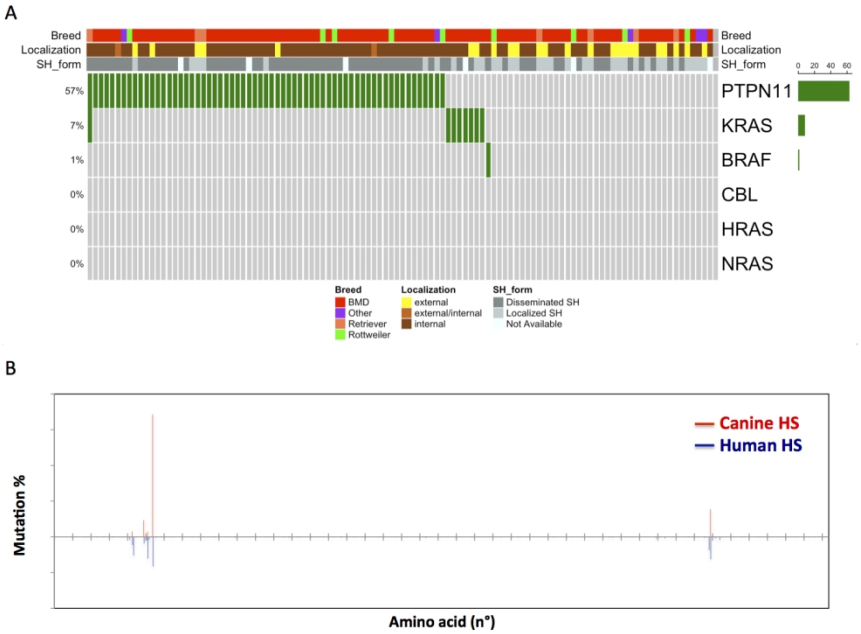
Figure and table legends

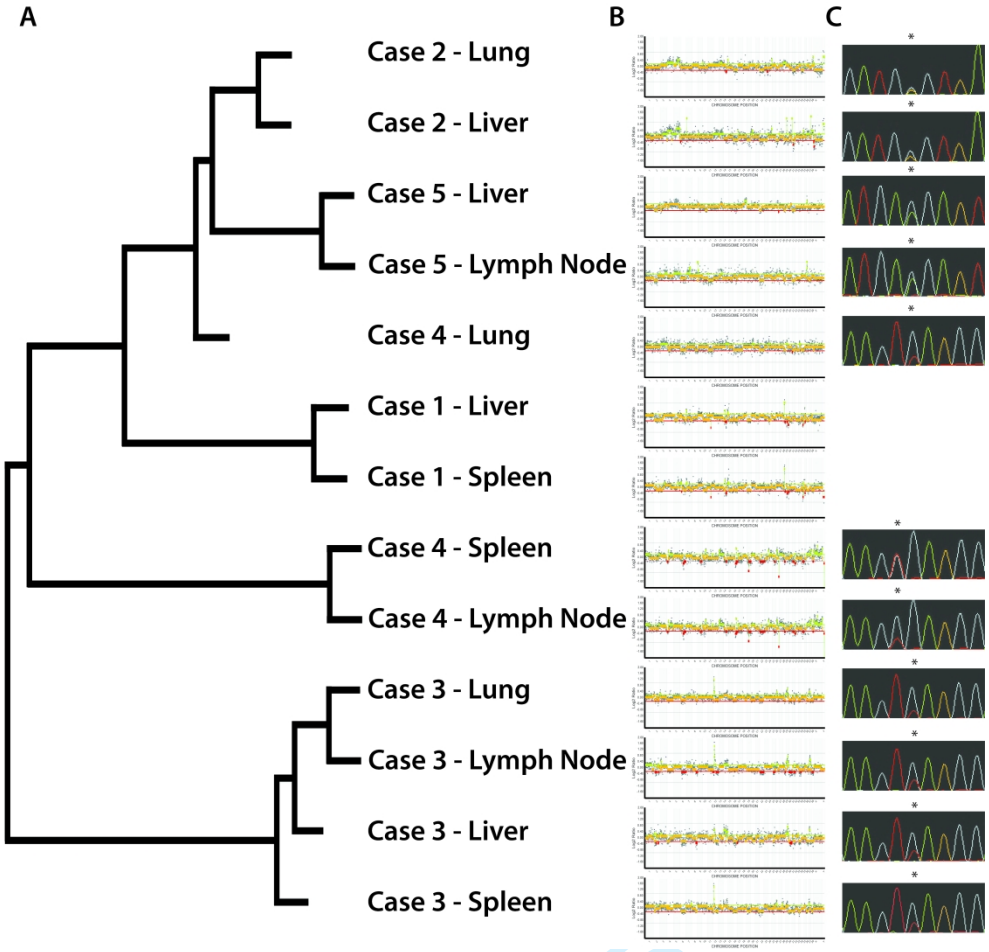
Figure 1: MAPK alterations in canine HS. A) Landscape of somatic mutations of several MAPK oncogenes in 111 cases of canine HS. *PTPN11* hotspots are mutated in 56,75% of the 111 cases of canine HS (63/111), *KRAS* hotspots are mutated in 7.2% of the cases (8/111), *BRAF* is mutated in 0.9%, while no mutation is found in *HRAS*, *NRAS* or *CBL* hotspots. B) Distribution of *PTPN11* mutations along the protein sequence in the canine HS cases compared to the *PTPN11* mutations reported for human cancers (COSMIC database).

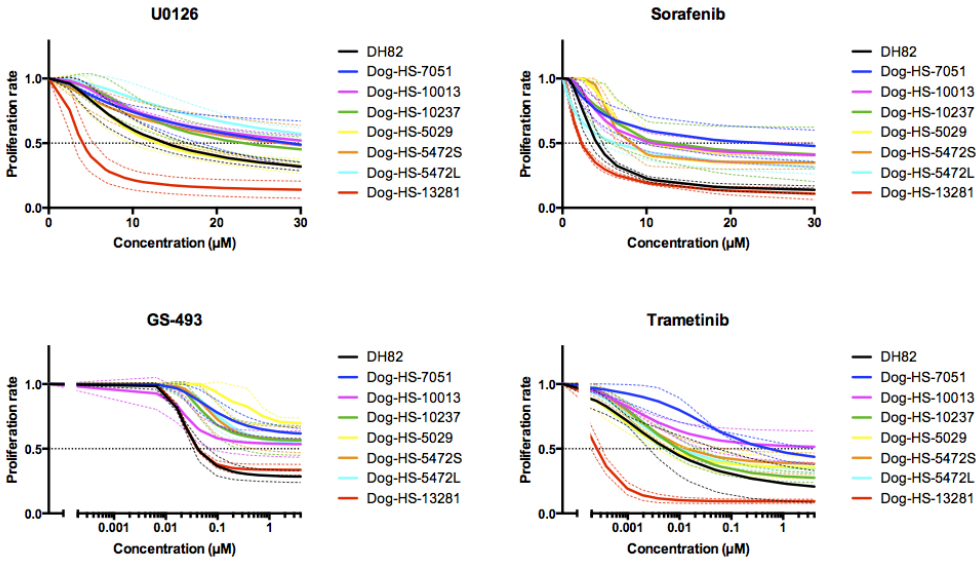
Figure 2: Hierarchical clustering of disseminated histiocytic sarcomas from five dogs. A) The algorithm summarizes relationships between the genomic aberrations of different tumors detected by CGH data in a dendrogram, in which pattern and length of the branches reflect the relatedness of samples. B) CGH profile of sarcoma cases: larger pictures of CGH profiles are available in supplementary Figure 2. C) *PTPN11* mutations. Mutations detected by Sanger sequencing are indicated by *. The mutations *PTPN11*^{G503V} , *PTPN11*^{E76G} and *PTPN11*^{G60D} were detected in Case 2, Case 3 and Case 5 respectively. The *PTPN11*^{E76G} was detected in the lung of Case 4 while *PTPN11*^{E76K} was detected in the spleen and lymph node tumors of Case 4.

Figure 3: Inhibitory effect of U0126, GS-493, Sorafenib and Trametinib on 72h cell proliferation of eight canine HS cell lines. All the cell lines are sensitive to the four inhibitors, with a higher sensitivity for the Trametinib.

Table 1: Human HS cases Summary. Cases were collected through the Reference Center for Histiocytoses, France and from the literature. Somatic alterations detected by NGS are indicated: mutations of *PTPN11* according to ENST00000351677, mutations of *KRAS* according to ENST00000256078.9, mutations of *BRAF* according to ENST00000496384.7.







Case No.	Age (years)	Sex	Tumor site	Outcome, follow-up	Prior/concurrent hematologic malignancy	mutations in PTPN11	Other Mutations	Previous reference (PMID)	Clonality
1	82	M	bone, orbit	Death 5.14 months		WT	NA	NA	
2	74	M	Lymph Node	Death 8.5 months		WT	NA	NA	
3	1	F	Lymph Node, lung	Death 8.16 months		WT	NA	NA	
4	63	F	liver	Death 13.96 months	CLL	PTPN11:chr12:112450451 A>G(91)	NA	NA	NA
5	25	M	NA	NA	NA	WT	NA	NA	
6	42	F	brain	Death 26.88 months		PTPN11:chr12:112450406 G>A(76)	NA	NA	
7	27	F	brain/spinal corde	Death 3.51 months		WT	NA	NA	
8	NA	F	NA	NA	NA	WT	NA	NA	
9	42	M	Lymph node	Death 60.91 months	LCH	WT	NA	NA	NA
10	61	F	Digestive tract	NA		WT	NA	NA	
11	21	M	Skin, lymph node	Death 14.26 months		WT	NA	NA	
12	74	M	Lymph node, digestive	Death 4.72 months		WT	NA	NA	

			tract						
13	8	M	Lymph Node	Death 24.98 months	pre B ALL	WT	NA	NA	NA
14	78	F	Bone, liver, brain	Death 3.05 months		PTPN11:chr12:112450385 G>A(69)	NA	NA	
15	81	M	Lymph node	Alive 5.5 years		WT	KRAS:chr12:25227341 T>G (61)	NA	
16	66	F	Lymph node, digestive tract, liver	Death 9.3 years		WT	WT	NA	
17	4	F	Lymphe node, lung. Spleen, liver	Death 9 days	T ALL	WT	KRAS:chr12:25245350 C>T (12)	NA	NA
18	7	M	BM, liver, spleen, LN	Death 6 weeks	T ALL	WT	KRAS:chr12:25245350 C>T(12) BRAF:chr7:140781617 C>A (464)	25833113	yes
19	12	M	BM, skin, liver, spleen	Death 10 months	T ALL	WT	WT	25833113	yes