Naturally occurring melanomas in dogs as models for non-UV pathways of human melanomas

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Summary

Spontaneously occurring melanomas are frequent in dogs. They appear at the same localizations as in humans, i.e. skin, mucosal sites, nail matrix and eyes. They display variable behaviors: tumors at oral localizations are more frequent and aggressive than at other anatomical sites. Interestingly, dog melanomas are associated with strong breed predispositions and overrepresentation of black-coated dogs. Epidemiological analysis of 2350 affected dogs showed that poodles are at high risk of developing oral melanoma, while schnauzers or Beauce shepherds mostly developped cutaneous melanoma. Clinical and histopathological analyses were performed on a cohort of 153 cases with a 4-yr follow-up. Histopathological characterization showed that most canine tumors are intradermal and homologous to human rare morphological melanomas types – 'nevocytoid type' and 'animal type'-. Tumor cDNA sequencing data, obtained from 95 dogs for six genes, relevant to human melanoma classification, detected somatic mutations in oral melanoma, in *NRAS* and *PTEN* genes, at human hotspot sites, but not in *BRAF*. Altogether, these findings support the relevance of the dog model for comparative oncology of melanomas, especially for the elucidation of non-UV induced pathways.

Introduction

Faced with the limitations of classical induced cancer models in rodents, innovative models, closer to humans and more effective in translational medicine are strongly needed. Similarly, the need for novel and more accurate prognosis factors and innovative treatment strategies calls for alternatives. An emerging concept is thus to consider species in which cancers naturally occur and progress with a physiopathology that is closer to that of humans. For this purpose, domestic animals, especially dogs, may help to bridge the gap.

During the last few years, the dog has emerged as an excellent model for genetic analyses of complex diseases

Significance

Epidemiological data identified high-risk canine breeds, with strong genetic predispositions to specific melanoma subtypes and anatomical localizations. Comparative histology and tumor sequencing data of relevant genes for human made it possible to define correspondances with the human histogenetic classification. These results led us to propose dog melanomas as relevant models for the study of genes involved in non-UV dependent pathways, such as mucosal and acral melanomas. This work paves the way for preclinical studies on naturally occurring melanomas in dogs to advantageously fill the gap between the classical rodent models and human patients.

(Galibert and Andre, 2008; Lindblad-Toh et al., 2005; Ostrander, 2012). Breeding practices aiming to select desired alleles involved in morphological and behavioral traits have also led to the co-selection of disease alleles in almost all dog breeds (Galibert and Andre, 2008; Parker et al., 2010). Because of limited breeding pools and sire effects, rare causal variants have been concentrated and are sometimes even fixed in certain breeds. The genetic heterogeneity between cases and controls is smaller within a given canine breed as compared to humans, thus facilitating gene discovery. Since the release of the canine genome sequence (Lindblad-Toh et al., 2005), the identification of millions of single nucleotide polymorphisms (SNPs) now allows genome-wide association studies (Cadieu et al., 2009; Grall et al., 2012; Karlsson et al., 2007; Merveille et al., 2011). Thus, dogs are commonly affected by many cancer types that strongly resemble human cancers and segregate in at-risk dog breeds. As examples, brachycephalic dogs have higher frequencies of glioma than any other breed (Thomas et al., 2009) and large dog breeds are prone to osteosarcoma (Phillips et al., 2010), Labrador retrievers commonly develop diffuse large B-cell lymphoma (DLCBL), whereas German shepherds are more prone to T cell lymphoma (Pastor et al., 2009). Bernese mountain dogs are severely affected by histiocytic sarcoma, with 20% affected at a mean age of 6 yrs (Hedan et al., 2011; Shearin et al., 2012). The high prevalence of some cancers in specific dog breeds reflects genetic predispositions that can be tracked down more easily than in the highly polymorphic human populations. In addition to classical family-based genetic analyses, genome wide association studies can be performed in dogs using either unique breed or multi-breed approaches (Lindblad-Toh et al., 2005; Parker et al., 2007). If a common ancestral founder mutation is suspected, the latter strategy is highly efficient (Cadieu et al., 2009; Karlsson et al., 2007). Thus, tracking predisposition genes in dog breeds should speed up their identification in human cancers. Moreover, treatment follow-up in dogs provides a unique opportunity to follow outcome and tumor progression in a naturally occurring cancer and also to perform clinical trials that are relevant for both species (Khanna and Gordon, 2009; Ostrander, 2012; Rowell et al., 2011).

In humans, melanoma is the most lethal skin cancer and its incidence has increased faster than any other cancer in the last 30 yrs (Rees, 2008). It is a highly heterogeneous cancer affecting numerous anatomical sites with different clinical behaviors. Cutaneous melanoma alone includes a large variety of neoplasms described in the latest World Health Organization (WHO) classification (Leboit et al., 2006). This classification, mainly based on histo-mophological criterias lacks prognostic value (Scolyer et al., 2011) and identification of ambiguous melanocytic tumors remains challenging (Vergier et al., 2011). Numerous efforts have been made to improve the classification taking molecular features into account (Broekaert et al., 2010; Whiteman et al., 2011). Melanoma's etiology is complex, associating genetic risk and environmental influences, with sun exposure identified as the major causal factor for cutaneous tumors. Most melanomas occur on light-exposed skin and are thought to be related to UV radiation. However, melanomas also occur in the eye and in non-exposed body sites like acral or mucosal sites. The distribution of melanoma subtypes among human populations is also meaningful as it depends upon their phototypes. Cutaneous melanoma is by far the most frequent subtype in Caucasian populations whereas acral and mucosal melanomas are most frequent in Asian populations and dark-skined populations of African origin (Bradford, 2009; Shoo and Kashani-Sabet, 2009). In addition to phototype, other host susceptibility factors include dysplastic nevi, increased number of nevi, freckling and familial history of melanoma (Russak and Rigel, 2012). Faced with this complexity, alternative model systems are highly desired to help improve our understanding of pathogenic pathways in melanomas (Flaherty, 2012). The dog is expected to be such a model. Indeed, melanomas spontaneously and frequently occur in dogs. They account for 7% of all malignant tumors and represent the most common malignant neoplasm of the oral cavity (Smith et al., 2002). Canine melanomas develop at the same anatomical sites as in humans, with differences in frequency and severity depending on their anatomical localization (Smith et al., 2002; Spangler and Kass, 2006). Strikingly, some breeds present a higher risk of developing a melanoma at specific anatomical localizations (Bergman, 2007; Ramos-Vara et al., 2000). Indeed, in the course of breed selection, genetic predispositions to melanomas have been strongly selected and as a consequence, characterizing these genetic factors should allow the identification of new driver mutations in human melanomas. However, the first step towards this aim is to define and characterize the correspondances between dog and human melanoma subtypes and their extent of homology.

The present study aims to compare canine and human melanoma subtypes in order to identify their homologies and differences through a thorough characterization of the different canine melanoma subtypes. The analysis was based on epidemiological data from 2350 canine melanoma cases and clinical outcomes and histopathological characterization from 153 cases. Somatic mutations were searched in six genes frequently mutated in human melanomas in a series of 95 canine melanoma cases. The results led us to propose a classification of canine melanomas according to the human histogenetic criteria, and showed that dogs and humans share common routes of melanoma development. We thus propose dog breeds as relevant natural models to decipher the non-UV dependent pathways in melanomas and to develop clinical trials based on homologous melanoma subtypes.

Results

Epidemiological and clinical data

Samples and data from canine melanocytic tumors were obtained through the Cani-DNA biobank (http://dog-genetics.genouest.org/) and epidemiology data were retrieved from 2350 melanocytic tumors. A subset of 153 cases with a 4-yr follow-up, was selected for a detailed epidemiological, clinical and histopathological characterization and genetic data were obtained from a total of 95 melanoma cases.

Of the 2350-case dataset, 70% were histologically diagnosed as malignant and 30% as benign tumors (so-called melanocytomas), following the WHO classification of melanocyte-derived skin tumors for domestic animals (Goldschmidt et al., 1998; Figure 1). While melanocytomas present as slowly growing pigmented nodules, mainly localized to the skin, that can be easily treated by surgical resection and remain, even if multicentric, of good prognosis (Ramos-Vara et al., 2000), melanomas are localized at different anatomical sites, with severe clinical outcome. In this dataset, we further analyzed the distribution per anatomical site for both benign melanocytic tumors (Figure 1A) and melanomas (Figure 1B). We showed that oral melanoma is the most frequent form of melanoma in canines (62%), whereas cutaneous melanoma is less frequent (27%). We observed that malignancy and severity is dependent on the anatomical localization; 97% of canine melanocytic tumors at oral sites are malignant (Figure 2), with a high metastatic behavior, as already reported (Ramos-Vara et al., 2000). In contrast, cutaneous melanocytic tumors appeared less aggressive, with only a 43% malignancy rate. Interestingly, canine digital and ungual localizations, presenting 84% and 100% malignancy, respectively, are far more aggressive than cutaneous tumors occurring on dog skin covered with fur. Finally, canine ocular melanocytic tumors are less aggressive than those from other sites, with only 29% of malignant forms (Figure 2). We observed no sex predilection for melanocytomas, but more affected males than females for melanomas (sex ratio: 1.4). In this set of dogs, the mean age of onset was late, in the last quartile of life (>0.75) showing that dog melanomas are old adult neoplasms, with a mean age of 11 yrs. For melanocytoma, the mean age of onset is earlier, 8.8 yrs.

In the subset of 153 dog cases (30 melanocytoma and 123 melanomas cases), the 4-yr follow-up allowed us to estimate the median survival time, based on death recorded from all causes. For melanocytoma, median survival time was 1463 days, since no dog died from its tumor and no recurrence was observed after surgery (to date). In the subset of 123 melanomas (85 mucosal and 18 cutaneous), median survival time was 200 days for mucosal melanoma and 384 days for cutaneous melanoma. In addition, we did observed a significant difference in the age of onset between oral melanomas, being 12.2 yrs and cutaneous melanomas, being 9.9 yrs. Metastasis at the time of diagnosis was noticed in 25% of melanoma cases, as in humans, local lymph nodes and lungs were primarily affected. Surgical resection was performed in most of the melanoma affected dogs and however recurrences were noticed in 36% of the cases and the median disease-free survival time was only 178 days; 104 days for oral melanomas and 384 days for cutaneous melanomas. The significative difference of outcome between melanocytoma and melanoma cases, correlates with the previous histological diagnosis of malignant versus begnin tumors and is concordant with previous literature (Ramos-Vara et al., 2000; Smedley et al., 2011; Spangler and Kass, 2006). Our results also highlight the severity of canine oral melanomas as compared to cutaneous melanomas.

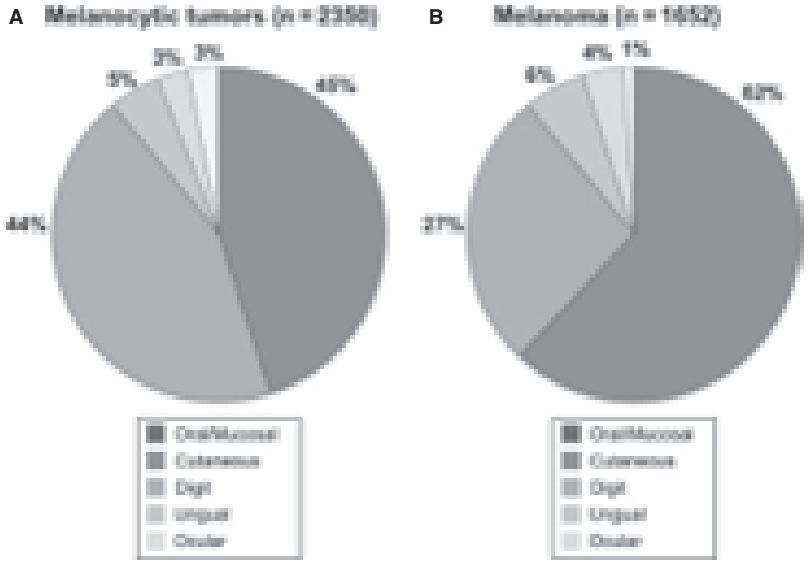

Figure 1. Distribution of the canine melanocytic tumors depending on the anatomical site. (A) Percentages of the 2350 melanocytic tumors, including benign tumors (30%; n = 698) and malignant tumors (70%; n = 1652), depending on the anatomical site. (B) Percentage of the 1652 melanomas, depending on the anatomical site.

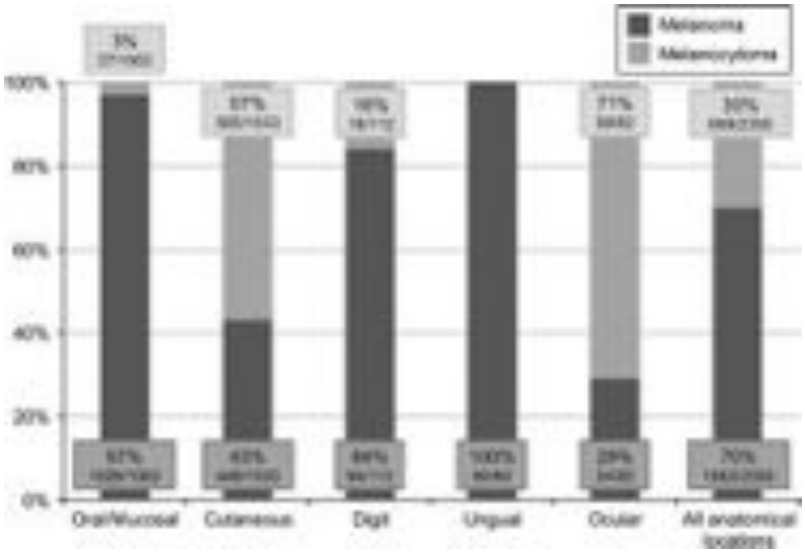

Figure 2. Distribution of 2350 canine melanocytic tumors per localization and correlation with malignancy: proportion of melanoma and melanocytoma (benign melanocytic tumors).

Table 1. Frequencies of melanocytic tumors in different dog breeds, highlighting breed predispositions. These frequencies were estimated from the histopathology lab data as the number of melanocytic tumors diagnosed per breed, as compared to the total number of dogs of this breed for which an histopathology diagnosis was performed, i.e. for poodles: 153 poodles had a diagnosis of a melanocytic tumor out of 3740 poodles with an other diagnosis

	Percentage of melanocytic tumors	Odds ratio
All breeds	2.6 (1726/65 580)	
Poodle	4.1 (153/3740)***	1.63
Beauce shepherd	6 (21/350)***	2.38
Rottweiler	3 (224/7437)*	1.17
Schnauzer	4.9 (13/263)*	1.93
Scottish terrier	8.5 (163/1911)***	3.71
Labrador retriever	4.4 (64/1466)***	1.72

Statistical significance, *: P-value < 0.05; ***: P-value < 0.001.

In the 2350-case dataset, we observed that specific dog breeds were significantly predisposed to melanocytic tumors (Table 1, Figure 3). We also observed that certain breeds are significantly predisposed to melanomas at specific anatomical sites (Table 2, Figure 3): out of all melanocytic tumors, poodles, present 77% of oral melanoma ($P < 0.001$), Beauce shepherd and rottweiller, respectively present 80% and 69% of cutaneous melanoma ($P < 0.001$), and a few breeds, such as Labradors, golden retrievers or boxers do not particularly display site specificity. More strikingly, considering all melanocytic tumors diagnosed in Poodles, 98% are at oral localization (data not shown). Among the predisposed dog breeds, we observed an overrepresentation of tumors in black-coated breeds, such as schnauzers, Rottweilers or Beauce shepherds for all anatomical localizations. On the contrary, pale-coated dogs, especially solid white-coated breeds, and even hairless dogs were underrepresented in the 2350-case dataset and are not prone to develop melanoma of any type. These observations and the protection role of fur against sun exposure, led us to hypothesize that UV radiations may not have a preponderant role in the development and/or progression of canine melanomas.

Genetic features for canine melanoma classification

To propose a histogenetic classification for canine melanomas based on the human classification (Whiteman et al., 2011), we screened six genes that harbor recurrent somatic mutations in different human melanoma subtypes, *BRAF, NRAS, PTEN, KIT, GNAQ* and *CDK4*. The genes were sequenced in canine tissue-derived cDNA generated from (i) tumor and healthy tissues from the same dog, for 35 affected dogs and (ii) healthy tissues only for 28 matched dog breed controls. Out of this set of 35 dog melanomas (27 oral and 8 cutaneous), no alteration was found in the *GNAQ*, *CDK4* and *BRAF* genes, nor in the *KIT* gene, concordant with the recent investigation of exon 11 *KIT* mutations in 39 canine oral melanoma cases (Murakami et al., 2011). However, two genes were found to harbor somatic variants, with a damaging effect on the protein: NRAS, at position Q61, and PTEN at position G251 (Table 3, Figure 4 and 5). Since both variants are precisely located at mutation hotspots of human tumors (COSMIC database), we re-sequenced *NRAS* Q61, *PTEN* G251 and *BRAF* V600 mutations in an additional set of 60 dog melanomas (50 oral and 10 cutaneous). In total, out of the 95 cases (77 oral and 18 cutaneous melanomas), three oral melanoma cases had a *NRAS* Q61 mutation and two oral melanoma cases had a mutation in PTEN at position G251, of which one case had both mutations; but still no alteration were

found in *BRAF*, as previously observed (Shelly et al., 2005).

The NRAS Q61 mutations were heterozygous and corresponded to the human activating mutations of codon 61 carrying most of the NRAS mutations of human melanomas (Figure 4). These mutations have been demonstrated to cause constitutive activation of the protein (Taparowsky et al., 1983).

The amino-acid 251 PTEN mutations are localized at a complex mutation hotspot site of small indels in humans that affect the open reading frame (Figure 5). The canine G251C mutation, observed at the homozygous state, was predicted to be damaging for the protein function (score: 1.0; Polyphen prediction; Adzhubei et al., 2010). The other mutation, an insertion at codon 251, was heterozygous and led to a premature stop codon at position 252. Together, these results allowed to identify somatic mutations in oral dog melanoma in *NRAS* and *PTEN* genes at human hotspots, suggesting common pathways in dog and human melanomas.

Table 2. Distribution of 2350 canine melanocytic tumors and correlation between the anatomical sites and breed predispositions calculated as the proportion of mucosal, cutaneous, or ocular melanocytic tumors. Poodles are predisposed to oral melanocytic tumors, whereas Beauce shepherds, rottweilers and schnauzers are mostly affected by cutaneous melanocytic tumors, and Labrador retrievers can be affected by both localisations

	Mucosal, %	Cutaneous, %	Ocular, %
All breeds	45 (1063/2350)	51 (1205/2350)	4 (82/2350)
Poodle	77 (181/237)***	22 (53/237)	1 (3/237)
Beauce shepherd	20 (15/76)	80 (61/76)***	0 (0/76)
Rottweiler	27 (53/193)	69 (133/193)***	4 (7/193)
Schnauzer	38 (12/32)	63 (20/32)	0 (0/32)
Labrador retriever	43 (94/217)	52 (112/217)	5 (11/217)

***Statistical signifiance, P-value < 0.001.

Histopathological comparison of canine and human melanomas

A thorough analysis of the histological features of the 153 melanocytic tumors, from oral, cutaneous and ocular localizations, was performed using malignancy criteria as previously defined by the WHO classification (Goldschmidt et al., 1998). However, this canine classification does not allow a classification per type, as it is the case in humans. We thus set up four major histological categories, based on histological correspondances with the human classification (Table 4). Analysis of the 153 cases was double–blinded and showed that all but one canine tumors were intradermal with generally no, or minimal epidermal component (junctional activity; Figure 6A, B). Among the four major histological categories observed, the first type, encountered in 72% of canine melanomas, presented cytological characteristics similar to human 'melanoma simulating nevus' with a 'congenital type' architecture resembling nevocytoid melanoma arising on a congenital nevus. The tumors presented a cytological spectrum from common nevi (high cellular density, round small cells with a small central nucleoli) to cellular blue nevi (small spindle cells containing several small nucleoli). These two entities were weakly or even not pigmented and generally displayed a high mitotic index (Figures 6C, D and 7A). The second type, observed in 16.5% of the cases, corresponded to the human 'animal type' melanoma, also called 'pigmented epithelioid melanocytoma' (Zembowicz et al., 2004). This entity, recently described in one case of canine cutaneous melanoma (Liu et al., 2011), presents as sheets or bundles of highly pigmented large neoplastic melanocytes, epitheloid to spindle-shaped (Figures 6E, F and 7B). It should be noted that the five ocular melanoma cases analysed corresponded to 'animal type' human melanomas (Table 4). A third 'composite type', consisted of both 'congenital type' and 'animal type' cell morphologies, it accounts for 4.5% of cases and suggests a common melanocytic origin followed by different clonal selections. The fourth type,

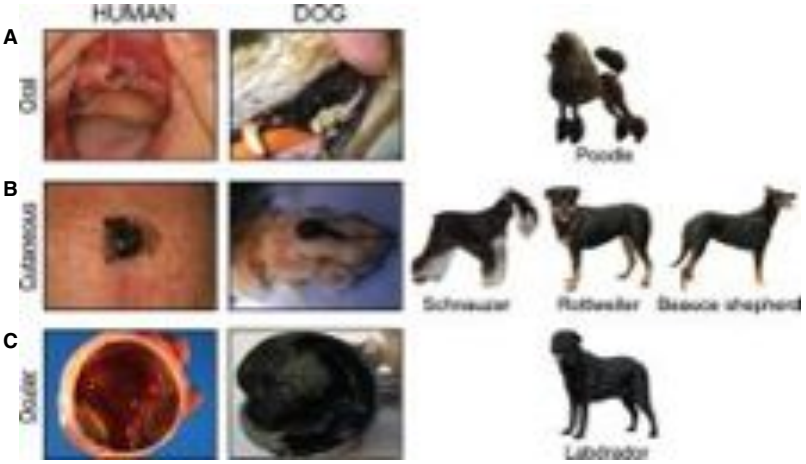

Figure 3. Clinical aspect of (A) oral, (B) cutaneous, (C) ocular melanoma types. Macroscopic comparison between human and dog melanoma tumors and illustration of major canine breed predispositions (A: Poodles; B: Schnauzer, Rottweiler, Beauce shepherd; C: Labrador retriever). Photographs are the courtesy of Pr. A. Dupuy (Human A and B), Pr. F. Leger (Human C), Dr. J-M. Péricard (Dog A), Dr. M. Delverdier (Dog B) and Dr. I. Raymond (Dog C).

Table 3. Somatic sequence variants identified in *NRAS* and *PTEN* genes in canine oral melanoma tumors

	Exon	Nucleotide alteration	Zygosity	Amino acid alteration	Number of cases	Effect[a]
NRAS	Exon 2	182A > G	Heterozygous	Q61R	2	Benign (Polyphen score: 0.085)
NRAS	Exon 2	183A > T	Heterozygous	Q61H	1	Benign (Polyphen score: 0.124)
PTEN	Exon 7	751G > T	Homozygous	G251C	1	Damaging (Polyphen score: 1)
PTEN	Exon 7	753insG	Heterozygous	D252Ter	1	Premature STOP

[a]Predicted effect on protein structure, Polyphen (http://genetics.bwh.harvard.edu/pph/).

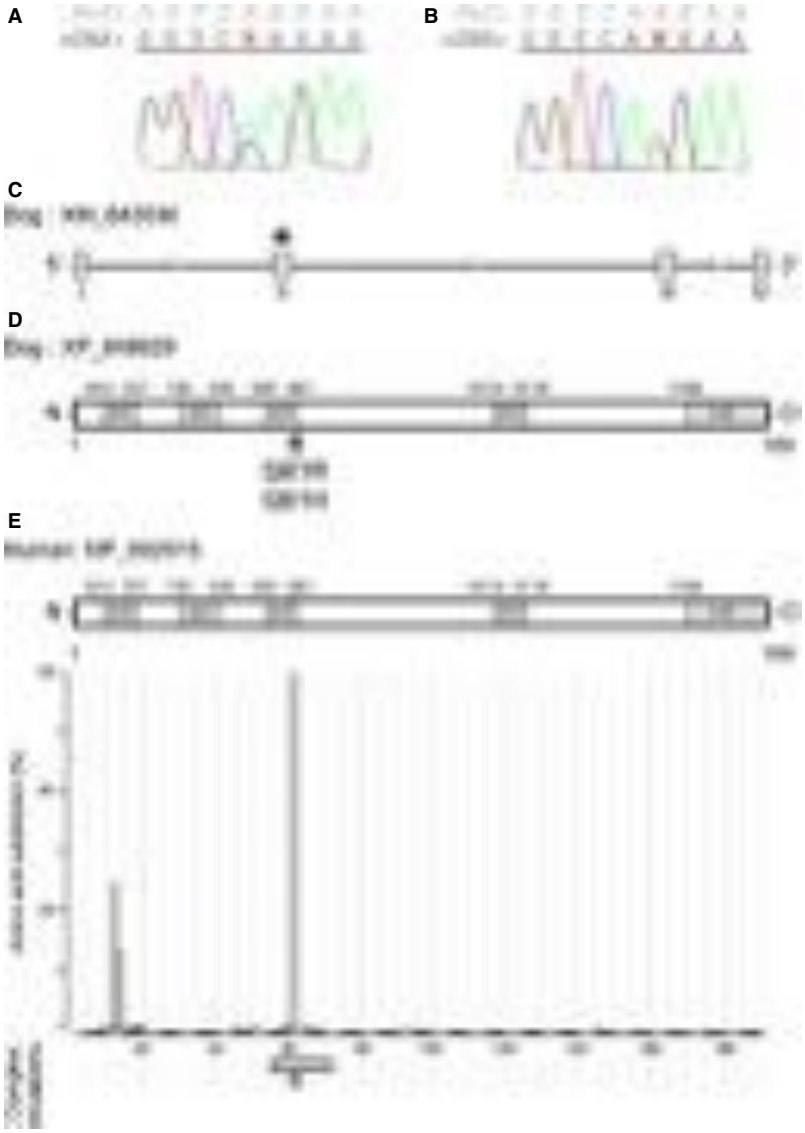

Figure 4. Mutation status of the canine *NRAS* cDNA sequence in three oral melanoma cases: (A) forward electrophoregram showing a 182A > G gene mutation, predicting a Q61R substitution in the NRAS protein, identified in one canine oral melanoma case; (B) forward electrophoregram showing a 183A > T mutation, predicting a Q61H substitution in the NRAS protein, identified in two canine oral melanoma cases; (C) structure of the dog *NRAS* gene (accession number XM_843536) with the position of the mutations in exon 2; (D) structure of the dog NRAS protein (accession number XP_848629) with the position of the two substitutions at codon Q61; (E) structure of the human NRAS protein (accession number NP_002515) with an histogram representing the frequency of the mutations along the NRAS protein (modified from COSMIC, Catalogue of Somatic Mutations In Cancer: http://www.sanger.ac.uk/genetics/CGP/cosmic). The canine mutations Q61R and Q61H are located at the main human hotspot of amino acid substitutions.

corresponding to 6.5% of the cases, remained unclassified: cases often presented epithelioid and pleomorphic features with the presence of balloon cell type (Figure 7C). Finally, a SSM type (superficial spreading melanoma), characterized by an intraepidermal lateral component, was observed in only one case out of 153 melanomas examined (Figure 6B). This comparative study highlighted that most canine melanomas were dermal, differently to human melanomas generally localized to the epidermis, and that their histological features corresponded to the morphology of rare types of human melanomas.

Discussion

The present study provides a detailed epidemiological characterization of 2350 canine melanocytic tumors, as well as a histogenetic classification on a 153-dog cohort with a 4-yr follow-up. First, in the set of 2350 dog melanocytic tumors, we observed that, similarly to humans, dogs develop both benign proliferations (melanocytomas) and melanomas. Dog melanomas share numerous similarities with human melanoma subtypes; particularly with mucosal, digital and ungual localizations (referred to as acral melanoma in humans), that usually display highly aggressive behavior and rapid growth characteristics in both species. In humans, these tumors, each accounting for about 5% of human melanomas, are poorly understood and in the need for models (Harbour, 2012; Leboit et al., 2006). Human mucosal melanoma has a very poor prognosis with 75% of the patients having lymph node metastases at diagnosis and survival of less than 25%, 5 yrs after diagnosis (Patrick et al., 2007). Similarly, in dogs, oral melanoma is highly aggressive, with propension to develop lung metastases, leading to a short survival time (median of 200 days after diagnosis) and a high mortality rate. These dog oral and acral melanomas thus constitute relevant models for their human homologs. However, we observed that cutaneous

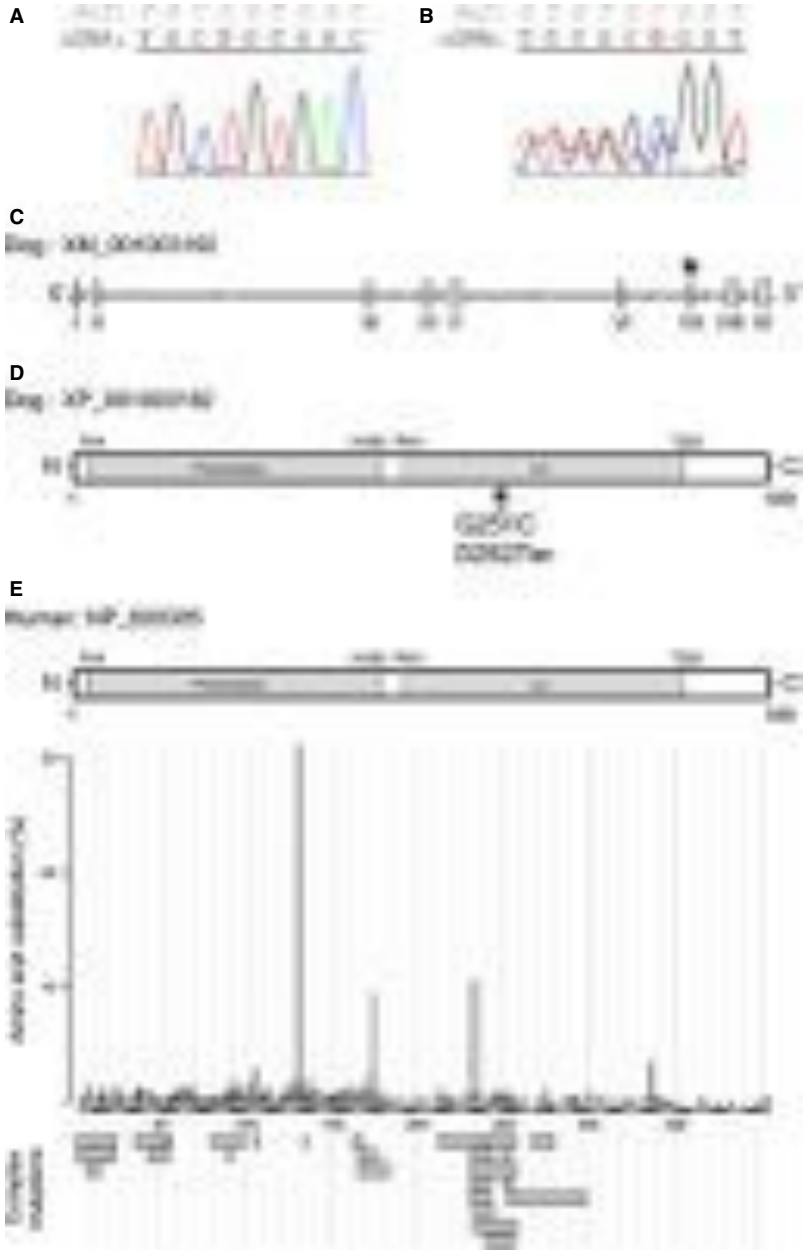

Figure 5. Mutation status of the canine *PTEN* cDNA sequence in two oral melanoma cases: (A) forward electrophoregram showing a 751G > T gene mutation, predicting a G251C substitution in the PTEN protein, identified in one canine oral melanoma case; (B) reverse electrophoregram showing a G insertion at position 750, leading to a superimposed sequence and predicting a stop codon at amino acid 252 in the PTEN protein, identified in one canine oral melanoma case; (C) structure of the dog *PTEN* gene (accession number XM_001003192) with the position of the mutations in exon 7; (D) structure of the dog PTEN protein (accession number XP_001003192) with the position of the two mutations at codon 251. The stop codon occurs at amino acid position 252 (D252Ter); (E) structure of the human PTEN protein (accession number NP_000305) with a histogram representing the frequency of the mutations along the PTEN protein (modified from COSMIC, Catalogue of Somatic Mutations In Cancer: http://www.sanger.ac.uk/genetics/CGP/cosmic/). The canine mutations in *PTEN* codon 251 are located at the main human hotspot of complex mutations.

Table 4. Distribution of the canine melanomas following the human histological classification and correlation with anatomical sites

	All anatomical sites, %	Mucosal, %	Cutaneous, %	Ocular, %
Nevocytoid type	72 (110/153)	77 (85/110)	66 (25/38)	0 (0/5)
Animal type	16.5 (25/153)	14.5 (16/110)	10.5 (4/38)	100 (5/5)
Composite type	4.5 (7/153)	5.5 (6/110)	2.5 (1/38)	0 (0/5)
Pleomorphic type	6.5 (10/153)	3 (3/110)	18.5 (7/38)	0 (0/5)
SSM type	0.5 (1/153)	0 (0/110)	2.5 (1/38)	0 (0/5)

SSM, superficial spreading melanoma.

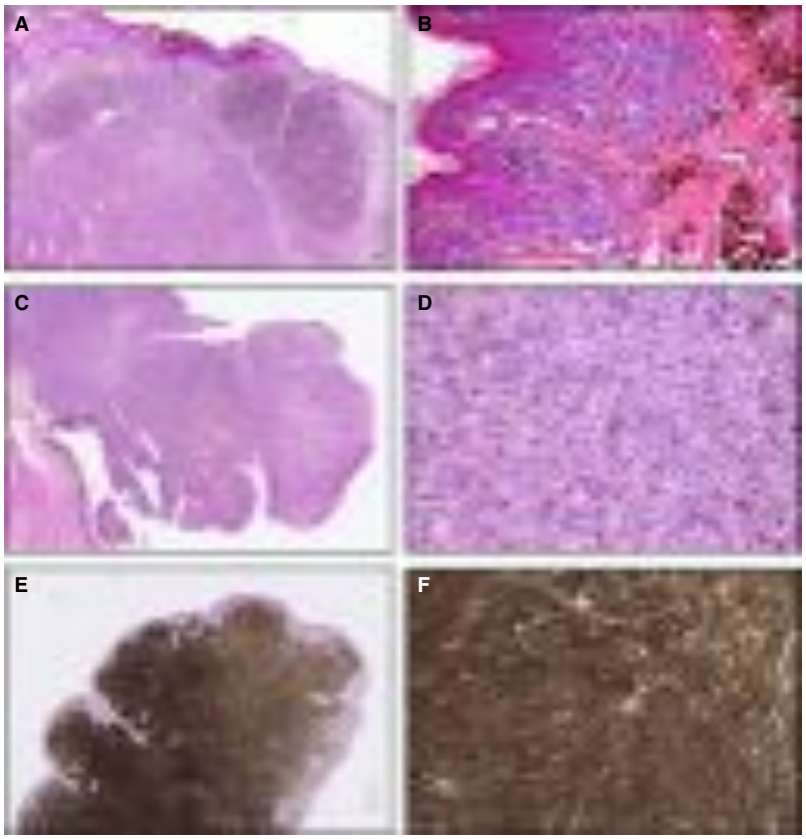

Figure 6. Histological comparison of dog melanoma subtypes, by Hematoxylin-Eosin-Saffranin (HES) staining of tumor biopsies: (A**,** ×20), melanoma showing a dermal invasive proliferation, without intraepidermal lateral component with ulceration of epidermis, representing the majority of dog melanoma architecture; (B, ×200) intraepidermal melanoma with a pagetoid architecture (SSM type), very rarely observed in dog melanomas; (C, ×20), (D, ×400) Congenital type nevocytoid melanoma, mimicking a nevocytoid melanoma arising on a congenital nevus with nevocytoid atypical melanocytes (many mitotic figures); (E, ×20), (F, ×100) Animal type melanoma, heavily pigmented and invasive dermal melanocytic proliferation with pigmented spindle melanocytes.

and ocular localizations are less frequent and less severe in dogs than in humans. Contrary to human cutaneous and uveal melanomas that are commonly highly aggressive, with frequent liver metastasis (Harbour, 2012), these tumors in dogs, are most often benign and rarely display metastatic behavior (Wilcock and Peiffer, 1986).

This is, so far, the first analysis of epidemiology data on such a large set of melanocytic tumors, which proposes a histogenetic classification of canine melanocytic tumors as compared to human melanomas. The dog genetic system, allowing the identification of constitutional genetic factors can be particularly powerful since significant breed predispositions to specific melanoma subtypes have been found. Indeed, the stricking predispositons of poodles to oral melanoma, of schnauzers, Beauce shepherd and rottweillers to cutaneous melanoma, especially at digit and ungual localizations are expected to allow the identification of specific allelic combinations that differently predispose the 'at risk' breeds to specific melanoma subtypes. In addition, breeds such as Golden or Labrador retrievers, affected by both oral and cutaneous melanomas suggest that common genetic factors can be tracked down in these breeds. These 'at risk' canine breeds, offer several relevant models to identify both specific and common melanoma-subtype factors (Cazenave et al., 2013) involved in homologous subtypes in humans. This is especially usefull since human mucosal or acral melanoma cohorts are not yet available for genome wide studies in humans.

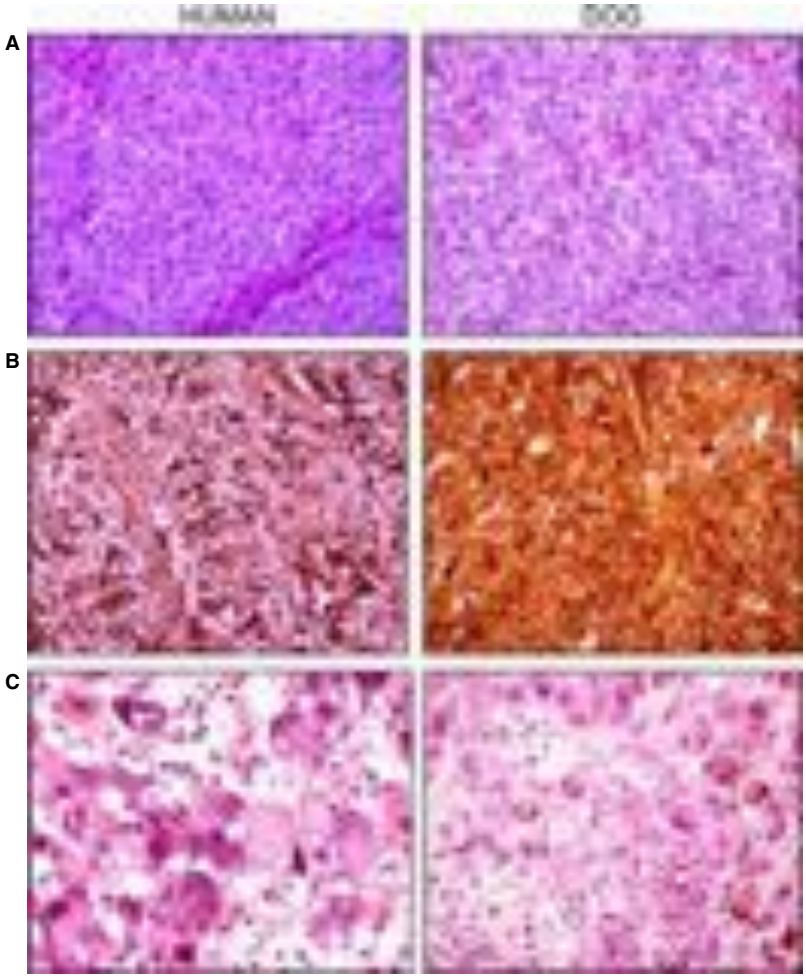

Figure 7. Histological comparison of human (left) and dog (right) melanoma subtypes, by Hematoxylin-Eosin-Saffranin (HES) staining of tumor biopsies. (A) nevocytoid type melanoma, (B) animal type melanoma (C) pleomorphic type melanoma. Original magnification ×200.

In addition to this constitutional genetic aspect of melanomas, the identification and characteristics of somatic alterations in canine melanomas have shed light on the pathways involved, respectively to human melanoma pathways. Here, we identified somatic mutations in dog oral melanoma cases, at human hotspot mutations: NRAS Q61 (3/95 dog melanomas: 3/77 oral and 0/18 cutaneous) and PTEN G251 (2/95 dog melanomas: 2/77 oral and 0/18 cutaneous), one dog harboring the 2 mutations. These four dogs were all affected by oral melanoma, all had surgery and all had recurrences 2–9 months following resection of the tumor, indicating the severity of the cases harboring the mutations. Interestingly, in the tumoral tissue, the G251C PTEN mutation was observed at the homozygous state (i.e. corresponds to 2 mutated alleles or to 1 mutated allele and a gene deletion), while the control healthy tissue was observed at the homozygous normal state. Since these mutations occur in a tumor suppressor gene, we hypothezise that one allele could be a constitutional deletion (loss of heterozygosity) and the other allele, a somatic mutation.

In humans, NRAS Q61 mutations are the most frequent somatic alterations found in *NRAS*-mutated melanomas, more frequently encountered in Chronic Sun Damage (CSD) melanomas, they occur in 20–40% of the cases (Fecher et al., 2008; (Scolyer et al., 2011; Whiteman et al., 2011), But, they also have been frequently found in congenital nevi almost exclusively at codon 61 (Bauer et al., 2007) and in nodular melanoma (Platz et al., 2008). These last data are in agreement with our findings of *NRAS* Q61 mutations in dermal non sun-exposed melanomas. In addition, in mice models, activating RAS mutations have long been established as important models for the genetics of melanoma, and the *NRAS* Q61K mutation is known to cooperate with *INK4A* or *ß-catenin* mutations to promote melanoma (Delmas et al., 2007). In human, PTEN G251 mutations are located at a complex mutation site, previously shown to be deleterious for the protein function and described in human glioma, lung and endometrial carcinoma (Fulci et al., 2000; Kohno et al., 1998; Moreno-Bueno et al., 2004). Interestingly, somatic mutations in the *PTEN* gene

and in other PI3K pathway genes have recently been identified in human mucosal melanoma (Shull et al., 2012; Turri-Zanoni et al., 2012), in agreement with our findings of *PTEN* mutations in dog mucosal melanoma. Finally, the absence of *BRAF* somatic mutation in canine cutaneous melanoma (0/18) is concordant with the fact that canine cutaneous melanoma behave differently than the human *BRAF*-mutated intermittently sun-exposed cutaneous melanoma (non-CSD types; e.g. SSM type), since canine cutaneous melanomas are mostly dermal and occur in non-UV exposed sites (skin covered with fur), like human rare dermal, acral or congenital type melanomas. The absence of *BRAF* somatic mutation in oral melanoma (0/77) is concordant with the fact that human mucosal melanoma do not either harbor *BRAF* mutations. These genetic results confirm the relevance of the study of canine mucosal and acral melanomas as natural homologous models for the human homologous melanoma subtypes.

Regarding histological features, the analysis of the 153 canine melanocytic tumor-set revealed that most of them were intradermal with no epidermal component, suggesting that dog melanomas may arise from dermal melanocytes. We also showed that most canine melanomas, independently of their anatomical localization, presented cytological characteristics similar to human 'melanoma simulating nevus' and 'animal type' melanomas. These melanomas in humans represent a group of rare tumors sometimes arising from pre-existing dermal lesions, like giant congenital nevi and blue nevi, in children and young adults (Heffel and Thaller, 2005; Miteva and Lazova, 2010; Zembowicz and Phadke, 2011). A better understanding of these melanomas and easier access to samples in dogs, is of great interest, as they represent rare entites in humans, but the majority of the melanomas affecting children.

Altogether, epidemiological and genetical characteristics, with morphological features, suggest classification of canine melanomas as dermal non-UV dependent melanomas. Comparing to the histogenetic classification (Broekaert et al., 2010; Whiteman et al., 2011), canine melanomas are closer to non-sun exposed melanomas in humans, such as acral (mucosal and ungueal), nodular melanoma (NM), acro-lentiginous melanoma (ALM) or rare congenital or blue nevi. The fact that (i) dog melanomas arise in non-sun exposed sites (mucosal sites, skin covered with fur and overrepresentation of black-coated breeds), (ii) the discovery of *NRAS* and *PTEN* somatic mutations in canine oral melanoma and (iii) the absence of *BRAF* somatic mutation in canine cutaneous and oral melanomas, are in agreement with the involvment of non-UV pathways in canine melanoma development and/or progression. In order to further decipher these not well known pathways, canine melanoma cases of relevant subtypes/breeds combinations will be sequenced through RNAseq, to obtain a better estimate of the occurrence and reccurence of these *NRAS* and *PTEN* somatic alterations, as well as other novel mutations in each dog melanoma subtype.

Conclusion

This study demonstrates the value of dogs as models for human melanomas and opens attractive prospects. The unique characteristics of dog breeds that develop specific melanoma subtypes in a context of multiple pigmentation patterns underlies major genetic predispositions that are theoretically simpler to track down in dog breeds. Faced with the complexity of human melanomas, the dog model offers a unique opportunity to estimate the relative causality of genetic and environmental features in as many systems as the canine breeds can offer. Thus, an essential step towards the use of these melanoma dog models, for comparative genetic and therapeutic applications, was first to characterize the extent of homology between dog and human melanoma subtypes.

Taken together, our findings lead us to propose canine melanomas as alternative models to speed up the identification of specific pathways in homologous human melanoma subtypes and particularly to decipher new pathogenetic factors in non-UV-linked pathways. We anticipate dog melanomas as powerful natural models for non-sun exposed melanomas such as mucosal, acral and rare dermal subtypes but also for sun-exposed melanomas that most probably also share non-UV-light exposure routes of development. Indeed, while sun exposure is a well known risk factor in the initial development of human cutaneous melanoma, particularly in people with pale skin, little is known about the factors involved in mucosal or acral lentiginous melanomas (ALM), that are not, or are less influenced by UV exposure and that mostly occur in pigmented phototype populations (Bradford, 2009; Papaspyrou et al., 2011; Shoo and Kashani-Sabet, 2009). Moreover, the finding of mutations in the same genes as in human melanoma subtypes, and even, at the human hotspot sites, opens the field for specific targeted clinical trials in dogs. We expect genetic and epidemiological approaches to reveal the constitutional and somatic events involved in dog melanomas, as well as environmental factors, considering each canine melanoma subtype in its predisposed breed as a piece of the complex human melanoma puzzle.

Methods

Sample collection

For this study, blood and tissue samples from the 153-dog cohort were collected through the Cani-DNA biobank (http://dog-genetics.genouest.org), thanks to the network including pathology laboratories, French National Veterinary Schools, as well as veterinarians oncologists and practitioners from all over France. Blood, tissue samples, clinical questionnaires and the corresponding dog pedigrees were collected by licensed veterinarians and complementary information was directly obtained from the owners or breeders with

the informed consent. All affected dogs had clinical evidence of melanoma and all cases were confirmed by pathology reports, including histopathological re-evaluation of biopsy samples by authors (J.A., L.D.). The histological parameters evaluated in all tumors included: mitotic rate, cellular atypies, inflammation, presence of ulceration and necrosis and presence of angiolymphatic invasion. All data and samples were entered into the Cani-DNA database and biobank. The use of dog samples was approved by the CNRS ethical board, France (35-238-13) for UMR6290. Genomic DNA was extracted from peripheral blood leucocytes using the NucleoSpin® Blood L kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. Tissue biopsy samples were stored in RNAlater (Qiagen, Courtaboeuf, France) at −20°C, and RNA was extracted from tissues using the NucleoSpin® RNA II kit (Macherey-Nagel) according to the manufacturer's instructions.

Epidemiological analyses

Using data from French histopathology laboratories (LHA, IDEXX, LAPVSO) recorded between January 2008 and April 2010, totalizing 2350 cases of melanocytic tumors, we obtained information about at-risk breeds, age of onset, sex and anatomical sites. This selection was considered to be representative of the occurrence of melanocytic tumors in the French canine population since they represent nearly one-third of the melanocytic tumour diagnoses and covers a large geographic area. A cohort of 153 sampled dogs were investigated using a questionnaire sent to each referring veterinarian requesting information about epidemiological, clinical and pathological features including: identification of the dog (identification, sex, gender, age, coat color, etc.), anamnestic data and case history, clinical presentation of melanoma at time of diagnosis (size, behavior, anatomical localization, etc.), biological evaluations (hematology and biochemistry), therapeutics and follow-up. Frequencies and distributions were compared with the Pearson chi-square test. Significance was taken as $P < 0.05$. Odds ratios with 95 percent confidence intervals were calculated to estimate the relative melanoma risk for the predisposed breeds. We used the Kaplan–Meier method to estimate disease-free survival time and survival time.

Genetic analyses

Exon sequencing was performed on six genes that frequently harbor recurrent somatic mutations in human melanoma subtypes: *BRAF, NRAS, PTEN, KIT, GNAQ* and *CDK4*. Mutations in these genes are described in human cutaneous melanoma (*BRAF, NRAS*), mucosal melanoma (*PTEN, KIT),* blue nevi and uveal melanomas (*GNAQ*) and in familial cutaneous melanoma (CDK4). The genes were sequenced in canine tissue-derived cDNA generated from tumor and healthy tissues (skin and oral mucosa) in 35 affected dogs. Sequencing was also performed in healthy tissues of 28 control dogs without any cancer history, from matched breeds. For each gene, the whole cDNA was sequenced in both tumor and healthy tissues, and was compared to the canine reference sequence (CanFam 2) and to the human sequence (GRCh37). Reverse transcription was performed on 0.5 μg of total RNA extracted form dog tissues using the high capacity cDNA Reverse Transcription kit (Applied Biosystems, Saint Aubin, France) according to the manufacturer's instructions. Primers were designed using the Primer3 program (http://frodo.wi.mit.edu/primer3/) based on the CanFam2 canine sequence. Products were amplified using polymerase chain reaction (PCR), from 1:40 diluted cDNA samples using standard PCR protocols with MJ-Research thermocyclers (Biorad, Marnes-la-Coquette, France). PCR products were purified by ExoSAP-IT (GE Healthcare, Orsay, France) and Sanger sequenced using the BidDye® Terminator v3.1 Cycle Sequencing Kit, using a capillary electrophoresis 3130XL Genetic Analyzer (Applied Biosystems). Data were analysed with the DNA Sequencing Analysis software v5.2 (Applied Biosystems). The presence of somatic mutations was assessed by comparison of melanoma with healthy tissue cDNA sequences from the same dog and from control dogs and compared to the CanFam 2 reference dog sequence, using the Seqscape software v2.5 (Applied Biosystems).

Histopathological analyses

Hematoxylin-Eosin-Saffranin (HES) stainings were performed following standard procedures on formalin-fixed paraffin embedded sections of skin and tumor biopsies of canine melanomas, obtained after surgery by DVM practicians. Tumors were classified after histopathological examination by two authors (J.A., L.D.) according the criteria defined by the WHO International Histological classification of Tumors of Domestic animals (Goldschmidt et al., 1998). According to this classification, canine melanocytic tumors may be classified in: (i) melanocytoma composed of cells of variable morphology (from small spindle cells with sparse melanin granules to large spindle and/or epithelioid, polygonal round cells, which often have a large amount of melanin within the cytoplasm) with low to moderate proliferative activity (<3 mitoses per 10 high-power fields) and (ii) melanoma, characterized by tumor cells of variable morphology (fusiform to epithelioid) with a variable degree of cellular and nuclear pleomorphism, often with large, prominent nucleoli and displaying numerous mitotic figures (>3/10 high power fields). In a comparative perspective, a subset of 153 canine malignant melanomas was examined by two observers (B.V. and J.A.) in order to apply the histological classification criteria and subtyping defined by the WHO for human melanomas (Leboit et al., 2006) to canine melanomas. Cases were firstly double blinded analyzed by a veterinary pathologist (J.A.) and a human dermatopathologist expert in human melanoma (B.V.), to confirm the diagnosis of malignant melanoma. Then, comparison between histopathological types of melanomas observed in dogs and in human was performed and four major types were defined: (i) 'congenital type nevocytoid melanoma' mimicking a nevocytoid melanoma and arising on a congenital nevus, (ii) 'animal type melanoma', (iii) 'composite melanoma', (iv) 'epithelioïd pleomorphic dermal melanoma'. After definition of these four major types, new double-blind analyses were performed.

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Competing interests statement

The authors declare that they have no competing financial interests.

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