Molecular Heterogeneity of Canine Cutaneous Peripheral Nerve Sheath Tumors: A Drawback in the Diagnosis Refinement

SÍLVIA TEIXEIRA^{1*}, IRINA AMORIM^{1,2,3*}, ALEXANDRA RÊMA¹,
FÁTIMA FARIA¹ and FÁTIMA GÄRTNER^{1,2,3}

¹Department of Pathology and Molecular Immunology of the Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal;

²Institute for Research and Innovation in Health, (i3S), University of Porto, Porto, Portugal;

³Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal

Abstract. *Background/Aim:* Peripheral nerve sheath tumors (PNSTs) belong to a very heterogeneous group of neoplasms occurring both in dogs and humans. The aim of the present study was to evaluate the histological and immunohistochemical features of canine cutaneous PNSTs contributing to further refine their diagnosis. *Materials and Methods:* The histopathological phenotype and biological behavior of 40 canine cutaneous PNSTs were evaluated and vimentin, S-100, glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), desmin, alpha-smooth muscle actin (α -SMA) and Ki-67 immunoreactivity were assessed. *Results:* Respectively, 17 and 23 lesions were classified as benign and malignant PNSTs. The malignant lesions were more often positive for S-100 and presented a proliferation index significantly higher when compared to the benign ones ($p < 0.05$). *Conclusion:* The differential diagnosis of PNST on routine stained samples is difficult and the immunohistochemical examination may contribute to the final diagnosis. However, these lesions present a complex histogenesis and show very variable individual features; thus, an unequivocally immunohistochemical panel that could have supported the PNST diagnostic was not achieved. Nevertheless, we concluded that Ki-67 can be a

useful marker helping to discriminate the biological behavior of canine PNST.

Peripheral nerve sheath tumors (PNSTs) are mesenchymal tissue-derived neoplasms that occur both in humans as in dogs (1). Cutaneous PNSTs belong to a very heterogeneous group of neoplasms that arise from neural crest-derived cells like Schwann cells, perineural cells or fibroblasts (2). In human medicine, these tumors have been subclassified as neurinomas, neurilemmomas, schwannomas, neurofibromas, neurofibrosarcomas and malignant peripheral nerve sheath tumors (MPNSTs) based on their presumed cell(s) of origin (3). However, in veterinary medicine, due to clinical and morphological similarities, the histogenesis of PNSTs is still not completely clarified and the nomenclature applied remains confusing. Thus, they are all broadly mentioned as PNSTs. The diagnosis of PNST is primarily based on the evidence of typical histological features, such as the presence of Antoni A and B growth patterns (2, 4), Verocay bodies (5) and pseudo-onion bulbs (6). They can exhibit a benign or malignant biological behavior (2, 7-10).

Several immunohistochemical markers have been suggested as supporting the differentiation of PNSTs (11-13). In human medicine, the presence of both Antoni A and Antoni B morphological patterns and S-100 immunopositivity supports the histopathological diagnosis of schwannoma (3, 4).

Immunohistochemical characterization of canine PNSTs has been performed by several research groups using broad panels that include different antibodies, such as protein gene product (PGP) 9.5, laminin (13, 14) and collagen IV (13). Apart from helping in the identification of the biological behavior of PNST (11), glial fibrillary acidic protein (GFAP) was also recognized as a useful tool in the differential diagnosis between PNST and other spindle cell neoplasms (8, 15, 16). Also nerve growth factor receptor (NGFR) might

*These Authors contributed equally to this study.

Correspondence to: Fátima Gärtner, ECVF (Dipl), PhD, MVD, Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Rua Júlio Amaral de Carvalho nr. 45, Porto, Portugal. Tel: +351 225570700, Fax: +351 225570799, e-mail address: fgartner@ipatimup.pt

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be helpful for differentiating canine PNSTs from other sarcomas (11).

However, in veterinary medicine, a sensitive and specific marker exclusively expressed by canine PNSTs is still not available. Thus, the differentiation of these tumors is mainly based on their histopathological phenotype, which is often challenging (17).

The aim of the present study was to evaluate the histological and immunohistochemical features of 40 canine cutaneous PNSTs, applying a different score evaluation system and additional markers, in order to further refine the diagnosis of these tumors.

Materials and Methods

Samples. The study was conducted using archival samples from 40 canine cutaneous tumors received between 2000 and 2011 in the Laboratory of Veterinary Pathology (ICBAS-UP), previously diagnosed as spindle cells tumors, consistent with PNSTs.

Histopathology. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Consecutive 2- μ m sections were cut, one being stained with hematoxylin and eosin (HE) for histological examination and the others used for the immunohistochemical study. Sections were independently examined by two pathologists being classified according to the morphological criteria used in canine (2, 9) and human classification schemes for PNSTs (3). Only clear examples of PNSTs were included in this study. Thus, all the lesions tested had to be completely composed of wavy spindle cells, arranged in bundles, palisades and whorls. Additionally, the identification of specific morphological growth patterns, such as Antoni type A or type B were evaluated. The first pattern comprised areas of densely packed spindle shaped cells. These were often arranged in interlacing bundles and fascicles with occasional storiform, palisading or concentric growth resembling Antoni A schwannoma patterns. The second pattern was characterized by less cellular, diffuse, loosely textured meshwork of more pleomorphic, round, fusiform or polygonal cells resembling the Antoni B schwannoma pattern (13). When there was a divergence of opinion, an agreed diagnosis was reached through simultaneous observations in a multi-head microscope.

The biological behavior of the tumors was assessed by the evaluation of some parameters commonly used, such as encapsulation/borders (present, absent, capsule invaded or discontinuous), cellular atypia (nuclear pleomorphism; number of nucleoli), necrosis (present/absent) and mitotic activity (present/absent, number).

The proportion of each growth pattern (Antoni A or Antoni B) was assessed semi-quantitatively on the following scale: (–) absence of a specific growth pattern, (+) <25% of the tumor sample displays the growth pattern, (++) 25-50% of the tumor displays the growth pattern, (+++) 50-75% of the tumor displays the growth pattern, (++++>) >75% of the sample displays the growth pattern (9).

Immunohistochemistry. With the exception of the slides designed for S-100 immunohistochemistry, antigen retrieval was performed on dewaxed sections by boiling in 10 mmol/l sodium citrate buffer (pH 6.0) for 3 min in a pressure cooker. Slides were cooled for 10 min

at room temperature and rinsed twice in triphosphate buffered saline (TBS) for 5 min. After blocking endogenous peroxidase activity with 3% hydrogen peroxide in methanol for 10 min, the sections were subjected to a panel composed by several antibodies incubated overnight (Table I). Secondary detection was with the Novolink™ Max-Polymer detection system (Novocastra, Newcastle, UK), according to the manufacturer's instructions. Sections were rinsed with TBS between each step of the procedure. Labeling was 'visualized' by incubation of the sections with a freshly prepared solution of 3, 3'-diamino-benzidine (DAB) for up to 3 min at room temperature. Finally, sections were lightly counterstained with hematoxylin, dehydrated and mounted.

Immunohistochemistry expression appeared as distinct brown labeling of cytoplasm, membrane and/or nucleus of neoplastic cells. The proportion of cells labeled by each marker was graded as: 0, 0% no labeling; 1, <25% of tumor cells labeled; 2, 26-50% of tumor cells labeled; 3, 51-75% of tumor cells labeled; 4, 76-100% of tumor cells labeled. The intensity of labeling was also recorded as: 0, absent; 1 weakly positive; 2, moderately positive; 3, intense staining. Finally, a semi quantitative estimation of this expression was made using a composite score obtained by multiplying the values of the grade and intensity of labeling. A mean score of ≥ 6 was considered positive and a score of < 6 as negative.

The Ki-67 proliferation index (PI) was defined as percentage of positive nuclei determined by counting up to 1,000 nuclei in the selected fields ($\times 400$).

Statistical analysis. The results were statistically analyzed with the SPSS software (version 19.0; SPSS Inc., Chicago, IL, USA).

Chi-square test was used to determine possible statistically significant associations between the biological behavior of lesions and the parameters age, breed and sex. Fisher's exact test was performed to verify if a statistically significant association between S-100 expression and the biological behavior of lesions occurs. Mann-Whitney non-parametric test was used to establish differences with statistical significance in PI and in the dimensions of lesions between malignant and benign cases. *p*-Values < 0.05 were considered as statistically significant.

Results

Clinical data and histological classification. Data regarding breed, age, sex, tumor location and dimensions are summarized in Table II. Eleven dogs were of mixed breed (27.5%) and the most commonly represented pure breeds were Boxers ($n=7$; 17.5%) and Poodles ($n=5$; 12.5%). The large standard was the most encountered by 14 dogs (35%). Ages ranged from 2 to 14 years (mean=9.55) and most dogs were adults (62.5%). Of the 40 dogs, 25 were male (62.5%) and 15 were female (37.5%).

The sample included 17 benign and 23 malignant lesions equally distributed in the trunk and limbs of the animals with an average size of 4.29 ± 2.84 cm. Statistical analysis attested an important association between the dimension of the lesions and the biological behavior of tumors; the malignant lesions were larger ($p < 0.05$).

Regarding the morphological pattern of lesions, both Antoni A and Antoni B patterns were identified in 26 out of

40 cases (65%). All lesions presented highly cellular areas that often disposed in a palisade pattern or arranged in whorls, with elongate to fusiform round dark nuclei, similar to Antoni A human schwannoma pattern. Of the total, 14 cases (35%) displayed exclusively this pattern. The poorly organized growth pattern consisting in lower cellular areas, resembling Antoni B human schwannoma pattern, was not identified in exclusively. However, it was present in 26 out of the 40 PNST cases (65%) (Figure 1).

Immunohistochemistry. The overall results of the immunohistochemical evaluation are summarized in Table III.

The majority of the tumors (38/40, 95%) were positive for vimentin. All benign lesions were positive, 10 of which (58.8%) displayed the maximum score value. Among the malignant lesions, 21 out of 23 (91.3%) were positive, 7 of which (33.3%) presented the maximum score value (Figure 2a and b). The remaining malignant cases were negative.

The decline of the vimentin score values was mainly due to a decrease in the labeling intensity observed in the malignant tumors when compared to the benign ones. Even though, these differences in vimentin intensity of labeling were not associated with a specific lesional morphological pattern.

S-100 immunopositivity was observed in 22 out of 40 tumors (55%). Six out of 17 (35.3%) benign lesions were positive, while, in malignant tumors, immunopositivity was observed in 16 out of 23 (69.6%). Benign tumors showed a weak labeling when compared to the malignant lesions included in this study, which justifies the decrease of the score values among this group. Additionally, a statistically significant association was found between the expression of S-100 and the biological behavior of the lesions: the malignant lesions were more often positive for this marker when compared to the benign ones ($p < 0.05$) (Figure 2c and d).

GFAP immunopositivity was identified in 37 out of 40 cases (92.5%). All benign lesions were positive (100%), with three cases presenting the maximum score value (17.6%). In malignant tumors, immunopositivity was observed in 20 out of 23 cases (87%), 10 of which (50%) presented the maximum score value (Figure 2e and f).

Neuron-specific enolase (NSE) immunopositivity was observed in 32 out of 40 lesions (80%). Twelve out of 17 benign lesions (70.6%) were positive and the remaining 5 (29.4%) were negative. Twenty out of 23 malignant lesions (87%) were positive, 5 of which (25%) presented the maximum score value and 7 (35%) the borderline value of 6 (Figure 3a and b).

Desmin immunopositivity was detected in 31 out of 40 tumors (77.5%). Fourteen out of 17 (82.4%) benign tumors were positive, 8 of which (57.1%) presented a score value of 6. Three benign lesions were negative (17.6%). Seventeen out of 23 malignant tumors were positive (73.9%), 6 of which presented the borderline value of 6 (35.3%). The remaining malignant lesions were negative (26.1%) (Figure 3c and d).

Table I. *Antibodies used in immunohistochemistry.*

Antibody	Clone	Supplier	Dilutions
Vimentin	V9	Dako (Glostrup, Denmark)	1:500
S-100	Polyclonal	Dako (Glostrup, Denmark)	1:500
GFAP	Polyclonal	Millipore (San Diego, CA, USA)	1:2,000
NSE	VI-H14	Imgenex (San Diego, CA, USA)	1:500
Desmin	D33	Dako (Glostrup, Denmark)	1:20
α -SMA	HHF35	Dako (Glostrup, Denmark)	1:300
Ki-67	MIB-1	Dako (Glostrup, Denmark)	1:500

Table II. *Clinical data of dogs with cutaneous PNSTs.*

Case number	Breed	Age (years)	Gender	Location of tumor	Dimensions of tumors (cm)*
1	Portuguese pointer	9	M	T	8x8
2	St. Bernard	10	F	L	10x10
3	German Shepherd dog	4	M	T	2.5x2
4	Mixed breed	12	M	T	4.3x3
5	Portuguese pointer	11	M	T	3x3
6	Boxer	7	F	T	6.5x6
7	Mixed breed	7	M	T	3x3
8	Mixed breed	12	F	T	14x11
9	Mixed breed	9	M	L	2.5x2
10	Boxer	8	M	L	NR
11	Mixed breed	12	M	L	3x2
12	Siberian husky	7	M	T	5x5
13	Siberian husky	13	F	L	4.5x4
14	Labrador Retriever	2	M	T	3x3
15	Mixed breed	12	F	T	10.5x8
16	Boxer	13	M	T	2x2
17	Samoyed	8	F	L	8x6
18	Boxer	7	M	L	4.5x3.5
19	Dalmatian	10	F	L	4x2.5
20	Boxer	8	F	L	4x2
21	Portuguese pointer	10	F	L	2.5x2
22	Whippet	12	M	L	9x6
23	Mixed breed	8	F	L	4.3x2.7
24	Miniature Schnauzer	13	M	T	2.5x2.5
25	Mixed breed	12	F	L	4x3.5
26	Mixed breed	9	M	NR	2x2
27	Poodle	9	M	NR	3x2.5
28	X Rottweiler	8	M	NR	NR
29	Pitbull	7	M	L	3x3
30	Poodle	13	F	T	1x1
31	Epagneul breton	8	M	L	5x3.5
32	Mixed breed	8	M	L	4x2.8
33	Poodle	9	M	T	4.5x4.5
34	Mixed breed	10	F	L	2x1
35	Boxer	10	M	T	2x2
36	German Shepherd dog	14	F	T	4x2
37	Poodle	13	M	L	1.2x1.2
38	Boxer	9	M	T	2x1
39	Poodle	10	M	NR	2.5x2.5
40	German Shepherd dog	9	F	L	2.2x2

Figure 1. Canine PNSTs displaying different morphological patterns. (a) Antoni A is characterized by densely packed spindle shaped cells, arranged in interlacing bundles with palisading or concentric growth (HE, 100 $\times$). (b) Cells in the Antoni B regions are often thin and wispy, separated from one another by microcystic spaces filled with basophilic mucin (HE 100 $\times$). (c) Antoni A tissue and Antoni B tissue within a canine PNST lesion. The highly cellular Antoni A region on the left of the field contrasts with the loosely organized hypocellular Antoni B region on the right (HE, 40 $\times$).

Figure 2. Expression of different immunomarkers in both benign (left row) and malignant (right row) canine PNSTs. (a) and (b) The majority of the lesions presented intense vimentin immunoreactivity (IHC, 200 $\times$). (c) and (d) Malignant PNSTs were more often positive for S-100 than the benign ones ($p < 0.05$) (IHC, 100 $\times$ and 200 $\times$, respectively). (e) and (f) GFAP was consistently expressed in both benign and malignant lesions (IHC, 200 $\times$).

Figure 3. Expression of different immunomarkers in both benign (left row) and malignant (right row) canine PNSTs. (a) and (b) The majority of the lesions presented NSE-immunopositivity (IHC, 200 $\times$). (c) and (d) Benign and malignant lesions presented identical desmin immunohistochemical scores. (IHC, 200 $\times$). (e) and (f) The majority of the lesions were α -actin negative (IHC, 200 $\times$).

Alpha-smooth muscle actin (α -SMA) immunopositivity was observed only in 4 out of 40 tumors (10%). Among the 17 benign lesions, two were positive (11.8%) and 15 were negative (88.2%). All the positive cases presented the borderline score value of six and 8 of the negative cases presented the minimum score value (0). Among the 23 malignant cases, only 2 were positive (8.7%) and the remaining 21 were negative (91.3%), 10 of which presented the minimum score (0) (47.6%) (Figure 3e and f).

Independently of their biological behavior, all tumors showed expression of Ki-67 and the proliferative index ranged from 0.6% to 80.9% (Figure 3). The average PI was 16.3 $\pm$ 17% in the benign tumors and 35.4 $\pm$ 27.8% in the malignant PNSTs. Among the benign lesions, six cases presented a Ki-67 PI higher than 21%. Apart from one case,

Figure 4. The picture shows a malignant canine PNST case presenting high proliferative index as observed by the moderate to strong nuclear expression of Ki-67 in the great majority of neoplastic cells (IHC, 200 $\times$).

Table III. Immunohistochemical final scores and panels of the canine PNSTs cases included in the study.

Classification	Cases		Immunohistochemistry results							
	Total nr.	Cases ID*	Vimentin	S-100	NSE	GFAP	Desmin	α -actin	Ki-67 PI (%)	
MPNST	8	5; 8; 9; 16; 19; 20; 21; 23	+	+	+	+	+	–	38.7; 80.9; 70.9; 25.9; 10.2; 2.8; 17.0; 27.2	<i>p</i> <0.05
	4	2; 7;13; 15	+	–	+	+	+	–	62.6; 42.4; 14.1; 6.4	
	3	6; 17; 18	+	+	+	+	–	–	21.4; 63.1; 26.2	
	2	10; 12	–	–	+	+	+	–	67.2; 6.1	
	1	1	+	+	–	+	–	–	38.9	
	1	3	+	+	–	–	+	–	68.2	
	1	4	+	+	+	–	–	+	18.6	
	1	11	+	+	–	+	+	–	54.0	
	1	14	+	–	+	–	–	–	23.5	
	1	22	+	+	+	+	+	+	28.6	
BPNST	4	24; 27; 29; 38	+	–	+	+	+	–	60.8; 36.3; 21.0; 2.8	NS
	5	28; 32; 36; 39; 40	+	+	+	+	+	–	4.3; 1.8; 14.1; 6.2; 0.6	
	3	25, 26, 31	+	–	–	+	+	–	15.8; 35.3; 6.8	
	1	30	+	–	–	+	–	–	5.6	
	1	33	+	–	–	+	+	+	32.0	
	1	35	+	–	+	+	+	+	28.2	
	1	37	+	+	+	+	–	–	1.2	
	1	34	+	–	+	+	–	–	3.8	

PNST, Peripheral nerve sheath tumors; MPNST, malignant PNST; BPNST, benign PNST; PI, proliferation index; NSE, neuron-specific enolase; GFAP, glial fibrillary acidic protein; (–) negative final immunohistochemical score, (+) positive final immunohistochemical score. NS, Not significant ($p > 0.05$).

all MPNSTs showed a Ki-67 proliferation index higher than 6%. The statistical analysis confirmed an important association between the proliferation index and the biological behavior of the neoplasms: when malignant, the tumor presented a proliferation index significantly higher ($p < 0.05$) (Figure 4).

Discussion

Tumors that originate from neural crest-derived cells, including benign and malignant forms of PNST, represent a heterogeneous group of neoplasms.

In the present study and in agreement with previously published reports, PNSTs affected mainly older dogs (8, 18). Similarly to previous reports, no predilection breed was identified in the present study (8, 13), although our series was comprised by a large number of Poodles and Boxers. This might be explained by the popularity of these specific breeds in our geographical localization rather than any racial predisposition. In veterinary medicine, no sex predilection has been yet established, but in our study 62.5% of the dogs affected were male. In opposition, in human medicine, malignant PNST (MPNST) tends to occur more frequently in women (3).

The histological evaluation allowed the distinction of the two different Antoni A and B morphological patterns, present

in different proportions. In our study and in accordance with Stoica *et al.*, these human typical growth patterns were not so clearly identifiable in the dog.

In agreement with their mesenchymal origin, PNSTs are positive for vimentin (11, 20). Also, in our study, all cases showed a consistently expression of vimentin. Previous investigations reported that, in veterinary medicine, vimentin is an immunohistochemistry marker invariably positive in all PNSTs regardless its biological behavior (5, 11, 13, 16, 19, 20-29). Interestingly, in our study, differences in the labeling intensity were observed between the benign and the malignant cases that beared on the decreased of the vimentin score values within the latter group. However, in previous studies, there is no mention of differences relative to intensity of labeling between benign and malignant lesions, preventing us to critically compare these results. Nevertheless, differences at this level were described in Antoni A and B areas of human schwannomas. Antoni B schwannoma pattern showed a weaker expression of vimentin when compared to Antoni A areas (30). Probably, the fact that the Antoni B areas are characterized by lower density of tumor cells randomly arranged in a loose matrix can result in a supposed visual fading of vimentin labeling intensity.

S-100 is an acidic protein that identifies various nervous tissue cells, including Schwann cells, and the majority of canine PNSTs diffusely expressed this molecule (10, 11, 14).

The association between S-100 immunorexpression and the biological behavior of PNST is controversial and still being debated by several authors (11, 24, 27, 29, 31, 32). In the present study, we observed S-100 positivity in 55% of all tumors. Additionally, benign forms showed a weaker labeling when compared to the malignant ones. Our results are in agreement with other investigations in which S-100 immunorexpression was considered a feature of canine and feline MPNSTs (24, 27, 29). Malignant transformation of benign peripheral nerve sheath tumors has been described in humans (33). Tumoral progression could explain the S-100 immunoreactivity in the malignant lesions included in this study. Whilst benign tumors showed no positivity for S-100. However, with tumor progression, these tumors probably acquire the expression of this protein. On the other hand, in humans, data describing a strong and uniform S-100 labeling in benign schwannomas is available (3) along with studies associating lack of S-100 expression and malignancy in MPNSTs (31). Furthermore, Pumarola *et al.* and Chijiwa *et al.*, concluded that lack of expression of S-100 in canine PNSTs supports the interpretation of malignancy. Stoica *et al.* also reported that animal MPNSTs appeared to be negative for S-100 in contrast to schwannomas that were S-100 immunopositive. The loss/absence of S-100 labeling in these MPNSTs may be related not to the malignant behavior of the lesions but its histogenesis. MPNSTs could arise from Schwann cells, perineural cells and fibroblasts (13, 34). In a tumor in which Schwann cells are not the main cellular component it is not expected to observe such a strong and diffuse S-100 immunoreactivity (34).

GFAP is the major component of the family of intermediate filaments found in glial cells; however, its expression is not restricted to these cells since Schwann cells also express this protein (35). Like in human medicine, GFAP expression is characteristic of canine benign PNSTs (11). Thus, this marker should be useful to distinguish the biological behavior of canine PNSTs (11). However, Gaitero *et al.* expressed doubts. In the present study, 92.5% tumors were positive for GFAP, including 100% of BPNSTs and 86.9% of MPNSTs. Although it has been identified as a useful tool in differential diagnosis between PNSTs and other spindle cell neoplasms (8, 15, 16), the usefulness of GFAP in the biological behavior characterization of these canine lesions was not so evident in our study.

Recently, Klopffleish *et al.* identified 25 genes with higher expression in canine PNSTs, 7 of which are associated with a neuroectodermal differentiation or neuronal function that could act as potential PNST markers. In our study, a glycolytic enzyme that allows the identification of neuroendocrine and neuronal related neoplasms - NSE - was also tested (2, 26). Concerning this marker, our results support previous studies: NSE immunoreactivity could be observed both in benign and malignant canine PNSTs (11,

13, 16, 25, 27). However, the frequent non-specific background staining of this marker is renowned rendering it to limited use (13).

Desmin is a protein of intermediate filaments and its expression is highly specific of muscle cells, as well as neoplasms of muscle origin tissues (36). More than three quarters (77.5%) of our sample showed an unexpected immunopositivity for this marker. As opposed, the few studies that tested desmin immunorexpression in PNSTs of some domestic animals did not show any reactivity (21, 25, 26). With this regard, both Nielsen *et al.* and Ahmadi *et al.* tested this antibody in bovine tissue; the first used the exact same clone that was used in this study, while the second used another antibody from a different company. Nevertheless, Kuwamura *et al.* performed their experiments in canine tissues but did not specify the clone used. Thus, the results herein obtained may highlight the non-specific reactivity of this particular clone of desmin in canine cutaneous PNSTs.

In attempt to further analyze and confirm/exclude the results obtained with desmin, we tested α -SMA immunorexpression, which, similarly to desmin, can be observed in cells of muscle origin. Only 10% of the total lesions were immunopositive for α -SMA. As similar, Dunder *et al.* described α -SMA labeling in 4 of 50 benign schwannomas and suggested that neural crest-derived tumors could occasionally present α -SMA expression. *In vitro*, neural crest stem cells can undergo multilineage differentiation and single cells could give rise to different types of cells, including neurons, Schwann cells and myofibroblasts (38). Several studies reported that neural crest-derived cells show some instability and plasticity resulting eventually in phenotype conversion between the different lineages (transdifferentiation), generating intermediate cell types that can express markers specific for both lineages (39). In our study, the unexpected finding of canine PNSTs expressing α -SMA could be the result of a divergent differentiation of some Schwann cells towards smooth muscle or myofibroblasts (37).

The expression of Ki-67 is strictly associated with cell proliferation. The Ki-67 antigen is present during all active phases of the cell cycle (G_1 , S, G_2 and M phases) but is absent from the resting phase (G_0), thus allowing the distinction between proliferating and quiescent cells (40). Ki-67 is tightly controlled and regulated being considered an excellent marker for cell proliferation and is assumed that high Ki-67 reveals tumor cell activity and, thus, predicts the further behavior of a specific pathology. In our study, 96% of the MPNSTs showed a Ki-67 proliferation index (PI) higher than 6%. Other investigations have shown that human MPNSTs display a Ki-67 index of 7-38% (with an average of 23%) (41) and of 5%-65% (42), as opposed to BPNSTs in which the Ki-67 index was lower than 5% (30, 42). In this study, which constitutes the first immunohistochemical investigation regarding Ki-67

in canine cutaneous PNSTs, the statistical analysis confirmed an important association between the proliferation index and the biological behavior of the neoplasms. When malignant, canine PNST proliferation index is significantly higher. In comparison, Mandara *et al.* did not identify any relationship between Ki-67 expression and the morphological malignancy in feline cutaneous PNSTs.

Peripheral nerve tumors show a significant microscopic and molecular heterogeneity, despite their common origin from the neural crest. The differential diagnosis of these tumors on HE stained samples may be sometimes difficult, in which case the immunohistochemical examination may contribute to the final diagnosis. In fact, Meyer and Klopfeish attempted unsuccessfully to conceive a practical polymerase chain reaction assay based on newly proposed mRNA markers for routine differentiation of canine PNSTs from fibrosarcomas and potentially from other tissue sarcomas. It seems that, despite their different morphology, PNSTs are more closely related to fibrosarcomas in terms of histogenesis than previously thought (14).

As in other investigations, we failed in determining an unequivocally immunohistochemical panel that could support the current diagnostic “gold standard” of histopathological appearance of canine PNSTs. These lesions present a complex histogenesis, show very variable individual features and the antibodies themselves revealed a non-specific level significant in canine tissues. Nevertheless, we conclude that Ki-67 can be a useful marker helping to discriminate the biological behavior of canine PNSTs.

Disclosures

The Authors have nothing to disclose regarding this article.

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References

- Kleihues P and Cavenue WK: Pathology and genetics of Tumors of the nervous system (2nd ed.). Lyon, IARC Press, 2000.
- Koestner A and Higgins RJ: Primary Tumors of the peripheral nervous system. *In: Tumors in Domestic Animals* (4th Edit., DJ Meuten, Ed.). Ames, Iowa State University Press, pp. 731-735, 2002.
- Scheithauer BW, Woodruff JM and Erlandson RA: Fascicle 24: Tumors of the peripheral nervous system. *In: Atlas of Tumors Pathology* (3rd Series). Washington DC, Armed Forces Institute of Pathology, pp. 105-176, 303-72, 1999.
- Joshi R: Learning from eponyms: Jose Verocay and Verocay bodies, Antoni A and B areas, Nils Antoni and Schwannomas. *Indian Dermatol Online J* 3: 215-219, 2012.
- Schulman FY, Johnson TO, Facemire PR and Fanburg-Smith JC: Feline peripheral nerve sheath tumors: Histologic, immunohistochemical and clinopathological correlation (59 Tumors in 53 cats). *Vet Pathol* 46: 1166-1180, 2009.
- Higgins RJ, Dickinson PJ, Jimenez DF, Bollen AW and Lecouter RA: Canine intraneural perineurioma. *Vet Pathol* 43: 50-54, 2006.
- Goldschmidt MH and Hendrick MJ: Benign peripheral nerve sheath Tumor (neurofibroma, schwannoma). *In: Tumors in domestic animals* (4th Edit., DJ Meuten, Ed.). Ames, Iowa State University Press, pp. 95-96, 2002.
- Gross TL, Ihrke P, Walder EJ and Affolter VK: Neural and perineural Tumors. Mesenchymal neoplasms and other tumors. *In: Skin diseases of the Dog and Cat. Clinical and Histopathological Diagnosis* (2nd ed.). Oxford, Blackwell Publishing., pp. 786-796, 2005.
- Hendrick MJ, Mahaffey EA, Moore FM, Vos JH and Walder EJ: *In: WHO Histological classification of mesenchymal tumors of skin and soft tissue of domestic animals* (Vol. II, FY Schulman, Ed.). Washington DC, Armed Forces Institute of Pathology, pp. 25-27, 1998.
- Koestner A, Bilzer T, Fatzer R, Schulman FY, Summers BA and Van Winkle TJ: *In: Histological classification of tumors of the nervous system of domestic animals* (Vol. 5, FY Schulman, Ed.) Washington DC, Armed Forces Institute of Pathology, pp. 37-38, 1999.
- Chijiwa K, Uchida K and Tateyama S: Immunohistochemical evaluation of canine peripheral nerve sheath tumors and other soft tissue sarcomas. *Vet Pathol* 41: 307-318, 2004.
- Dennis MM, McSparran KD, Bacon NJ, Schulman FY, Foster RA and Power BE: Prognostic factors for cutaneous and subcutaneous soft tissue sarcomas in dogs. *Vet Pathol* 48: 73-84, 2011.
- Gaitero L, Añor S, Fondevila D and Pumarola M: Canine cutaneous spindle Cell Tumors with features of peripheral nerve sheath Tumors: a histopathological and immunohistochemical study. *J Com Pathol* 139: 16-23, 2008.
- Meyer A and Klopfeisch R: Multiple polymerase chain reaction markers for the differentiation of canine cutaneous peripheral nerve sheath Tumors versus canine fibrosarcomas. *J Comp Path* 150: 198-203, 2014.
- Sarlomo-Rikala M, Tsujimura T, Lendahl U and Miettinen M: Patterns of nestin and other intermediate filament expression distinguish between gastrointestinal stromal Tumors, leiomyomas and schwannomas. *APMIS* 110: 499-507, 2002.
- Sawamoto O, Yamate J, Kuwamura M, Hagiwara R and Kurisu K: A canine peripheral nerve sheath Tumor including peripheral nerve fibers. *J Vet Med Sci* 61: 1335-133, 1999.
- Klopfeisch R, Meyer A, Lenze D, Hummel M and Gruber AD: Canine cutaneous peripheral nerve sheath Tumors versus fibrosarcomas can be differentiated by neuroectodermal marker genes in their transcriptome. *J Comp Path* 148: 197-205, 2013.
- Scott DW, Miller Jr WH and Griffin CE: Neoplastic and non-neoplastic tumors. *In: Muller & Kirk's small animal dermatology* (6th ed.). Philadelphia, W.B. Saunders Company, pp. 1236-1414, 2001.
- Stoica G, Tasca SI and Kim HT: Point mutation of neu oncogene in animal peripheral nerve sheath tumors. *Vet Pathol* 38: 679-688, 2001.

- 20 Pérez J, Bautista MJ, Rollón E, de Lara FC, Carrasco L and Martín de las Mulas J: Immunohistochemical characterization of hemangiopericytomas and other spindle cell tumors in the dog. *Vet Pathol* 33: 391-397, 1996.
- 21 Ahmadi N, Oryan A, Ghane M and Daneshbod Y: Cutaneous schwannoma in a cow. *Braz J Vet Pathol* 5: 81-85, 2012.
- 22 Bergmann W, Burgener IA, Roccabianca P, Rytz U and Welle M: Primary splenic peripheral nerve sheath tumor in a dog. *J Comp Pathol* 141: 195-198, 2009.
- 23 Brower A, Salamat S, Crawford J and Manley P: Unilateral limb enlargement in a dog with a malignant peripheral nerve sheath tumor. *Vet Pathol* 42: 353-356, 2005.
- 24 Buza EL, Menzies RA, Goldschmidt MH and Durham AC: Malignant peripheral nerve sheath tumors in a cat with nodal and pulmonary metastases. *J Vet Diagn Invest* 24: 781-784, 2012.
- 25 Kuwamura M, Yamate J, Kotani T, Takeuchi T and Sakuma S: Canine peripheral nerve sheath tumor with eosinophilic cytoplasmic globules. *Vet Pathol* 35: 223-226, 1998.
- 26 Nielsen AB, Jansen ECL, Leifsson PS and Jensen HE: Immunoreactivity of bovine schwannomas. *J Comp Path* 137: 224-230, 2007.
- 27 Park JW, Woo GH, Jee H, Jung DW, Youn HY, Choi MC and Kim D: Malignant peripheral nerve sheath tumor in the liver of a dog. *J Comp Pathol* 144: 223-226, 2011.
- 28 Pavarini SP, Gomes DC, Bandinelli MB, Wouters F, Sonne L, Driemeier D and da Cruz, EF: Malignant peripheral nerve sheath tumor as a cause of chronic insufficiency in cattle. *Acta Vet Scand* 55: 7, 2013
- 29 Tavasoly A, Javanbakht J, Khasi F, Hosseini E, Bahrami A, Hassan MA and Mirabad M: Ulnar malignant peripheral nerve sheath tumor diagnosis in a mixed-breed dog as a model to study human: Histologic, immunohistochemical, and clinicopathologic study. *Diagn Pathol* 8: 86, 2013.
- 30 Ghiluşi M, Pleşea IE, Comănescu M, Enache SD and Bogsan F: Preliminary study regarding the utility of certain immunohistochemical markers in diagnosing neurofibromas and schwannomas. *Rom J Morphol Embryol* 50: 195-202, 2009.
- 31 Hoang MP, Prasanna S and Albores-Saavedra J: Expression of protein gene product 9.5 in epithelioid and conventional malignant peripheral nerve sheath Tumors. *Arch Pathol Lab Med* 125: 1321-1325, 2001.
- 32 Pumarola M, Añor S, Borrás D and Ferrer I: Malignant epithelioid schwannoma affecting the trigeminal nerve of a dog. *Vet Pathol* 33: 434-436, 1996.
- 33 Weiss SW and Goldblum JR: Malignant Tumors of peripheral nerves. In: Enzinger and Weiss's soft tissue tumors (4th edn.). St. Louis, Mosby, pp. 1209-1264, 2001.
- 34 Takeuchi A and Ushigome S: Diverse differentiation in malignant peripheral nerve sheath tumors associated with neurofibromatosis-1: An immunohistochemical and ultrastructural study. *Histopathology* 39: 298-309, 2001.
- 35 Jessen KR, Thorpe R and Mirsky R: Molecular identity, distribution and heterogeneity of glial fibrillary acidic protein: an immunoblotting and immunohistochemical study of schwann cells, satellite cells, enteric glia and astrocytes. *J Neurocytol* 13: 187-200, 1984.
- 36 Ellis I: Immunocytochemistry in diagnostic pathology. In: Bancroft's Theory and practice of histological techniques (4th ed.). Philadelphia, Churchill Livingstone, pp. 471-487, 1996.
- 37 Dundr P, Povýšil C and Tvrdík D: Actin expression in neural crest cell-derived tumors including schwannomas, malignant peripheral nerve sheath tumors, neurofibromas and melanocytic tumors. *Pathol Int* 59: 86-90, 2009.
- 38 Joseph NM, Mukoyama Y, Mosher JT, Jeagle M, Crone SA, Dormand E, Lee K, Meijer D, Anderson DJ and Morrison SJ: Neural crest stem cells undergo multilineage differentiation in developing peripheral nerves to generate endoneurial fibroblasts in addition to Schwann cells. *Development* 131: 5599-5612, 2004.
- 39 Real C, Glavieux-Pardanaud C, Vaigot P, Le Douarin N and Dupin E: The instability of the neural crest phenotypes: Schwann cells can differentiate into myofibroblasts. *Int J Dev Biol* 49: 151-159, 2005.
- 40 Scholzen T and Gerdes J: The Ki-67 protein from the known to the unknown. *J Cell Physiol* 182: 311-322, 2000.
- 41 Ogoe A, Hotta T, Morita T, Higuchi T, Umezaki H, Imaizumi S, Hatano H, Kawashima H, Gu W and Endo N: Diagnosis of peripheral nerve sheath tumors around the pelvis. *Jpn J Clin Oncol* 34: 405-413, 2004.
- 42 Kindblom LG, Ahlden M, Meis-Kindblom JM and Stenman G: Immunohistochemical and molecular analysis of p53, MDM2, proliferating cell nuclear antigen and Ki67 in benign and malignant peripheral nerve sheath tumors. *Virchows Arch* 427: 19-26, 1995.
- 43 Mandara MT, Fabiani E, Pavone S and Pumarola M: Feline cutaneous nerve sheath tumors: histological features and immunohistochemical evaluations. *Res Vet Sci* 95: 548-555, 2013.

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