Study of canine cutaneous melanocytic tumours: evaluation of histological and immunohistochemical prognostic criteria in 65 cases

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SUMMARY

Canine cutaneous melanocytic tumours account for 10 to 15% of skin neoplasms in this species. Despite the existence of a prognostical classification established by Bostock in 1979, based on the number of mitotic figures in the neoplastic cell population, the histological prognosis given by the pathologist is often considered as poorly reliable. No architectural or cytological criterion other than mitotic index is currently considered for the establishment of the melanocytic tumours prognosis in dog. Immunohistochemical markers, such as nuclear proliferation antigen (Ki-67 antigen), have been shown to improve the prognostic quality in both dogs and men. More recently, markers related to cellular differentiation (Melan-A) or the adhesion abilities (CD44) of the neoplastic melanocytic cells were proposed as elements related to the prognosis. In this study, using 65 cases of canine cutaneous melanocytic tumours, observed during a 12 months post surgical period, 13 architectural and cytological criteria and 3 immunohistochemical markers (Ki-67 antigen, CD44 and Melan-A) were investigated. Several major (neoplasm size, growth type, symmetry of the lesion, mitotic index and lymphatic / vascular invasion) and minor (tumour shape, invasion level and shape of the neoplastic cells) histological criteria were proposed, but the use of Ki-67 and CD44 for the establishment of a more reliable prognosis was also recommended.

Keywords: Dog, cutaneous melanocytic tumour, histopathology, immunohistochemistry, prognosis, Ki-67, Melan-A, CD44.

RÉSUMÉ

Etude des tumeurs mélanocytaires cutanées du chien : évaluation des critères pronostiques histologiques et immunohistochimiques à partir de 65 cas

Les tumeurs mélanocytaires représentent 10 à 15 % des tumeurs cutanées dans l'espèce canine. En dépit d'une classification pronostique établie par Bostock en 1979 basée sur le nombre de figures de mitose au sein de la population tumorale, le pronostic histologique est souvent considéré comme peu fiable. Actuellement, le seul critère histologique utilisé pour établir le pronostic des tumeurs mélanocytaires du chien est l'index mitotique. L'utilisation de certains marqueurs immunohistochimiques, et notamment les marqueurs de prolifération tels que l'antigène Ki-67, peut améliorer la qualité du pronostic dans l'espèce humaine comme dans l'espèce canine. Plus récemment, l'expression de marqueurs de différenciation cellulaire (Melan-A) ou d'adhésion (CD44) des cellules mélanocytaires tumorales a été utilisée pour affiner le pronostic. Au cours de cette étude, 13 critères architecturaux et cytologiques, ainsi que l'expression de 3 marqueurs immunohistochimiques (Ki-67, CD44, Melan-A) ont été étudiés à partir de 65 cas de tumeurs mélanocytaires cutanées canines, suivis pendant 12 mois après exérèse chirurgicale. Plusieurs critères histologiques majeurs (taille du néoplasme, mode de croissance, symétrie de la lésion, index mitotique et invasion lymphatique / vasculaire) ou mineurs (aspect de la tumeur, degré d'expansion et type des cellules néoplasiques) sont proposés, mais l'utilisation des marqueurs Ki-67 et CD44 est également recommandée pour affirmer le pronostic.

Mots clés : Chien, tumeur mélanocytaire cutanée, histopathologie, immunohistochimie, pronostic, Ki-67, Melan-A, CD44.

Introduction

Melanocytic tumours in animals are mainly found in the skin, the oral cavity and the ocular structures. In dogs, these neoplasms account for 10-15% of all skin tumours [4, 10, 11, 15]. Lately, a close attention has been paid to these lesions because of their increasing occurrence in human beings and because of the fact that domestic animals and man may be exposed to similar environmental factors. Furthermore, many clinicopathological studies concerning canine cutaneous melanocytic tumours are performed with the aim to identify predictive factors of the biological behaviour of these neoplasms [4, 8, 11, 13, 17-19].

Some clinical and histopathological factors have a significant prognostic value. Many cutaneous melanocytic tumours in dogs follow a benign course but some localisations are known to carry a worse prognosis, particularly the mucocutaneous junctions (lips) and the distal extremities. In digital localization, the nail bed involvement could be associated with malignancy [8]. In various studies, different clinical features, such as degree of pigmentation, dermal vs. hypodermal location, ulceration, volume, have been supposed to have predictive values. In a few studies, some of these factors seemed to be correlated with survival but the same prognostic factors were not identified in these different studies, leading to a lack of agreement [17].

The first histopathological classification of melanocytic tumours in dogs was proposed by Weiss and Frese in 1974 [20]. In 1979, Bostock evaluated the prognostic significance of this classification [4]. He came to the conclusion that it is of great value as a morphological guide and may allow to identify categories for histopathological benign lesions and malignant tumours. Yet its use as a prognostic indicator seemed to be unreliable since in his study a small group (about 10%) of apparently histologically benign lesions had a malignant behaviour, and 55% of dogs with histologically malignant tumours were completely cured by surgical excision. In Bostock's study, a comparison was made between the behaviour of skin melanocytic tumours with different mitotic index, and mitotic index was found to be of great prognostic value. Consequently, Bostock proposed to simplify the classification by dividing all skin melanocytic tumours into either "well-differentiated" or "poorly-differentiated" categories without further subdivision. Well-differentiated tumours have a mitotic index of maximum 2 (estimated on 10 randomly selected high power fields), which is often closely associated with a heavy pigmentation, an abundant stroma and a lack of invasion. Poorly-differentiated tumours include all tumours with a mitotic index of 3 or more. Based on these criteria, in Bostock's study, well differentiated melanocytic tumours carried a favourable prognosis, with a 90% 2-years cure rate after surgery. Conversely poorly-differentiated tumours had a poor prognosis with almost 70% of dogs dying from their tumour and 50% being dead within the 7 months following surgery. For a long time, the criteria proposed by Bostock remained the single histopathological factors of prognosis. In 1998, the revised World Health Organization classification system recommended to use the term 'melanocytoma' to encompass all variants of congenital and acquired benign neoplasms arising from melanocytes, 'melanoma' being used synonymously with malignant melanoma [8].

The current development of immunohistochemistry in veterinary pathology supplies with new tools for prognosis. In some studies, measurement of tumour proliferation for canine cutaneous melanomas was correlated with biological behaviour. In a study including 20 canine melanocytic tumours [14], it has been shown that lesions histologically classified as either benign or malignant differed significantly in respect of Ki-67 epitope and PCNA (Proliferating Cell Nuclear Antigen) positivity. In another study that we have performed (with 68 canine cutaneous melanocytic neoplasms) [11], high Ki-67 proliferative index (more than 15%) and histopathological malignancy were both associated with significantly poorer 2-years survival rate. The predictive value of the Ki-67 proliferative index (97%) was mildly higher than the predictive value of classical histopathology (91%). In this study, the Ki-67 proliferative index identified a subgroup of cases (4 cases) with a proliferative index of less than 15% but having histopathological criteria of malignancy (vesicular and nucleolated nuclei with marked anisokaryosis, mitotic index > 2). All of these cases showed a significantly longer survival time, which would have been anticipated, had the prognosis been determined by the proliferative index. In other studies, it has been shown that specific melanocytic epitopes as Melan A/MART-1 (Melanoma Antigen Recognized by T-cells-1) are expressed by normal and neoplastic canine melanocytes [5, 9, 12, 13]. For some authors [10], Melan A/MART-1 may be informative regarding the biological behaviour of canine melanocytic tumours.

In the present study, we report the results of a histopathological and immunohistochemical study of 65 cutaneous melanocytic tumours in dogs. The 2 main objectives of this study were to evaluate the prognostic value of histocytopathological features of the neoplasms as well as Melan A/MART-1 and CD44 expression by neoplastic cells, in comparison with Ki-67 expression. CD44 is a cell surface glycoprotein implicated in multiple cellular functions including cell-cell adhesion, cell-substrate interactions and cell activation [1]. In some clinical studies, a high level expression of CD44 in human malignant melanoma has been associated with increased metastatic risk and reduced survival [2, 3, 6].

Material and Methods

RETROSPECTIVE CASE MATERIAL

Sixty-five cases of canine cutaneous melanocytic neoplasms were included in the present study. Tumour specimens were retrieved from archived material submitted to the Laboratoire d'Anatomie Pathologique Vétérinaire du Sud-Ouest (Toulouse, France), over a period of 3 years. All tumour specimens were formalin fixed and paraffin embedded samples. Complete information about clinical and biological behaviour was available for all cases selected for the study. All of these cases had been followed up for at least 12 months after excision. Clinicopathological features of these dogs were given by the veterinary surgeons who had made the excision of tumours and who had watched over clinical evolution (dead with inoperable recurrence or metastatic extension, alive with local recurrence, alive with no evidence of disease).

In this study, a favourable outcome was characterized by absence of local recurrence and absence of metastases a year after surgical excision. An unfavourable outcome is characterized by local recurrence and/or death of the animal due to a metastatic spread during the year following surgical excision of the primary neoplasm.

HISTOLOGICAL EVALUATION

Three-micrometer thick sections of the tumours were stained with haematoxylin and eosin. Before staining, a melanin bleaching was performed on heavily pigmented lesions (dewaxed paraffin sections were treated in 0.25% potassium permanganate 30 minutes at room temperature, followed by 1% oxalic acid for 1 minute).

Histological examination was independently performed by two observers. For each case, pattern and cytological features were analysed. All the features with variable intensity were semi-quantitatively scored on a scale from 1 to 3 (mild, moderate and marked).

The main histological features evaluated were the symmetry of the lesion, the level of invasion (epidermis, dermo-epidermal junction, dermis, subcutis), the growth (expansive or infiltrating growth), the shape (nodular, multinodular…), the presence of

lymphatic and/or vascular invasion, the presence and distribution of stroma reaction (tumour associated lymphoid cells) and the epidermal lesions (ulceration, acanthosis, hyperkeratosis…).

Concerning the cytological features, the following features were studied: the shape of neoplastic cells (fusiform, epithelioid, mixed…), the shape and location of nuclei and nucleoli, the nucleocytoplasmic ratio, the nuclear abnormalities (anisocaryosis, giant cells…), the pigmentation (score of 0 for amelanocytic tumours and 3 for heavily pigmented neoplasms) and the mitotic index (total of mitosis evaluated on 10 fields, x 40 objective, randomly selected).

IMMUNOHISTOCHEMISTRY

Three primary monoclonal antibodies were used in this study:

1) a monoclonal antibody against the Ki-67 epitope (isotype IgG1, Dako, ref. M7240); Ki-67 epitope is expressed exclusively in the nuclei of cycling cells (from G1 to M phase in the cell cycle). As others [14], we have used this marker in a previous study on canine cutaneous melanocytic tumours [11],

2) a monoclonal antibody against Melan A (isotype IgG1, clone A103, Dako, ref. M7196); this epitope has been proven to be a specific and sensitive marker for canine melanomas [10, 12],

3) a monoclonal antibody against CD44 named MCA1449 (isotype IgG1, Serotec, ref. MAC 329); it has been shown that this antibody recognizes porcine and canine CD44 [1, 16, 21].

Two different pre-treatment methods were used to unmask the epitopes:

1) for Ki-67 epitope, pre-treatment with phosphate buffered saline solution (PBSS) at pH 7.6 containing 0.1% trypsin (200 UI/g) during 5 minutes and then heating in a microwave oven in a citrate buffer 10 mM at pH 6.15 [11];

2) for Melan A and CD44, pre-treatment with a steamer, heating the slides in antigen retrieval EDTA buffer solution at pH 8.0 [12, 13].

Concerning immunohistochemical staining procedure, the following protocol was used: 1) primary monoclonal antibody (MAb) (Mouse IgG), 2) biotin conjugated goat anti-mouse IgG, 3) peroxidase conjugated to streptavidin, 4) aminoethylcarbazole (AEC) as enzyme substrate. Except for the MAb against CD44, all reagents were from Dako (France). Stained slides were first evaluated by two independent pathologists in a blind manner. When readings were not concordant, cases were then re-evaluated. Negative controls included omission of the primary antibody and its substitution by an irrelevant antibody of the same IgG1 subclass.

The assessment of immunoreactivity has slightly varied according to the marker used. For the Ki-67 epitope, counting was performed on aggregates containing positive nuclei for focal immunoreactivity pattern or on fields randomly selected when the immunoreactivity pattern was diffuse. Areas under ulcerated and secondarily infected zones were avoided because of the numerous positive inflammatory cells. Counting was done on 500 tumour cells, at a magnification of a x 40 objective with the assistance of an eyepiece graticule. Any nucleus with evidence of immunoreactivity (even weak) was considered as positive. A red granular cytoplasmic staining was considered to be positive for Melan A expression. For each tumour, the ratio of positive cells was calculated for 1000 cells (five high power fields of 200 cells). CD44 is expressed by a great range of mammalian cells [7]. The intensity of CD44 expression on malignant melanocytic cells was semi-quantitatively scored on a scale of 0 to 2, as it was described by DIETRICH *et al*. [6]. To palliate the staining differences between specimens, the level of CD44 expression of malignant melanocytes was compared to that in the overlying epidermis in the same sections since keratinocytes are known to express CD44: 0, no expression of neoplastic cells but staining of keratinocytes in the same section; 1, neoplastic cells staining is weaker than in the overlying epidermis; 2, neoplastic cells staining is identical to or stronger than in the overlying epidermis. A diffuse, red staining of membranes was considered as positive.

STATISTICAL ANALYSIS

Chi 2 t tests were used to compare percentages (Software STATITCF). The differences and correlation between mean values were statistically analysed by the Mann-Whitney non parametric U test (Software SIMSTAT). A P value of ≤ 0.05 was considered significant in all tests.

Results

The clinical, histopathological and cytopathological features of the 65 patients included in the study are summarized in Tables I, II, III and IV.

CLINICOPATHOLOGICAL FEATURES (TABLES I AND II)

The mean age at diagnosis was 9 years-old (from 5 to 13 year-old). No breed was over represented in this study.

Nineteen of the 65 dogs (29.2%) developed local recurrence (5 cases: 7.7%) and/or metastasis (14 cases: 21.5%) within one year. The other 46 cases (70.8%) presented no sign of recurrence and all of them were alive 1 year after surgery.

Concerning the anatomical location of tumours, the digits (13 cases) and eyelids (11 cases) are frequently affected sites (36.9% of cases in this study). Two locations were often associated with an unfavourable behaviour: 62% (8/13) of digital and ungueal tumours and 50% of labial tumours showed signs of recurrence or metastasis. For other locations, 1 year after surgery, the outcome was favourable in 78% of cases.

The tumour size was measured on histological sections. For each lesion, the size was evaluated by measuring the most important thickness of the lesion perpendicular to the epidermis. For lesions with a favourable outcome, the mean thickness was 0.73 cm (SD: 0.60 cm). For tumours with unfavourable behaviour, it was 1.42 cm (SD: 0.88 cm). Using the Mann and

Cases	Breed	Sex	Age (year)	Location	Thickness (cm)	Clinical outcome[1]
1	Pointer dog	F	11	Vulva	0.3	Favourable
2	Shepherd	F	6	Nose	0.9	Favourable
3	Spaniel	M	5	Periocular (Eyelid)	0.1	Favourable
4	Basset	M	13	Digit	1.3	Unfavourable (Met.)
5	Scottish Terrier	M	6	Shoulder	1.7	Favourable
6	Pyrenean Shepherd	F	8.5	Tail	0.5	Favourable
7	Shepherd	F	6.5	Digit	2.4	Favourable
8	Irish Setter	M	12	Eyelid	0.5	Unfavourable (Rec.)
9	Cross breed	F	10	Anterior limb	0.3	Favourable
10	Spaniel	F	8	Lip	0.5	Favourable
11	Boxer	M	10	Posterior limb	1.9	Favourable
12	Labrador Retriever	M	11	Digit	1.9	Unfavourable (Met.)
13	Boxer	F	9	Abdomen	0.4	Favourable
14	Scottish Terrier	M	9	Digit	0.7	Unfavourable (Met.)
15	Cross breed	F	6	Posterior limb	0.3	Favourable
16	Cross breed	M	8	NA	1	Favourable
17	Setter	M	13	Digit	2.9	Unfavourable (Death)
18	Doberman	F	13	Cheek	1.5	Unfavourable (Death)
19	French Bulldog	F	8.5	Back	0.3	Unfavourable (Rec.)
20	Daschund	M	12	Digit	1.1	Favourable
21	Shepherd	M	10	Eyelid	0.9	Favourable
22	Rottweiler	F	12	Abdomen	1.4	Unfavourable (Death)
23	Poodle	F	12	Tail	1.3	Favourable
24	Bouvier des Flandres	M	9	Digit	0.4	Unfavourable (Met.)
25	Cross breed	M	5	Eyelid	0.3	Favourable
26	Briard Sheepdog	M	11	Digit	2.1	Favourable
27	Cross breed	F	12	Eyelid	0.3	Favourable
28	Brittany Spaniel	M	9	Head	0.6	Favourable
29	Yorkshire	F	7	Anterior limb	0.4	Favourable
30	Boxer	F	9	Neck	1.3	Favourable
31	Husky	M	7	Pinnae	0.2	Favourable
32	Pyrenean Shepherd	F	8	Abdomen	0.4	Favourable
33	Cross breed	M	9	Eyelid	0.5	Favourable
34	Yorkshire	M	7	Eyelid	0.6	Favourable
35	Chow Chow	M	NA	Eyelid	1.2	Unfavourable (Met.)
36	Beauceron	F	13	Vulva	0.3	Favourable
37	Brittany Spaniel	F	NA	Anus	0.1	Favourable
38	Beauceron	M	NA	Digit	2.6	Unfavourable (Met.)
39	WHWT	F	10	Shoulder	0.3	Favourable
40	Cross breed	M	11.5	Digit	1.7	Favourable
41	Briard Sheepdog	M	6	Digit	2.8	Unfavourable (Met.)
42	Cocker Spaniel	M	13	Lip	1.9	Unfavourable (Met.)
43	Braque	F	11	Lip	1.4	Unfavourable (Met.)
44	Labrador Retriever	F	5	Nose	0.3	Favourable
45	Pinscher	F	7.5	Abdomen	0.9	Favourable
46	Cross breed	M	10.5	Nose	0.7	Unfavourable (Rec.)
47	Bernese Mountain dog	F	7.5	Lip	0.9	Favourable
48	Boxer	M	7	Abdomen	0.2	Favourable
49	Belgian Shepherd	F	13	NA	1	Unfavourable (Death)
50	Spaniel	M	5	Eyelid	0.4	Favourable
51	Spaniel	M	7	Nose	0.3	Favourable
52	Doberman	M	12	NA	0.8	Favourable
53	Cross breed	M	7	Eyelid	0.3	Favourable
54	Welsh Terrier	M	10	Flank	0.8	Favourable
55	Cross breed	F	9	Anus	0.5	Favourable
56	Bernese Mountain dog	F	5	Vulva	0.6	Favourable
57	Irish Setter	F	13	NA	3	Unfavourable (Death)

Cases	Breed	Sex	Age (year)	Location	Thickness (cm)	Clinical outcome[1]
58	Poodle	M	8	Lip	0.7	Unfavourable (Rec.)
59	Yorkshire	F	8	Lip	0.3	Favourable
60	Spaniel	M	10	Anus	2.4	Favourable
61	Bernese Mountain dog	F	12	Head	0.7	Favourable
62	Scottish Terrier	F	9	Digit	0.7	Unfavourable (Rec.)
63	Briard Sheepdog	M	6	Anterior limb	0.8	Favourable
64	Cross breed	F	8.5	Eyelid	0.2	Favourable
65	Poodle	M	9	Digit	0.5	Favourable

[1]Clinical outcome on 12 months post surgery; WHWT: White highland white Terrier; M: male; F: female; NA: Not available; Rec.: Recurrence; Met.: Metastases.

TABLE I: Canine cutaneous melanocytic tumours (n = 65): signalment, location and outcome.

	Case number (%)
Sex	
Male	34 (52%)
Female	31 (48%)
Site	
Head	4 (6%)
Eyelid	11 (17%)
Lip	6 (9%)
Nose	4 (6%)
Trunk	5 (8%)
Limb	5 (8%)
Digit	13 (20%)
Perianal / perivulval skin	6 (9%)
Abdominal skin	5 (8%)
Tail	2 (3%)
Unknown	4 (6%)
Outcome	
DWD	14 (21.5%)
AWD	5 (7.7%)
ANED	46 (70.8%)

DWD: Dead With Disease (inoperable recurrence or metastatic extension); AWD: Alive With Disease (local recurrence within one year after the first surgery); ANED: Alive With No Evidence of Disease.

TABLE II: Clinicopathological features in dogs with cutaneous melanocytic tumours (n = 65).

Whitney test (U=206.5; p = 0.004), these results appear significant but there were not enough cases in the present study to identify a threshold of thickness that would be of prognostic significance.

HISTOPATHOLOGY (TABLE III)

Symmetry

In this study, 47 tumours (72.3% of cases) had a symmetrical pattern, the others (27.7%) being classified as asymmetrical. This feature correlated well with the clinical behaviour. 89.4% of tumours with a symmetrical pattern follow a favourable course and 77.8% of tumours with an asymmetrical pattern showed an unfavourable behaviour (Chi 2 t test with t = 28.361 and $P < 0.001$).

	Case number (%)
Symmetry of the tumour	
Symmetrical tumours	47 (72%)
Non symmetrical tumours	18 (28%)
Invasion level	
In situ lesions confined to the epidermis	0 (0%)
Superficial dermis	32 (49%)
Only deep dermis	1 (2%)
Superficial and deep dermis	12 (18%)
Dermis and subcutis	1 (2%)
Mixed (dermo-epidermal junction and dermis or hypodermis)	19 (29%)
Type of growth	
Expansive	43 (66%)
Infiltrative	22 (34%)
Shape of the tumour	
Nodular	52 (80%)
Multinodular	13 (20%)
Epidermal ulceration*	
Present	29 (46%)
Absent	34 (54%)
Junctional activity*	
Present	14 (22%)
Absent	49 (78%)
Lymph / vascular invasion	
Present	6 (9%)
Absent	59 (91%)
Stroma reaction	
Present	16 (25%)
Absent	49 (75%)

** No epidermis on histological sections for two cases*

TABLE III: Histopathological features in dogs with cutaneous melanocytic tumours (n = 65).

Level of invasion

Concerning the level of invasion (in cutaneous and subcutaneous tissues), tumours confined to the superficial dermis were associated with a benign course in 94% of cases. On the other hand, tumours reaching the deep dermis and the subcutis showed a malignant behaviour but the cases were too few in the study to allow for a conclusion.

Type of growth

An expansive growth has a positive influence on the clinical outcome (43 cases, in which 93% were associated with a favourable outcome). Infiltrative tumours showed an unfavourable outcome in 73% of cases (16/22 cases). Growth was significantly associated with the outcome (Chi 2 t test with t = 30.415 and $P < 0.001$).

Shape

The tumour shape was nodular in 80% of cases and multinodular for 20% of cases. Multinodular lesions were often associated with an unfavourable outcome (10/13 cases). These tumours were large sized lesions and this feature is correlated with a malignant behaviour (Chi 2 t test with t = 17,868 and $P < 0.0001$). This result is probably linked to the big size of the multinodular lesions.

Other features

Other histological features such as the presence and the density of tumour associated lymphoid cells, ulceration or junctional activity were not significantly associated with clinical behaviour of neoplasms. Concerning the presence of lymphatic and/or vascular invasion of neoplastic cells, it was observed in 6 cases. One year after surgery, 5 of these 6 cases were dead with metastatic extension of the neoplastic process.

CYTOPATHOLOGY (TABLE IV)

In most cases (33 cases, 50.7%), the shape of neoplastic cells was a mixture of epithelioid and spindle cells (figure 1). Most often tumours with large round cells, spindle cells and mixed cells had a favourable outcome (4/4 cases for large round cells; 12/13 cases for spindle cells; 23/33 cases for mixed cells). On the contrary, the epithelioid shape was associated with an unfavourable course, for 8/15 cases. The difference seems to be significant (Chi 2 t test with t = 8.80 and $P = 0.03$).

Concerning nuclear abnormalities (anisokaryosis and others), there was no correlation between these cytopathological features and clinical behaviour of the neoplasms. In a similar way, the degree of pigmentation of neoplastic cells was not an indicator of prognosis.

Finally, the mitotic index was strongly correlated with the clinical outcome of tumours. For tumours with a favourable outcome, the mean value of the number of mitosis (on 10 randomly selected high power fields) was 1.98 (from 0 to 27).

	Case number (%)
Shape of neoplastic cells	
Large round cells	4 (6%)
Epithelioid	15 (23%)
Spindle cell	13 (20%)
Mixed	33 (51%)
Anisokaryosis	
Mild	35 (54%)
Moderate	20 (31%)
Marked	10 (15%)
Pigmentation	
Mild	30 (46%)
Moderate	9 (14%)
Marked	23 (35%)
None	3 (5%)
Mitotic index	
Mean value for tumours with long course	1.98
Mean value for tumours with malignant behaviour	18.53

TABLE IV: Cytopathological features in dogs with cutaneous melanocytic tumours (n = 65).

For tumours with a malignant behaviour, it was 18.53 (from 0 to 75). The difference was highly significant with Mann and Whitney non parametric U test (U = 119.5 and $P < 0.001$).

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For each antibody, the staining of controls was in accordance with the expected results.

The proliferative index evaluated by the Ki-67 epitope expression (figure 2) was a good prognostic variable (Table V). For lesions with a proliferative index of less than 15%, the outcome is favourable in 95% of cases, with a small subgroup (5%) having an unfavourable course. When the proliferative index was more than or equal to 15%, an unfavourable outcome was observed in 64% of cases. The difference between the 2 subgroups of Ki-67 epitope expression was statistically significant (Chi 2 t test with t = 24.278 and $P < 0.01$).

When staining with the monoclonal antibody against Melan A, the labelled neoplastic cells showed a granular cytoplasmic staining (figure 3). All the cases of the study were labelled with this antibody. The mean ratio of positive cells for 1000 cells was 61.22%. This value for amelanocytic tumours was

	Ki-67 < 15%	Ki-67 ≥ 15%
Favourable outcome	36 cases	8 cases
Unfavourable outcome	2 cases	14 cases

TABLE V: Expression of Ki-67 epitope and clinical outcome in dogs with cutaneous melanocytic tumours (n = 60, because 5 cases were without immunostaining because of decalcifying pre-treatment).

14.7% and this ratio was correlated with the intensity of pigmentation (Table VI). There was no significant difference between the mean ratio for tumours with a favourable outcome (62.3%) and the mean ratio for tumours with a malignant behaviour (58.6%) (Mann and Whitney non parametric U test with U = 473.5 and *P = 0.599*).

When the levels of expression of CD44 by neoplastic cells were low, the clinical outcome was favourable (11/12 cases (92%)). For high levels of CD44 expression (figure 4), results were not significant (Table VII). The level of CD44 expression and the clinical outcome were not statistically significantly associated (Chi 2 t test with t = 2.578 and *P = 0.104*).

The results of the combination of the proliferative index and the expression of CD44 are summarized in Table VIII. For lesions with a proliferative index (Ki-67 epitope expression) inferior to 15%, a low level expression of CD44 was always associated with a favourable outcome. For lesions with a proliferative index equal or superior to 15%, expression of CD44 was less informative. A high level of CD44 expression was associated with an unfavourable outcome in 68% (13/19) of cases.

Intensity of pigmentation	Absent	Mild	Moderate	Marked
Ratio of Melan A positive cells	14.7%	53.6%	47.2%	78.0%

TABLE VI: Expression of the Melan A epitope and the intensity of pigmentation in dogs with cutaneous melanocytic tumours (n = 60).

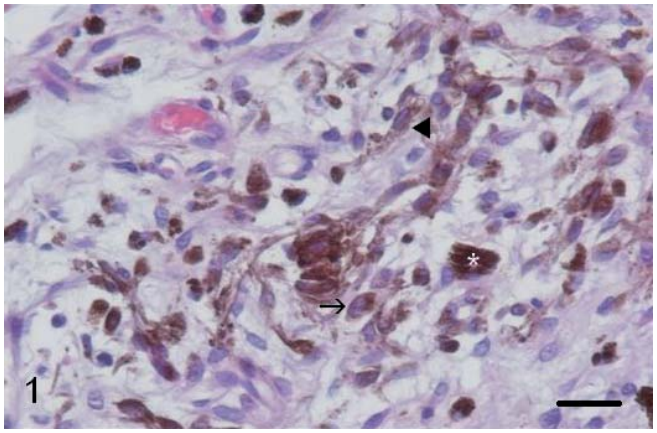

FIGURE 1: Cutaneous melanoma in a dog (case n°11, haematoxylin and eosin x 400). The neoplasm is composed of ovoid (arrow) and spindle (arrowhead) neoplastic cells. Heavily pigmented cells (white star) are present. Bar: 50 µm.

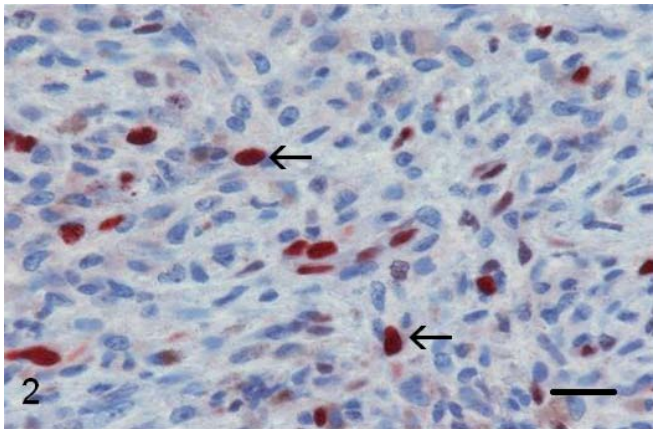

FIGURE 3: Cutaneous melanoma in a dog (case n°16, Ki-67, x 400). Expression of Ki-67 is nuclear (arrow). In this case, the proliferation index is superior to 15%. Immunoperoxydase with AEC substrate, haematoxylin counterstain. Bar: 50 µm.

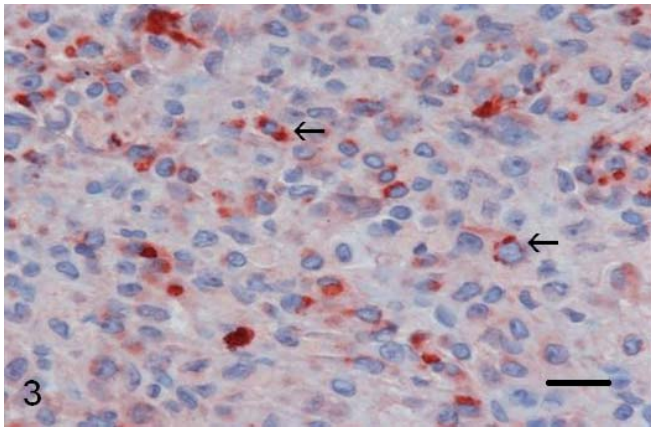

FIGURE 3: Cutaneous melanoma in a dog (case n°34, Melan-A, x 400). Staining of Melan-A is red, granular, and cytoplasmic in neoplastic cells (arrow). Immunoperoxydase with AEC substrate, haematoxylin counterstain. Bar: 50 µm.

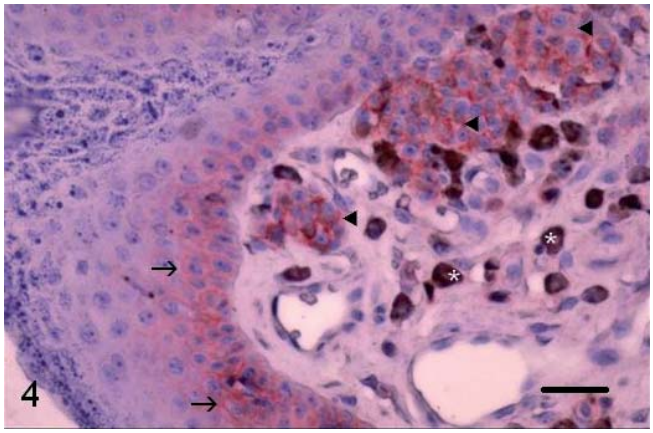

FIGURE 4: Cutaneous melanoma in a dog (case n°63, CD44, x 400). Staining is diffuse, membranous and red, in neoplastic cells (arrowhead) and keratinocytes (arrow) of the overlying epidermis. In this case (high CD44 expression), the staining of neoplastic cells (arrowhead) is stronger than in the epidermis. Note the presence of numerous melanophages in the dermis (white star). Immunoperoxydase with AEC substrate, haematoxylin counterstain. Bar: 50 µm.

	Low CD44 expression	High CD44 expression
Favourable outcome	11 cases	33 cases
Unfavourable outcome	1 case	15 cases

TABLE VII: Expression of CD44 by neoplastic cells and clinical outcome in dogs with cutaneous melanocytic tumours (n = 60, because 5 cases were without immunostaining because of decalcifying pre-treatment).

Proliferative index	**Ki-67 < 15%**		**Ki-67 ≥ 15%**	
CD44 expression	**Low**	**High**	**Low**	**High**
Favourable outcome	9 cases	27 cases	2 cases	6 cases
Unfavourable outcome	0 case	2 case	1 case	13 cases

TABLE VIII: Combination of the proliferative index (Ki-67 epitope expression), the CD44 expression and the clinical outcome in dogs with cutaneous melanocytic tumours (n = 60).

Discussion

Sixty-five cases of canine cutaneous melanocytic tumours were included in this study. This number of cases is limited in comparison to other studies because many cases that were not in agreement with selection criteria have been eliminated. Unlike in other studies, only cutaneous tumours were selected. Histopathological and histochemical procedures were only performed on cases for which a strict post-surgical survey, a certain histopathological diagnosis and appropriate characteristics (shape or symmetry of the tumours and level of invasion...) were available. Concerning the duration of post-surgical follow-up, this period was restricted to one year because it allowed collecting more precise data from veterinary practioners. Moreover, in most studies about canine cutaneous melanocytic tumours, an unfavourable outcome is observed for malignant lesions during the first year following surgical excision [4, 11].

The neoplasm location in our study is an important feature for canine melanocytic tumours. As already reported in previous data [4, 8], the digital and labial locations are more often associated with malignancy.

Concerning the morphological features of neoplasms, the main objective of the work was to evaluate the correlation between these features and the clinical behaviour of tumours. In the present study, some features seem to be very relevant to the prognosis: i) the size of the neoplasm although there were not enough cases in this study to identify a threshold of thickness with a prognostic significance, ii) the growth (expansive vs. infiltrative), iii) the symmetry of the lesion, iv) the mitotic index, and v) the presence of lymphatic and/or vascular invasion of neoplastic cells. These features, which are named "major" features, show a high correlation with the clinical outcome for the cases in this study. Several other features, shape of tumours, level of invasion and the shape of the neoplastic cells (the epithelioid shape was associated with malignancy), seem to be interesting but the sample size of clinical cases was not sufficient to conclude on their importance and are named 'minor' features.

The morphological features pointed out by the present study show some similarities with criteria used in human pathology as a prognostic system. But it is well established in veterinary pathology that important lesional differences between human and canine species exist for melanocytic tumours [8]. Particularly, the level of invasion used in human prognostic systems (as Clark's levels or Breslow's thickness) is useless in canine tumours since most of them are within the deep dermis or subcutis at the time of diagnosis. Two other important differences between human and canine tumours were noted here: firstly, the ulceration of canine tumours was not significantly associated with clinical behaviour as it is in humans, and secondly, a symmetrical shape of the canine tumours was often associated with a favourable course whereas this feature seems without any influence in human tumours.

The results of immunohistochemistry confirm previous works about expression of Ki-67 epitope and Melan A in canine melanocytic tumours. The proliferative index evaluated by the Ki-67 epitope expression is a good prognostic variable. A proliferative index inferior to 15% was predictive of a favourable outcome in 95% of cases. In the present study (as in previous works [11]), it has been noted that for a small proportion of tumours, there is no correlation between Ki-67 expression and clinical outcome. This finding justifies the search for additional features of prognosis (Melan A and CD44 in this study).

For all the cases of the study, neoplastic cells showed a cytoplasmic expression of Melan A. In several studies, it has been shown that Melan A is expressed by neoplastic cells in a large proportion of canine melanocytic tumours [10, 13]. But, in contrast with the results of some of these studies, no correlation was found between the expression of this epitope and the clinical outcome of tumours in the current study.

The expression of CD44 in canine melanocytic tumours has been documented in some studies [1, 16]. In this work, a weak expression seems to indicate a favourable outcome but when this marker is used alone, results are not significant. More interestingly, the combination of two markers, namely Ki-67

epitope and CD44 gave more precise results about clinical outcome than when they were used separately: the combination of a Ki-67 epitope expression less than 15% and a low CD44 expression was always associated with a favourable outcome. In tumours with a Ki-67 expression of more than 15%, the combination with CD44 expression allows a more precise predictive value than with the Ki-67 expression alone. Despite their interest, more clinical cases from future studies are needed to validate these features in view of inclusion in diagnosis procedures for canine melanocytic tumours.

As a conclusion, it seems that the histopathological analysis of canine melanocytic tumours could be improved by a systematic control of some morphological features (size of the neoplasm, growth, symmetry, mitotic index, lymphatic or vascular invasion by neoplastic cells). At that time, immunohistochemistry may supply with additional information for difficult cases with ambiguous histopathological features.

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