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MIB-1 immunoreactivity correlates with biologic behaviour in canine cutaneous melanoma

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Abstract The growth fraction of 68 canine cutaneous melanomas was determined by immunostaining with MIB-1, a monoclonal antibody to a Ki-67 epitope that recognizes all proliferating cells. Fifty tumours were classified histologically as benign and 18 as malignant. The Ki-67 proliferative index (percentage of positive cells over 500 neoplastic cells) was low ($< 15\%$) in 55 cases and high ($\geqslant 15\%$) in 13 cases. High Ki-67 proliferative index and histological malignancy were both associated with significantly poorer 2-year survival ($P < 0.0001$). However, the predictive value of the Ki-67 proliferative index (97%) was higher than the predictive value of classical histology (91%). The evaluation of the growth fraction by the Ki-67 proliferative index is highly predictive of the biological behaviour of canine cutaneous melanoma.

Keywords: cutaneous melanoma, dog, immunohistochemistry, Ki-67 epitope, prognosis.

INTRODUCTION

Melanomas account for 6% of all skin tumours in the dog.[1] The predictive value of classical histology for canine cutaneous melanomas has been reported to be 89%[2] in a review including oral melanomas and 86%[3] in a study of distal extremity melanomas. The mitotic rate appears to be the best prognostic histological criterion, a mitotic count of three or more being associated with a statistically significant shorter survival.[4] As the mitotic count gives a rough estimation of the tumour proliferative rate, a more precise determination of this biologic variable is needed.

In human pathology, the assessment of cell proliferation in a neoplasm gives useful information to histologically-based tumour classification and shows a real prognostic significance.[5,6,7,8] Proliferation markers allow the evaluation of the growth fraction (number of cycling cells per total number of cells). The MIB-1, an IgG1 mouse monoclonal antibody,[9] detects the epitope Ki-67 which has been discovered by Gerdes *et al.* in 1983.[10] Ki-67 is expressed exclusively in the nuclei of cycling cells[11] (from G1 to M phase in the cell cycle) and has been defined as a nonhistone protein with apparent molecular weight of 345 and 395 kiloDaltons.[12] Located predominantly in the nucleoli, this epitope may be associated there with nucleolar RNA.[13] The proliferation index determined by the number of positive MIB-1 cells per total number of tumour cells counted has been shown to be a prognostic factor in numerous human neoplasms including cutaneous melanoma.[14,15,16]

Conserved during evolution, the Ki-67 epitope is also expressed in proliferating cells of several mammalian species including the dog.[17,18] The Ki-67 proliferative index has been successfully used on normal and neoplastic animal tissues[19,20,21] and has been valuable to detect the proliferative potential of canine neoplasms such as testicular tumours[22,23] and, recently, cutaneous plasmocytomas[24] and mast cell tumours.[25]

The MIB-1 antibody can be used on formalin fixed and paraffin embedded tissues[26] without requirement of additional sophisticated skill. This is a real advantage over the flow cytometry method, which has been used previously to study cell proliferation[2] on canine cutaneous melanoma. The aim of the present investigation was to test for a correlation between MIB-1 immunoreactivity and the behaviour of cutaneous melanomas.

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METHODS

Retrospective case material

Sixty-eight cases of cutaneous melanomas were included in the present study. Tumour specimens were obtained from archived material submitted to the Laboratoire d'Histocytopathologie Vétérinaire (Maisons-Alfort, France) during the period 1992–94. Information about clinical and biological behaviour was available in all cases.

Histological evaluation

Two-micrometer thick sections of the tumours were stained with haematoxylin and eosin (H & E). Before staining, a melanin bleaching was performed on heavily pigmented lesions. Dewaxed paraffin sections were treated in 0.25% potassium permanganate for 30 min at room temperature, followed by 1% oxalic acid for 1 min. After a brief wash, H & E staining was performed. Achromic melanomas were stained with a Fontana Masson Silver stain. Tumours were then diagnosed as benign or malignant using classical histological criteria, i.e. type of growth (expansive vs. infiltrative), anisokaryosis (evaluated between 1 (low) to 4 (marked)), mitotic index (total of mitoses evaluated on 10 fields $\times$ 40 objective, selected randomly, excepted for the fields located below ulcerated zones which were avoided). Other parameters were also studied including histological subtype (fusiform, epithelioid or mixed), epidermal ulceration, localization (dermal vs. hypodermal), pigmentation (0/achromic lesion to 2/highly pigmented tumour) and junctional activity. The mitotic index was considered determinant, knowing that a mitotic index superior to 2 was statistically indicative of malignant tumour.[4] However, other relevant histological criteria such as atypia and type of growth were considered also.

Immunohistochemistry

Sections were placed on slides coated with silane (Superfrost Plus, CML, Nemours, France), dried at 56 °C overnight, dewaxed in toluene and acetone. Slides were then immersed in phosphate-buffered saline solution (PBSS) pH 7.6 containing 0.1% trypsin (Merck, Darmstadt, Germany) for 6 min at 37 °C. Endogenous peroxidase activity was blocked by immersing the slides in methanol containing 1% hydrogen peroxide (30 vol) for 30 min. Following a brief wash in PBSS, sections were placed in two coplin jars of microwaveable plastic filled with 250 mL of citrate buffer (10 mM) pH 6. Jars were heated in a microwave oven at 700 watts for 10 min. Afterwards, the fluid level was checked and evaporated buffer was supplemented. Two additional heating cycles of 5 min followed in the same manner. The plastic jars were allowed to cool for 20 min at room temperature. The sections on the slides were surrounded by water repellent Dako Pen (Dako SA, Trappes, France) and rinsed in PBSS. To reduce nonspecific staining, slides were incubated for 20 min in normal goat serum diluted 1 : 5 with PBSS. Primary monoclonal antibody was applied (MIB-1, code 0505, Immunotech, Marseille, France) diluted 1 : 50 in PBSS containing 1% albumin (bovine serum albumine BSA) (Sigma, St Quentin Fallavier, France) for 30 min. Sections were washed in PBSS and incubated for 30 min in biotinylated goat antimouse IgG (Dako, code K492) diluted 1 : 100 in PBSS with 1% BSA. After washing in PBSS, streptavidin peroxidase conjuguate (Dako, code K492) diluted 1 : 100 in PBSS with 1% BSA was applied for 30 min. Sections were then incubated with 3-amino 9-ethylcarbazole (AEC, Dako, code K3464), counterstained with Harris haematoxylin and slides were mounted with an aqueous gel (Glycergel, Dako, code C0563). Positive nuclei stained red. Staining of basal keratinocytes of the epidermis served as an internal positive control. Negative control included omission of the primary antibody and its substitution by an irrelevant antibody of the same IgG1 subclass (CBF78,[27] kindly donated by Prof. Delsol, CHU Purpan, Toulouse, France).

Assessment of MIB-1 immunoreactivity

Counting was performed on aggregates containing positive nuclei for focal immunoreactivity pattern or on fields selected at random when the immunoreactivity pattern was diffuse. Areas under ulcerated zones and secondarily infected were avoided because of the numerous positive inflammatory cells. Counting was done at a magnification of a $\times$40 objective with the assistance of an eyepiece graticule (working from left to right and downwards). The first observer (C. L.) calculated two ratios per tumour: one for 500 tumour cells and one for 1000 tumour cells. Cell counting on 1000 tumour cells was also performed independently by a second observer (M. D.). MIB-1 activity was assessed without knowledge of clinical outcome. All nuclei with any evidence of immunostaining, even weak immunostaining, were considered as positive.

Statistical analysis

All statistical analyses were performed with the Abacus concepts, Survival tools for software programme (Abacus concepts, Inc., Berkeley, CA, 1994). Differences in MIB-1 counts between populations (i.e. survivors vs. dead dogs) were statistically analysed by Student's *t*-test and chi-square test. Survival times (i.e. time from surgery to death or the end of the study) for dogs based on mitotic index and on Ki-67 proliferative index were plotted by use of the Kaplan-Meier method for censored variables. Survival times were compared by the Mantel-Haenszel log-rank test. Multivariate analysis of the association of potential prognostic factors was assessed by Cox regression analysis. To determine the independent effect of the Ki-67 proliferative index, we included in the multivariate analysis all factors that could be predictive for outcome in

cutaneous melanomas. These prognostic factors included sex, site, histological subtype, depth, ulceration, degree of pigmentation, junctional activity, mitotic index, Ki-67 proliferative index (PI) as determined on 500 and 1000 neoplastic cells. For all analyses, a value of $P < 0.05$ was considered statistically significant.

RESULTS

The clinical and pathologic features of the 68 patients of the study are summarized in Table 1. The prognostic value of all the evaluated criteria are shown in Table 2.

Fifteen of the 68 dogs (22%) developed local recurrence and/or metastasis within 2 years. In 14 of these 15 dogs (20.5% of the 68 dogs), death related to cutaneous melanoma occurred within 7 months after surgery, most dogs being humanely euthanased. The other 53 cases (78%) presented no sign of recurrence and all were alive 2 years after surgery.

In all the cases but one (67/68), the histopathological classification was correlated with the mitotic count. Only one tumour with a mitotic count of 2 was classified as malignant due to marked atypia and infiltrative growth. Histological examination correctly predicted biologic behaviour in 63/68 melanomas (93%) (Table 2). The histologically benign melanomas had a statistically significant higher 2-year survival rate than the malignant melanomas ($P < 0.0001$). The mitotic index alone gave almost similar results with a predictive value of 91% (62/68 cases accurately predicted). A mitotic count of three or more was significantly ($P < 0.0001$) correlated to a low 2-year survival (Fig. 1).

In some tumours, the immunopositive nuclei were diffusely distributed throughout the section whereas, in others, they were present in focal aggregates. The number of MIB-1 positive nuclei/500 tumour cells was highly correlated ($r = 0.981$; $P < 0.0001$) with number of MIB-1 positive nuclei/1000 tumour cells. The difference between the mean number of the Ki-67 proliferative index counted on 1000 neoplastic cells by the two observers was less than 10% (8.2 for C.L. and 7.6 for M.D.). The correlation of mitotic count with Ki-67 proliferative index was significant ($r = 0.596$; $P < 0.0001$). The mean PI was 25% for histologically malignant lesions (29% for behaviourally malignant lesions) and 2% for histologically benign lesions (3% for animals still alive 2 years after surgery). The difference was statistically significant ($P < 0.0001$). The scores of Ki-67 index appear to be divided in three major groups: a low proliferative index population ($0.2\% < PI < 5\%$), an intermediate Ki-67 group ($10\% < PI < 18\%$), and a high proliferative index population ($PI > 30\%$). When comparing these scores with the biologic behaviour, we noticed that none of the behaviourally benign neoplasms had a proliferative index superior to 15%, so we decided to check if this 15% value had a statistically significant influence on survival. The Ki-67 proliferative index (PI) on 500 cells were then defined as follows: low ($< 15\%$) in 55 cases (Fig. 2) and high ($\geqslant 15\%$) in 13 cases (Figs 3 and 4). There was a statistically significant ($P < 0.0001$) lower survival rate for dogs with tumours with a Ki-67 proliferative index $\geqslant 15\%$, as assessed by comparison

Table 1. Clinicopathologic features of dogs with cutaneous melanomas

Clinical study	
Sex	
Male	33
Female	35
Site (known for 65 cases)	
Head	14
Eyelid	12
Ear	9
Trunk	11
Limb	7
Digit	12
Follow up time (months)	24
*Outcome**	
DWD	14
AWD	1
ANED	53
Histology	
Type of growth	
Expansive	36
Infiltrative	32
Histological subtype	
Epithelioid	13
Spindle cell	20
Mixed	35
Pigmentation	
Low	17
Marked	48
None	3
Junctional activity	
Present	28
Absent	40
Epidermal ulceration	
Present	24
Absent	44
Mitotic Index	
< 3	51
$= 3$	17
Anisokaryosis	
Low	51
Marked	17
Tumour classification	
Benign	50
Malignant	18
Tumour extension	
Dermal with limits included	44
Dermal with limits not included	9
Dermal and hypodermal	15
Immunohistochemistry	
Proliferative index	
$< 15\%$	55
$= 15\%$	13

*(DWD, dead with disease (inoperable recurrence or metastatic extension); AWD, alive with disease (local recurrence within two years after the first surgery); ANED, alive with no evidence of disease).

Biologic behaviour		Histology		Mitotic index		Proliferative index	
Benign	53 cases	Benign	49	< 3	49	< 15%	53
		Malignant	14	= 3	4	= 15%	0
Malignant	15 cases	Benign	1	< 3	2	< 15%	2
		Malignant	14	= 3	13	= 15%	13

Table 2. Prognostic value of all the evaluated criteria for cutaneous melanoma

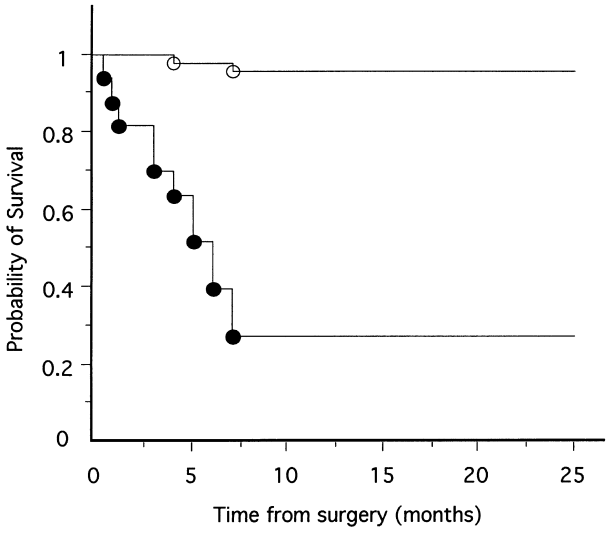

Figure 1. Kaplan-Meier survival curves for dogs with cutaneous melanoma. Dogs were grouped on the basis of mitotic index (MI) evaluated on 10 high power field selected randomly (< 3 [-○-], and = 3 [-●-]).

of Kaplan-Meier survival curves (Fig. 5). The Ki-67 proliferative index appears able to predict the biologic course in 66 of the 68 cases (predictive value of 97%).

Heavy pigmentation, junctional activity, and strictly dermal position have a positive influence on the survival time (respectively $P < 0.0001$, $P = 0.0046$, $P < 0.0001$). On the contrary, there is a significantly lower survival time when the lesion is ulcerated ($P = 0.0023$) or when the neoplastic cells show marked anisokaryosis ($P < 0.0001$). The 2-year survival rate for digital tumours was lower (56%) than for tumours located at other sites (83.8%), the difference approaching statistical significance ($P = 0.0635$). Three of the five histologically malignant digital melanomas derived from ungual epithelium. One of them showed no sign of recurrence or metastasis. The histologically benign digital lesions did not show any relation with the ungual epithelium. However, one of these histologically benign melanomas displayed malignant behaviour.

The results of the multivariate analysis with the Cox regression model are shown in Table 3. Variables that were statistically significant on survival are mitotic index ($P < 0.0001$) and MIB-1 proliferative index, assessed on 500 and 1000 tumour cells ($P < 0.0001$ in both cases). Other variables, such as deep location ($P = 0.0012$), ulceration ($P = 0.0065$), pigmentation ($P = 0.0007$) and junctional activity ($P = 0.0239$) are also independent prognostic factors.

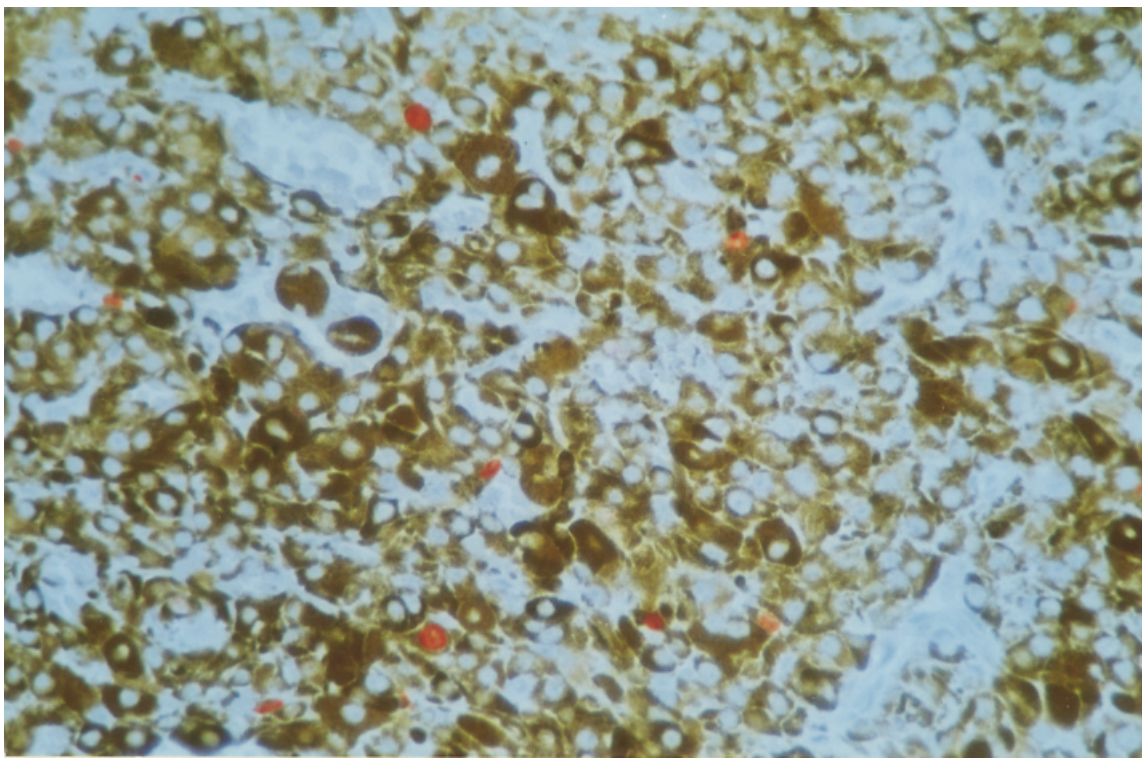

Figure 2. Photomicrograph of a section of a benign melanoma stained with MIB-1 antibody. The few positive nuclei are visible even on heavily pigmented tumour (×200).

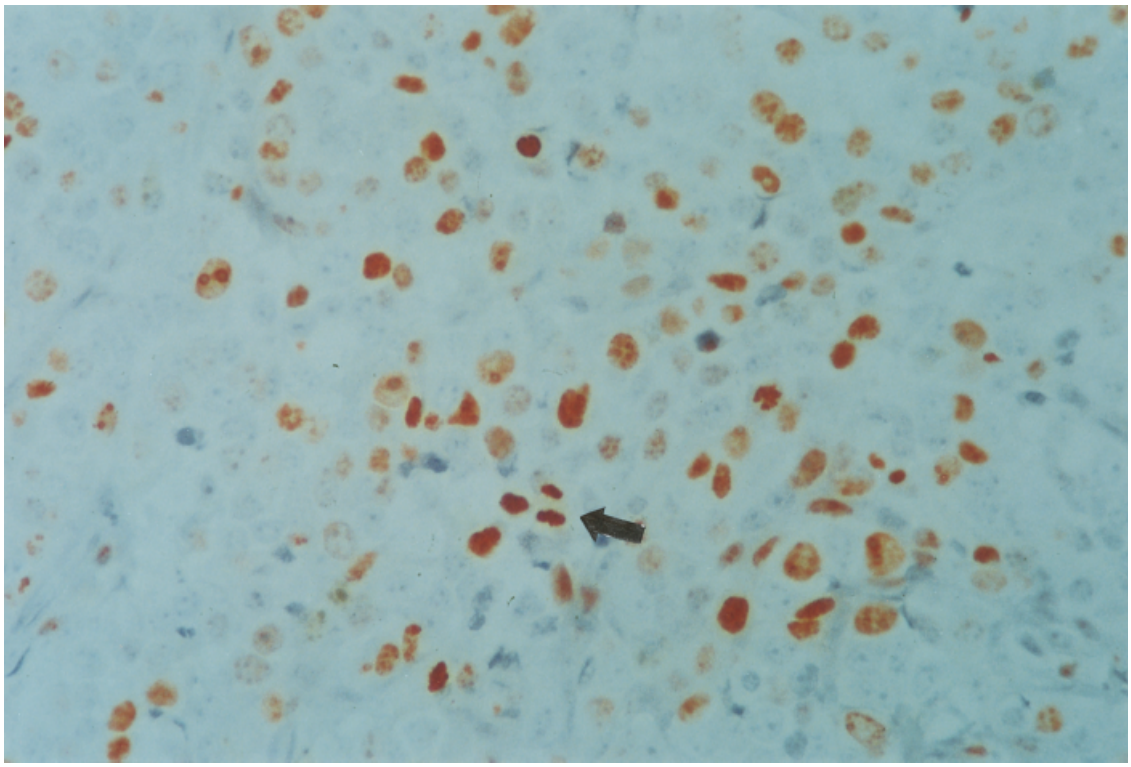

Figure 3. Photomicrograph of a section of a malignant achromic melanoma stained with MIB-1 antibody. Note the numerous positive nuclei and the positive labelling of mitotic figures (arrow, × 400).

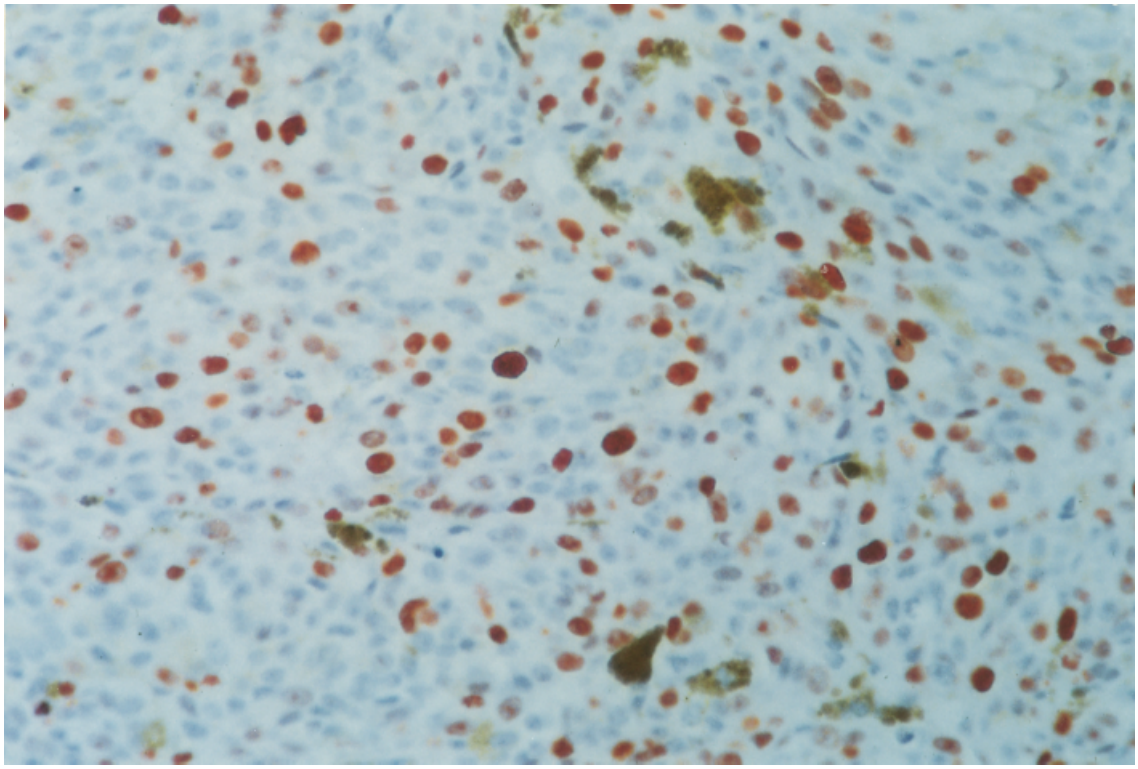

Figure 4. Photomicrograph of a section of a slightly pigmented malignant melanoma stained with MIB-1 antibody (× 200).

DISCUSSION

We report for the first time that the tumour proliferation rate assessed by MIB-1 immunohistochemistry is an accurate prognostic factor for canine cutaneous melanoma. A similar correlation has been observed in humans on primary thick cutaneous melanoma.[14] Moreover, we have shown that a proliferative index (PI) $< 15\%$ vs. $\geqslant 15\%$ is a significant and independent prognostic variable for

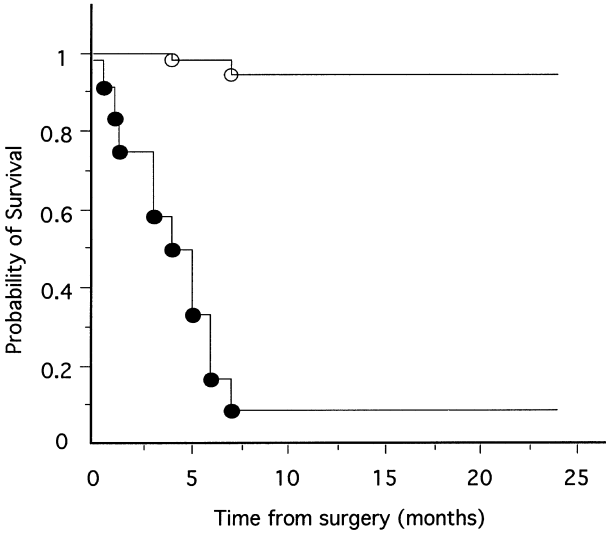

Figure 5. Kaplan-Meier survival curves for dogs with cutaneous melanoma. Dogs were grouped on the basis of proliferative index, as assessed by number of MIB-1 positive nuclei/500 tumour nuclei (< 15% [-○-], and = 15% [-●-]).

Table 3. Multivariate analysis of prognostic factors with survival

Variables	
Proliferation	
Mitotic index	< 0.0001
Index MIB-1/500	< 0.0001
Index MIB-1/1000	< 0.0001
Pathology	
Pigmentation	0.0007
Depth	0.0012
Ulceration	0.0065
Histological subtype	0.2245
Junctional activity	0.0239
Clinical	
Sex	0.4616
Site	0.66

these tumours when compared with other predictive variables. This value enabled us to predict the behaviour of 97% of the tumours. Additionally, this MIB-1 proliferative index identified a subgroup of four cases with PI < to 15% but having histopathological criteria of malignancy (vesicular and nucleolated nuclei with marked anisokaryosis, mitotic index = 3). All of these cases showed a significantly longer survival time which would have been anticipated if prognosis were determined by proliferative index alone. This unpredictable behaviour of some melanomas based on the mitotic index alone could be suggestive of an immunogenic cancer inducing some form of host control, as suggested in humans.[28] It seems inconsistent that those tumours showed a low proliferative index whereas the mitotic index was suggestive of a high proliferative activity, especially since mitotic index only takes into account cells in the M phase whereas the MIB-1 antibody detects cells from the G1 to the M phase of the cell cycle. Paradoxically, low mitotic rates might arise if mitoses proceeded to completion after tissue sampling but before tissue fixation, as this is thought to occur in tissues harvested post-mortem.[29] Another explanation is that these particular tumours were perhaps overfixed, and were therefore a poorer substrate for immunohistochemistry, resulting in falsely low immunopositive cell counts. However, the counting of mitoses in routine paraffin sections is difficult and lacks standardization and reproducibility. Mitotic figures are sometimes difficult to differentiate unequivocally from pycnotic nuclei and apoptotic figures, particularly in highly pigmented tumours. Furthermore, some authors suggest that expressing mitotic activity in terms of area (number of mitoses per high power field) is poorly standardized because of variation in field size between different microscopes and fails to accommodate differences in cell size, the amount of intervening stroma and the space occupied by lymphoid infiltrates or necrotic tissues.[6,30] It has thus been suggested that a mitotic index determined on 1000 cells could be more reliable.[30] It has also been shown that there are considerable subjective variations in the assessment of histomorphological features of malignant human melanomas, resulting in a congruence rate of only 60–70%.[31] This could explain the variation in the predictive value between the different surveys of canine cutaneous melanomas, although the same histological criteria were studied.[2–4] The determination of the proliferative index might be a more objective aid for assessing the grade of malignancy in cutaneous melanoma.

In the present study, one histologically benign lesion with a PI < 15% led, however, to the death of the animal. It was possible that the local recurrence of the tumour which had not been analysed showed marked atypia and a higher proliferative activity. In humans, it has been reported in a series of thin melanomas that the tumours of 15% of the deceased patients did not contain any histologically detectable mitotic[32] and overall survival in patients with thick melanomas and metastases did not correlate with MIB-1 reactivity.[16] As in our case, this lack of correlation between aggressive behaviour and proliferative activity, could be attributed to a variable proportion of blocked noncycling cells in neoplastic tissue due to hypoxia, nutritional deprivation or cell-mediated immunity.[33]

Cellular counting was performed on aggregates containing positive nuclei for focal pattern of MIB-1 immunoreactivity because some authors have suggested that focal aggregates of PCNA reactivity, another proliferation marker, may reflect tumour heterogeneity or clones more likely to invade or metastatize.[34] The difference in the selection of the fields between evaluation of mitotic index (fields chosen at random except for areas under ulcerated zones which were avoided) and proliferative index could be a bias and thus explains the differences in predictive values by leading the counting toward

more proliferating areas. This point is interesting for the subgroup of four cases in which proliferative index and mitotic index have different predictive values from the other groups. In these groups,. low proliferative and low mitotic index lesions and high proliferative and high mitotic index tumours, both parameters being in agreement with biologic behaviour. When counting the immunopositive cells on the same fields where mitotic cells were observed, we found similar results for these four cases. It seems consistent with the fact that the mitotic cells counted on fields chosen for the mitotic index (even when counting was done on fields selected at random for mitotic index and on focal proliferating areas for proliferating index) belonged to the relatively small amount of immunopositive cells on the slides ($10\% < PI < 15\%$). Thus, in our opinion, it is more simple to choose the more proliferating areas to evaluate the proliferative index when the immunoreactivity pattern is multifocal, provided that this does not bias the counting.

In humans, MIB-1 staining is nuclear but heterogeneous in strength, in a granular pattern with accentuation of the nucleoli, dark red with prominent nucleoli and strongly positive during mitotic division.[35] We also see those variations in the dog, which could also correspond to those different cell cycle phase (Fig. 3). The scoring method of MIB-1 positive nuclei chosen in this study could be validated by the correlation between counts on 500 and 1000 neoplastic cells and by the small difference between the mean numbers of Ki-67 proliferation index counted on 1000 tumour cells by the two observers.

As in previous surveys,[2–4] this study confirms that the mitotic index (MI) and histological criteria (nuclear dedifferentiation, type of growth...) are significantly correlated to survival and that mitotic index (< 3 vs. $6 = 3$) is an independent prognostic factor. But the predictive value of those parameters stayed however, weaker than the Ki-67 proliferative index (respectively 91% and 93% vs. 97%). Other variables which showed a statistical influence on survival were degree of pigmentation, dermal vs. hypodermal position, ulceration, junctional activity. In other studies, the degree of pigmentation was not of value in predicting biologic behaviour for Bostock[4] nor was ulceration for Aronsohn.[3] On the other hand, sex and morphological variable did not show any influence on survival as previously described.[1,36] The digital site was associated with a shorter survival time at two years (56% vs. 83.8% for others sites) and digital melanomas were diagnosed as malignant tumours in 59% of the cases. This result was in agreement with previous reports: 50% of the digital tumours were malignant in a study of digital melanomas[3] and 49% for Bostock.[4] Three of the five malignant digital tumours had nail bed involvement while none of the benign tumours had this finding. However, because of the small size of this sample, the relationship between subungual origin and malignancy could not be unequivocally confirmed. In humans, melanomas affecting nail bed, feet and hands are considered a special form of melanoma,[37] quite distinct from melanomas occurring at other skin sites, and prognosis is worse than for cutaneous melanomas at other sites (less than 50% 5-year survival vs. 70%).

The Ki-67 proliferative index has several obvious advantages in dogs. We have obtained a combination of strong immunoreactivity with optimal preservation of morphology and have demonstrated that the proliferative index is a useful prognostic factor. Even if this MIB-1 technique offers a small increase in the predictive value compared with mitotic counts, it is the only reproducible and valuable method to improve prognostic value when histology fails in predicting the biological behaviour. We haven't counted cells on an automatic computerized image analysis system but on a simple microscope with the help of a squared objective. Although this technique was rather time consuming, it may be performed in a routine diagnostic laboratory. The MIB-1 is widely used in routine pathology in human medicine. This method is highly reproducible, easy to perform and no additional technical skill is needed because routine immunohistochemical methods are used after a simple microwave treatment. We hope it will soon be also widely used in veterinary cancerous pathology, where numerous studies confirm its utility as a prognostic significant tool. In conclusion, the MIB-1 immunohistochemistry technique appears to be a useful complement to histopathology for improving the prognosis of cutaneous melanomas, thus giving better information to the clinicians.

REFERENCES

1. Bostock, D.E. Neoplasia of the skin and mammary glands in dogs and cats. In: Kirk, R.W., ed. *Current Veterinary Therapy VI*. Philadelphia: W.B. Saunders, 1977: 493–505.
2. Bolon, B., Calderwood Mays, M.B., Hall, J.B. Characteristics of canine melanomas and comparison of histology and DNA ploidy to their biologic behavior. *Veterinary Pathology* 1990; **27**: 96–102.
3. Aronsohn, M.G., Carpenter, J.L. Distal extremity melanocytic nevi and malignant melanomas in dogs. *Journal of the American Animal Hospital Association* 1990; **26**: 605–12.
4. Bostock, D.E. Prognosis after surgical excision of canine melanomas. *Veterinary Pathology* 1979; **16**: 32–40.
5. Guinebretière, J.M. Sabourin J.C. Ki-67, marqueur de proliferation. *Annales de Pathologie* 1997; **17**: 25–30.
6. Kaudewitz, P., Braun-Falco, O., Ernst, M., Landthaler, M., Stolz, W., Gerdes, J. Tumor cell growth fractions in human malignant melanomas and the correlation to histopathologic tumor grading. *American Journal of Pathology* 1989; **134**: 1063–8.
7. Hall, P.A., Levison, D.A. Review: Assessment of cell proliferation in histological material. *Journal of Clinical Pathology* 1990; **43**: 184–92.
8. Quinn, C., Wright, N. The clinical assessment of proliferation and growth in human tumours:

Evaluation of methods and applications as prognostic variables. *Journal of Pathology* 1990; **160**: 93–102.
9. Key, G., Becker, H.G., Baron, B. *et al*. New Ki-67 equivalent murine monoclonal antibodies (MIB-1, MIB-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. *Laboratory Investigation* 1993; **68**: 629–36.
10. Gerdes, J., Schwab, U., Lemke, H., Stein, H. Production of a mouse monoclonal antibody reactive with a monoclonal nuclear antigen associated with cell proliferation. *International Journal of Cancer* 1983; **31**: 13–20.
11. Gerdes, J., Lemke, H., Baisch, H., Wacker, H., Schwab, U., Stein, H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *Journal of Immunology* 1984; **133**: 1710–5.
12. Gerdes, J., Li, L., Schlüter, C. *et al*. Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. *American Journal of Pathology* 1991; **138**: 867–73.
13. Szekeres, G., Le Tourneau, A., Benfares, J., Audouin, J., Diebold, J. Effect of ribonuclease A and deoxyribonuclease I on immunostaining of Ki-67 in fixed-embedded sections. *Pathology Research Practitioner* 1995; **191**: 52–6.
14. Ramsay, J., From, L., Iscoe, N., Kahn, H. MIB-1 proliferation activity is a significant prognostic factor in primary thick cutaneous melanomas. *Journal of Investigative Dermatology* 1995; **105**: 22–6.
15. Vogt, T., Zipperer, K.-H., Vogt, A., Hölzel, D., Landthaller, M., Stolz, W. p53-protein and Ki-67-antigen expression are both reliable biomarkers of prognosis in thick stage I nodular melanomas of the skin. *Histopathology* 1997; **30**: 57–63.
16. Böni, R., Doguoglu, A., Burg, G., Müller, B., Dummer, R. MIB-1 Immunoreactivity correlates with metastatic dissemination in primary thick cutaneous melanoma. *Journal of American Academy of Dermatology* 1996; **35**: 416–8.
17. Falini, B., Flenghi, L., Fagioli, M. *et al*. Evolutionary conservation in various mammalian species of the human proliferation-associated epitope recognized by the Ki-67 monoclonal antibody. *Journal of Histochemistry and Cytochemistry* 1989; **37**: 1471–8.
18. Teter, K.P., Holloway, D.C., Sandusky, G.E. Assessment of PCNA (19A2) and Ki-67 (MIB-1) cell proliferation markers in formalin fixed tissues. *Journal of Histotechnology* 1995; **18**: 99–103.
19. Laprie, C., Abadie, J., Amardeilh, M.F., Raymond, I., Delverdier, M. Detection of the Ki-67 proliferation associated nuclear epitope in normal canine tissues using the monoclonal antibody MIB-1. *Anatomia Histologia Embryologia* 1998; **27**: 251–6.
20. Fournel, C., Magnol, J.P., Marchal, T. *et al*. Detection of the Ki-67 proliferation-associated nuclear antigen in normal canine lymphoid lymph node by the use of the monoclonal antibody MIB-1. *Revue de Médecine Vétérinaire* 1994; **145**: 749–53.
21. Wrobel, K.H., Kujat, R., Lutz, R. Expression of the proliferation-associated Ki-67 antigen in bovine testicular tubular cells during the seminiferous epithelial cycle, demonstrated with the MIB-1 antibody. *Andrologia* 1993; **25**: 301–5.
22. Sarli, G., Benazzi, C., Preziosi, R., Marcato, P.S. Proliferative activity assessed by anti-PCNA and Ki-67 monoclonal antibodies in canine testicular tumours. *Journal of Comparative Pathology* 1994; **110**: 357–68.
23. Fournel-Fleury, C., Magnol, J.P., Chabanne, L. *et al*. Growth fractions in canine non-Hodgkin's lymphomas as determined *in situ* by the expression of the Ki-67 Antigen. *Journal of Comparative Pathology* 1997; **117**: 61–72.
24. Platz, S.J., Breuer, W., Pfleghaar, S., Minkus, G., Hermanns, W. Prognostic value of histopathologic grading in canine extramedullary plasmocytomas. *Veterinary Pathology* 1999; **36**: 23–7.
25. Abadie, J., Amardeilh, M., Delverdier, M. Prognosis relevance of Ki-67 and PCNA as cell proliferation markers for canine cutaneous mast cell tumors: special consideration of moderately-differentiated neoplasms. *Journal of the American Veterinary Medical Association* 1999; **215**(11): 1629–34.
26. Cattoretti, G., Becker, H.G., Key, G. *et al*. Monoclonal Antibodies against recombinant parts of the Ki-67 antigen (MIB-1 and MIB-3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *Journal of Pathology* 1992; **168**: 357–63.
27. Saati, T., Tkaczuk, J., Krissanssen, G. *et al*. A novel antigen detected by the CBF.78 antibody further distinguishes anaplastic large cell lymphoma from Hodgkin's disease. *Blood* 1995; **86**: 2741–6.
28. Stuhmiller, G.M. Immunobiology of melanoma. In: Seigner, H.F., ed. *Clinical Management of Melanoma*. The Hague: Martinus-Nijhoff, 1982: 233–66.
29. Underwood, J.C.E. *Introduction to Biopsy Interpretation and Surgical Pathology,* 2nd edn. London: Springer-Verlag, 1987: 204.
30. Evans, A.T., Blessing, K., Orrell, J.M., Grant, A. Mitotic indices, Anti-PCNA immunostaining, and AgNORs in thick cutaneous melanomas displaying paradoxical behaviour. *Journal of Pathology* 1992; **168**: 15–22.
31. Suffin, S.C., Waisman, I., Clark, W.H. Jr, Moston, D.L. Comparison of the classification by microscopical level (stage) of malignant melanoma by three independant groups of pathologists. *Cancer* 1977; **40**: 3112–4.
32. Kuehnl-Petzoldt, C., Keil, H., Schoepf, E. Prognostic significance of patient's sex, tumor site, and mitotic rate in thin (< 1.5 mm) melanoma. *Archives of Dermatological Research* 1984; **276**: 151–5.
33. Ostmeier, H., Suter, L. The Ki-67 antigen in primary human melanomas: its relationship to mitotic rate and tumor thickness and its stability. *Archives of Dermatologic Research* 1989; **281**: 173–7.
34. Takashi, H., Strutton, G.M., Parsons, P.G. Determination of proliferating fractions in malignant melanomas by anti-PCNA/cyclin monoclonal antibody. *Histopathology* 1991; **18**: 221–7.
35. Seigneurin, D., Guillaud, P. L'antigène Ki-67, marqueur du cycle cellulaire et de la prolifération tumorale. *Pathologie Biologie* 1991; **39**: 1020–8.
36. Gross, T.L., Ihrke, P.J., Walder, E.J., eds. *Veterinary Dermatopathology: a Macroscopic, Microscopic Evaluation of Canine, Feline Skin Disease*. St Louis: C.V. Mosby, 1992: 451–64.
37. Clark, W.H. Jr, Goldman, L.I., Mastrangelo, M.J., eds. *Human Malignant Melanoma*. New York: Grune and Stratton, 1979: 109–24.

Résumé L'indice de prolifération de 68 mélanomes cutanés canins a été déterminé en utilisant un immunomarquage par le MIB-1, un anticorps monoclonal d'un épitope du Ki-67, qui reconnait les cellules prolifératives. Cinquante tumeurs étaient classées histologiquement bénignes et 18 malignes. L'index de prolifération au Ki-67 (pourcentage de cellules positives sur 500 cellules néoplasiques) était faible ($< 15\%$) dans 55 cas et élevé ($\geqslant 15\%$) dans 13 cas. La présence d'un index de prolifération Ki-67 et de signes histologiques de malignité étaient associées avec un taux de survie plus faible à 2 ans ($P < 0.0001$). Cependant, la valeur prédictive de l'indice de prolifération Ki-67 (97%) était supérieure à celle de l'histologie classique (91%). L'étude de la prolifération néoplasique par l'indice de prolifération Ki-67 est très fortement corrélée avec le comportement biologique des mélanomes cutanés canins. [Laprie, C., Abadie, J., Amardeilh, M.-F., Net, J.-L. L. E., Lagadic M., Delverdier M. *MIB-1 immunoreactivity correlates with biologic behaviour in canine cutaneous melanoma*. (L'immunoréactivité au MIB-1 est un bon marqueur d'évolution du mélanome cutané canin.) *Veterinary Dermatology* **12:** 139–147.]

Resumen El índice de crecimiento de 68 melanomas cutáneos caninos fue determinado mediante inmunotinción con MIB-1, un anticuerpo monoclonal contra el epitopo Ki-67, que reconoce todas las células proliferantes. Se clasificaron histológicamente cincuenta tumores como benignos y 18 como malignos. El índice de proliferación Ki-67 (porcentaje de células positivas sobre 500 células neoplásicas) fue bajo ($< 15\%$) en 55 casos y alto ($\geqslant 15\%$) en 13 casos. El índice de proliferación Ki-67 y la malignidad histológica se asociaban ambos a un supervivencia de 2 años significativamente peor ($P < 0.0001$). Sin embargo, el valor predictivo del índice de proliferación Ki-67 (97%) fue superior al valor predictivo de la histología clásica (91%). La evaluación del crecimiento del índice de proliferación Ki-67 es altamente predictivo del comportamiento biológico del melanoma cutáneo canino. [Laprie, C., Abadie, J., Amardeilh, M.-F., Net, J.-L. L. E., Lagadic M., Delverdier M. *MIB-1 immunoreactivity correlates with biologic behaviour in canine cutaneous melanoma*. (La inmunoreactividad a MIB-1 se correlaciona con el comportamiento biológico del melanoma cutáneo canino.) *Veterinary Dermatology* **12:** 139–147.]

Zusammenfassung Die Wachstumsfraktion von 68 kutanen Melanomen beim Hund wurde durch Immunfärbung mit MIB-1, einem mononukleären Antikörper gegen ein Ki-67 Epitop, das proliferierende Zellen erkennt, bestimmt. Fünfzig Tumoren wurden histologisch als gutartig und 18 als bösartig eingeteilt. Der Ki-67 Proliferationsindex (Prozentsatz positiver Zellen von 500 neoplastischen Zellen) war niedrig ($< 15\%$) in 55 Fällen und hoch ($\geqslant 15\%$) in 13 Fällen. Hoher Ki-67 Proliferationsindex und histologische Bösartigkeit waren jeweils mit einer signifikant schlechteren Überlebenszeit nach 2 Jahren verbunden ($P < 0.0001$). Allerdings war der prädiktive Wert des Ki-67 Proliferationsindexes (97%) höher als der prädiktive Wert der klassischen Histologie (91%). Die Bewertung der Wachstumsfraktion durch den Ki-67 Proliferationsindex sagt das biologische Verhalten von Hautmelanomen beim Hund sehr gut voraus. [Laprie, C., Abadie, J., Amardeilh, M.-F., Net, J.-L. L. E., Lagadic M., Delverdier M. *MIB-1 immunoreactivity correlates with biologic behaviour in canine cutaneous melanoma*. (MIB-1 Immunreaktivität korreliert mit dem biologischen Verhalten von kutanen Melanomen beim Hund.) *Veterinary Dermatology* **12:** 139–147.]