DNA ploidy and nuclear morphometric variables for the evaluation of melanocytic tumors in dogs and cats

Stefan L. M. F Roels, DVM; André J. Van Daele; Erik A. Van Marck, MD, PhD; Richard V. A. Ducatelle, DVM, PhD

Objective—To determine the prognostic importance of the DNA content and nuclear morphometric variables in melanocytic tumors of cats and dogs.

Sample Population—27 melanocytic tumors of dogs and cats.

Procedures—Biopsy specimens were investigated by quantitative image analysis after the Feulgen staining method. The DNA content (index), nuclear diameter, ploidy balance, proliferation index, hyperploidy, and growth fraction (Ki67) were measured. Using 1-way ANOVA and a Pearson correlation test, the relationships between the different variables were tested. Their role in the prognosis in affected dogs and cats was estimated using the Cox regression test with respect to 6 months postoperative survival rate.

Results—Significant correlations were found between DNA index and ploidy balance and proliferation index. A significant correlation was also found between hyperploidy and DNA index, and between ploidy balance and proliferation index. Significant differences were found between histologically malignant and benign melanocytic tumors but not between primary malignant tumors and metastatic malignant tumors for DNA index and ploidy balance. No correlation was found between DNA variables and survival time.

Conclusion and Clinical Relevance—In melanocytic tumors of cats and dogs, DNA index and ploidy balance can be used to differentiate histologically benign from malignant tumors. However, DNA content and nuclear morphometric variables have little value in predicting survival time. The DNA index and ploidy balance provide an additional tool to evaluate melanocytic tumors of cats and dogs. Survival in dogs and cats with melanocytic tumors, however, is not dependent on DNA content or nuclear variables including DNA content, ploidy, nuclear size, and proliferation index.

Melanocytic tumors in dogs are usually solitary, and the skin is the most common site, followed by the oral cavity. There is a striking difference in behavior of canine melanocytic tumors depending on their location. At least 90% of oral melanocytic tumors are malignant, whereas most cutaneous melanocytic tumors are benign, with the notable exception of the digits.

In cats, melanocytic tumors are comparatively rare, constituting <1% of all feline tumors and approximately 2% of all skin tumors in this species. Most common clinical finding in cats is the intraocular melanocytic tumor, generally arising from theuveal tract, or occasionally from the nictitating membrane.

Melanocytic tumors of the haired skin usually occur on the head (particularly the ear), tail, or limbs. Intraocular melanocytic tumors in cats generally are considered to have greater malignant potential than dermal melanocytic tumors, although their latency period is reported to be long.

Prognostic factors of relevance for canine melanocytic tumors are the location of the tumor, its size, mode of growth, and number of mitoses.

Because melanocytic tumors in cats are rare, prognostic factors have not been clearly established. It is commonly accepted that degree of pigmentation is not of prognostic value. Nuclear pleomorphism has been put forward as a prognostic factor in domestic animals, especially for the recognition of malignant transformation of dermal melanocytoma, but it remains controversial. A major drawback in the prognosis of melanocytic tumors in dogs and cats is the lack of well-documented prospective studies using survival rates as objective criteria for tumor behavior.

The purpose of the study reported here was to test the hypothesis that quantitative measurement of nuclear variables including DNA content, ploidy, nuclear size, and proliferation index could be used as objective prognostic criteria for melanocytic tumors in dogs and cats. This hypothesis was tested in a sample population of feline and canine tumors for which survival rates of the affected dogs and cats were determined.

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Table 1—Information on melanocytic tumors from dogs and cats surviving greater than six months after an initial diagnosis of malignant melanoma

<table>
<thead>
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<td>Dog</td>
<td>Dog</td>
<td>Dog</td>
<td>Cat</td>
<td>Cat*</td>
<td>Cat''</td>
<td>Cat''</td>
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</table>

*Different tumor sites on 1 cat (Roels and Ducatelle, 1998). †Metastatic tumors of biopsy specimen No. 8. §Amelanotic.

See Table 1 for key.

Materials and Methods

Tumors—Tumors from 20 dogs and 4 cats (Tables 1 and 2) were identified as melanocytic tumors on the basis of histologic criteria, including evidence of melanin pigment in the proliferating cells and immunocytochemical labeling for S100. In most instances, these tumors were classified as melanomas, because histologically melanocytes (single or in nests) could be seen spreading from the epithelium into the surrounding or underlying stromal tissue. Aggressiveness of the tumors was determined from cellular pleomorphism and atypia, anisokaryosis, mitotic rate (malignant = more than 2 mitoses/10 HPF [40X magnification]), and histologic infiltrative nature. Metastasis was considered to be an important indicator of malignancy. In addition, the size and macroscopic invasive growth of the tumor was recorded at the time a biopsy specimen was obtained. Proliferative activity in these tumors has been studied in detail and reported elsewhere.8 In most instances, the samples for histologic evaluation were obtained during removal of the tumor. Treatment for malignant and benign melanocytic tumors consisted of wide excision of the tumor. Ocular melanocytic tumors were treated by enucleation. Digital melanocytic tumors were treated by amputation of the toe. In 1 dog (biopsy specimen No. 6; Table 2) with an amelanotic melanocytic tumor of the corpus, the affected limb was amputated. In 2 affected cats (No. 7 and 8, 9, 10, 11; Table 1) the cats were euthanatized at the time of histopathologic diagnosis for humane reasons. Criterion for biological behavior of the tumor was the 6 months survival rate. Therefore, retrospectively, veterinarians were contacted and asked about the condition of the animal 6 months after initial diagnosis. Animals euthanatized soon after diagnosis were classified in the group surviving <6 months.

Histologic evaluation—For histologic examination, all biopsy specimens were fixed in a 4% phosphate-buffered formaldehyde solution, processed routinely, paraffin-embedded, and sectioned at 5-µm thickness. Sections were stained with H&E.

Immunohistochemistry—Immunohistochemical labeling was performed on sections of formalin-fixed paraffin wax-embedded tissue. Sections were mounted on amino-propyltrioxyxilane coated glass slides to ensure tissue adherence. Sections were microwaved in a 2% ureum-citrate solution up to 100°C and boiled for 10 minutes. All antibodies were used, and treatments of sections were done according to the instructions of the manufacturers. The antibody used for the identification of the tumors was S100p, a polyclonal antibody raised in mice against human S100 protein with known cross reactivity in many species, including human, cow, mouse, and chicken,8 diluted at 1 to 200. Clone MB1, raised in mouse against human Ki67,5 diluted at 1 to 20 was used for evaluation of growth fraction. All incubations with antibodies were performed at room temperature (approx 25°C). Incubations with primary antibodies were done overnight.

As a second step, biotinylated goat anti-mouse antibodies were used followed by incubation of a peroxidase-conjugated streptavidin; these were applied for 30 minutes. Labeling was performed with hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride as chromogen.

DNA staining—For the Feulgen staining method,11,12 5-µm-thick paraffin sections were cut and mounted on slides. Slides were dewaxed in xylene (2 times for 5 minutes) followed by rehydration in a decreasing series of ethanol solutions (100, 95, and 70%) and distilled water. Subsequently, the sections were rinsed in 1 M HCl for 1 minute and placed in 1 M HCl for 1 hour at 25°C. Sections were then rinsed in 1 M HCl for 1 minute and transferred to Schiff’s reagent for 1 hour at 25°C.

After rinsing the sections 3 times for 2 minutes each in a 10% bisulphite solution they were extensively rinsed in demineralized water, dehydrated through graded solutions of ethanol (70, 95, and 100%) for 5 minutes each and finally cleared in xylene.

Bleaching of melanin—For bleaching tissue sections, the method of Kivelä8 was used with minor modifications as described.8 Sections were incubated for 42 hours, instead of 18 hours, at 25°C with a bleaching solution containing
3.0% (vol/vol) hydrogen peroxide, 2.0% (wt/vol) bovine serum albumin, and 10 g of disodium hydrogen phosphate/L. Sections were covered with 300 μL of this solution in a moist chamber. This procedure followed the immunohistochemical labeling and preceded the Feulgen staining method.

Quantitative image analysis—Images were obtained with a light microscope, equipped with a 100X objective (numeric aperture 1.25), a green filter of 540 nm, and a video camera. Images were digitized with an image processing system under standardized optical and electronic conditions.

Briefly, gain and black level of the camera and analogue video camera were equipped with a 100X objective (numeric aperture 1.25), a green filter of 540 nm, and a video camera. Images were digitized with an image processing system under standardized optical and electronic conditions.

Table 3—Mean (± SEM) growth fraction (Ki67), DNA index, and morphometric variables of tumor biopsy specimens from dogs and cats surviving less than 6 months after initial diagnosis

<table>
<thead>
<tr>
<th>Variables</th>
<th>1</th>
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<td>319</td>
<td>309</td>
<td>296</td>
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<td>319</td>
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<td>ND (μm)</td>
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<td>7.15 ± 0.14</td>
<td>7.57 ± 0.13</td>
<td>7.09 ± 0.12</td>
<td>6.62 ± 0.12</td>
<td>6.19 ± 0.12</td>
<td>7.26 ± 0.13</td>
<td>6.89 ± 0.11</td>
<td>7.86 ± 0.10</td>
<td>6.39 ± 0.12</td>
<td>6.18 ± 1.07</td>
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<td>HG (%)</td>
<td>1.09 ± 0.41</td>
<td>1.30 ± 0.44</td>
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<td>1.27 ± 0.54</td>
<td>1.23 ± 0.50</td>
<td>1.10 ± 0.47</td>
<td>1.27 ± 0.48</td>
<td>1.28 ± 0.59</td>
<td>1.82 ± 0.65</td>
<td>1.14 ± 0.48</td>
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<td>PB (%)</td>
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<tr>
<td>Hyperploidy (%)</td>
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<td>5.37</td>
<td>1.86</td>
<td>4.83</td>
<td>3.00</td>
<td>1.54</td>
<td>2.51</td>
<td>7.14</td>
<td>18.8</td>
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Table 4—Mean (± SEM) growth fraction (Ki67), DNA index, and morphometric variables of tumor biopsy specimens from dogs and cats surviving more than 6 months after initial diagnosis

<table>
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<tr>
<td>ND (μm)</td>
<td>7.46 ± 0.10</td>
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<td>6.89 ± 0.11</td>
<td>7.86 ± 0.10</td>
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<tr>
<td>HG (%)</td>
<td>1.09 ± 0.41</td>
<td>1.30 ± 0.44</td>
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<td>1.27 ± 0.54</td>
<td>1.23 ± 0.50</td>
<td>1.10 ± 0.47</td>
<td>1.27 ± 0.48</td>
<td>1.28 ± 0.59</td>
<td>1.82 ± 0.65</td>
<td>1.14 ± 0.48</td>
<td>1.12 ± 0.37</td>
<td>2.06 ± 0.38</td>
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<td>2.01 ± 0.36</td>
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<tr>
<td>PB (%)</td>
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<td>17.2</td>
<td>43.6</td>
<td>12.6</td>
<td>13.4</td>
<td>37.4</td>
<td>18.6</td>
<td>18.8</td>
<td>30.0</td>
<td>30.8</td>
<td>11.9</td>
<td>30.6</td>
<td>30.5</td>
<td>30.4</td>
<td>30.3</td>
<td>30.2</td>
</tr>
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<td>6.50</td>
<td>0.4</td>
<td>6.90</td>
<td>20.1</td>
<td>0.05</td>
<td>7.11</td>
<td>5.95</td>
<td>19.3</td>
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<td>0.67</td>
<td>6.50</td>
<td>6.49</td>
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<tr>
<td>Hyperploidy (%)</td>
<td>2.06</td>
<td>5.37</td>
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See Table 3 for key. See Table 2 for histopathologic diagnoses of biopsy specimens.
Table 5—P Values for malignant versus benign in the canine and feline melanocytic tumor biopsy specimens (n = 27) determined by one-way ANOVA test

<table>
<thead>
<tr>
<th>Variable</th>
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<th>Malignant*</th>
<th>P value</th>
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<td>DNA index</td>
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<tr>
<td>Ploidy balance</td>
<td>51 ± 8</td>
<td>21 ± 4</td>
<td>0.0019</td>
</tr>
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<td>Ploidy index (Feulgen)</td>
<td>5 ± 1</td>
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<td>Hyperploidy</td>
<td>1.4 ± 0.9</td>
<td>5 ± 1</td>
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</table>

*Mean (= SEM) values from 8 benign and 19 malignant tumors.

content greater than 5c (pentaploid). As an internal reference, cell nuclei of liver tissue from clinically normal cats and dogs were measured. Two hundred to 400 tumor nuclei and 50 to 150 reference nuclei were measured for each animal.

Statistical analysis—Relationships among the nuclear diameter, DI, PB, PI, and growth fraction (Ki67) were tested, using 1-way ANOVA and a Pearson correlation test.18

Magnitude of the difference in prognosis of the different variables was estimated, using the Cox proportional hazards regression test (censoring at 6 months with continuous independent variables).19

Results

Histologic findings and immunohistochemistry—Tumor cells were either fibroblast-like spindle cells arranged in interfacing fascicles or more epithelioid cells arranged in clusters (Table 1 and 2). Tumors containing mixed populations of spindle cells as well as epithelioid cells were also grouped as a separate type. Briefly, the histologic classification determined on the basis of satisfying the following criteria for malignancy: nuclear pleomorphism, nuclear hyperchromasia, and mitotic figures (> 2 mitoses/10 HPF [40x magnification]). Of the 27 biopsy specimens, 26 (96%) stained S100 positive. The 1 melanocytic tumor that was S100 negative could be identified on the basis of pigment content. The S100 staining in all positive tumors was diffuse and mainly cytoplasmic, but nuclear reactivity could also be found. Of the animals with histologically malignant tumors, 8 of 19 (42%) survived more than 6 months. All animals with histologically benign tumors survived more than 6 months.

DI and morphometric variables—No significant differences in DI and morphometric variables were found between the melanocytic tumors of dogs and cats (Table 3 and 4). The DI ranged from 0.95 to 1.82. Mean DI of the histologically benign melanocytic tumors (range, 0.95 to 1.38) differed significantly from that of malignant tumors (range, 1.00 to 1.82).

The PB, which ranged from –21 (most aneuploid) to 79.4% (most euploid), also differed significantly between benign (range, 79.4 to –0.7%) and malignant (range, 58.0 to –21.0%) melanocytic biopsy specimens. The degree of hyperploidy was not significantly different between histologically benign and malignant tumors.

A significant difference could not be detected between the histologically malignant and benign group for the nuclear diameter nor for the PI (Table 5).

Finally, no significant differences were found, with any of the variables used, between the primary malignant and metastatic melanocytic tumors. The DI was significantly correlated with PI (r = 0.47; P = 0.013), PB (r = –0.67; P < 0.001), and the hyperploidy (r = 0.95; P < 0.001). Hyperploidy was significantly correlated with the PB (r = –0.46; P = 0.016) and the PI (r = 0.53; P = 0.004). No correlation could be found between the different DNA variables and the growth fraction (Ki67) or survival time.

Discussion

Histologic findings indicate that melanocytic tumors have a continuous spectrum, ranging from strictly benign to highly malignant. Thus, the borderline between the 2 categories may be rather broad. We preferred, however, not to classify any of the tumors as borderline but to group them histologically in one or the other category. Although classification of tumors on the basis of cytologic malignant characteristics inevitably contains a degree of uncertainty, there is a useful relationship to clinical outcome.20

Traditionally, Fontana and Masson stains for melanin have assisted in the diagnosis of melanocytic tumors but have now been supplanted by immunocytochemistry.20 Useful markers include antibodies to S100 and to melanosomes (HMB45 and HMSA).21 None of these markers are absolutely specific.19 Markers for melanosomes were found to be of limited sensitivity or even nonreacting in certain animal tissues.20,22 Therefore, in our study, S100 immunocytochemistry was used to confirm that the tumors were melanocytic tumors. Usually S100 expression in melanocytic tumors is found in the nucleus and in the cytoplasm of the tumor cells.21 In our study, labeling was mostly observed in the cytoplasm.

Only 1 tumor (biopsy specimen No. 8; Table 2) did not react with S100 antibody. It was concluded that this tumor belonged to the small group (17%) of non-S100-reacting melanocytic tumors in dogs. On the basis of histopathologic features including a diffuse deposit of melanin pigment, Canine amelanotic tumors of the gingiva and carpus (No. 7 and 13) reacted with S100 antibody; in these 2 tumors, S100 immunocytochemistry was essential for the identification of the tumor.23

Computerized DNA cytometric image analysis has been proven to be of prognostic value in certain types of tumors in humans.24 In melanomas in humans, however, it seems not useful for predicting the metastatic potential of primary melanomas,25 and its value for the prognosis of melanocytic tumors is still largely controversial.26,27 In our study, the DI and nuclear morphometric variables were not significantly correlated with survival, whereas in another study7 histologic classification was significantly correlated with survival. Therefore quantitative DNA analysis cannot be considered as an alternative prognostic tool for the time being. Conventional histologic evaluation has been used for a long time and is therefore still used in many studies as the standard. However, other studies mention that sometimes conventional histologic evaluation can give rise to misinterpretations with regard to survival.28 Histologic evaluation requires an experienced pathologist for reading the different histologic diagnostic and prognostic variables, which to some extent are inherently subjective. Once standardized, the comput-
erized image analysis of DNA profiles should be objective. Standardization of DNA profile analysis may turn this technique into a supplementary tool that may help in better understanding the biological nature of melanomas in dogs and cats.

Quantification of nuclear DNA is increasingly used in research and for clinical applications. Various stains and chromogenic reagents for quantitative staining of DNA have been recommended in literature, but only 1 of them gathered worldwide acceptance for DNA cytometry, namely the Feulgen staining method.31,32 This no doubt constitutes a serious onset to standardization.

The DNA densitometry results in 2 types of variables.9 The first type contains the distributional variables. The main and most popular variable of this group is the DI. Usually, melanocytic tumors with DI less or equal to 1 are considered diploid, and tumors with DI higher than 1 are considered aneuploid. However, El Naggar33 proposed a classification of diploid (DI ≤ 1), low aneuploid (DI > 1 but ≤ 1.5), and high aneuploid (DI > 1.5). Conflicting results have been reported with regard to the prognostic value of this variable in tumors. Results of some studies indicate that DI is a valuable prognostic factor in humans,34,35, whereas others imply that it has little or no value in predicting the survival time.36-38

The second type contains interpretative variables and is considered helpful for understanding the biological rationale behind any relationship with tumor prognosis. The PB and PI belong to this second type of variables. The PB better indicates which cells have abnormal DNA content. It varies from +100% (all the cells are euploid) to −100% (all of the cells are aneuploid). The PI indicates which cells have DNA content intermediate between G0 to G1 and between G2 to M growth phases. When associated, the PB and PI provide the 2 main features of neoplasia accessible by DNA staining, the degree of aneuploidy and the proliferation, respectively.17 In our study, however, these variables were not correlated with survival, indicating that in canine and feline melanocytic tumors abnormalities in DNA content does not influence survival time.

In human melanocytic tumors, significant differences are found between primary malignant and benign melanocytic tumors on the basis of nuclear ploidy, but no significant differences are found between primary malignant and metastatic melanocytic tumors.39,40 Similarly, in our study, in dogs and cats no significant differences were found in DI and PB between histologically malignant and benign melanocytic tumors. These similarities make it worthwhile to consider melanocytic tumors in these companion animals as a model for the study of melanocytic tumors in humans.

References