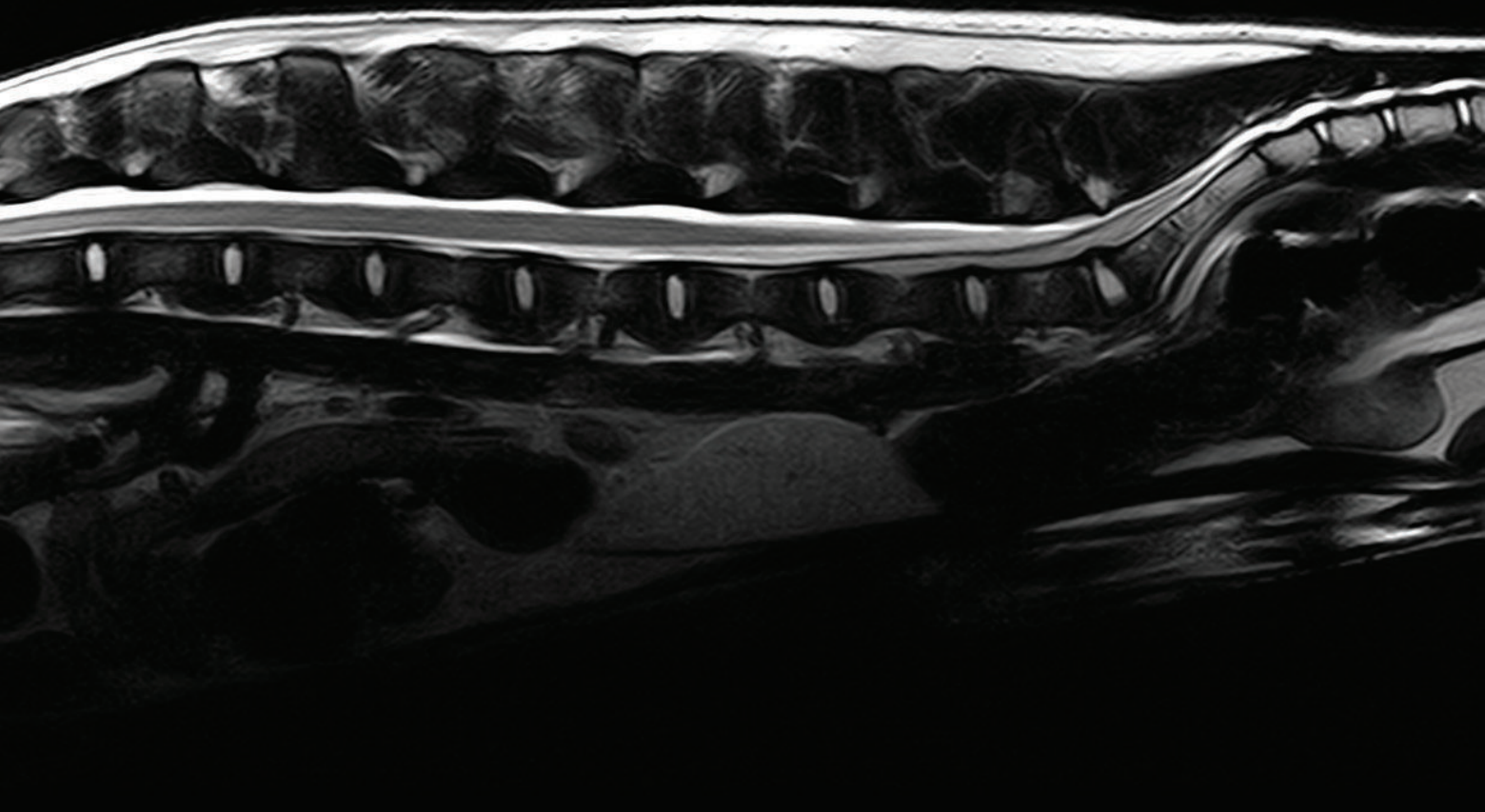


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Canine Mast Cell Tumors: Correlation of Apoptosis and Proliferation Markers with Prognosis

Timothy J. Scase, David Edwards, Jodi Miller, William Henley, Ken Smith, Anthony Blunden, and Sue Murphy

The Patnaik histologic grading system is commonly used to predict the behavior of cutaneous mast cell tumors (MCTs) in dogs, but it is less useful for grade 2 MCTs because they exhibit considerable variation in biological behavior. In this retrospective study, immunohistochemical staining for Ki-67, proliferating cell nuclear antigen (PCNA), and survivin and a standardized argyrophilic staining of nucleolar organizer regions (AgNOR) protocol were performed on 121 archived paraffin-embedded specimens of canine cutaneous MCTs, for which clinical follow-up data were available. Cox regression models indicated that the Ki-67 score (hazard ratio, 1.92; $P < .001$) and mean AgNOR score (hazard ratio, 2.57; $P < .001$) were significantly associated with Patnaik grade and survival time. A binary Ki-67 variable (cutoff point Ki-67 score = 1.8) was a significant predictor of survival for dogs with grade 2 MCTs. The estimated 1-, 2-, and 3-year survival probabilities for dogs with grade 2 MCTs and Ki-67 scores less than 1.8 were 0.92, 0.86, and 0.77, respectively (SEs, 0.08, 0.14, and 0.23, respectively; median not estimable). The corresponding survival probabilities for dogs with grade 2 MCTs and Ki-67 scores higher than 1.8 were 0.43, 0.21, and 0.21, respectively (SEs, 0.19, 0.18, and 0.18, respectively; median survival time, 395 days). No significant association was identified between survival and survivin score or PCNA score. This study shows that both mean AgNOR score and Ki-67 score are prognostic markers for canine MCTs. The Ki-67 score can be used to divide Patnaik grade 2 MCTs into 2 groups with markedly different expected survival times.

Key words: AgNOR; Inhibitor of apoptosis; Ki-67; PCNA; Survivin.

Mast cell tumors (MCTs) are the most frequently diagnosed skin tumors of dogs, occurring at an estimated incidence of 129 per 100,000 dogs.¹ The Patnaik histologic grading scheme² is the most frequently used method to predict the biological behavior and prognosis of an individual MCT. This grading scheme is useful in predicting the behavior of generally benign (grade 1) and malignant forms (grade 3) of the disease. However, the Patnaik histologic grading scheme is less useful for predicting the behavior of the intermediate grade (grade 2) MCT because of the wide range of behavior exhibited by these tumors. Most intermediate grade MCTs have a benign clinical course and can be cured by wide local excision. However, a proportion of intermediate grade MCTs will metastasize, with estimates of the metastatic rate varying between 5%³ and 22%⁴ and the rates of local recurrence varying between 5%³ and 11%.⁴ Most MCTs fall into this intermediate grade category,⁵ and it is still difficult to accurately predict how most MCTs are likely to behave and what additional therapy, if any, should be given to these dogs. For instance, if all intermediate grade tumors are treated by local excision alone, a considerable number of the tumors are being undertreated, whereas if all intermediate grade MCTs are treated with adjuvant systemic treatment, then a large proportion of dogs would receive unnecessary, and potentially harmful, therapy.

Veterinary pathologists may interpret the Patnaik histologic grading scheme in different ways, and different pathologists may assign different grades to the same MCT. A quantitative and less subjective grading scheme would therefore be of great clinical value.

Several studies have been performed in an attempt to develop less subjective methods of predicting MCT behavior and prognosis from analysis of formalin-fixed, paraffin-embedded tumor tissue. These studies have used assessment of various markers of cellular proliferation, including argyrophilic staining of nucleolar organizer regions (AgNOR),^{6–8} proliferating cell nuclear antigen (PCNA),^{6,9} and Ki-67 indices.⁹ Of these proliferation markers, only the Ki-67 index has been shown to be a prognostic indicator for canine MCTs independent of Patnaik grade.⁹

All of these proliferation markers can be used to estimate the numbers of cells that are undergoing cell division. This estimate has been shown to be more accurate than counting mitotic figures alone, because these markers identify cells in the S phase and the M phase of the cell cycle and have been shown to have prognostic value in a number of different studies in both humans and animals. PCNA is a member of the DNA sliding clamp family of proteins, which are essential components of the DNA replication system. PCNA helps to coordinate the DNA replicating protein complex at the replication fork and enhances DNA processivity for replicative DNA polymerases, including the delta subunit of DNA polymerase.¹⁰ This protein is expressed during both the S and M phases of the cell cycle.

Ki-67 is a large (395-kDa) protein that, despite extensive study, has no known function in the cell.¹¹ Its subcellular localization and expression are tightly regulated during the cell cycle, and it appears to be necessary for progression of the cell cycle, because inhibition of Ki-67 expression by antisense nucleotides halts cell proliferation.¹² The monoclonal antibody MIB-1 is used to detect Ki-67 protein expression and

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Submitted January 4, 2005; Revised April 29, 2005; Accepted July 6, 2005.

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0891-6640/06/2001-0019/\$3.00/0

has been used in studies in both dogs^{13,14} and humans, as a measure of cell proliferation rate (as reviewed by Brown and Gatter¹⁵). In some canine tumors, the frequency of Ki-67 expression by neoplastic cells is significantly associated with tumor prognosis.^{13,16–20}

AgNOR counts have been used in many studies and have been shown to provide clinically useful prognostic information in both veterinary^{6,21–24} and human^{25,26} oncology. However, because many different histochemical AgNOR staining techniques were used, it is impossible to directly compare the results of these studies among different laboratories. This difficulty has prevented widespread adoption of this staining technique in routine diagnostic pathology practice, because each laboratory would be required to validate its own staining technique. To address this issue, an international AgNOR standardization committee²⁷ was formed and published standardized and reproducible AgNOR staining methods for a wide range of clinical samples. To date, no studies in veterinary medicine have used these standardized methods; therefore, the results of previous studies of MCT prognosis and AgNOR staining counts cannot be reliably compared or reproduced.

Tumor growth depends not only on the rate of neoplastic cell proliferation but also on the rate of neoplastic cell death. A tumor with a low proliferation marker index and a low rate of cell death may be just as aggressive as one that is proliferating quickly but has a higher rate of cell death. Indeed, because carcinogenesis is a multistep process that requires multiple genetic mutations in individual cells, the inhibition of apoptosis in the mutated cell is a necessary step for that transformed cell to survive and continue to replicate. Therefore, there has been an increased interest in human oncology in the use of markers of apoptosis to determine whether they too can be used to predict tumor prognosis. Such has proved to be the case with a recently identified member of the inhibitors of apoptosis family, survivin.²⁸ The inhibitors of apoptosis function as endogenous inhibitors of caspase 9 and caspase 3, but activation of other intracellular signaling pathways also may be involved. Evidence is accumulating that indicates that survivin is involved in the maintenance of chromosomal stability during mitosis,²⁹ and that this may be the main regulatory effect of survivin on the cell cycle.³⁰ Despite being largely undetectable in most normal differentiated human tissues,³¹ survivin has proved to be an almost universal tumor antigen, being expressed in most human cancers that have been examined for its presence.³² In many of these cancers, it has proved to be an independent prognostic marker. For example, patients with glioma that express survivin at high concentrations have a much poorer prognosis than those that express survivin at low concentrations.³³

In this study, we performed immunohistochemical and histochemical staining of a large number of canine MCTs to determine whether the frequency of expression of inhibitor of apoptosis, survivin, and a number of markers of cell proliferation (PCNA, Ki-67, and AgNOR) can accurately predict canine MCT behavior.

Materials and Methods

Dog Selection

A retrospective study was performed on tissues submitted by veterinary practices that were examined by 1 of 2 pathologists (K.S., A.B.) at the Diagnostic Pathology Service of the Animal Health Trust between 1997 and 1999 and resulted in a diagnosis of a cutaneous MCT. Data retrieved from archived reports included breed, sex, and age of the dog, grade of tumor, site of the primary tumor, and histologic margins.⁵ The MCTs were graded according to the system of Patnaik,² and all histopathologic grading was performed blinded to clinical outcome. Inclusion criteria were based on those previously published.⁵ All dogs were treated by veterinarians in general practice. All tumors were treated by surgery, except for 5 dogs, who also received corticosteroid therapy. Of these 5 dogs, 3 died as a result of their MCT. In total, 121 formalin-fixed, paraffin-embedded MCT tissue blocks were identified and had sufficient tissue for additional sections to be taken for analysis, and for which complete clinical follow-up data were available.

For calculation of survival times, the date of MCT diagnosis, rather than the date that the tumor was first noticed, was used as the entry point, because for many dogs the date the owner noticed the tumor was unknown. The date of censoring was September 1, 2000. Dogs who were reported to be dead from a MCT before that date were recorded as events. Any animal that was reported to be alive after that date, lost to follow-up before that date, or dead from unrelated causes was censored. If there were multiple submissions from a single dog, these submissions were carefully evaluated. For a MCT from which more than 1 biopsy specimen had been taken, relating either to its local regrowth or metastasis, only the first report from that particular tumor was included in the analysis. In the case of dogs with multiple MCTs (ie, neither metastases nor local tumor regrowth), survival data were analyzed considering each tumor as a separate event and by analyzing the data with only the first tumor that was diagnosed.

Immunohistochemical Staining

Five-micrometer sections were taken from each paraffin block and mounted onto positively charged, capillary gap glass slides. Immunohistochemical staining for PCNA, Ki-67, and survivin immunoreactivity was performed with an automated staining system.^a The positive control tissues for PCNA and Ki-67 were formalin-fixed, paraffin-embedded sections of canine hyperplastic lymph node. Formalin-fixed, paraffin-embedded sections of a canine oral squamous cell carcinoma, a tumor that frequently expresses survivin in humans,³⁴ were used as positive controls for survivin immunohistochemistry. For negative controls, the primary antibody was replaced with antibody diluent. Antigen retrieval was performed by incubating the mounted sections in a microwave oven for a total of 10 minutes in a citrate buffer,^b pH 6.0 (survivin and PCNA), or EDTA buffer,^c and pH 9.0 (Ki-67). Endogenous peroxidase activity within the tissue sections was blocked with hydrogen peroxide,^d and sections were incubated for 30 minutes, with the primary antibodies diluted^e at the dilutions outlined in Table 1. Detection of primary antibody binding was performed by a 2-layer method and chromogen developed with diaminobenzidine. Slides were counterstained with hematoxylin.

Histochemical Staining

The AgNOR staining technique used in this study was approved by the International AgNOR Standardization Committee.^{27,35} Five-micrometer sections were made from each paraffin block and mounted onto positively charged glass slides. Sections were dewaxed, rehydrated, and immersed in 0.01M sodium citrate

Table 1. Antibody clone, source, and dilution used for immunohistochemical staining.

Antibody	Clone	Supplier	Dilution	Positive Control Tissue
PCNA	PC10	Dako Corporation, Carpinteria, CA	1:2,000	Lymph node
Ki-67	MIB-1	Dako Corp	1:150	Lymph node
Survivin	NB500-201	Novus Biologicals, Littleton, CO	1:600	Oral squamous cell carcinoma

PCNA, proliferating cell nuclear antigen.

monohydrate, pH 6.0, and heated in a wet autoclave at 120°C for 20 minutes. Cooled sections were then washed in distilled water. Silver stains were performed in the dark (at 37°C) by immersing the slides in a 33% silver nitrate, 0.6% gelatin, and 0.33% formic acid solution for 13 minutes. After staining, slides were washed, dehydrated, and mounted.

Image Capture and Cell Counting

Histologic images were captured digitally at a magnification of 200× from equivalent regions of each stained tissue section, such that the site selected was approximately one third of the distance from the deep and lateral margins of the tumor mass. A computer program (Tag and Count) was designed and written⁸ to enable reliable counting of positive cells from each captured image. Cells were counted with a pen and tablet input system. Counting was performed without prior knowledge of clinical outcome.

Survivin Scoring

Positive survivin expression was assessed after optimization of the technique with formalin-fixed, paraffin-embedded specimens of canine oral squamous cell carcinoma, a tumor that expresses survivin with high frequency in people.³⁴ For survivin characterization, positive immunohistochemical staining was assessed by a numeric scoring system for each captured image. Both nuclear and cytoplasmic staining were assessed independently according to the following scale: 0 indicates no positive staining cells; 1, less than 0.1% of neoplastic mast cells exhibit positive staining; 2, 0.1–1% of neoplastic mast cells exhibit positive staining; 3, 2–10% of neoplastic mast cells exhibit positive staining; 4, 11–50% of neoplastic mast cells exhibit positive staining; and 5, 51–100% of neoplastic mast cells exhibit positive staining. This scoring system was used because most MCTs exhibited low concentrations of survivin immunoreactivity. Statistical analysis was performed with individual categories on the scale and also by grouping the scales into 0–10%, 11–50%, and 51–100% categories.

Proliferation Marker Scoring

For evaluation of PCNA and Ki-67 expression, the total numbers of mast cells and the total number of immunohistochemical staining-positive mast cell nuclei were counted per captured image. PCNA and Ki-67 scores were calculated as the percentage of mast cell nuclei that exhibit positive immunohistochemical staining within the image. The total number of AgNOR dots per neoplastic mast cell nucleus was counted for each captured image, up to a maximum of 200 mast cell nuclei, and the mean AgNOR score per neoplastic cell nucleus was calculated.

Statistical Analysis

A preliminary examination of the data was performed with frequency tables, histograms, and the medians and ranges for different variables. Estimates of the survivor function were displayed by Kaplan-Meier plots. Survival distributions were

summarized by the median survival time where estimable and, if not estimable, by estimated 1-, 2-, and 3-year survival probabilities.

Cox regression analysis was used to determine any association between time to death associated with MCTs (ie, death or euthanasia of the animal as a direct result of MCT presence) and possible prognostic indicators (ie, Patnaik grade and AgNOR, survivin, PCNA, and Ki-67 scores). Survivin score was coded with hierarchical indicator variables to reflect the inherent ordering of the categories. Age at diagnosis was examined as a potential confounder.

Variables were selected for inclusion in the model if they significantly improved the fit (likelihood ratio χ^2 statistic $P < .05$). The relationship between survival time and prognostic indicator was displayed by Kaplan-Meier plots.³⁶ Smoothing splines were used to explore the functional form of the relationship between time to death associated with MCTs and the continuous prognostic indicator variables (AgNOR, PCNA, and Ki-67 scores).³⁶ All Cox regression computations were performed by S-PLUS 6 software.^h

Results

Descriptive Statistics

The estimated 1-, 2-, and 3-year survival probabilities were 0.83, 0.75, and 0.64, respectively (SEs, 0.17, 0.25, and 0.36, respectively). The mean age of the dogs was 8.3 years (median, 8 years; range, 1–15 years). Nineteen of the 121 dogs had multiple cutaneous MCTs. A total of 121 dogs were analyzed after data checking: 72 of these dogs were still alive at the end of follow-up, 31 deaths were associated with the MCT, and 18 died of causes unrelated to the MCT and were censored. Of 5 dogs who received prednisolone in addition to surgery, 2 were alive at the end of the study period and 3 were dead. Of the living dogs, 1 had a grade 1 MCT and the other had a grade 2 MCT. Of the 3 dead dogs, 2 had grade 3 MCTs and 1 had a metastatic grade 2 MCT.

Patnaik Histologic Grade and Survival

Of the 121 dogs, 16 had grade 1 MCTs, 86 had grade 2 MCTs, and 19 had grade 3 MCTs; 17 dogs had 2 MCTs and 1 dog had 3 MCTs. Patnaik histologic grade was found to be a highly significant predictor of death associated with MCTs ($P < .001$). However, because none of the dogs with Patnaik grade 1 tumors died of the MCT during follow-up, the Cox regression model gave unstable coefficients for grade 1 tumors. The Kaplan-Meier survival plot for Patnaik grade indicates that the ability of the different grades to predict survival of this subset of dogs was consistent with the originally published report² and with other studies^{3,4,6} (Fig 1).

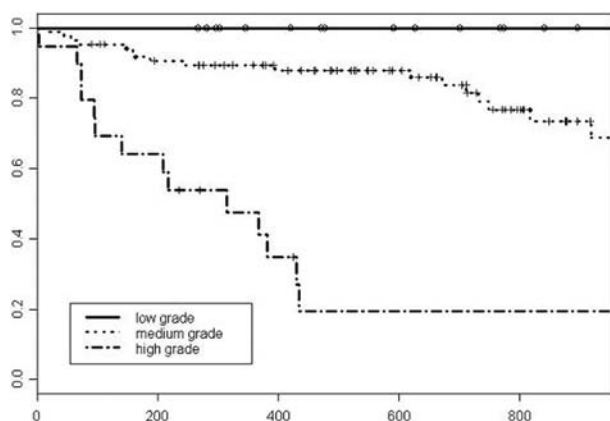


Fig 1. Kaplan-Meier survival plot for canine mast cell tumor categorized by Patnaik grade.

Frequency of Proliferation Marker Expression by Neoplastic Mast Cells

The Ki-67 scores ranged from 0–22.2% (median, 1.0%). The mean AgNOR scores ranged from 1.13–4.1 (median, 1.7). The PCNA scores ranged from 3.2–83% (median, 48.6%).

Univariable Cox regression models were used to assess associations between the frequency of proliferation marker expression by neoplastic mast cells and survival of dogs with cutaneous MCTs. This analysis showed a significant association between the Ki-67 and AgNOR scores and survival (Table 2). No significant association occurred between PCNA score and survival. The final multivariable Cox regression model for continuous proliferation marker expression and survival indicated that Ki-67 score (hazard ratio, 1.92; 95% confidence interval, 1.44–2.56; $P < .001$) and mean AgNOR score (hazard ratio, 2.57; 95% confidence interval, 1.44–4.60; $P < .001$) were significantly associated with survival time of dogs with MCTs.

Smoothing splines indicated that the hazard ratio increased after 1% for Ki-67 score and that the hazard ratio increased after a mean of 1.8 was reached for mean AgNOR score (data not shown). With these values as cutoff points, binary variables for Ki-67 and AgNOR scores were placed into a Cox regression model. This analysis indicated that although both scoring systems were significantly associated with survival, Ki-67 expression (hazard ratio, 3.77; 95% confidence interval,

Table 2. Univariate results for proliferation markers calculated by Cox regression analysis.

	Coefficient (SE)	Hazard Ratio (95% CI)	P Value ^a
Ki-67 score	0.241 (0.051)	1.27 (1.15–1.41)	< .001
Mean AgNOR score	0.982 (0.285)	2.67 (1.53–4.67)	.002
PCNA score	0.019 (0.012)	1.02 (1.00–1.04)	.108

AgNOR, argyrophilic staining of nucleolar organizer regions; CI, confidence interval; PCNA, proliferating cell nuclear antigen.

^a P values calculated using the likelihood ratio test.

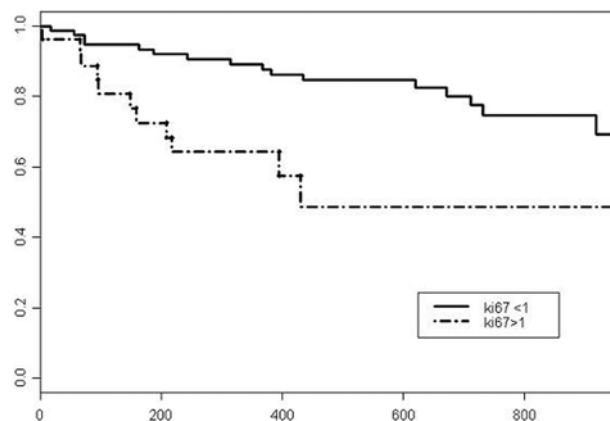


Fig 2. Kaplan-Meier survival plot for canine mast cell tumors categorized by binary Ki-67 score with a cutoff value of 1%.

1.65–8.64; $P = .002$) was a better predictor of death associated with MCT than AgNOR score (hazard ratio, 2.59; 95% confidence interval, 1.14–5.85; $P = .022$). Four (27%) of 15 Patnaik grade 1 tumors had a Ki-67 value greater than 1.8, compared with 14 (20%) of 70 Patnaik grade 2 tumors, and 8 (50%) of 16 grade 3 tumors. Ki-67 values were only available for 101 dogs because of tissues repeatedly floating off the slides during antigen retrieval. Kaplan-Meier survival plots for canine MCTs categorized by binary variables for Ki-67 score with a cutoff value of 1% (Fig 2), and for mean AgNOR score with a cutoff 1.8 (Fig 3), are shown.

The prognostic value of Ki-67 scores and mean AgNOR scores were determined for a subset of the MCTs that represented Patnaik grade 2 MCTs (85 dogs). Smoothing splines indicated that within the Patnaik grade 2 subset, in contrast to the smoothing splines described herein that were created for the dog population that covered all Patnaik grades, a more suitable cutoff point for Ki-67 was 1.8 (data not shown). In this Patnaik grade 2 subset, the AgNOR count cutoff point remained 1.8. Cox regression analysis indicated that only the binary Ki-67 variable was a significant predictor of survival of dogs with medium grade MCTs (hazard ratio, 11.1; 95% confidence interval, 3.41–36.2; $P < .001$). The estimated

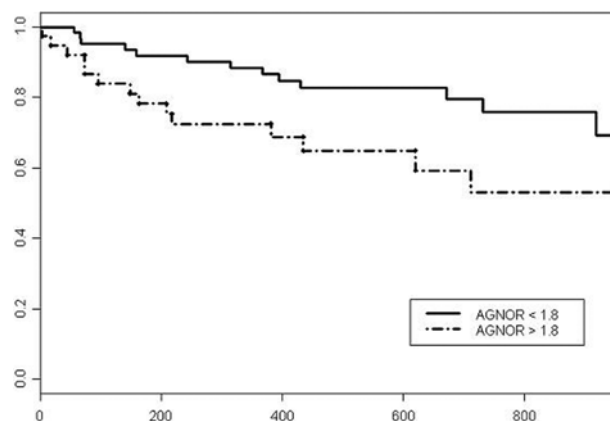


Fig 3. Kaplan-Meier survival plot for canine mast cell tumors categorized by binary mean argyrophilic staining of nucleolar organizer regions score with a cutoff value of 1.8.

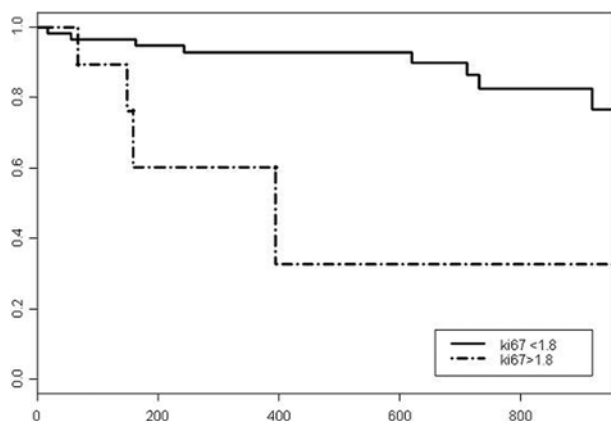


Fig 4. Kaplan-Meier survival plot for Patnaik grade 2 mast cell tumors categorized by binary mean Ki-67 score with a cutoff value of 1.8.

1-, 2-, and 3-year survival probabilities for dogs with grade 2 MCTs and Ki-67 scores greater than 1.8 were 0.92, 0.86, and 0.77, respectively (SEs, 0.08, 0.14 and 0.23, respectively; median not estimable). The corresponding survival probabilities for dogs with grade 2 MCTs and Ki-67 scores greater than 1.8 were 0.43, 0.21, and 0.21, respectively (SEs, 0.19, 0.18, and 0.18, respectively; median survival, 395 days). A Kaplan-Meier survival plot for Patnaik grade 2 MCTs categorized by binary Ki-67 score with a cutoff value of 1.8 is shown in Figure 4.

Expression of Survivin by Neoplastic Mast Cells

Immunohistochemical staining of a variety of formalin-fixed, paraffin-embedded canine tissues with anti-survivin antisera was performed with an array of 20 histologically normal canine tissues. As expected, this procedure identified positive cytoplasmic staining in small populations of lymphocytes within lymphoid follicles of the spleen and lymph nodes, and within epithelial cells at the base of mucosal crypts within the small and large intestine. As expected, no positive immunohistochemical staining was evident within tissue sections of heart, lung, liver, kidney, brain, pancreas, thyroid gland, adrenal gland, stomach, skeletal muscle, peripheral nerve, salivary gland, skin, tongue, esophagus and trachea. Neoplastic epithelial cells throughout a sample of oral squamous cell carcinoma exhibited moderate to intense positive cytoplasmic and variable nuclear immunohistochemical staining for survivin (Fig 5). This finding was subsequently used as a positive control in later experiments. Negative controls were performed by omitting the primary antibody.

Neoplastic mast cells in most dogs with MCT exhibited positive nuclear survivin staining, cytoplasmic survivin staining, or both (Fig 6; Table 3).

Cox regression analysis of this immunohistochemical data for survivin expression indicated no significant association between survival and the frequency of survivin expression by neoplastic mast cells, or between survival and subcellular location of survivin expression within the neoplastic mast cells (data not shown).

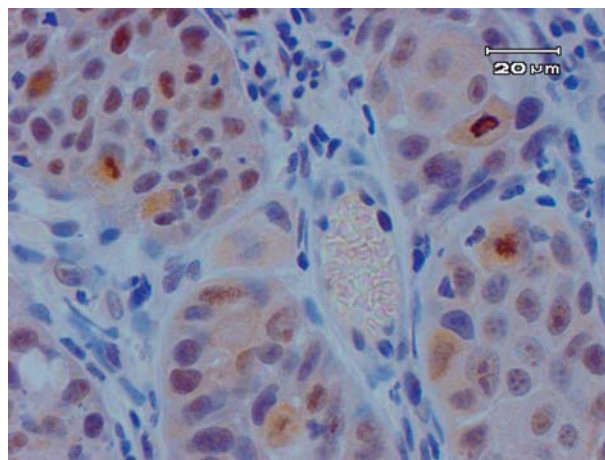


Fig 5. Survivin immunoreactivity by neoplastic epithelial cells of a canine oral squamous cell carcinoma. Neoplastic cells exhibit moderate positive immunohistochemical staining for survivin within the nucleus and cytoplasm (rabbit anti-human survivin, counterstained with hematoxylin, $\times 40$ objective lens).

Association Between Age and Multiplicity of Tumors and Survival of Dogs with Cutaneous MCTs

In this population of dogs with MCTs, a significant association between survival and age at diagnosis was identified (hazard ratio, 1.19; 95% confidence interval, 1.03–1.38; $P = .015$). However, no association was found between survival and the number of MCTs that any 1 dog developed (hazard ratio, 0.91; 95% confidence interval, 0.32–2.59; $P = .850$).

Discussion

A large group of canine cutaneous MCTs were examined, with a number of markers of cell proliferation and an inhibitor of apoptosis (survivin), to determine whether these markers would have prognostic value for survival after local treatment of MCTs. This was a large study, and the methods used should be reproducible and repeatable in any laboratory suitably equipped for performing immunohistochemical staining of paraffin-embedded tissues. Indeed, by assessing the proliferation marker index in a single digital microscopic image obtained from a standardized, selected area of the tumor, rather than obtaining multiple digital microscopic images of the tumor mass as has been performed in other studies,⁹ these techniques should be relatively simple to incorporate into the routine diagnostic pathology laboratory workflow.

The most important finding of this study was that it is possible to divide intermediate MCTs on the basis of Ki-67 scores. Among dogs with Patnaik grade 2 MCTs, those with a Ki-67 score greater than 1.8 had substantially lower estimated survival probabilities at 1, 2, and 3 years than dogs with a Ki-67 score less than 1.8. This finding confirms and extends similar findings that were evident in a smaller study by Abadie et al.⁹ However, the data reported represents the first study to directly assess survival for a large numbers of dogs with MCTs with a broad range of different

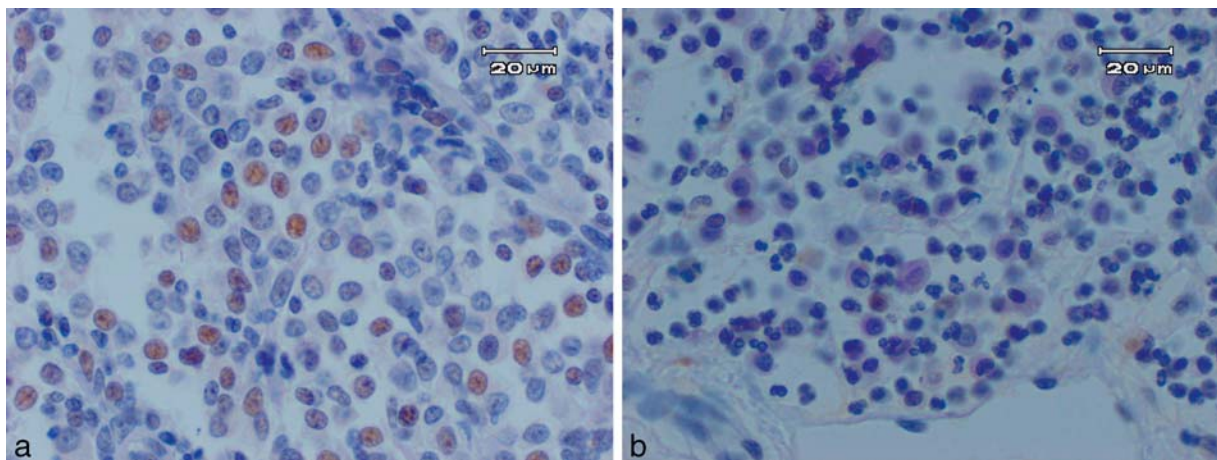


Fig 6. Survivin immunoreactivity in a subset of neoplastic mast cells from a Patnaik grade 2 mast cell tumor. A subset of neoplastic mast cells exhibit mild to moderate positive immunohistochemical staining of nuclei (a) and weak staining of the cytoplasm (b) for survivin (rabbit anti-human survivin, counterstained with hematoxylin, $\times 40$ objective lens).

markers. We are currently performing a prospective study to determine whether a Ki-67 score of 1.8 can be used as a cutoff value in a larger series of intermediate grade MCT.

Interestingly, there were more grade 1 MCTs above the Ki-67 score cutoff point of 1 than grade 2 MCTs. Of these dogs with grade 1 MCTs, most had multiple MCTs, but all survived beyond the end of the study. Therefore, it is possible that the neoplastic cells in the dogs with multiple grade 1 MCTs are different from those lesional neoplastic mast cells in dogs with solitary MCTs. For instance, there may be different cellular signaling pathways (eg, tyrosine kinase receptor-mediated pathways other than c-KIT) affected within these 2 groups of neoplastic cells that could account for the differences in growth fraction, without increasing the likelihood of them recurring or metastasizing. Alternatively, the rate of tumor cell death by apoptosis may be higher in grade 1 MCTs than in some grade 2 MCTs. Additional work is needed to dissect the signaling pathways involved in MCT carcinogenesis, and different signaling pathways may be altered in different MCT subsets.

Similar to previous studies,^{6,7} lower AgNOR scores were associated with longer survival times in dogs with MCTs, but could not predict clinical behavior independent of the Patnaik histologic grade. This finding greatly reduces the usefulness of the assay, because Patnaik grade 2 MCTs are the most frequently encountered histologic subtype and AgNOR scores do

not provide more prognostic information than is already provided by the Patnaik histologic scoring system. However, this is the first companion animal study to use the standardized, internationally recognized AgNOR staining protocol.^{27,35} The need for a standardized AgNOR staining protocol has been recognized in human medicine, in which it was found that AgNOR score values could not be replicated among different laboratories due to differences in AgNOR staining techniques. Without standardization of this technique, these studies cannot be translated from a research environment into widespread clinical diagnostic practice, because each laboratory would be required to perform their own studies to determine the equivalent AgNOR scores for their specific staining protocol. The results of this study will be directly comparable to any future studies that also use the standardized AgNOR staining protocols. Failure to find any association between PCNA score and MCT prognosis confirms similar findings in smaller previous studies.^{6,9}

In the study population, only 5 dogs received adjunctive chemotherapy (ie, prednisolone). Of these dogs, 2 lived and 3 died. The 3 who died had much more aggressive disease (grade 3 MCTs or metastatic grade 2 MCTs), compared with those who were given adjunctive chemotherapy and lived (a grade 1 MCT and a grade 2 MCT). Therefore, the use of chemotherapy in these dogs is unlikely to have skewed the results of these analyses.

Table 3. Immunohistochemical detection of survivin expression in neoplastic cells of canine mast cell tumors.

	Frequency of Survivin-Positive Neoplastic Cells ^a					
	0	1	2	3	4	5
Nuclear survivin expression	22	12	31	28	18	3
Cytoplasmic survivin expression	44	20	11	13	12	14

^a For frequency of survivin-positive neoplastic cells, 0 indicates no positive staining cells; 1, less than 0.1% of mast cells exhibit positive staining; 2, 0.1–1% of mast cells exhibit positive staining; 3, 2–10% of mast cells exhibit positive staining; 4, 11–50% of mast cells exhibit positive staining; and 5, 51–100% of mast cells exhibit positive staining.

This study is the first to demonstrate immunohistochemical expression of survivin by neoplastic cells in a companion animal species. We have found that the antisera used in this study cross-reacts with a recombinant canine survivin by Western blot analysis (data not shown), which is consistent with the demonstration of the expected tissue staining profile within multiple normal canine tissues and in dogs with canine oral squamous cell carcinoma. In some human cancers, survivin expression by the neoplastic cells is closely associated with prognosis and, in some cases, response to treatment.³⁷ In this study, survivin expression was not found to be an independent prognostic factor for canine cutaneous MCTs and had no value in predicting the clinical outcome of disease. The overall concentration and frequency of expression of survivin by the neoplastic mast cells was low. Therefore, it is unlikely that any survivin-targeted therapies that are developed in the future will be of clinical benefit in the treatment of canine MCTs. However, this study showed that neoplastic cells (including neoplastic epithelial cells within squamous cell carcinoma), and some normal adult cells, exhibit immunoreactivity for survivin in dogs. Additional studies will be necessary to determine whether expression of survivin is associated with clinical behavior or response to treatment in other cancers of companion animal species.

Clearly, there is still a need to use the standard Patnaik histologic grading scheme to identify the grade (1–3) of an individual MCT, because this is the most rapid and cost-effective way to broadly predict clinical behavior of these tumors. However, this study indicates that if the tumor is of intermediate grade, additional prognostic information may be gained by determining the Ki-67 score. Additional studies are in progress in this laboratory, with larger numbers of intermediate grade MCTs, to determine the clinical usefulness of Ki-67 expression in predicting the clinical behavior of these tumors.

Footnotes

- ^aDako Techmate, Dako Corporation, Carpinteria, CA
^bChemMate Target Retrieval Solution, Dako Corporation, Carpinteria, CA
^cChemMate Target Retrieval Solution, Dako Corporation, Carpinteria, CA
^dChemMate Target Retrieval Solution, Dako Corporation, Carpinteria, CA
^eChemMate Antibody Diluent, Dako Corporation, Carpinteria, CA
^fEnvision system, Dako Corporation, Carpinteria, CA
^gRealbasic software, version 5.2.4, Mac OS10.2, Austin, TX
^hInsightful, 2001, Seattle, WA
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Acknowledgments

This project was generously supported by an RCVS Trust 2003 award. We thank Mrs Sue Gower for

technical advice and Dr Andy Sparkes for helpful review of the manuscript. We thank the many veterinarians in private practice who provided detailed clinical history for the dogs involved in the study.

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