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Cytoplasmic and nuclear morphometric parameters in cytologic preparations of canine cutaneous mast cell tumors

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Abstract

Thirty fine-needle biopsy (FNB) samples from 28 dogs subjected to surgical resection of cutaneous mast cell tumors (MCTs) were stained with Giemsa. At least 100 neoplastic cells from each cytology slide were evaluated by morphometric analysis. The parameters were: area, perimeter of the cell, cytoplasm, nucleus and circumference factor. MCTs of grade III had a mean cellular area of 231.70 μm^2 ± 57.1, and grade II had a mean of 252.30 μm^2 ± 55.0. Cellular perimeter was 61.20 ± 7.1 in grade II and 59.1 ± 8.6 in grade III. Cellular parameters were not statistically different between grades (p> .05). Mean nuclear area was 88.90 μm^2 ± 19 in grade III and 72.30 μm^2 ± 13.9 in grade II, with statistical difference between grades (P = .011). Mean nuclear perimeter was 32.40 µm ± 3.0 in grade II and 35.70 µm ± 4.0 in grade III, with statistical difference between grades (P = .018). Mean nuclear circumference factor was 1.0 ± 0.33 in grade II and 1.1 ± 0.28 in grade III, with no statistical difference between grades (P = 0.78). Nuclear-to-cytoplasmic ratio in grade II was 0.29 ± .07 and 0.39 ± .08 in grade III, with statistical difference (P = .02). The number of binucleated and multinucleated cells and mitotic figures was significantly increased in grade III MCTs (P < .001). In conclusion, the number of mitotic figures, presence of binucleation and multinucleation, and nuclear-to-cytoplasmic ratio can help to guide a profile of MCT aggressiveness in cytologic preparations.

Key Words: cytopathology, morphometry, image analysis, mast cell tumor

Introduction

Cutaneous mast cell tumors (MCTs) are one of the most common tumors in dogs, accounting for 7-21% of all skin tumors (3, 18, 22). Affected animals have a mean age of 9 years at the time of diagnosis. Boxers, Boston terriers, Labrador retrievers, and Bulldogs have an increased risk of MCT development (2, 18, 22). Most dogs with MCTs will present with solitary lesions, but approximately 11-21% of animals will present with multiple lesions (8, 9). Grossly, the appearance of mast cell tumors is variable, ranging from soft, slow-growing masses to rapidly enlarging, ulcerated masses (18, 22). Metastatic sites include the regional lymph nodes, spleen and liver (18, 22). The process by which cutaneous mast cells transform into a MCT remains largely unknown (18, 21, 23). Clinically, these tumors range from a benign mass that can be cured by surgical excision alone, to potentially fatal metastatic disease (2, 8, 9, 10, 12, 18, 22). Dogs with high-grade MCTs and those with a history of disseminated disease have a short survival time (2, 10, 12, 18, 22).

Attempts have been made to predict the behavior of these tumors with criteria such as clinical stage (8, 9), growth rate (2), proliferative activity (1, 7, 15, 21), intratumoral angiogenesis (13), c-KIT expression and mutation (22, 23), and histological grade (2, 12). The results of these studies have been highly variable, and no single parameter has been shown to clearly predict the biological behavior of canine cutaneous MCTs. However, histological grade is the most frequently used criterion in routine diagnostic procedures because it is accepted as

predictive, is easily applied to stained histological sections, and does not require more expensive and complicated techniques (2, 12, 18, 22). The histological grading system proposed by Patnaik et al. (12) is based on certain histological features (extent of tumor, cellularity, cellular morphology, and mitotic index) and is the most commonly used method (18, 22). Although this grading system is the accepted method for predicting MCT behavior, it is not reproducible, with as much as 50–60% discordance between experienced pathologists (11). The Patnaik grading system is based on information derived from analysis of different histological features, some of which may be strongly influenced by subjective inter-observer differences as well as intratumoral heterogeneity (11, 12).

Computerized morphometric techniques were used in three studies, two of which evaluated cytology samples (16, 17) while another assessed histologic samples of MCTs (6). The computerized morphometric technology was applied in an attempt to minimize subjective inter-observer variation and, consequently, to aid in accurate diagnostic classification.

The goal of the present study was to determine whether moderately (grade II) and poorly differentiated (grade III) MCTs have different cellular, cytoplasmic and nuclear morphometric measurements, by combining the precision of computerized morphometric analysis with the rapidity and practicality of cytopathologic examination. The study also evaluated the contribution of number of mitotic figures, and of binucleated and multinucleated cells in grade II and III MCTs as criteria to correlate histiologic grade with cytologic characteristics of MCT.

Material and Methods

Source of MCTs and cytology samples

Thirty cutaneous MCTs from 28 dogs were obtained from the Department of Veterinary Surgery and Anesthesiology, School of Veterinary Medicine and Animal Science, São Paulo State University, Botucatu, Brazil. These tumors were subjected to fine-needle biopsy (FNB), using a fenestration (non-aspiration) method (20) prior to surgical excision. Cases were excluded if the dog had been treated with corticosteroids or any other chemotherapeutic drug previously the diagnosis by FNB. After surgical excision, the tumors were fixed in 10% formalin and routinely processed for histopatology. Two 5-µm sections were obtained from each tumor, then stained with hematoxylin-eosin (HE) and submitted to 3 experienced pathologists for histopathologic grading according to Patnaik´s system (12). The grade was established by consensus of 2 or more observers.

The cytologic smears obtained by FNB were air-dried and fixed in methanol for five minutes and stained with Romanowsky-type stain (Giemsa). Tumors were selected for further evaluation by one author (RTN), on the basis of the high quality of the cytologic preparations (cellularity, cellular integrity, cellular basophilia, absence of artifacts).

Evaluation of mitotic figures, binucleation and multinucleation on cytologic smears

The number of mitotic figures, and binucleate and multinucleate cells on cytologic preparations stained with Giemsa were determined by counting these cells within twenty high power fields (x400; HPF) in a contiguous manner. All mitotic figures, binucleated and multinucleated cells were counted with the assistence of a morphometric computer system (Leica – QWin).

Morphometric analysis

The material obtained by FNB was analyzed by a computer system composed of an optical microscope (Leica-DMR) coupled to a digital camera (Leica-DFC500) that transfered the images to a computer. The images were analyzed with an image analysis software program designed for quantitative microscopy (Leica - QWin). The measurements were calibrated with the aid of a micrometric ruler (Pyser-SGI) at 640x magnification. For each cytologic smear, at least 100 cells (nucleus and cytoplasm) were isolated using a Trust 1200-V2 Wireless Scroll Tablet (Dell). The cells analyzed had distinct cytoplasmic and nuclear borders to avoid evaluation of overlapping cells. First the nuclei were outlined, followed by the cytoplasm border of each cell of the field. The image analysis QWin software automatically calculated the morphometric parameters of these structures outlined in the neoplastic cells. Binucleate and multinucleate cells were not included in the morphometric measurement, nor were any mitotic figures. The morphometric parameters measured were: area of the cell, perimeter of the cell, cytoplasm and nucleus circumference factor. This cytoplasm and nucleus circumference factor represented the regularity of the nucleus and cytoplasm, in which a calculated value near 1.0 is a perfect circle. To calculate the nuclear-to-cytoplasmic ratio" the nucleus area divided by the respective cytoplasm area obtained by the image analysis software was determined.

Statistical Analysis

The cytology results obtained by computerized morphometry were compared and correlated with the histologic grades using software (GraphPad Software, Inc., San Diego, CA). Statistical significance was set at $P < 0.05$. The comparison between the morphometric parameters, nuclear-to-cytoplasmic ratio and histologic grades of the MCTs was assessed by Student's t-test and Mann Whitney test for mitotic figures, binucleated and multinucleated cells analysis.

Results

The age of the 28 affected dogs in this study ranged from 2 to 17 years with a mean age of 9 years. Fourteen (50%) were intact females and 14 (50%) were intact males. A total of nine breeds were represented including boxers (n = 12; 42.8%), mixed breed dogs (n = 7; 25%), pit bulls (n = 2; 7.1%), American cocker

spaniel (n = 1; 3.6%), miniature pinscher (n = 1; 3.6%), a poodle (n = 2; 7.1%), a German shepherd (n = 1; 3.6%), a rottweiler (n = 1; 3.6%) and a Brazilian terrier (n = 1; 3.6%). From the 28 affected dogs, two dogs with grade III MCTs had two tumors (multiple MCTs), distant from each other by more than 10cm, which made a total of 30 tumors. Of these, 20 (66.7%) were grade II mcts and 10 (33.3%) were grade III MTCs. Seven (23.4%) tumors were located on head or neck, 15 (50%) were on the trunk, and eight (26.6%) were on the extremities.

Binucleation, multinucleation, mitotic figures and tumor grade

Binucleated cells on cytologic smears were present in all grade III MCTs (n = 10/10 – 100%) (range, 3-47 per 20/HPFs). Three grade II MCTs (n = 3/20 – 15%) did not show binucleation (range, 0-14 per 20/HPFs) (Fig. 1A, 1B). Multinucleated cells were present in seven (n = 7/10 – 70%) grade III MCTs (range, 0-13 per 20/HPFs) and in four (n = 4/20 – 20%) grade II MCTs (range, 0-5 per 20/HPFs (Fig. 1C, 1D). Mitotic figures were present in all grade III tumors (n = 10/10 – 100%) (range, 2-45 per 20/HPFs) . Three grade II tumors (n = 3/20 – 15%) had mitotic figures (range, 0-2 per 20/HPFs) (Fig. 1E, 1F). The number of binucleate or multinucleate cells and cells undergoing mitosis was significantly increased as the grade of the tumor increased ($P < .001$). Results of binucleation, multinucleation, mitotic figures between tumor grades are summarized in Table 1.

Cytomorphometry

A total of 3106 cells in the 20 grade II tumors were analyzed. A total of 1409 cells from the 10 grade III tumors were evaluated. Morphometric parameters are summarized in Tables 2, 3 and 4.

Cellular area ranged widely within cells from grade II (163.21 μm^2 to 353.46 μm^2) and grade III (159.94 μm^2 to 313.72 μm^2) tumors. Grade III mean cellular area (231.70 $\mu m^2 \pm 57.1$) was smaller than mean cellular area in grade II tumors (252.30 $\mu m^2 \pm 55.0$). Cellular perimeter ranged from 49.35 µm to 75.16 µm in grade II tumor cells, while in grade III the cellular perimeter ranged from 47.29 µm to 654.47 µm. Mean cellular perimeter was 61.20 ± 7.1 in grade II and 59.1 ± 8.6 in grade III tumors. Cellular circumference factor ranged from 1.050 to 1.283 in grade II tumor cells, while in grade III ranged from 1.082 to 2.067. Mean cellular circumference factor in grade II tumor cells was 1.15 ± .006 and in grade III was 1.22 ± .30. All cellular parameters were not statistically different between grades (p> .05).

Cytoplasmic area ranged widely within cells from grade II tumors (95.61 μm^2 to 267.35 μm^2) and also from grade III (87.13 μm^2 to 221.36 μm^2) tumors. Mean cytoplasmic area was 177.70 $\mu m^2 \pm 54.1$ in grade II tumor cells and 138.20 $\mu m^2 \pm 49.9$ in grade III tumors, again without statistical difference ($P > .05$).

Nuclear area ranged widely from 53.64 μm^2 to 110.52 μm^2 in grade II MCTs and from 52.39 μm^2 to 121.06 μm^2 in grade III MCTs. Mean nuclear area was 72.30 $\mu m^2 \pm 13.9$ in grade II tumor cells and 88.90 $\mu m^2 \pm 19$ in grade III, with a statistical difference observed between grades ($P = .011$). Nuclear perimeter measurement ranged from 27.86 µm to 40.11 µm in grade II tumors and 27.20 µm to 41.59 µm in grade III. Mean nuclear perimeter was 32.40 µm ± 3.0 in grade II and 35.70 µm ± 4.0 in grade III, with statistical difference detected between grades ($P = .018$). Nuclear circumference factor ranged from 1.051 to 1.177 in grade II tumor cells and 1.076 to 1.177 in grade III. Mean nuclear circumference factor was 1.0 ± 0.33 in grade II and 1.1 ± 0.28 in grade III, with no statistical difference between grades ($P = 0.78$).

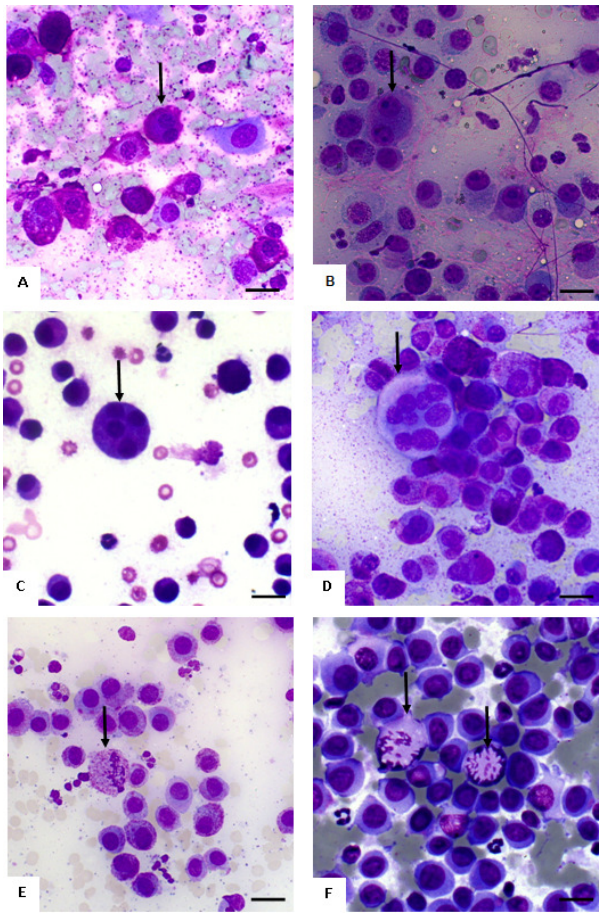

Figure 1. Skin, dog, fine needle biopsy (Giemsa stain; bar = 20µm): (A) a few, small, atypical, well granulated mast cells are present, including a binucleated cell (arrow), MCT grade II. (B) large numbers of poorly granulated mast cells are present. There is also a large binucleated cell (arrow) with prominent nucleoli, MCT grade III. (C) multinucleated cell on the left center (arrow) and few small well granulated mast cells, MCT grade II. (D) multinucleated cell on the left center (arrow) with variability in nucleus size, MCT grade III. (E) irregular mitotic figure in a granulated mast cell, MCT grade II (arrow). (F) large numbers of poorly granulated mast cells are present, along with 2 irregular mitotic figures (arrows), MCT grade III.

Nuclear-to-cytoplasmic ratio in grade II tumor cells was 0.29 ± .07 and 0.39 ± .08 in grade III, with statistical difference (P = .02).

Table 1. Summary of median and quartile interval of the number of mitotic figures, binucleation and multinucleation from grade II and grade III MCTs in cytologic preparations.

	MTCs grade II			**MTCs grade III**			
	Median	1^0 quartile	3^0 quartile	Median	1^0 quartile	3^0 quartile	*P*
Mitotic figures	0.0[a]	0.0	0.0	9.0[b]	4.0	19.0	< .001
Binucleation	1.0 [a]	0.0	3.0	21.5 [b]	5.0	35.0	< .001
Multinucleation	0.0 [a]	0.0	0.0	2.5 [b]	0.0	5.0	< .001

Results with different letters in the same line are statistically significant (P < .001)

Table 2. Summary of mean, standard deviation (sd) of the cellular parameters from grade II and grade III MCTs in cytologic preparations.

	MTCs grade II		**MTCs grade III**		
	Mean	sd	Mean	sd	*P**
Area (µm^2)	252.3	55.0	231.7	57.1	.35
Perimeter (µm)	61.2	7.1	59.1	8.6	.47
Circumference factor	1.15	0.06	1.22	0.30	.32

*Not statistically significant (P > .05)

Table 3. Summary of mean, standard deviation (sd) of the cytoplasmic parameters from grade II and grade III MCTs in cytologic preparations.

	MTCs grade II		**MTCs grade III**		
	Mean	sd	Mean	sd	*P**
Area (µm^2)	177.7	54.1	138.2	49.9	.063
Perimeter (µm)	93.7	8.7	92.1	10.0	.66

*Not statistically significant (P > .05)

Table 4. Summary of mean, standard deviation (sd) of the nuclear parameters from grade II and grade III MCTs in cytologic preparations.

	MTCs grade II		**MTCs grade III**		
	Mean	sd	Mean	sd	*P*
Area (µm^2)	72.3[a]	13.9	88.9[b]	19.0	.011
Perimeter (µm)	32.4[a]	3.0	35.7[b]	4.0	.018
Circumference factor	1.0[a]	0.33	1.1[a]	0.28	.78

Results with different letters in the same line are statistically significant (P < .05)

Discussion

The clinical demographic data obtained in this study are in agreement with the published literature with regard to breed (boxers 42.8%) and the average age of the affected dogs (9 years) (2, 18, 22). Mixed breed dogs were the second most frequently seen, which is consistent with their prevalence in our hospital population.

Due to the variable biologic behavior of canine MCTs and the potentially fatal outcome associated with this disease, an accurate diagnosis and prognostication is critical in order to determine the most appropriate therapeutic strategy for a given tumor. Histopathology can be routinely used to diagnose canine MCTs without much difficulty, but accurate prognostication of these tumors can be more challenging. Currently, histologic grading is the most commonly used prognostic and therapeutic determinant

for canine MCTs, as several studies have found a significant association between histologic grade and survival (10, 12). Cytologic evaluation of Giemsa-stained FNB samples can be used to establish the diagnosis of MCTs in dogs and cytologic criteria of malignancy on cytologic smears is relatively easy to recognize in cutaneous round cell neoplasms (14, 18, 20). Purple granules are characteristic of mast cells and neoplastic cells with poor cytoplasmic granulation, such as poorly differentiated MCTs (grade III), are more easily recognized cytologically than in histopathologic sections (14).

In previous histopathological studies of substantial numbers of canine MCTs, the proportions of moderately and poorly differentiated tumors identified has varied (2, 8, 9, 10, 12) In our study, 66.7% of our patients had grade II tumors and 33.3% grade III. The higher incidence of grade II tumors is consistent with the literature (2, 9, 10, 12). In our study we did not observe or include any grade I MCTs. These tumors are rarely presented to our clinical service, lesions are often advanced by the time owners seek veterinary care. The Patnaik classification system for histologically grading canine MCTs (12) defines grade I MCTs as being well-differentiated tumors located in the superficial dermis. This superficial localization makes it challenging to obtain sufficient cellular yield on cytologic smears to provide a definitive cytologic diagnosis of MCT.

Cytologic evaluation of Giemsa-stained FNB samples can be used to establish the diagnosis of moderately and poorly differentiated MCTs in dogs; cytologic criteria of malignancy on cytologic smears is relatively easy to recognize (14, 19, 20). Purple granules are characteristic of mast cells and neoplastic cells with poor cytoplasmic granulation, such as poorly differentiated MCTs of grade III, may even be more easily recognized cytologically than in histopathologic sections (14).

The importance of the mitotic index (MI) as a prognostic factor in MCTs has been emphasized recently (15). Romansik et al. (15) showed a significant association between mitotic index (MI) in histologic samples and overall survival, which could be important for clinical decision making, especially in the case of Patnaik grade II tumors. The median survival time for dogs with a tumor MI ≤ 5 was significantly longer than for those with a MI > 5, regardless the grade. In our study, we found mitotic figures in three cytologic smears of grade II MTCs (3/20). Two of them had one mitotic figure per 20 HPFs, and one had two mitotic figures per 20 HPFs. According to Patnaik´s original article (12), grade II MCTs have zero to two mitotic figures per HPF and grade III MCTs have three to six mitotic figures per HPF. Our results are in accordance to Patnaik findings, since most of grade II tumors did not have mitotic figures, and the ones that had it (3/20), ranged from one to two mitotic figures per 20 HPFs.

All grade III MTCs had mitotic figures in cytologic smears, ranging from two to 45 per 20 HPFs, with a median of four mitotic figures per 20 HPFs. The mean mitotic index in grade III tumors are in accordance with Patnaik´s results, but we found seven (7/10 - 70%) tumors with 5 or more mitotic figures, which suggest that they are in a more aggressive clinical category, as demonstrated by Romanski et al. (15).

There was statistical difference in number of mitotic figures between grade II and III tumors. However, we cannot assume, for example, that two mitotic figures per HPF indicated grade II and five mitotic figures per HPF indicates a grade III tumor, since one of our grade III MCTs (1/10 – 10%) had two mitotic figures. One might anticipated longer survival for this individual, as described by Romansiki et al.[16] Critically, the number of mitotic figures seen in our study is variable, regardless the grade.

The median number of binucleated cells was 21.5 for grade III tumors (minimum three binucleated cells and maximum 47) and 1.0 for grade II MCTs, but one tumor had 14 binucleated cells (1/20 - 5%). The median of multinucleated cells was zero for grade II tumors, but it ranged from zero to five. For grade III tumors, the median number of multinucleated cells was five, with a range from zero to 13. Binucleated cells were more frequent than multinucleated cells in both grades II and III MCTs, and there was no correlation between presence or number of binucleated cells and multinucleated cells among each case.

While there was a statistical difference between grade II and III MCTs regarding the number of mitotic figures, binucleated and multinucleated cells, these parameters cannot be used exclusively to predict a “cytologic grade.” The minimum and maximum values for each of these parameters overlap, and they do not correlate with each other. This renders these factors invalid as predictive or prognostic features on cytologic evaluation.

Morphometry has been used previously to establish the diagnostic and prognostic importance of cytologic cell features in veterinary medicine, including studies of MCTs (4, 5, 16, 17) . Strefezzi et al. (17) described cytopathologic morphometric data indicating a correlation between mean nuclear area and patient survival. In this study, Kaplan-Meier analysis demonstrated that survival times were significantly shorter as nuclear area increased (17).

In our study, the cellular morphometric parameters (area, perimeter and circumference factor) (Table 2) did not differ statistically between grade II and III MCTs, although the mean cellular area and perimeter was bigger in grade II tumors than grade III. One explanation for this could be based on the presence of cytoplasmic granules, since grade II tumors have more intracytoplasmic granules than undifferentiated mast cells of grade III tumors (12). Abundant granulation could expand the cytoplasm, inducing larger cytoplasmic parameters in better differentiated cells.

The cytoplasmic area of grade II and III mast cells were widely variable (Table 3), and grade II tumors had statistical higher mean area than grade III MCTs. Despite the fact that mast cell granules may obscure cytoplasmic membrane and nucleus, we did

not encounter difficulty in defining these cellular structures, making it possible to determine cytoplasmic and nuclear morphometric parameters. To the authors knowledge, this is the first time QWin image capture software was used for assessment of canine mast cell tumors on cytologic preparations stained by Giemsa.

Nuclear area and perimeter were statistically different between grades II and III MCTs (Table 4). These results corroborate previous data that using nuclear parameters in an independent manner can predict the behavior of MCTs (17).

Grade III neoplastic mast cell had larger nuclear area and smaller cytoplasmic area, which made the nuclear-to-cytomasmic ratio higher in poorly differentiated tumors (0.39) than in moderately differentiated tumors (0.29). The nuclear-to-cytoplasmic ratio is a good criterion of malignancy in different types of neoplastic cells (19), including MCTs (2).

The nuclear-to-cytoplasmic ratio was statistically different between grades (Table 4), but taking into consideration the standard deviation (sd), values may overlap in MCTs grade II and III. Nuclear-to cytoplasmic values ranging from 0.22 to 0.30 were found only in grade II tumors and from 0.37 to 0.47 only in grade III tumors.

In conclusion, our data indicate that mitotic figures, binucleation, multinucleation, and nuclear-to-cytoplasmic ratio in cytologic preparations can correlate with MCTs grade II and III. Furthermore, computer-assisted nuclear morphometry in cytologic preparations from MCTs can also correlate with histopathologic grading. This potentially increases the utility of easily obtained cytologic specimens for predicting the behavior of mast cell tumors in dogs.

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