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Mutations of the *c-KIT* gene in canine mast cell tumors and respective nodal metastases classified according to mast cell infiltration[1]

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ABSTRACT.- Rassele A.C., Souza L.M., Gorza L.L., Giuliano A., Flecher M.C. & Horta R.S. 2023. **Mutations of the *c-KIT* gene in canine mast cell tumors and respective nodal metastases nodes classified according to mast cell infiltration.** *Pesquisa Veterinária Brasileira 43:e07140, 2023.* Departamento de Clínica e Cirurgia Veterinária, Universidade Federal de Minas Gerais, Campus Pampulha, Av. Pres. Antônio Carlos 6627, São Luiz, Belo Horizonte, MG 31270-901, Brazil. E-mail: rodrigohorta@ufmg.br

The molecular background of canine mast cell tumors (MCT) has been extensively investigated; however, the dynamic molecular changes that occur during carcinogenesis and metastasis are not fully understood. This study aimed to evaluate the incidence of mutations in the *c-KIT* proto-oncogene in canine MCTs and relative draining regional lymph nodes. Suspected or confirmed lymph node metastasis was classified accordingly to the HN Weishaar classification. The study included 34 dogs diagnosed with MCT; 19 patients were enrolled prospectively. These dogs had the primary MCT and regional lymph node resected and analyzed simultaneously. The second group was evaluated retrospectively and included fifteen patients resectioning the primary MCT without evaluation of regional lymph node. Analyzes of *c-KIT* mutation were performed for all primary MCTs and, in the first group, compared between primary MCT and HN-classified metastasis. Internal tandem duplications (ITD) in exon 11 of the *c-KIT* gene were detected in 20% of patients. Ten of the nineteen patients (52%) in the first group presented mast cell infiltration in the regional lymph node, and ITD in exon 11 of the *c-KIT* gene was detected in five and two dogs from Groups 1 and 2, respectively. ITD *c-KIT* mutations are common in canine MCT and may be found in the draining lymph node metastases/mast cell infiltrates in the absence of mutation of the primary tumor. Evaluation of *c-KIT* mutation in the primary tumor and metastases may be informative for defining both prognosis and therapeutic options in MCT cases.

INDEX TERMS: Dog, cancer, oncogene, internal tandem duplication.

RESUMO:- [**Mutações no gene *c-KIT* em mastocitomas caninos e respectivas metástases nodais classificados segundo a infiltração de mastócitos.**] O perfil molecular do mastocitoma (MCT) tem sido bastante investigado, no entanto as dinâmicas moleculares que ocorrem durante a carcinogênese e metástase desta neoplasia não estão bem esclarecidas. O objetivo desse estudo foi avaliar a incidência de mutações no proto-oncogene *c-KIT* em MCTs caninos e respectivos linfonodos regionais. Os casos suspeitos ou confirmados de metástase para os linfonodos, foram classificados de acordo com a classificação HN de Weishaar. O estudo incluiu 34 cães diagnosticados com MCT e, desses, 19 pacientes foram avaliados de maneira prospectiva, em que o tumor primário e o linfonodo regional foram ressecados e analisados simultaneamente. O segundo grupo foi avaliado retrospectivamente e incluiu quinze pacientes que tiveram ressecção do MCT primário sem avaliação de linfonodo regional. A análise da mutação *c-KIT* foi realizada para todos

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os MCTs primários e, no primeiro grupo, comparados entre MCT primário e metástase classificada pelo sistema HN. Duplicações internas em tandem (DIT), no exon 11 do gene *c-KIT*, foram detectadas em um total de 20% dos pacientes. Dez dos dezenove pacientes (52%) do primeiro grupo apresentavam infiltração de mastócitos no linfonodo regional, e DIT no exon 11 do gene c-KIT foram identificadas em cinco e dois cães dos Grupos 1 e 2, respectivamente. Mutações do tipo DIT no gene *c-KIT* são comuns no MCT canino e podem estar presentes nas metástases/infiltrados de mastócitos na ausência de mutação do tumor primário. A avaliação da mutação no gene *c-KIT* no tumor primário e metástases pode ser informativa para definir tanto o prognóstico quanto as opções terapêuticas em casos de MCT.

TERMOS DE INDEXAÇÃO: Caninos, câncer, oncogene, duplicação inerna em tandem.

INTRODUCTION

Mast cell tumor (MCT) is dogs' most common cutaneous neoplasm (Strefezzi et al. 2009, London & Thamm 2013). Several studies have evaluated MCTs' morphological, genetic and molecular characteristics (Giuliano et al. 2017, Horta et al. 2018, Nardi et al. 2022). However, despite several prognostic factors, there is no reliable parameter to predict treatment outcomes. Abnormalities in the *c-KIT* oncogene, especially internal tandem duplications (ITD) in the exon 11 are implicated in the development and progression of canine MCT (Zemke et al. 2002, Letard et al. 2008, Welle et al. 2008, Avery 2012, Amagai et al. 2013). Patients with macroscopic disease and ITD in the *c-KIT* oncogene may present a better response to tyrosine kinase inhibitors. However, the efficacy of TKIs in the adjuvant treatment of patients with high-risk MCTs is not proven (Horta et al. 2018).

Metastases are the main cause of death in patients with cutaneous MCT; however, the molecular changes in cancer cells undergoing metastasis are still poorly understood (Amagai et al. 2013, Marconato et al. 2014). The most common metastatic sites in dogs are the regional lymph nodes (Dobson & Scase 2007). It can be very difficult to differentiate true lymph node MCT metastasis from "trafficking" non-metastatic mast cells, which are found regularly in the draining lymph node of MCT (Bookbinder et al. 1992). To solve this problem, Weishaar et al. (2014) published a new histopathological classification of MCT regional lymph node involvement. In this classification system, MCT regional lymph node involvement is classified from HN0 to HN3 based on the number and distribution of mast cells and the architectural disruption of the lymph node. HN0 represents a lack of infiltration in this classification, and HN3 is an evident MCT nodal metastasis.

Marconato et al. (2014) observed 100% concordance in the *c-KIT* mutational status, in exons 8, 9 and 11, of primary and metastatic MCTs in twenty-one prospectively enrolled canine patients. However, in this study, the classification of MCT lymph node metastasis based on HN system was not performed. Only one MCT was found to carry exon 8 mutation, and two MCTs presented exon-11 *c-KIT* mutations. Amagai et al. (2013) reported discordance between ITD mutation and relative metastasis in two patients. The ITD mutations in the primary tumors were not found in the relative metastasis, suggesting a non-clonal expansion.

This study aimed to evaluate the incidence of ITD in the *c-KIT* oncogene in canine mast cell tumors and the respective HN system-classified regional lymph node metastases and to compare the incidence of mutations and survival in patients submitted or not to regional lymph node excisional biopsy.

MATERIALS AND METHODS

Ethics Committee. This research was performed at the Veterinary Hospital "Prof. Ricardo Alexandre Hippler", at "Universidade Vila-Velha" (UVV), Espírito Santo, Brazil. This study was approved by the Ethics Committee on Animal Use from UVV, number 376/2016-attachment 1.

Inclusion and exclusion criteria. This study included prospective and retrospectively enrolled dogs diagnosed with MCT stages I and II. For the prospective segment, the dog's owners signed a consent form for participation in the study. In total, 34 dogs with MCT were evaluated from June 2016 to December 2017. However, 11 dogs were excluded from the study due to the presence of inoperable MCT (unresectable or presenting with multiple nodules or distant metastases), and four dogs, due to the pet owner´s lack of compliance, resulting in 19 dogs in the prospective group. For the retrospective study, histopathological records of dogs diagnosed with MCT were retrieved from the Laboratory of Animal Pathology, from the same university, from 2003 to 2015. Four were excluded from the 30 cases selected due to multiple MCTs and/or distant metastases. The other seven and four cases were excluded due to the impossibility of recovering the medical and histopathological records, resulting in 15 cases.

Clinical and surgical evaluation of prospectively analyzed dogs. Patients enrolled prospectively were screened previous to anesthesia with electrocardiogram, complete blood count, coagulation profile (activated partial thromboplastin time – APTT and prothrombin time – PT) and serum biochemistry (urea, creatinine, alkaline phosphatase – ALP and alanine aminotransferase – ALT). All dogs were staged with abdominal ultrasound (Warland et al. 2014), but the liver and spleen cytology was not performed. Thoracic radiographs were performed in cases located in the skin of the trunk above the diaphragm. Lymph node cytology was not always performed in the cohort of retrospectively enrolled patients. However, the regional lymph node was always removed during surgery of the primary tumor in the prospectively enrolled patients. Stage II patients included those with HN1, HN2 or HN3 Weishaar lymph node classification. All the dogs were treated with neoadjuvant Prednisolone (40mg/m2, every 24h) for 7-10 days to reduce the tumor size before surgical resection.

According to the pre-surgical evaluations, the general anesthetic protocol was defined by the anesthetist. Surgery was performed to achieve clean histological margins, removing 2-3cm of skin for the lateral margin and at least one fascial plane for the deep margins (Fulcher et al. 2006). The removed tissues were fixed in 10% formalin for histopathological evaluation and detection of mutations in the *c-KIT* exon 11 by the polymerase chain reaction (PCR).

Histopathological evaluation. Histopathological specimens retrieved at the Animal Pathology Laboratory and from the prospectively enrolled patients' samples were evaluated by the same pathologist using hematoxylin-eosin (HE) and Toluidine blue stains. The histopathological grading of MCT was performed according to the systems proposed by Patnaik et al. (1984), and Kiupel et al. (2011). The tumors were classified as grades I, II and III, according to the skin localization (confined to the dermis, interfollicular spaces, lower dermal, subcutaneous, or deep tissues), mast cells morphology (pleomorphism, distinct or indistinct cytoplasm, amount of intracytoplasmic granules), and mitotic figures in 10

high-power fields), criterions described by Patnaik et al. (1984). Using the system proposed by Kiupel et al. (2011), the MCTs were graded in high grade if any one of the following characters were present: seven or more mitotic figures in 10 high-power fields, three or more multinucleated cells in 10hpf, three or more bizarre nuclei in 10hpf, and karyomegaly and anisokaryosis with nuclear diameters of at least 10% of neoplastic mast cells varying by at least two-fold).

The edges of the primary tumor fragment were marked with India ink and used for microscopic surgical margin evaluation, according to Meuten (2017): clean (2-5mm), close (1-2mm), without margins (<1mm) or compromised (infiltrated). Lymph nodes (LN) were classified based on the microscopical evaluation of node metastases in dogs with MCT, according to Weishaar et al. (2014). The classification included four classes: 1) HN0/non-metastatic (0-3 scattered, individualized mast cells in sinuses and/or in parenchyma per X 400 field), or when does not meet criteria for any other classification below); 2) HN1/pre-metastatic or suspicious metastasis (More than three individualized mast cells in sinuses and/or parenchyma in a minimum of four X 400 fields); 3) HN2/initial metastasis (Clusters of mast cells (≥3 associated cells) in sinuses and/or parenchymal, or sinusoidal sheets of mast cells), and 4) HN3/overt or evident metastasis (disruption of normal nodal architecture by foci, nodules, sheets or overt masses composed of mast cells).

Detection of mutations in *c-KIT* gene exon 11. Mutation in *c-KIT* gene exon 11 was evaluated in all the primary tumors and regional lymph nodes in which metastases or infiltrated mast cells were detected. Even tumors classified as HN0 with rare mast cells were evaluated.

The research for mutation in *c-KIT* gene exon 11 (nucleotides 1661 to 1787 of chromosome 13) was performed by PROGEN Biotechnology in the VETPAT laboratory. Four and eight samples, with 5µm each, were sectioned from the respective paraffin-embedded blocks and used for DNA extraction using the proteinase K protocol with commercial kit ReliaPrep™ FFPE gDNA Miniprep System (Promega). The deparaffinization was previously performed by adding 300µL of mineral oil and incubating at 80°C for a minute.

The oligonucleotides primers used in flanking amplification of the fragment in the *c-KIT* gene (exon 11-nucleotides 1661 to 1787 of chromosome 13) were designed using BLAST software (Basic Local Alignment Search Tool®, NCBI) and manufactured by Invitrogen, being *c-KIT* F: 5'-ATCTGTCTCTCTTTTCTCCCCC-3', sense, involving nucleotides 1633 to 1655 (intron 10 of chromosome 13) and *c-KIT* R: 5'-TGGGGTTCCCTAAAGTCATTGT-3, antisense, involving nucleotides 1857 to 1835 (end of exon 11and beginning of intron 11). Reactions were carried out in a GenPro thermocycler (BIOER Technology), with maintenance at 95oC for 5 minutes, then 30 cycles of 94oC for 45 seconds for denaturation, 63oC for 45 seconds for annealing, 72oC for 1 minute for extension, maintained at 72oC for 10 minutes for molecular stabilization. The amplified material was separated by electrophoresis at 100V with free amperage. The positive and negative controls were canine healthy skin samples and water. The product obtained by these pairs has 225pb in the absence of mutations (native *c-KIT*).

Statistical analysis. Statistical analysis was performed with Graph Pad Prism version 6.02, *p*-value less than 0.05 were considered significant. Chi-Square's test was used to compare both groups' clinical, histopathological, and molecular characteristics. Cohen's Kappa index was applied in the prospective group of patients to assess the concordance of *c-KIT* ITD mutation in the primary tumor and lymph node. Kappa value was indicative of no concordance ($\kappa<0$), slight ($0.01<\kappa<0.20$), fair ($0.21<\kappa<0.40$), moderate ($0.41<\kappa<0.60$), substantial ($0.61<\kappa<0.80$) or almost perfect ($0.81<\kappa<1.00$) concordance. The Disease-free-interval (DFI) and overall survival (OS) were estimated by Kaplan-Meier curves, and the log-rank test of Cox-Mantel was used to compare the curves. The normality of weight, age and the mitotic count was assessed using the Kolmogorov-Smirnov test. The comparison of means was made by the t-Student test (normal distribution-weight and age), and the Mann-Whitney test was used for median comparisons (non-normal distribution-mitotic count).

RESULTS

Thirty-four dogs with MCT were included, 19 from the prospective cohort (Group 1), with surgical removal of the primary tumor and regional lymph node, and 15 retrospectively enrolled (Group 2), with only primary tumor resection, without regional lymph node evaluation.

The clinical and histopathological characteristics in each of these two groups are shown in Table 1. Considering the two groups (n=34), mixed-breed dogs (17.6%) were predominant, followed by Pinscher (11.7%), Dachshund (11.7%), and Pit-bull Terrier (8.8%). Other less common breeds (each representing 5.8%), were Boxer, American Staffordshire, Poodle and Brazilian Terrier. Females were more common (67.6%). Age ranged from 1.5 to 15 years (8.6±3.2).

In the present study, primary MCT was located predominantly in the limbs (26.4%), trunk (20.6%), region of the mammary gland (17.6%), and scrotum (14.7%). The surgical margins were evaluated in thirty cases. Histopathological free, close, and compromised margins were obtained in 38.2%, 8.8%, and 47%, respectively. According to the histopathological grading system proposed by Patnaik et al. (1984), it was observed that among 34 evaluated dogs, the majority (73.5%) were classified as grade II, grades I (17.6%), and III (8.8%). According to Kiupel et al. (2011), 29 (85.3%) MCT evaluated in the present study were classified as low-grade and 5 (14.7%) as high-grade. The mitotic count was ranged from 0 to 25 (1.8±4.4, average = 10). However, thirty MCT (88.2%) remained below five mitotic figures in 10 fields of high magnification (400x, 2.37mm2).

The groups were considered homogeneous in gender, age, weight, tumor's anatomic localization, surgical margins, histopathological grade, and presence of the mutation in exon 11 of the *c-KIT* gene. Regarding the clinical staging, eight dogs (42.1%) in Group 1 were classified as stage I, and 11 (57.9%) in stage II, according to World Health Organization.

ITD in exon 11 of the *c-KIT* gene was detected in 7 (20%) of the dogs, five (26%) from Group 1, and two (13%) from Group 2, as shown in Table 1. This difference was not statistically significant in Chi-Square's test (p=0.4). When considering only the *c-KIT* gene status of the primary tumor, mutations were detected in four (11%).

In Group 1, lymph nodes were classified according to Weishaar et al. (2014) in HN3 (n=3), HN2 (n = 2), HN1 (n=3), and HN0 (n=11). A few scattered mast cells were also noted in two of the 11 HN0 lymph nodes in the subcapsular sinus or lymphatic vessels (Fig.1 and 2). The tumors classified as Kiupel low-grade had their lymph nodes classified as HN0 in 10 cases (66.6%), HN1 in 3 cases (20%), HN2 and HN3 in one case each (0.6%), while high-grade tumors had their lymph nodes classified as HN0 in one case (25%), HN2 in only one case (25%), and HN3 in two lymph nodes (50%).

Other cases (n=9) were classified as HN0 and have not shown mast cell infiltration. On physical examination, the regional lymph node was found enlarged in only four dogs. On histopathology, two of these enlarged lymph nodes were not metastatic; one was considered HN1 and another HN3 (Fig.3-6).

The search for *c-KIT*/ITD was performed in all primary tumors and 10 lymph nodes, as presented with identifiable mast cells (2 HN0, 3 HN1, 2 HN2 and 3 HN3). ITD was detected in the primary tumor and its respective metastases in two cases and only in the lymph node in three dogs (HN1, HN2 and HN3). Furthermore, in the present study, the agreement among mutational status in primary tumor and lymph node by Cohen's Kappa index (k=0,496; SE=0.290) was weak (fair). In two dogs, the detected mutation on the primary tumor was not searched in the lymph node because they were classified as HN0 and did not show infiltrated mast cells.

In the present study, only 25 dogs (73.5%) were followed within 724.8 days. The median of DFI and OS was not reached in all groups, and there was no significant difference by the log-rank test of Cox-Mantel. Only one patient had tumoral recurrence and death in each group, within seven and 30 days, in Group 1 and 130 and 150 days, respectively, in Group 2. Regarding adjuvant treatment, two dogs were treated only with prednisone (Group 1), 12 dogs with prednisone associated with vinblastine (2mg/m2, IV, every seven days for four weeks, and every 14 days, in the next four sessions

Table 1. Clinical and histopathological characteristics of Groups 1 and 2

Characteristics		Group 1 (n = 19)	Group 2 (n = 15)	Total (n = 34)	Statistic (*p* value)
Sex	Females	14 (73.6%)a	9 (60%)a	23 (67.6%)	Chi-Square ($p = 0.4$)
	Males	5 (26.4%)a	6 (40%)a	11 (32.4%)	
Age (years; average ± standard deviation)		8.9 ± 3.3a	8.2 ± 3.2a	8.6 ± 3.2	t student ($p = 0.5$)
Weight (Kg; average ± standard deviation)		15.6 ± 9.5a	21.4 ± 13.4a	18.2 ± 11.6	t student ($p = 0.2$)
Clinical staging	Stage I	8 (42.1%)a	15 (100%)b*	23(67.6%)	Chi-Square ($p = 0.03$)
	Stage II	11 (57.9%)a	0 (0%)b	11 (32.4%)	
Anatomic localization	Ear	0 (0%)	1 (6.6%)	1 (2.9%)	Chi-Square ($p = 0.1$)
	Limb	5 (26.3%)	4 (26.6%)	9 (26.4%)	
	Axilar region	1 (5.2%)	0 (0%)	1 (2.9%)	
	Abdominal	1 (5.2%)	1 (6.6%)	2 (5.9%)	
	Dígits	1 (5.2%)	0 (0%)	1 (2.9%)	
	Lombar region	1 (5.2%)	0 (0%)	1 (2.9%)	
	Back	0 (0%)	1 (6.6%)	1 (2.9%)	
	Trunk	1 (5.2%)	6 (40.0%)	7 (20.6%)	
	Scrotum	3 (15.7%)	2 (13.3%)	5 (14.7%)	
	Mammary gland	6 (31.6%)	0 (0%)	6 (17.6%)	
Surgical margins (Meuten 2017)	Clean (>5 mm)	8 (42.1%)	5(33.3%)	13 (38.2%)	Chi-Square ($p = 0.6$)
	Close (1-2 mm)	1 (5.2%)	2 (13.3%)	3 (8.8%)	
	Infiltrated (compromised)	9 (47.3%)	7 (46.6%)	16 (47.0%)	
	Margin not evaluated	1 (3.2%)	1 (6.6%)	2 (5.9%)	-
Grading (Patnaik et al. 1984)	I	5 (26.3%)	1 (6.6%)	6 (17.6%)	Chi-Square ($p = 0.06$)
	II	11 (57.9%)	14 (93.3%)	25 (73.5%)	
	III	3 (15.7%)	0 (0%)	3 (8.8%)	
Grading (Kiupel et al. 2011)	Low grade	15 (78.9%)	14 (93.3%)	29 (85.3%)	Chi-Square ($p = 0.2$)
	High grade	4 (21%)	1 (6.6%)	5 (14.7%)	
Mitotic index (10 fields of high magnification)	Average ± standard deviation	2.5 ± 6.0	1.1± 1.1	1.8 ± 4.4	-
	Median	1.0	1.0	1.0	Mann-Whitney ($p = 0.9$)
<5		15 (78.9%)	15 (100%)	30 (88.2%)	Chi-Square ($p = 0.1$)
>5		2 (10.5%)	0 (0%)	2 (5.9%)	
Mitotic index not evaluated		2 (10.5%)	0 (0%)	2 (5.9%)	-
C-KIT gene status (primary tumour)	Native	17 (89.5%)	13 (86.7%)	30 (88.2%)	Chi-Square ($p = 0.8$)
	Mutant	2 (10.5%)	2 (13.3%)	4 (11.8%)	
C-KIT gene status (primary tumor + lymp node)	Native	14 (73.7%)	13 (86.7%)	30 (79.4%)	Chi-Square ($p = 0.4$)
	Mutant	5 (26.3%)	2 (13.3%)	7 (20.6%)	

* In the Group 2, lymph node metastasis occurrence was not evaluated and staging was incomplete; a,b = Different lowercase letters on the same line indicate statistically significance difference ($p<0.05$).

(six in the Group 1 and four in the Group 2), and one patient in each group received lomustine (70-90mg/m2, VO, every 21 days). However, in this study, 58.8% of the dogs (20/34, 10 animals from each group) did not receive any treatment other than surgery.

DISCUSSION

Dysregulation of KITr and mutations of its proto-oncogene are among the most described molecular abnormalities in canine MCT (Strefezzi et al. 2009, London & Thamm 2013). In the present study, ITD in exon 11 of the *c-KIT* oncogene occurred

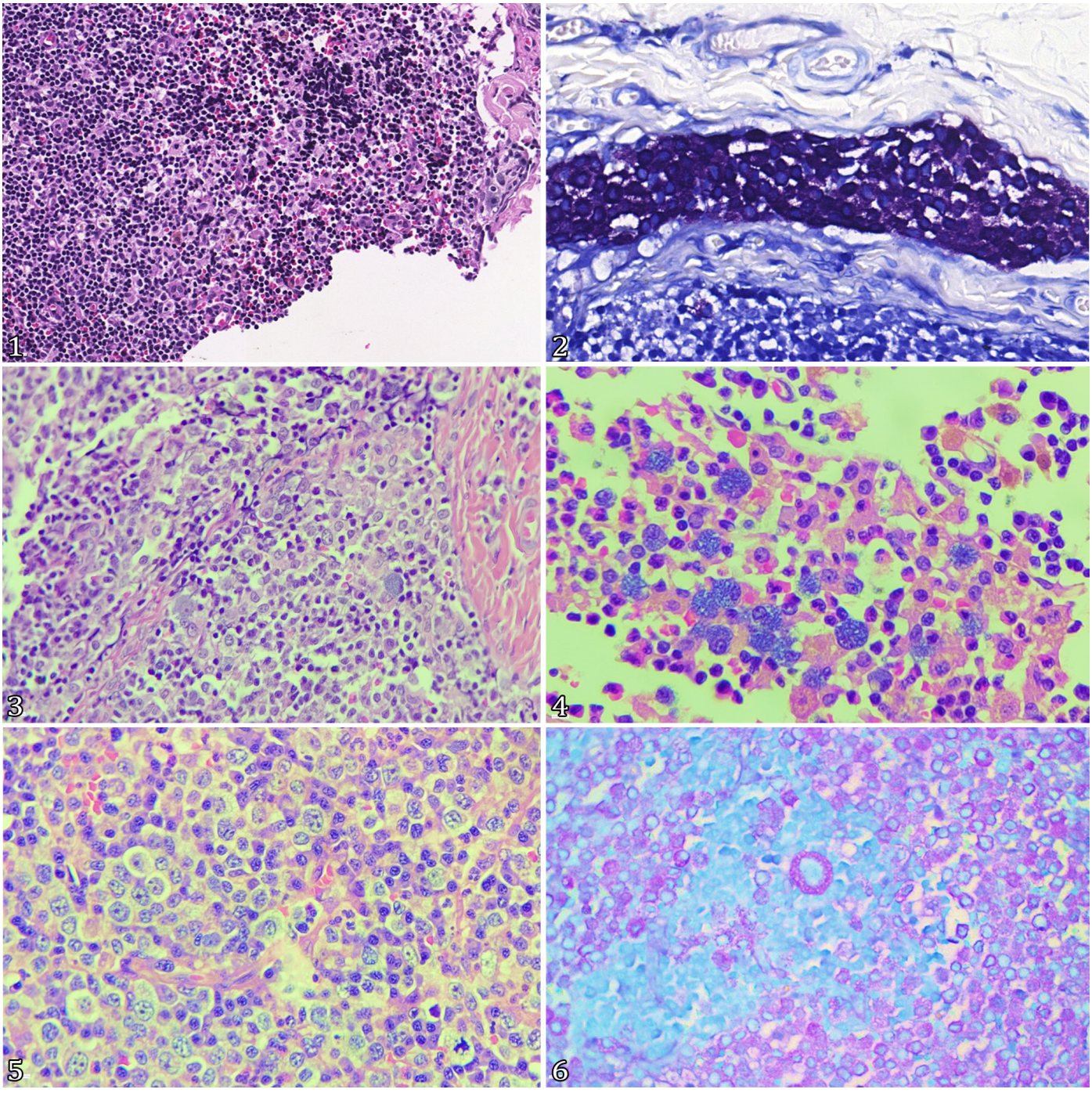

Fig.1-6. Histopathologic characteristics of the dog lymph nodes, with metastases from canine cutaneous mast cell tumor, according to the classification system proposed by Weishaar et al. (2014). (**1**) HN0 popliteal lymph node with preserved parenchyma, lymphocytes, and rare infiltration of mast cells. HE, obj.10x. (**2**) HN0 lymph node in high magnification, showing mast cells within the lymphatic vessel of the subcapsular sinus. Toluidine blue, obj.40x. (**3**) HN1 lymph node with single mast cell infiltration in the subcapsular sinus. HE, obj.20x. (**4**) HN2 axillary lymph node with a moderate proliferation of mast cells and partial loss of the nodal architecture. HE, obj.40x. (**5**) HN3 lymph node with disruption of the nodal architecture by intense sheets of mast cells, showing prominent anisokaryosis. HE, obj.20x. (**6**) HN3 lymph node with intense mast cell proliferation and histamine granules. Toluidine blue, obj.20x.

in 7/34 (20%) patients. These numbers agree with Letard et al. (2008) and Weishaar et al. (2018), which reported an incidence of 20-29%. Other authors have reported a lower incidence in their studies, 9% in Zemke et al. (2002) and 15% in Webster et al. (2006). Recently, Horta et al. (2018) demonstrated ITD in exon 11 of the *c-KIT* by PCR in 10.7% (16/149) of dogs; this was associated with an increased risk of metastases and mortality related to MCT. It is known that a higher occurrence of mutations is expected in high-grade advanced-stage MCT or those with multiple negative prognostic factors (Welle et al. 2008, Horta et al. 2018). Differences in *c-KIT* mutation incidence in different studies are likely related to the inclusion of different dog populations with different inclusion criteria.

In this study, a higher mutation incidence was found in the lymph node compared to the primary tumor. Although not statistically significant, most likely due to the small number of cases, our finding could highlight the importance of evaluating *c-KIT* in both the primary tumor and the metastatic lymph node. Mutation of *c-KIT* could be a marker of MCT progression, and evaluation of *c-KIT* in both primary tumors and metastasis could influence the prognosis and therapeutic decision. However, a study with a higher number of primary MCTs and HN1-HN3 classified metastasis is necessary to prove this assumption (Strefezzi et al. 2009, Amagai et al. 2013, London & Thamm 2013, Horta et al. 2018).

ITD, in exon 11, is the most frequent mutation detected in MCTs, and although other types of *c-KIT* mutations have been reported, their clinical relevance is unknown. In a study performed in 149 dogs, two patients (1.3%) had internal tandem deletions in exon 11 of the *c-KIT* gene, lymph node metastases were confirmed in both cases, and the dogs died within 30 days and 134 days of treatment (Horta et al. 2018). In the present study, these mutations did not occur. Out of the 10 lymph nodes evaluated (in Group 1), one presented 0-3 mast cells in the subcapsular sinus, and another had mast cells in the lymphatic vessels of the capsule. These two lymph nodes were sent for genetic evaluation without detecting mutations in exon 11, and both patients were still alive at the end of the study. Although they were classified as HN0 and mutation was not detected, it is not possible to determine the clinical relevance of these cells in the regional lymph nodes (Weishaar et al. 2014). Mutation in a few cells in the lymph node could also pass undetected by current PCR evaluation methods, further complicating the significance of a few mast cells in the draining lymph node of MCT.

Mast cells are natural residents of the connective tissue and may be found in cytological and/or histopathological examination of lymph nodes in up to 24% of dogs without MCT (Bookbinder et al. 1992). However, it is speculated that neoplastic mast cells in a primary site might release chemotactic agents and growth factors that stimulate the proliferation and migration of normal mast cells in the regional lymph node, causing the lymph node to be classified as HN0 and HN1 (Bookbinder et al. 1992, Weishaar et al. 2014).

In the remaining eight lymph nodes with mast cell infiltration, three showed ITD in *c-KIT* exon 11, and in one of these cases, metastasis was only suspicious (HN1). Marconato et al. (2014) observed concordance in the *c-KIT* mutational status in 100% of the cases when comparing the primary MCT and its lymph nodes with metastases in 21 cases; however, only two patients had mutations. Amagai et al. (2013) reported discordance of ITD between the primary tumor and the respective cutaneous metastases, suggesting a non-clonal expansion. This finding is similar to our study, proving that MCTs with different genotypes can be found in the same patients. Mutation can occur on the primary lesion and dispel during the tumor progression and metastasis. However, new mutations that give a survival advantage, like *c-KIT*, can be acquired later in the progression and metastasis of the tumor.

Various studies have proven an association between the histological grade and MCT's biological behavior. High-grade Kiupel and Grade III Patnaik tumors have a more aggressive behavior with an increased risk of regional lymph node metastases (Weishaare et al. 2014, Marconato et al. 2020). In Group 1, three of the four evaluated lymph nodes in patients with high-grade MCTs showed mast cell infiltration. These findings suggest a worse prognosis due to the higher risk of metastases, as observed by Kiupel et al. (2011). In the same group, 50% (5/10) of the grade II MCT showed mast cells infiltration on the lymph nodes, with two lymph nodes classified as HN1 (20%), two HN2 (20%) and one HN3 (10%). These results were compatible with Weisse et al. (2002) and Séguin et al. (2006), which reported a metastatic rate of 5-45% in grade II MCT, and 55-96% in grade III MCT. According to Welle et al. (2008), lymph node metastases are rare, lower than 10% in grade I MCT. Among MCT grade I, only 1/5 had mast cell infiltration in the lymph node, classified as HN1.

In the present study, 59% of the dogs received surgical treatment only. The use of adjuvant therapy in cases of low-grade MCT with HN2 lymph nodes is a matter of debate. Marconato et al. (2020) retrospectively included 73 dogs diagnosed with low-grade cutaneous mast cell tumors (Kiupel)/I and II (Patnaik), with initial metastases (HN2) in the lymph nodes. In this study, 42 cases (57%) received adjuvant therapy after the surgical resection of both primary tumor and regional lymph node, and thirty-one (42%) dogs only surgical treatment. This study showed no benefit of adjuvant chemotherapy compared to surgery alone (Marconato et al. 2020). However, larger prospective studies with long follow-ups are needed to establish the benefit of adjuvant chemotherapy in low-grade MCT with early lymph node metastatic disease (HN2).

According to the European consensus of canine and feline MCT, systemic therapy is considered appropriate for patients with a higher risk of metastases (Blackwood et al. 2012). In the present study, patients diagnosed with high-grade (Kiupel)/grade III (Patnaik) MCT confirmed metastasis in lymph nodes (HN1, HN2 and HN3), high mitotic count (>5 in 10 high-power fields (400x, 2.37mm2), and with MCT located in the pinna, muzzle, scrotum, mammary gland and prepuce, with incomplete surgical margins, were associated with a higher risk of metastases, as proposed previously by Blackwood et al. (2012), Miller et al. (2016) and Horta et al. (2018). Those patients were treated with adjuvant lomustine or vinblastine, and achieved the median survival was not reached. Various studies have reported successful treatment of canine MCT with tyrosine kinase inhibitors, especially with the presence of ITD of exon 11 of the *c-KIT* gene (London 2009, Avery 2012), although Weishaar et al. (2018) has demonstrated a similar response to therapy with vinblastine. This treatment was not performed in the present study, as TKI drugs were not licensed or available in Brazil during the

study. Even though there was no difference in mutations and survival between Groups 1 and 2, the excisional biopsy of the regional or sentinel lymph node is highly recommended in dogs with MCT. Such procedure, along with the lymph node classification and determination of *c-KIT* mutational status, may provide valuable information for prognosis and adjuvant treatment of those patients with individual gain on disease-free interval and survival (Nardi et al. 2022).

CONCLUSIONS

The study demonstrated that in the same patient, ITD in exon 11 of the *c-KIT* gene might be present in the lymph node metastasis without mutation in the primary tumor. Mutation of *c-KIT* can be detected more frequently in metastatic lymph nodes compared to the primary tumor. Investigations of *c-KIT* in primary tumors and regional lymph nodes could be useful in prognostication and therapeutic planning.

The mutation in the mast cell infiltrates in the lymph node may indicate the occurrence of metastases, even if the histopathological classification is not conclusive. However, the significance of small numbers of mast cells in the draining lymph nodes, whether carrying or not *c-KIT* mutated, is still unknown.

More prospective studies are needed to evaluate the significance of our findings.

Conflict of interest statement.- The authors declare that they have no competing interests.

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