Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2021.058

RESEARCH ARTICLE

Division of Canine Mast Cell Tumors According to Tryptase and Chymase Expression using Immunohistochemistry

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ARTICLE HISTORY (20-266)

Received: May 23, 2020
Revised: July 06, 221
Accepted: July 08, 2021
Published online: August 04, 2021

Key words:
CD2
CD25
Dog
Mast cell chymase
Mast cell tryptase
Mast cell tumor
Mast cell type

A B S T R A C T

The study aimed at morphological and immunohistochemical analysis of 53 mast cell tumors in dogs. Immunohistochemical studies included determination of cell markers for CD2, CD25, mast cell tryptase and mast cell chymase in canine MCTs and comparison of their expression levels with parameters of malignancy (grade, mitotic index, Ki-67 labelling index and CD117 staining pattern). Patnaik grading system identified 20 dogs with grade I MCTs, 17 with grade II and 16 with grade III. According to Kiupel grading, 34 dogs had low-grade MCT and 19 high-grade tumors. Mitotic index was between 0 and 2.4 (mean 0.4). The CD2 expression was high in 32 of 53 cases, CD25 in 20, tryptase in 44, and chymase in 15. The Ki-67 labelling index ranged between 0 and 55% (mean 11%). Majority of MCTs revealed the second staining pattern of CD117 (n=21). We found that expression of CD2 is linked with tumor malignancy, while CD25 determined tumor development. The tumor division into MCT_T and MCT_{TC} may be used as prognostic factor. MCT tryptase positive and chymase positive are less malignant than tryptase positive and chymase negative ones.

To Cite This Article: Kandefer-Gola M, Dzimira S, Janus I, Ciaputa R, Madej JA and Prządka P, 2021. Division of canine mast cell tumors according to tryptase and chymase expression using immunohistochemistry. Pak Vet J, 41(3): 446-450. http://dx.doi.org/10.29261/pakvetj/2020.058

INTRODUCTION

Mast cell tumors (MCTs) mainly occur in middle-aged. Predisposed breeds include i.e. Labrador Retrievers, Boxers, Boston Terriers, and Pugs; the tumors are gender independent (Kandefer-Gola *et al*., 2015; Pulz *et al*., 2019). Biological behavior of different histological grades of MCTs is extremely variable. Several prognostic factors may foretell the disease but histological grading, however imperfect, is still the most important diagnostic procedure. In dogs, there is no specific prognostic panel. Recent studies identified CD117 and Ki-67 as suitable in a more precise evaluation of MCT proliferation rate (Kiupel *et al*., 2004; Kandefer-Gola *et al*., 2015).

CD2 is usually expressed in T-cell lymphocytes and natural killer cells (Yang *et al*., 2001). CD25 (Interleukin-2 Receptor) is present on maturing T and B-cell lymphocytes, fibroblasts and endothelial cells (Malek, 2008; Létourneau *et al*., 2009). Its expression was detected in bone marrow, skin and gastrointestinal track (Hahn and Hornick, 2007; Hollmann *et al*., 2008). CD2 and/or CD25 allow for differentiation of neoplastic and non-neoplastic mast cells (Meyer *et al*., 2012). CD2 and/or CD25 allow for differentiation of neoplastic and non-neoplastic mast cells in SM humans (Cherian *et al*., 2016). Whether CD2, or CD25 can be employed as diagnostic criteria for canine mast cell tumours remains so far unknown (Willmann *et al*., 2019).

Mast cells (MC) release tryptase and chymase as mediators (Galli *et al*., 2011; Ribatti, 2018). Human mast cells can be divided into MC_T type that contains mainly tryptase, and MC_{TC} type that contains tryptase and chymase (Galli *et al*., 2011). In animals there is no tendency to divide mast cells into MC_T and MC_{TC} types. Mast cell tryptase acts as an important marker of mast cell activation. Tryptase and chymase are essential mediators of inflammation, angiogenic factors and play a crucial role in tumor growth, invasion and metastasis. Tryptase participates also in extracellular matrix damage (Stack and Johnson, 1994).

The aim of this study was to evaluate the utility of immunohistochemical assessments in the diagnosis of mast cell tumors. The expression level of CD2, CD25, mast cell tryptase and mast cell chymase in canine MCTs were compared to parameters of malignancy (grade, mitotic index (MI), Ki-67 labelling index and CD117 staining pattern). We also divided MCTs into MCT_T and MCT_{TC} types and compared the results with malignancy rates. We hypothesized that the expression of mast cell tryptase and chymase determines tumor malignancy and invasiveness, and CD2 and CD25 expression could be involved in MC differentiation and tumor development and may simplify identification of undifferentiated mast cell tumors. It may allow the development of a cost-effective stepwise approach for the complementary immunohistochemical analysis.

MATERIALS AND METHODS

Animals: Fifty-three samples of cutaneous mast cell tumors were retrieved from the Department of Pathology, Wroclaw University of Environmental and Life Sciences.

As per Polish law, standard diagnostic procedures and studies conducted on animal tissues do not require permission from the Ethical Board. To ensure animal welfare, all surgical procedures were performed under full anesthesia. Tumor resection was part of the therapy. None of the patients received any chemotherapeutic treatment prior to the surgery.

Histopathological examination: A section of each sample was collected, dehydrated and embedded in paraffin. Four micrometer thick paraffin sections were routinely stained with hematoxylin and eosin (HE), and additional toluidine staining was used if necessary, to confirm the diagnosis. MCT grading was based on Patnaik and Kiupel grading systems (Kiupel *et al*., 2011). MI was determined as an average number of cells in mitosis per 10 randomly selected high-power fields.

Immunohistochemical examination: Immunohisto-chemical (IHC) studies were performed in 4 μM-thick paraffin sections placed on silanized slides (Dako, Denmark). The sections were cleared in xylene and passed through a series of decreasing alcohol concentrations to water. Then they were incubated with 3% hydrogen peroxide to block endogenous peroxidase. Epitope retrieval was performed in a microwave (650 W for 10 min) with citrate buffer (pH 6.0). After that, the sections were overlaid with primary antibodies, specific for: CD2 (1C5, Abcam, United Kingdom), CD25/IL-2R (OX-39, Invitrogen, USA), Mast Cell Chymase (CC1, Invitrogen, USA), Mast Cell Tryptase (AA1, Dako, Denmark), CD117 (Polyclonal Rabbit Anti-Human CD117, Dako, Denmark) and Ki-67 (MIB-1, Dako, Denmark), and incubated overnight at 4°C. A negative control was set up by replacing the primary antibody with phosphate buffered solution. Following incubation, the slides were washed in EnVision™ FLEX Wash Buffer (Dako, Denmark), the reagents of EnVision™ FLEX/HR SM802 (Dako, Denmark) visualization system were applied and the slides were incubated at room temperature for 20 min. 3,3'-diaminobenzidine (DAB; for 7 min, room temperature) was used as the reaction substrate. All the sections were counterstained with Mayer hematoxylin. Each immunohistochemical test had a positive control – tonsil tissue for CD2, CD25 and chymase, and normal skin for Ki-67, CD117 and tryptase. Sections immunostained in the absence of a primary antibody were used as negative controls.

The slides (10 random areas) were examined under a binocular light microscope at 400X magnification. Expression was scored in a semiquantitative manner. For tryptase, chymase, CD2 and CD25 the number of positive cells was classified as follows: negative = no positive cells, +/low = less than 10% of positive cells; ++/medium = 10-50% of positive cells; +++/high = 50-100% of positive cells. CD2 and CD25 were considered highly positive; when above 50% of cells were positive. Mast cell tryptase and chymase were considered highly positive, when above 10% of cells were positive, as MCs are the source of these mediators. Staining intensity variations within a sample and stroma labelling were recorded but not included in the scoring system. Necrotic areas and slide margins were omitted.

Antibodies against chymase and tryptase were used to distinguish mast cell types into MCT_T and MCT_{TC} type. Expression level of Ki-67 was evaluated in 10 power-fields containing at least 1000 cells each. Labelling index was defined as the percentage of Ki-67 positive cells. CD117 was evaluated as proposed by Kiupel *et al.* (2004). Microphotographs of all examined specimens were taken with Olympus BX53 optical microscope (Olympus, Japan), equipped with a Color View IIIa digital camera (Olympus, Japan).

Statistical analysis: Normality of the data was evaluated using Shapiro-Wilk analysis. The data were subjected to Kruskal-Wallis rank analysis, Mann-Whitney U-test and Spearman's correlation test using Statistica PL for Windows (StatSoft, Kraków, Poland). Statistical significance was set at $P \leq 0.05$.

RESULTS

Animals: A total of 53 MCTs were investigated. Tumors affected dogs of various ages (from eight months to 18 years; median 8.5 years) with a morbidity peak between seven and 10 years of age (29 cases), and of both sexes (27 males and 26 females). The breed breakdown included mixed breed dogs (14), Labrador Retrievers (12), American Staffordshire Terriers (4), Boxers (3), followed by Golden Retrievers, Bull Terriers, Beagles, Cane Corso and Yorkshire Terriers (two dogs of each breed), and 10 other breeds represented by single animals. Tumors involved head and neck (8), trunk (21), inguinal region (7) or the limbs (17).

Histopathological grading and mitotic index (MI): According to the Patnaik grading system, 20 dogs (38%) had grade I MCT, 17 dogs (32%) suffered from grade II MCT, and 16 dogs (30%) from grade III MCT. Kiupel grading identified 34 (64%) low-grade MCTs and 19 (36%) had high-grade MCTs. The grading systems correlated strongly ($P<0.05$; $r = 0.77$).

Mitotic index ranged from 0 to 2.4 (mean 0.4), with an average of 0.06 in grade I, 0.17 in grade II, and 0.96 in

grade III, as well as 0.05 in low-grade and 0.94 in high-grade lesions (Table 1). We observed a strong positive correlation between the MI and Patnaik grading system ($P<0.05$; $r = 0.72$) and Kiupel grading system ($P<0.05$; $r = 0.85$). MI was the highest in III MCTs and high-grade MCTs ($P<0.001$).

Table 1: Parameters of malignancy in mast cell tumors. Average mitotic index (MI) and Ki-67 labelling index compared to MCT grading

	MI	Ki-67 (%)
MCT I	0.06	2.85
MCT II	0.17	11.24
MCT III	0.96	19.81
Low-grade MCT	0.5	6.09
High-grade MCT	0.94	18.84

Immunohistochemical examination: Tables 2 and 3 summarize the results of IHC investigation, and Fig. 1 presents immunoreactivity of the studied proteins.

CD2 and CD25: CD2 expressed abundantly in 32 of 53 cases (60%) and CD25 in 20 of 53 cases (38%). Negative expression of CD2 occurred in one case (2%), and of CD25 in two cases (4%). The percentage of CD25 positive cells correlated weakly with Kiupel grading system ($P<0.05$; $r = -0.31$) and CD117 staining pattern ($P<0.05$; $r = -0.28$). There were no significant correlations between the malignancy parameters and CD2 expression.

Mast cell tryptase and chymase: Expression of mast cell tryptase was high in 44 of 53 cases (83%) and of chymase in 15 of 53 cases (28%). Mast cell tryptase labelled all MCTs, while negative expression of chymase was observed in 23 cases (43%). There was a weak negative correlation between CD117 staining pattern and the percentage of mast cell tryptase positive cells ($P<0.05$; $r = -0.28$). We found no correlation between expression of mast cell tryptase and chymase and the other malignancy parameters. In 57% of dogs the main type of cells within the tumor were MCT_{TC} cells, and in 43% MCT_T cells.

Ki-67: Ki-67 labelling index ranged between 0 and 55% (mean 11%). In MCT grade I its mean value reached 2.85%, in grade II 11.24%, in grade III 19.81%, in low grade MCT 6.09% and in high grade MCT 18.84% (Table 1). Ki-67 positive cells correlated moderately with Patnaik grading system ($P<0.05$; $r = 0.47$) and Kiupel grading system ($P<0.05$; $r = 0.4$). There was also a moderate positive correlation between MI and Ki-67 expression ($P<0.05$; $r = 0.47$). The values of Ki-67 were significantly higher in III MCTs than in I MCTs ($p = 0.004$), and in high-grade than in low-grade MCTs ($p = 0.005$).

CD117: Majority of MCTs (40%) revealed the second staining pattern of CD117. The first staining pattern was observed in 34% of cases, and the third in 26% (Table 1). There was a strong positive correlation between CD117 staining pattern and Patnaik grading system ($P<0.05$; $r = 0.76$), Kiupel grading system ($P<0.05$; $r = 0.68$), and MI ($P<0.05$; $r = 0.64$).

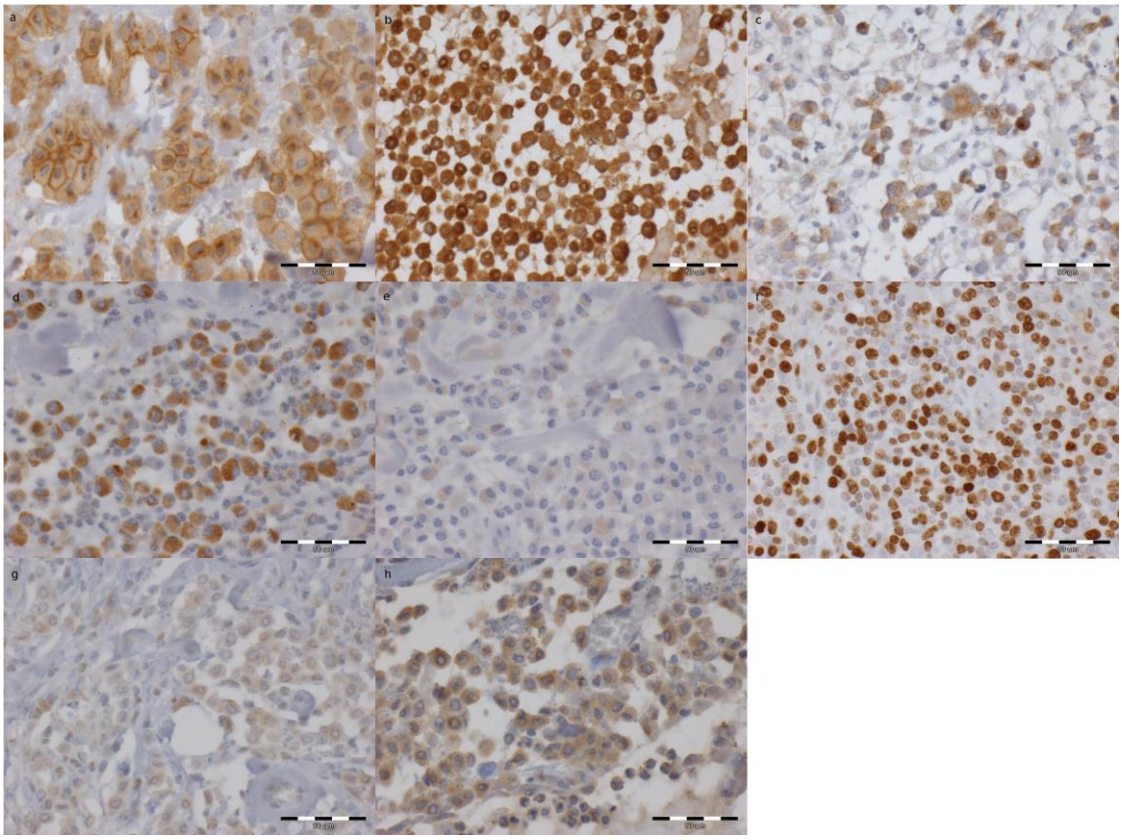

Fig. 1: Staining pattern of examined antibodies in mast cell tumor. (a–c) CD117 expression pattern was different, depending on malignancy: a: grade I/low-grade. Pronounced membranous reaction is visible. × 400; b: grade II/low-grade. Intense, focal staining of cytoplasm. × 400; c: grade III/high-grade. Delicate, diffuse staining of cytoplasm. × 400. (d–e) Tryptase and chymase expression in grade II/low-grade MCT – in the same tumor: d: highly positive, intense reaction of mast cell tryptase. × 400; e: highly positive, delicate reaction of mast cell chymase. x 400; f. Ki-67 expression in MCT grade III/high-grade; (g-h) CD2 and CD25 expression in grade II/low-grade MCT – in the same tumor: d: highly positive, delicate reaction of CD2. × 400; e: highly positive, delicate reaction of CD25. x 400.

Table 2: Number of cases with high/low expression levels of CD2, CD25, tryptase and chymase according to the grading systems

	CD25 n (%)		CD2 n (%)		Tryptase n (%)		Chymase n (%)		MCT type	
	High	Low	High	Low	High	Low	High	Low	T	TC
MCT I (n=20)	8 (40)	12 (60)	11 (55)	9 (45)	18 (90)	2 (10)	5 (25)	15 (75)	6 (30)	14 (70)
MCT II (n=17)	8 (47)	9 (53)	9 (53)	8 (47)	13 (76)	4 (24)	8 (47)	9 (53)	8 (47)	9 (53)
MCT III (n=16)	4 (25)	12 (75)	12 (75)	4 (25)	13 (81)	3 (19)	2 (12)	14 (88)	9 (56)	7 (47)
Low-grade MCT (n=34)	15 (44)	19 (56)	19 (56)	15 (44)	28 (82)	6 (18)	11 (32)	23 (68)	13 (38)	21 (62)
High-grade MCT (n=19)	5 (26)	14 (74)	13 (68)	6 (32)	16 (84)	3 (26)	4 (21)	15 (79)	10 (53)	9 (47)

Table 3: Number of cases with specific CD117 staining pattern compared to MCT histological grading systems

	CD117		
	1st n (%)	2nd n (%)	3rd n (%)
MCT I (n=20)	14 (70%)	6 (30%)	0
MCT II (n=17)	4 (23%)	11 (65%)	2 (12%)
MCT III (n=16)	0	4 (25%)	12 (75%)
Low-grade MCT (n=34)	18 (53%)	14 (41%)	2 (6%)
High-grade MCT (n=19)	0	7 (37%)	12 (63%)

DISCUSSION

We hypothesized that CD2, CD25, mast cell tryptase, chymase, as well as mast cell type, may determine MCT malignancy and invasiveness. In this study, MCTs expressed all examined cell markers. Expression profiles varied in individual dogs. Two different histological grading systems allowed us to compare the relationships between expression of cell markers and tumor malignancy.

CD2 showed higher expression in more aggressive lesions, and lower in benign tumors, while CD25 expression was high in benign cases and decreased with increasing MCT malignancy. This may suggest that CD25 mediates transformation of non-neoplastic mast cells to neoplastic ones, and CD2 may be an important factor in malignancy progression. Only Meyer *et al*. (2012) published similar findings. They also suggested that high expression of CD25 in benign lesions may be necessary for mast cell activation. Our results indicate that higher expression of CD25 may be linked to neoplastic activation of mast cells and CD2 may be responsible for malignant behavior.

Previous studies did not demonstrate a significant association between malignant MCTs and tryptase-staining pattern or cells were positive for both tryptase and chymase (Kiupel *et al*., 2004). Lack of positive staining was justified by either a failure of these two stains or an incorrect histologic diagnosis. In our study, we decided to divide MC into two types – MC_T and MC_{TC}. We hypothesized, that lower tumor differentiation is linked with fewer cytoplasmic granules and decreased tryptase and chymase expression. Tryptase-positive mast cells dominate in canine MCTs. In benign lesions (70% of MCT grade I and 62% of low-grade MCT) the most common type of mast cells is MCT_{TC}. Low or none chymase expression is detected in more malignant tumors, in MCT grade III and high-grade MCT the most common type of mast cells is MC_T. Tryptase appears to play a more significant role in tumor progression than chymase. Similar conclusions were reported by de Souza Junior *et al*. (2015), who presented the influence of mast cell chymase and tryptase in tumor angiogenesis.

Mast cells tryptase and chymase have similar physiological roles. Both of them stimulate endothelial cells to form vessels, as well as induce inflammation and degrade extracellular matrix (Sammarco *et al*., 2018). Mast cell tryptase additionally stimulates fibroblast chemotaxis and collagen production (Bagher *et al*., 2018). Mast cells express fibroblast growth factor 2 (FGF-2), through which they might directly promote growth and differentiation of fibroblasts. Conversely, mast cell growth is influenced by fibroblasts (Artuc, 2002). Cancer associated cells (CAFs) are stromal cells that play a crucial role in carcinogenesis. Suh *et al*. (2020) demonstrated that CAFs express FGF-2 in breast cancer. CAFs, their native ECM and mast cell-derived tryptase, rendering them important microenvironmental drivers of prostate cancer progression (Pereira *et al*., 2019). Tryptase probably stimulate tumor stroma cells and proliferation of fibroblast that affect tumor growth and development. Further studies on chymase and tryptase expression in MCTs are necessary to demonstrate the impact of these enzymes on cancer associated fibroblasts.

Conclusions: In conclusion, we demonstrated that expression of all examined proteins can be used as a prognostic factor of MCTs. Higher expression of CD2 is linked with tumor malignancy, while CD25 determine tumor development. We found that mast cells tumor division into MCT_T and MCT_{TC} may be successfully used; those tryptase positive and chymase negative (MCT_T) are more malignant than tryptase and chymase positive ones.

Authors contribution: MKG contributed to data collection, data analysis interpretation and manuscript drafting. MKG, SD, JAM designed the study and participated in data analysis and interpretation. IJ contributed statistical analysis and participated in data analysis. JAM and RC contributed revision of manuscript. PP collected samples. All authors read and approved the final manuscript.

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