Eurasia Specialized Veterinary Publication

International Journal of Veterinary Research and Allied Science
ISSN:3062-357X

2022, Volume 2, Issue 2, Page No: 73-81

Available online at: www.esvpub.com/

Toluidine Blue-Enhanced Immunohistochemistry: A Cost-Effective Method for Mast Cell Co-Localization in Canine Skin Pathology

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ABSTRACT

Immunohistochemistry (IHC) remains a cornerstone method in diagnostic pathology; however, the concurrent visualization of multiple antibodies using various chromogens is typically labor-intensive, technically challenging, and relatively costly. To simplify mast cell (MC) recognition during immunohistochemical evaluation of membrane or nuclear antigens, we introduce a novel staining protocol combining IHC with toluidine blue as a counterstain. This approach was applied to assess c-kit, Ki67, and cannabinoid receptor 2 expression across several cases of canine cutaneous mast cell tumors (MCTs), mastocytosis, and atopic dermatitis. Our findings indicate that this dual-staining approach—though applicable only to non-cytoplasmic markers and less effective in poorly differentiated MCTs where MC metachromasia is indistinct—offers practical value in analyzing membranous and nuclear markers in canine skin diseases, especially those containing sparse MC populations. It aids in differentiating neoplastic MCs from infiltrating inflammatory cells during Ki67 index determination and serves as a valuable tool for investigating new molecular markers in both veterinary and human mast cell–related conditions.

Keywords: Canine mast cell tumors, Co-localization, Immunohistochemistry, Toluidine blue stain

Received: 29 January 2022
Revised: 03 April 2022
Accepted: 04 April 2022

How to Cite This Article: Anita C, Federico S, Vittoria M, Niccolò DL. Toluidine Blue-Enhanced Immunohistochemistry: A Cost-Effective Method for Mast Cell Co-Localization in Canine Skin Pathology. Int J Vet Res Allied Sci. 2022;2(1):73-81.
https://doi.org/10.51847/4dXAQoMlm4

Introduction

Within diagnostic pathology, immunohistochemistry (IHC) enables the detection of cellular markers in tissue sections, providing essential insights that influence disease interpretation and clinical management [1]. A comprehensive understanding of different disorders also requires identifying the specific cells that express these markers or receptors, thereby offering potential therapeutic targets.

Mast cells (MCs) are examined across multiple veterinary diseases, including canine atopic dermatitis (AD), cutaneous mastocytosis, and mast cell tumors (MCTs) [2, 3]. Their hallmark feature is the metachromatic violet-red granules visualized using stains such as toluidine blue, May-Grünwald Giemsa, or Leishman [4]. Such staining reliably highlights MCs in tissue and is recommended for routine diagnostic use [4]. Nonetheless, when immunohistochemistry is used to assess antigen expression in normal or neoplastic MCs, verifying that the observed antigen truly belongs to MCs can be challenging. Cytoplasmic detection of granule components such as chymase and tryptase is considered a definitive indicator of mast cell phenotype [5–8]. Both enzymes are employed in evaluating normal [8, 9] and neoplastic [10–12] states, and chymase activity, in particular, has been linked with inflammatory disease progression [8].

Canine AD represents a chronic inflammatory skin condition marked by barrier dysfunction and inflammation [13]. In affected skin, perivascular infiltrates composed of T lymphocytes, dendritic cells, eosinophils, and MCs are more abundant than in healthy skin [14]. Cutaneous mastocytosis—a condition similar to human urticaria pigmentosa (UP)—is rare in dogs [15, 16], presenting with uniform, well-differentiated MC infiltrates extending through the dermis [7]. In humans, UP forms of mastocytosis often involve c-kit oncogene mutations, and exon 11 mutations of this gene have likewise been identified in canine mastocytosis [17].

MCTs rank as the second most common malignancy in dogs [18], representing about 20% of all cutaneous canine tumors [19]. Their prognosis is assessed through histological parameters used in the Patnaik three-tier grading system (grades 1–3) [20] and the Kiupel two-tier system (low vs. high grade) [21], alongside c-kit expression abnormalities [22], activating c-KIT mutations [23, 24], and Ki67 index evaluation [25]. C-kit, also known as CD117 or mast/stem cell factor receptor (SCFR), encodes a type III transmembrane tyrosine kinase receptor [26] expressed in both normal and malignant canine MCs [27, 28]. In MCTs, its expression pattern—membranous, cytoplasmic, or perinuclear (Golgi-like)—correlates with histological grading parameters [29–32]. Among proliferation markers, Ki67 is widely utilized for assessing canine MCTs, appearing exclusively in cell nuclei during interphase [33, 34].

Cannabinoid receptor 2 (CB2R), an endocannabinoid type 2 receptor, is localized in both the cytoplasm and nucleus of various cell types [35, 36]. In the canine gastrointestinal tract, CB2R-positive cells include lamina propria MCs, immune cells, endothelial, and smooth muscle cells [37]. Within the skin of healthy and AD-affected dogs, CB2R immunoreactivity is noted in perivascular MC-like cells, fibroblasts, and endothelial cells [19], suggesting CB2R as a promising therapeutic target for AD and other inflammatory disorders in companion animals.

During histological evaluation of MCTs, AD, and mastocytosis, determining the presence of mast cells (MCs) in tissues is often valuable, and the simultaneous expression of multiple markers can be analyzed through multiplex immunohistochemistry or immunofluorescence assays [38]. These advanced methods permit the concurrent visualization of several antigens, serving as fundamental tools for studying the organization of intrinsic cellular components within individual cells [39, 40]. However, these techniques are typically intricate, costly, require considerable time, and are difficult to interpret. In addition, immunofluorescence staining demands a fluorescence microscope, which is rarely available in routine diagnostic laboratories.

In this study, we aimed to evaluate and confirm the efficiency of a modified immunohistochemical counterstaining technique applicable when MC identification is challenging or when marker expression in MCs needs to be clearly demonstrated. The method integrates immunohistochemistry with toluidine blue (TB) as a counterstain. Using this approach, we examined various canine MC-related conditions by employing markers of diagnostic, prognostic, and therapeutic significance. The purpose was to validate the method, illustrate its practical advantages, and recommend its potential for future use with additional membranous or nuclear immunohistochemical markers.

Materials and Methods

Sample collection

Samples were obtained from the archive of the Pathology Service, Department of Veterinary Medical Sciences, University of Bologna (Ozzano dell'Emilia, Bologna, Italy). The material included 17 canine cutaneous MCTs (classified per the WHO histological classification of domestic animals [18]), four AD cases, and two cutaneous mastocytosis cases previously diagnosed as described by Gross *et al*. (2005) [7].

Histologic grading of all MCTs was performed following both the Patnaik system [19]—six grade 1, four grade 2, and seven grade 3—and the Kiupel system [20], which classified 10 as low-grade and seven as high-grade.

Immunohistochemistry and toluidine blue counterstaining

Immunohistochemical analyses for c-kit, Ki67, and CB2R were carried out on all samples, replacing the usual Harris hematoxylin (HH) with TB as the counterstain.

Sections (2 µm thick) were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched using 0.3% H_2O_2 in methanol for 30 minutes at room temperature (RT). Primary antibody specifications, dilutions, retrieval conditions, and positive controls are listed in **Table 1**. Following antigen retrieval and 20 minutes of

cooling at RT, nonspecific binding was blocked by incubating slides in 10% normal goat serum in PBS for 30 minutes at RT, after which they were incubated overnight (ON) at 4 °C with the primary antibodies.

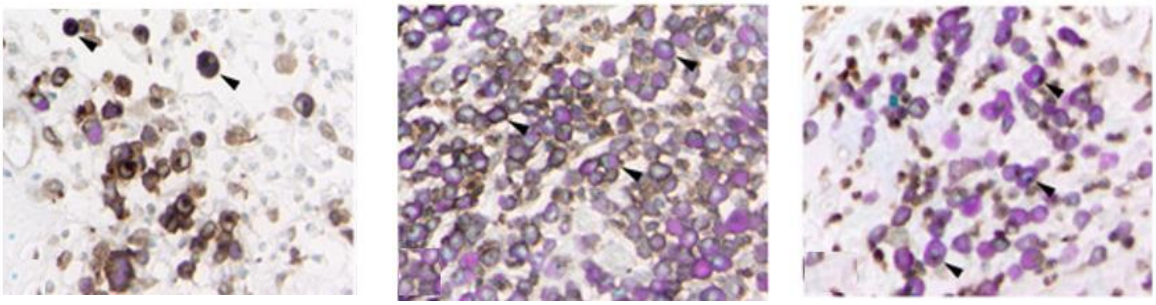
Table 1. Immunohistochemistry materials and procedures. Abbreviations: MW, microwave; ON, overnight; Ag, antigen [references of cross-reactivity with canine MCs/tissues].

Marker	Antibody Type, Clone, Reference	Supplier	Dilution/Incubation	Antigen Retrieval
c-kit (CD117)	Rabbit polyclonal anti-c-kit (A4502) [41]	Dako, Glostrup, Denmark	1:300 ON	10′ Citrate pH6 MW: 750 W
Ki67	Mouse monoclonal anti-Ki67 (MIB1) [41]	Dako, Glostrup, Denmark	1:600 ON	20′ Citrate pH6 MW: 750 W
CB2R	Rabbit polyclonal anti-Cannabinoid Receptor II (ab45942) [37]	Abcam, Cambridge, UK	1:500 ON	10′ EDTA pH8 MW: 750 W

Detection was achieved with a biotinylated secondary antibody and an avidin–biotin–peroxidase complex (ABC Kit Elite, Vector, Burlingame, CA, USA). The chromogen used was 3,3′-diaminobenzidine (0.05%) (Histo-Line Laboratories, Emergo, Europe). Counterstaining was done with toluidine blue (0.03%) (MERCK & Co., Readington, NJ, USA) for 7 minutes, followed by a 5-minute rinse in tap water. Sections were then briefly dehydrated (10–20 seconds per step) through 80%, 95%, and two changes of 100% ethanol, cleared in xylene, and permanently mounted in DPX medium (Fluka, Riedel-de Haen, Germany).
To compare TB with conventional HH counterstaining, c-kit and Ki67 immunostaining was also performed on MCTs of various Patnaik and Kiupel grades using HH under standard conditions.

Results and Discussion

All analyzed specimens were successfully evaluated and included in the study.
Immunohistochemical detection of c-kit in canine MCTs revealed three distinct expression patterns. Metachromatic staining was distinctly visible in membranous and Golgi-like expression types **(Figures 1b, 1c and 2c)**, whereas it was masked in diffuse cytoplasmic c-kit expression **(Figure 1a)**, particularly in poorly differentiated tumors (Patnaik grade III, Kiupel high grade) **(Figure 3f)**. Dermal MCs in AD exhibited both cytoplasmic and membranous c-kit localization **(Figure 2a)**, similar to well-differentiated MCs in mastocytosis **(Figure 2b)**.



a) b) c)

Figure 1. Association between toluidine blue and c-kit immunohistochemistry. Canine mast cell tumors with cytoplasmic (a), membranous (b), and Golgi-like (c) c-kit expression. The red metachromatic cytoplasmic staining is evident in (b) and (c) but obscured in (a) when diffuse cytoplasmic immunostaining is present. Scale bar = 50 μm.

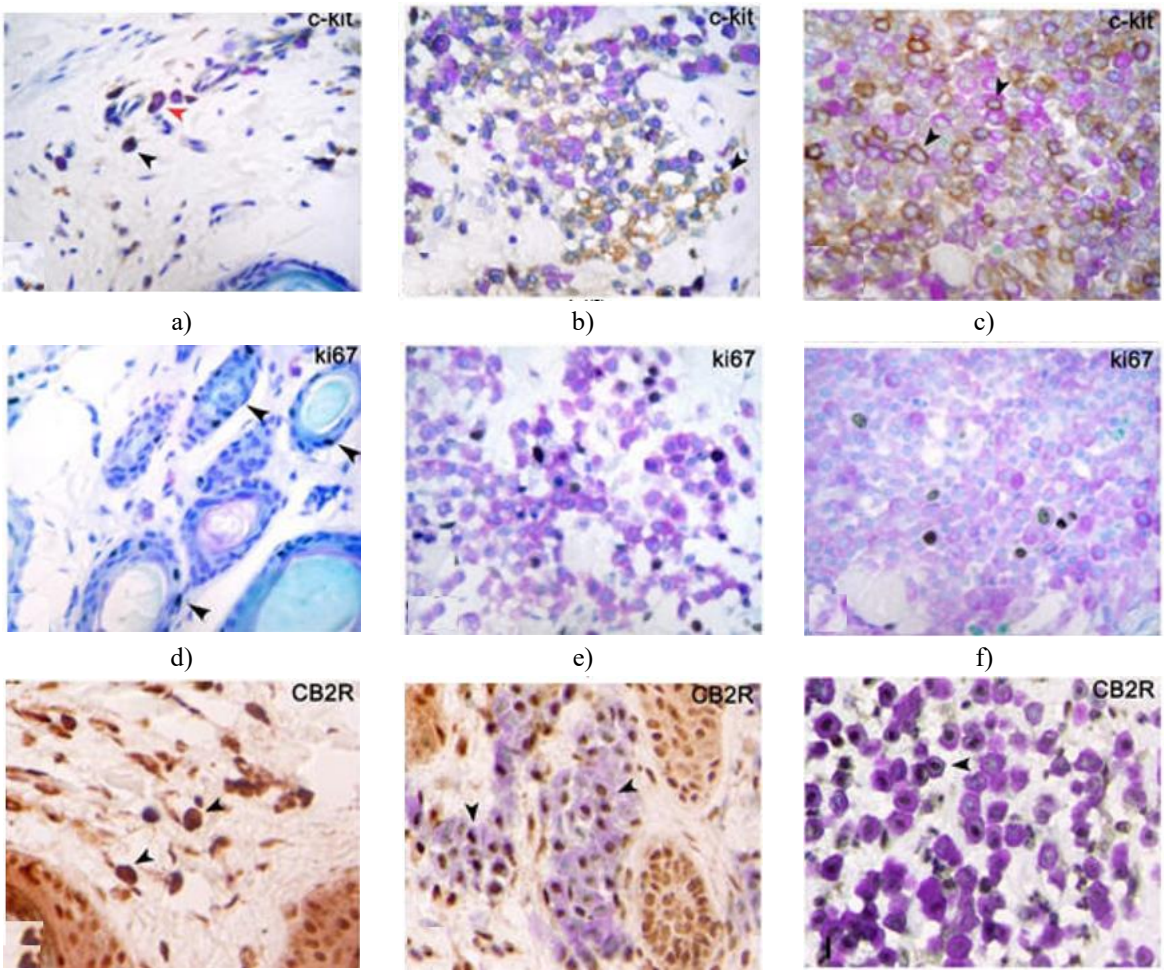
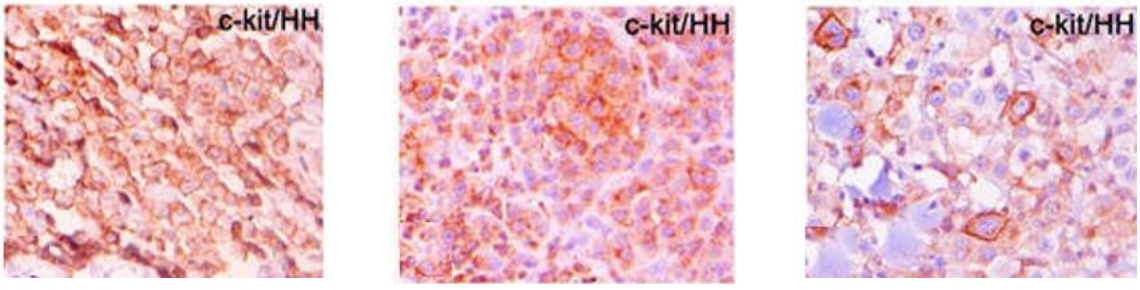


Figure 2. Immunostaining for c-kit, Ki67, and CB2 receptor using toluidine blue (TB) as the background stain in atopic dermatitis (AD), cutaneous mastocytosis, and cutaneous mast cell tumors (MCTs). In AD (a), mastocytosis (b), and MCTs (c), c-kit staining appeared both in the cytoplasm (black arrow) and along the cell membrane (red arrow) for (a,b), while in (c) only surface staining (black arrow) was visible on mast cells (MCs). Ki67 labeling in mastocytosis (e) and MCTs (f) showed distinct nuclear activity within MCs (e, f) highlighted by TB, whereas in AD, mast cells were quiescent, and Ki67 reactivity was observed exclusively in hair follicle epithelial cells (black arrows) (d). Nuclear CB2 receptor staining (black arrows) was evident in mastocytosis (h) and MCTs (i), but in AD (g), the CB2 receptor was detected in the cytoplasm (black arrow), obscuring the TB metachromatic hue. Scale bar = 50 µm.



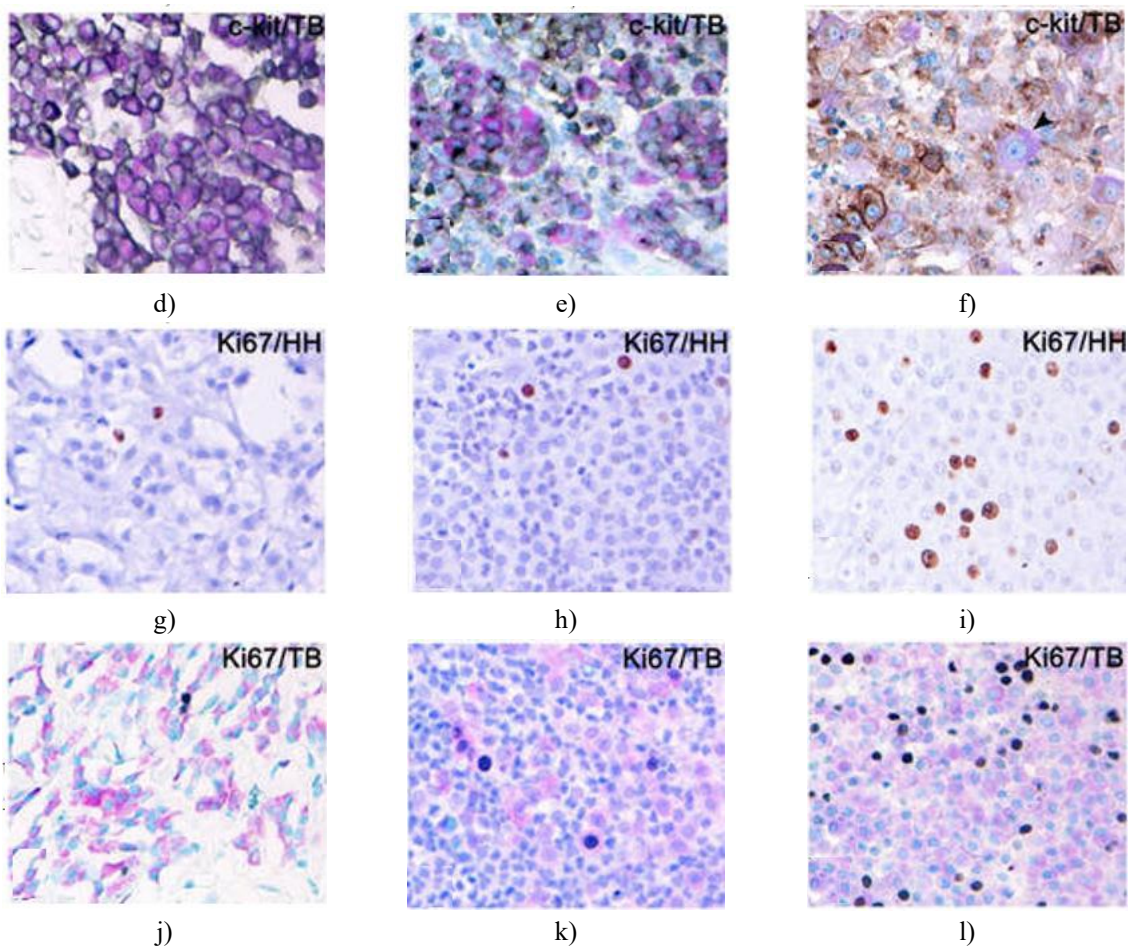


Figure 3. Immunolabeling for c-kit and Ki67 counterstained with Harris hematoxylin (HH) or toluidine blue (TB) in cutaneous MCTs. In Patnaik grade 1 and Kiupel low-grade tumors, c-kit staining using HH (a) or TB (d) revealed a clear membranous pattern, preserving TB metachromasia. For Patnaik grade 2 and Kiupel low grade, c-kit showed both membranous and Golgi-like distribution in HH (B) or TB (e) sections, with TB metachromasia still apparent. In Patnaik grade 3 and Kiupel high-grade MCTs, c-kit was mainly cytoplasmic, and some MCs lacked c-kit signal (black arrow); thus, metachromasia persisted only in c-kit–negative cells (c, f). Ki67 staining of Patnaik grade 1 (G,J), grade 2 (h, k), and grade 3 (i, l) MCTs, counterstained either with HH (g–i) or TB (j–l), is presented. Scale bar = 50 µm.

During the Ki67 evaluation, mastocytosis and all MCT samples exhibited simultaneous nuclear Ki67 positivity and cytoplasmic TB metachromasia within the same cells **(Figures 2d–2f)**. Conversely, in AD, Ki67 reactivity was restricted to the hair follicle epithelium, indicating an absence of active proliferation among mast cells.
CB2 receptor immunostaining showed nuclear localization within MCs of mastocytosis **(Figure 2h)** and MCTs **(Figure 2i)**, allowing the TB metachromatic tone to remain visible. However, in AD, CB2 receptor labeling was cytoplasmic **(Figure 2g)**, which masked the TB reaction due to the chromogenic deposit.
A comparative view of HH and TB counterstains **(Figure 3)** demonstrated that both membranous **(Figure 3a and 3d)** and Golgi-like **(Figures 3b and 3e)** KIT staining patterns, typical of Patnaik grades 1–2 or Kiupel low-grade tumors **(Figures 3a, 3b, 3d and 3e)**, could be clearly visualized regardless of the counterstain. In Patnaik grade 3 or Kiupel high-grade MCTs, the cytoplasmic distribution of c-kit concealed TB metachromasia **(Figure 3c)**, whereas mast cells lacking c-kit expression retained visible metachromatic granules **(Figures 3f).**
Ki67 staining revealed minimal nuclear labeling in Patnaik grades 1–2 or Kiupel low-grade MCTs **(Figure 3g, 3h, 3j and 3k)**, whereas Patnaik grade 3 or Kiupel high-grade lesions displayed a strong proliferative index **(Figures 3i and 3l)**. Comparison of counterstains confirmed that TB provided clearer visualization of mast cells than HH **(Figures 3g–3l)**.

Discussion

The detection of antigen expression in mast cells (MCs) is most commonly achieved through double immunofluorescence analysis [39], using anti-tryptase and/or anti-chymase antibodies to confirm their identity. In both human and veterinary studies, alternative counterstains to Harris hematoxylin (HH) for immunohistochemistry (IHC) are scarcely described. Conventional histochemical stains are primarily employed to identify MCs in light microscopy by their structural features. However, since HH does not emphasize the cytoplasmic granules characteristic of MCs, distinguishing them from other mononuclear cells such as plasma cells or histiocytes remains challenging.

In this research, we introduced and evaluated a modified staining protocol that merges IHC with a histochemical counterstain specific for MCs. This approach effectively reveals nuclear and membranous antigens in MCs but is not suitable for cytoplasmic markers, as chromogenic deposits may conceal the metachromatic granules. Although this limitation persists, the proposed staining technique represents a practical and low-cost substitute for immunofluorescence when demonstrating nuclear or surface antigens in MCs.

In canine pathology, mast cell tumors (MCTs) are the second most common malignant neoplasm derived from MCs [42]. These belong to the group of round-cell tumors of connective origin [43], which also includes plasmacytomas, lymphomas, histiocytomas, and transmissible venereal tumors. For diagnostic confirmation, immunolabeling of either membrane antigens (lymphoid or histiocytic markers) or nuclear markers such as MUM-1 (Multiple Myeloma 1) for plasma cells is often necessary [44, 45]. The staining technique proposed here could simplify this process, as the detection of cytoplasmic metachromasia rapidly supports an MCT diagnosis through a single test. Although poorly differentiated MCTs may not consistently display the metachromatic pattern typical of toluidine blue (TB) [5, 46], in our study, even Patnaik grade 3 or Kiupel high-grade tumors retained visible metachromasia **(Figures 3f and 3l)**, confirming the method's reliability in these cases.

MCTs exhibit considerable variability in biological behavior and clinical outcome [47]. The Ki67 proliferation index is routinely used to predict recurrence [18] and survival [23]. Determining the proportion of Ki67-positive nuclei can be more precise with this combined staining method, particularly in tumors with heavy inflammatory infiltrates, as it enables clear visualization of MCs through their metachromatic cytoplasm. When evaluating the c-kit expression pattern—an important prognostic marker correlated with tumor grade and surgical outcome [29]—this dual technique is especially beneficial for identifying membranous or Golgi-like localization (KIT patterns I and II), as observed in this work. However, it is less effective for assessing cytoplasmic c-kit distribution in highly malignant, poorly differentiated MCTs (KIT pattern III). Additionally, this approach could also be valuable for detecting other nuclear antigens in canine MCTs, such as p21 [48], p27 [48], p53 [49, 50], and Mdm2 [49].

In cutaneous inflammatory diseases like atopic dermatitis (AD) and mastocytosis, MCs play a pivotal diagnostic role [14]. Nonetheless, our findings demonstrated that the described method is unsuitable for assessing c-kit in non-neoplastic MCs, as they typically exhibit both membrane and cytoplasmic staining. The technique, however, remains applicable for nuclear and membranous marker evaluation. Consequently, it may be advantageous for visualizing MCs when investigating new nuclear or surface targets of potential therapeutic relevance, such as the CB2 receptor (CB2R) [19, 36].

Conclusion

The technique proposed in this study, which integrates immunohistochemistry and histochemistry to leverage the intrinsic cytoplasmic metachromasia of mast cells, provides a straightforward and efficient method for identifying MCs during IHC analysis involving nuclear or membrane-associated markers. Although its use is limited to non-cytoplasmic antigens, it offers a reliable and cost-effective alternative to multiplex IHC or dual immunofluorescence methods, suitable for both veterinary and human research applications.

Further validation using larger sample sets and additional molecular markers will be essential to broaden the applicability and confirm the diagnostic potential of this staining strategy.

Acknowledgments: None

Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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