Case Report

Association between histological grading, Ki-67, AgNOR, KIT expression pattern and survival time in a dog with mast cell Tumour: A case report

Ploen Nganthavee* Paitoon Srimontri Tanit Kasantikul Namphung Suemanotham

999 Faculty of Veterinary Science, Mahidol University, Salaya, Phutthamonthon district, Nakhon Pathom, 73170 Thailand

***Corresponding author, E-mail address: ploennganthavee@hotmail.com**

Abstract

A 13-year-old entire male Thai Ridgeback dog presented to Prasu-Arthorn Animal Hospital, Faculty of Veterinary Science, Mahidol University with a mass on the caudal right flank. Fine needle aspiration (FNA) revealed round cells with intracytoplasmic granules consistent with mast cell tumour (MCT). Surgical excision was performed. Histological grading revealed poorly-differentiated, high grade MCT with 18 mitotic figures per 10 high power-fields (HPFs) and KIT staining was consistent with KIT pattern II (focal or stippled cytoplasmic staining); both of which are associated with poor survival. This contrasted with the better survival time implicated by low argyrophilic nucleolar organiser region (AgNOR) and Ki-67 scores, which were all below the cut-off values, suggesting low cellular proliferation. A chemotherapy protocol of vinblastine and prednisolone was subsequently commenced. Distant recurrence at the neck occurred at 67 days, confirmed to be MCT by histopathology. The dog later died, with a total survival time of 90 days from diagnosis. The actual survival time closely aligned with the estimated survival time based on histological grading. With few multivariate survival analyses available, this case demonstrates the use of AgNOR, Ki-67, and KIT localisation to complement histological grading for further studies to compare different variables in a clinical setting in Thailand.

Keywords: Mast cell tumour, Grading, Ki-67, AgNOR, KIT, survival time

ความสัมพันธ์ระหว่างเกรดของเนื้องอก ค่าดัชนีการเพิ่มจำนวนของเซลล์ Ki-67 การแสดงออกของ AgNOR และ KIT ต่อระยะเวลารอดชีวิต ในสุนัขที่มีเนื้องอกมาสต์เซลล์: รายงานสัตว์ป่วย

เพลิน งานทวี* ไพฑูรย์ ศรีมนตรี ฐานิต กสานติกุล น้ำผึ้ง สื่อมโนธรรม

999 คณะสัตวแพทยศาสตร์มหาวิทยาลัยมหิดล ต.ศาลายา อ.พุทธมณฑล จ.นครปฐม 73170

***ผู้รับผิดชอบบทความ E-mail address: ploennganthavee@hotmail.com**

บทคัดย่อ

สุนัขพันธุ์ไทยหลังอาน เพศผู้ ยังไม่ทำหมัน อายุ 13 ปี เข้ารับการตรวจวินิจฉัยที่โรงพยาบาลสัตว์ประศุอาทร คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล เนื่องจากพบก้อนเนื้อบริเวณลำตัวด้านขวา เมื่อทำการเก็บตัวอย่างเซลล์ก้อนเนื้อไปตรวจพบว่าเซลล์มีลักษณะกลมมีไซโทพลาสมิกแกรนูล สอดคล้องกับเนื้องอกมาสต์เซลล์ สุนัขได้รับการผ่าตัด ผลการตรวจทางจุลพยาธิวิทยาสรุปได้ว่าเนื้องอกมีเกรดสูง เซลล์ส่วนใหญ่มีพัฒนาการต่ำ และมีการแบ่งตัว (mitotic figure) จำนวน 18 ตัวต่อกำลังขยายของกล้องจุลทรรศน์ขนาดสูง ผลย้อมสีอิมมูโนฮิสโตเคมีและฮิสโตเคมีพบว่า ค่า AgNOR และ Ki-67 ต่ำกว่าค่าจุดตัด (cut-off value) และมีรูปแบบของการแสดงออกของ KIT แบบ II หรือมีการติดสีในไซโทพลาสซึมลักษณะเป็นหย่อมเฉพาะที่ไม่แพร่กระจาย ผลการตรวจทางพยาธิวิทยาว่าด้วยเกรดและรูปแบบการแสดงออกของ KIT บ่งชี้ถึงการพยากรณ์โรคที่ไม่ดี ส่วนผล AgNOR และ Ki-67 ที่มีค่าต่ำกว่าจุดตัดบ่งบอกถึงการพยากรณ์โรคที่ดี สุนัขได้รับการรักษาโดยใช้เคมีบำบัดด้วย vinblastine ร่วมกับ prednisolone หลังจากได้รับการวินิจฉัย 67 วันพบการเกิดก้อนเนื้อซ้ำบริเวณคอ โดยผลตรวจทางพยาธิวิทยารายงานเป็นเนื้องอกมาสต์เซลล์ สุนัขเสียชีวิตในเวลาต่อมา รวมระยะเวลาทั้งหมด 90 ในกรณีศึกษานี้พบว่าระยะเวลาการรอดชีพมีความสัมพันธ์กับเกรดของเนื้องอกมากที่สุด และเนื่องจากขณะนี้ยังมีการศึกษาวิเคราะห์การรอดชีพโดยตัวแปรเชิงพหุเป็นจำนวนน้อย กรณีศึกษานี้จึงบ่งบอกถึงความสำคัญในการใช้ AgNOR Ki-67 และ รูปแบบของการแสดงออกของ KIT เพื่อส่งเสริมการแปลผลเกรดของเนื้องอกสำหรับการเปรียบเทียบเพื่อนำไปประยุกต์ใช้ในทางคลินิกในประเทศไทย

คำสำคัญ : เนื้องอกมาสต์เซลล์, เกรด, Ki-67, AgNOR, KIT, ระยะเวลารอดชีพ

Introduction

Mast cell tumour (MCT) or mastocytoma, a tumour arising from neoplastic transformation of mast cell is a commonly encountered tumour in dogs, making up 16 to 21% of canine cutaneous neoplasms (Blackwood et al., 2012). MCT primarily occurs in older dogs (mean age 8 to 9 years) and there is no predilection for sex (Blackwood et al., 2012; Rothwell et al., 1897). Breeds such as Boston Terrier, Boxer, Pug, Labradors, Golden Retrievers and Pitbull are at a significant risk of developing MCT (Blackwood et al., 2012; Rothwell et al., 1897).

In dogs, MCTs can occur anywhere on the body though are commonly found in cutaneous or subcutaneous tissues (Blackwood et al., 2012). Tumours at mucosal sites and in unresectable locations are associated with poorer outcome (Hillman et al., 2010; London and Thamm 2020). A diagnosis of MCT can be made via fine needle aspiration (FNA) and excisional biopsy revealing the characteristic round cells with fine purple metachromatic cytoplasmic granules (Blackwood et al., 2012; Bostock 1986).

The presentation and biological behaviour of canine MCTs can be highly heterogeneous with variable outcome and metastasis, making prognostic factors essential in predicting the clinical course (London and Thamm 2020). Amongst multiple prognostic factors, histological grading remains the single most important prognostic factor as it is strongly correlated with survival (Bostock 1986; London and Seguin 2003; Patnaik et al., 1984). High-grade MCTs corresponding to the current two or three-tiered grading systems are associated with shorter survival time and shorter time to metastasis; with undifferentiated tumours found to have a metastatic rate of 55 to 96% (Blackwood et al., 2012; Bostock 1973; Patnaik et al., 1984). The most common metastatic sites include local lymph nodes, liver, spleen and bone marrow (Blackwood et al., 2012; O'connell and Thomson 2013). Other diagnostic tools that are not routinely used in practice but have been promising in providing valuable prognostic information include the evaluation of cellular proliferation markers (Ki-67 and argyrophilic nucleolar organiser region (AgNOR)) and receptor expression (KIT localisation pattern and c-KIT mutation) (Smith et al., 2017).

While surgery alone can be curative for low grade MCTs, only 6 to 27% of dogs with high grade tumours treated with surgery alone survive longer than 1 year (Bostock 1973; Bostock et al., 1989; Patnaik et al., 1984). Thus, radiotherapy or systemic chemotherapy should be considered in combination with surgery in high grade MCTs or in patients with negative prognostic factors (Blackwood et al., 2012; Sledge et al., 2016). This report describes a case of MCT in a dog and compares the association between prognostic factors (namely histological grading, Ki-67, AgNOR, and KIT receptor expression) with clinical survival time, and survival times in literature.

Case Description

A 13-year-old entire male Thai Ridgeback dog presented to Prasu-Arthorn Animal Hospital, Faculty of Veterinary Science, Mahidol University with a mass on the caudal right flank. The mass was noticed six months prior to presentation as a hard firm 1 x 1 x 1 cm nodule and was reported to be fast-growing, with pruritus and licking observed. No gastrointestinal abnormalities were reported. At presentation, a firm non-mobile, well-circumscribed mass at the right flank measuring 5 x 5 x 3 cm with alopecia, and ulcerated suppurative surface was identified (Figure 1A). Right popliteal lymph node was enlarged, measuring at 2 x 2 x 2 cm.

Cytological evaluation by FNA and Diff-Quick™ of the mass revealed a population of discrete round cells with pale basophilic cytoplasm, large nuclei and purple intracytoplasmic granules with some degenerate neutrophils. The cytological appearance of these round cells was consistent with MCT with a background of infection. FNA of the right popliteal lymph node revealed low number of mast cells with accompanying lymphocytes and lymphoblasts, suggestive of reactive lymphoid hyperplasia. A routine complete blood count, biochemistry panel and 3-view thoracic radiographs revealed no abnormalities (Table 1). Abdominal ultrasound study was unremarkable. The decision was made to perform a wide surgical mass excision followed by chemotherapy.

To excise the mass, the dog was premedicated with 0.3 mg/kg IM morphine with 0.2 mg/kg IV diazepam, followed by induction with 4 mg/kg IV propofol, and maintained with isoflurane. Before surgery, 0.4 mg/kg chlorpheniramine was also given. After excision, the mass was fixed in 10% neutral buffered formalin, and sectioned for histopathological examination, margin analysis, and staining for AgNOR, Ki-67 and KIT pattern localisation. Closure was performed using a unilateral advancement flap using a cruciate pattern with 3-0 nylon (Ethilon® Ethicon) suture (Figure 1B).

Paraffin-embedded samples were cut to 5 μm and submitted to Mahidol University Veterinary Diagnostic Center for histopathology and margin analysis, to Vet Clinical Center for AgNOR staining, and to Thailand's Institute of Pathology for Ki-67 staining and KIT pattern localisation. For histopathology and margin analysis, the slides were stained with routine toluidine blue. To perform modified AgNOR staining, samples were deparaffinised in xylazine and rehydrated in ethanol. To evaluate AgNORs, samples were incubated with silver nitrate at 37 °C for 35 minutes and 0.1% gold chloride before dehydration. The AgNORs were counted in 100 cells and averaged.

Ki67 immunostaining was performed with the Benchmark staining platform (Ventana, Tucson, AZ). Sections were incubated with murine monoclonal anti-Ki67 primary antibodies (MIB1; Dako Cytomation) at a 1:400 dilution, detected using a commercial alkaline phosphatase and secondary antibody system. All slides were counterstained with haematoxylin. The slides were compared to a negative control (canine MCT incubated with a buffer) and a positive control (the epidermal basal layer). Counting was performed at 100 X magnification, where immunopositive cells per area were averaged in 5 HPFs.

Immunohistochemical analysis for KIT was performed on the Leica Microsystems Bond maX System (Leica Microsystems, Bannockburn, IL). Slides were deparaffinised with dewax Solution (Leica Microsystems) before incubating with rabbit polyclonal anti-KIT antibody (Dako Cytomation) at a 1:750 dilution for 30 minutes in Bond Epitope Retrieval Solution 2 (Leica Microsystems). Immunohistochemical analysis was performed using the Bond Polymer Refine Detection kit (Leica Microsystems) and a 3-step indirect immunoperoxidase technique. Samples were referenced against a negative control (Canine MCT with only added buffer) and a positive control (known MCT slide).

Histopathology revealed highly cellular, poorly delineated mast cells arranged in sheets with scattered cells present in the dermis and subcutis. Neoplastic cells were round, had distinct borders and moderate amounts of cytoplasm with intracytoplasmic basophilic granules (Figures 2 and 3). There was moderate anisokaryosis

with some multinucleated cells having 1 to 2 nucleoli. There were 18 mitoses/ 10 HPFs. Extensive necrosis and oedema were noted. Complete surgical excision of the mass could not be determined as there were rare aggregates of mast cells within the deep dermis and subcutis of the caudal tangential margin. Immunohistochemical labelling showed Ki-67 of 16.8 cells per grid (cut-off 23), histochemical staining revealed AgNOR of 2.16 cells per nucleus in neoplastic cells (cut-off 2.25), and AgNOR x Ki-67 of 36.29 (cut-off 54) (Figures 4 and 5) (Webster et al., 2007). KIT pattern labelling revealed loss of perimembrane labelling and perinuclear or stippled cytoplasmic labelling in greater than 10% of neoplastic cells which are characteristic of cytoplasmic focal (KIT pattern II) (Figures 6 and 7). Detection of internal tandem duplication mutations of c-KIT gene is not yet available in Thailand.

Chemotherapy regimen with vinblastine and prednisolone was administered after complete wound healing. Two mg/ m^{-2} IV vinblastine (Vilban®) was given weekly for 4 weeks with 2 mg/ kg PO prednisolone twice daily for 2 weeks, followed by 2 mg/ kg once daily for 4 weeks. Follow-up monitoring revealed that the patient developed a 2 x 2 x 1 cm dark-coloured mass at the ventral neck after 4 chemotherapy treatments confirmed to be an MCT by FNA (67 days after first presentation). Cytology of the left submandibular lymph node revealed multiple aggregates of mast cells infiltration. Wide surgical excision of the mass and lymphadenectomy were performed. Histopathology revealed round cells with toluidine blue positive cytoplasmic granules in the superficial to deep dermis. A large population of neoplastic cells were seen in the lymph node medullary sinus.

Despite adequate surgical wound healing, the dog started to exhibit signs of depression and anorexia at 10 days post-surgery. At 22 days post-surgery the patient died at home; the total survival time was 90 days.

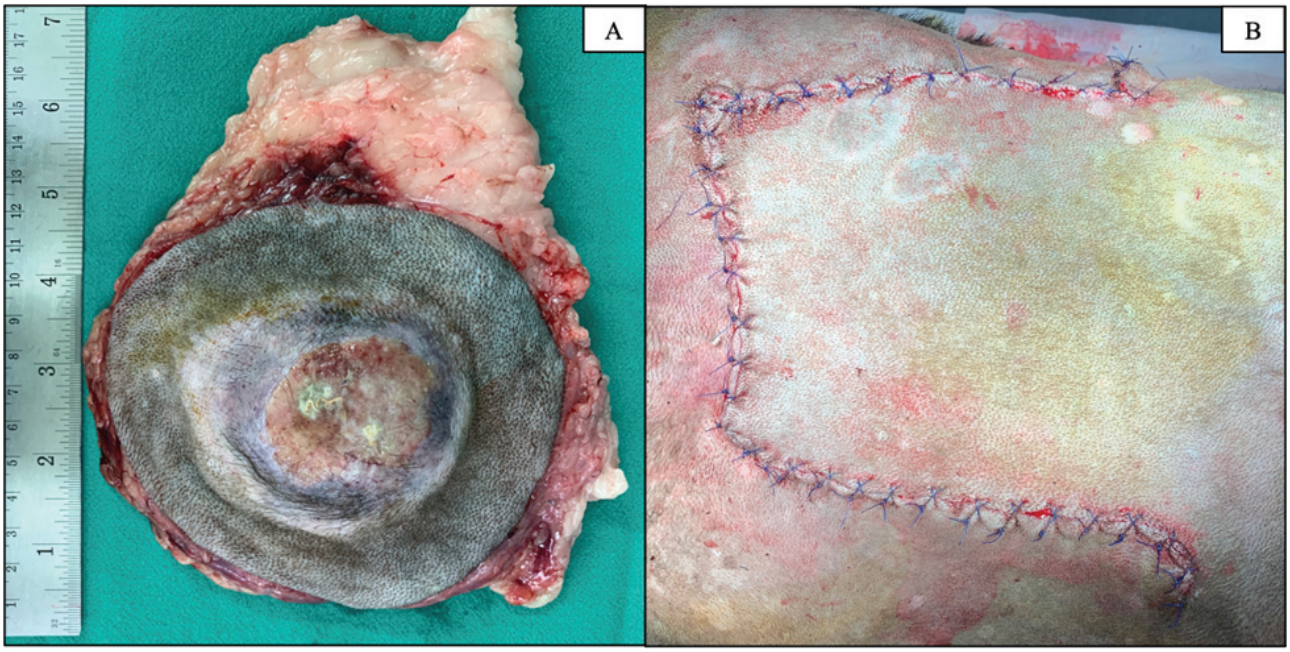

Figure 1. Photograph of the lesion. A) Skin mass excised from the right flank containing a well-circumscribed superficial dermal to deep subcutaneous, 5 x 5 x 3 cm, off-white/ pink ulcerated suppurative mass surrounded by normal skin B) Surgical wound following closure with a unilateral advancement flap.

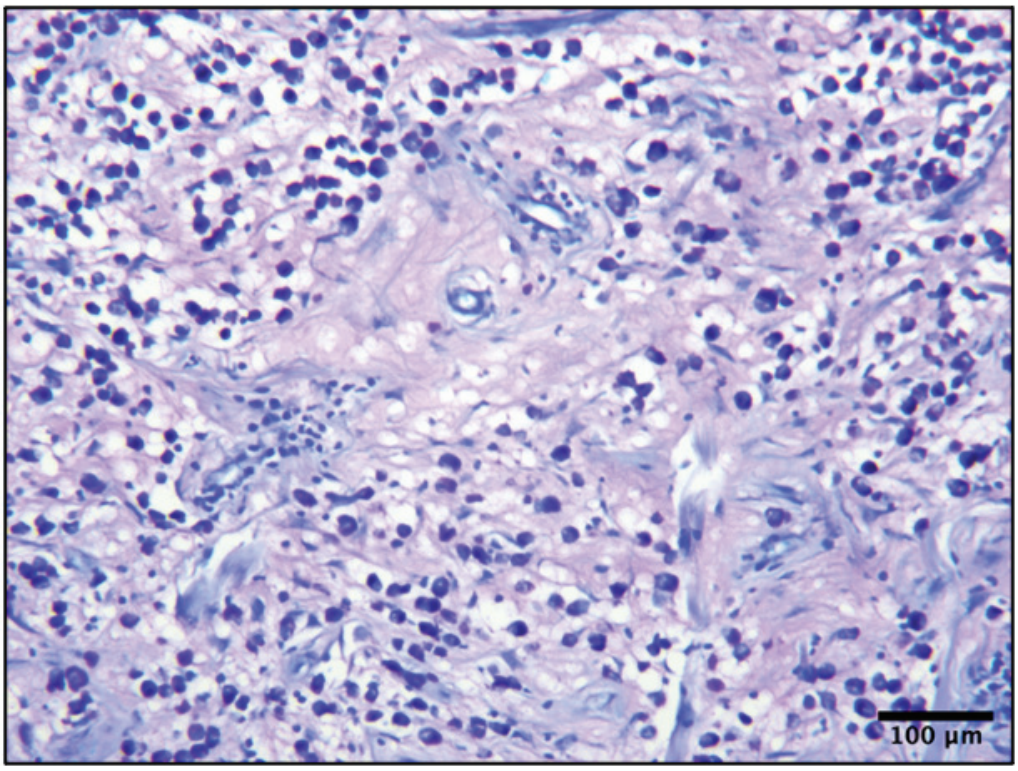

Figure 2. Mast cell tumour (MCT), dog, skin. Toluidine blue. Neoplastic cells containing dense metachromatic granules at 10x magnification.

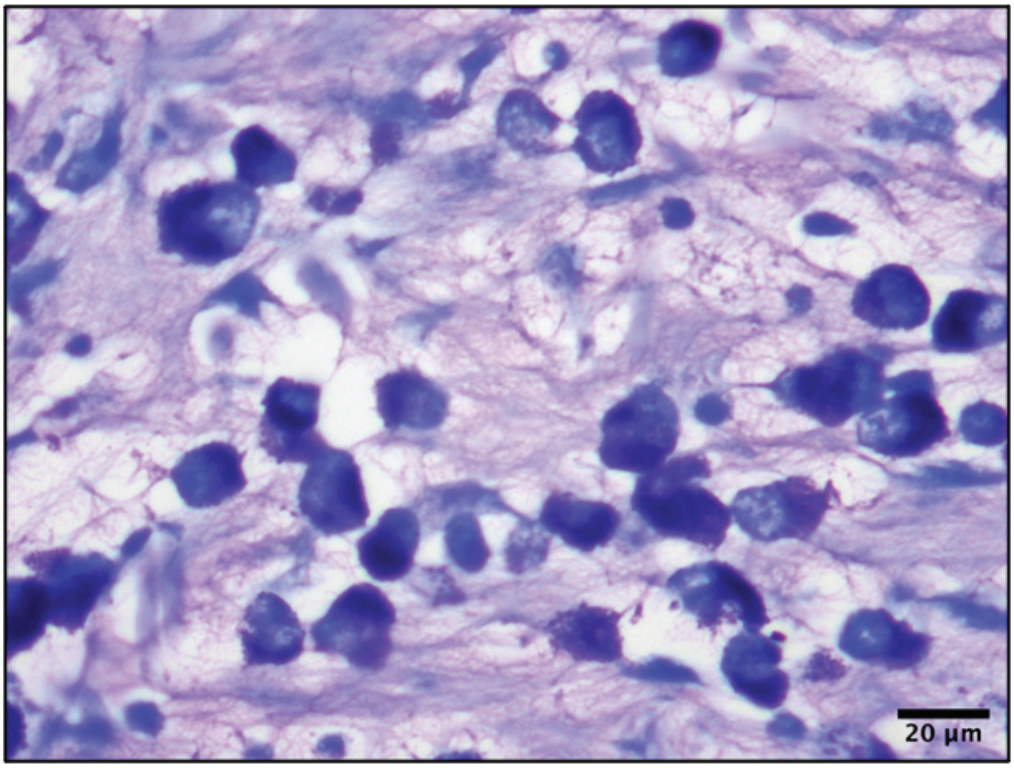

Figure 3. Mast cell tumour (MCT), dog, skin. Toluidine blue. Neoplastic cells containing dense metachromatic granules at 40x magnification.

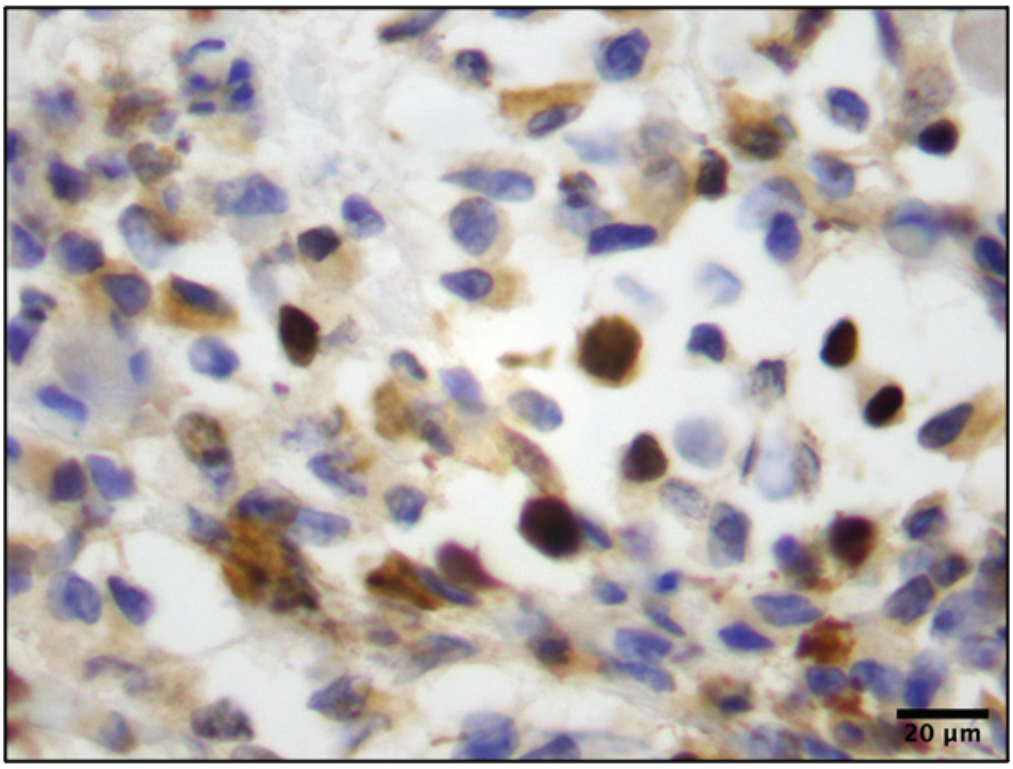

Figure 4. Mast cell tumour (MCT), dog, skin. Immunohistochemistry labelled for Ki-67 reveals occasional nuclear labelling. 40x magnification.

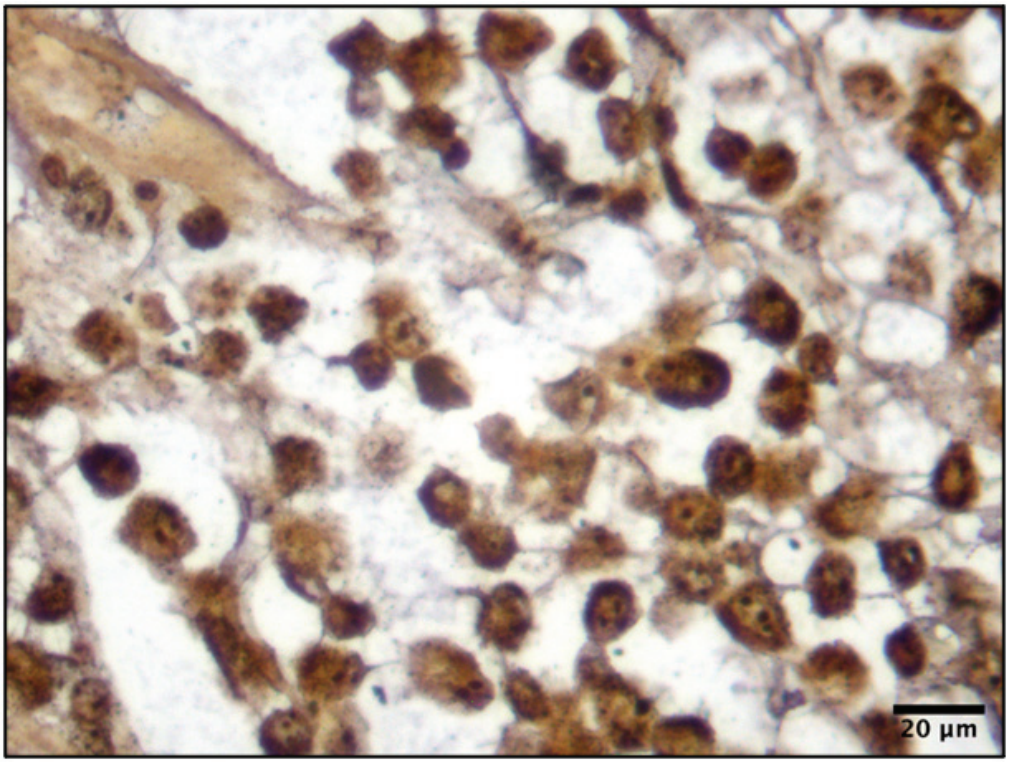

Figure 5. Mast cell tumour (MCT), dog, skin. Histochemical staining for AgNOR reveals variable numbers of Nucleolar organiser regions (NORs). 40x magnification.

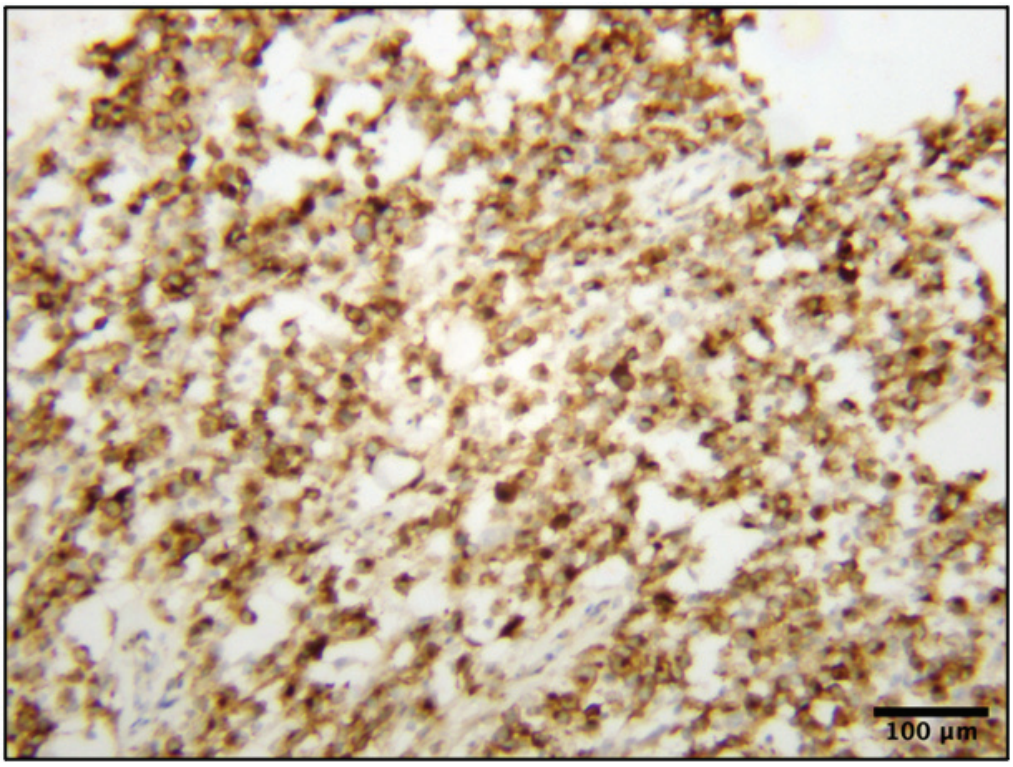

Figure 6. Mast cell tumour (MCT), dog, skin. Immunohistochemistry labelled for KIT at 10x magnification.

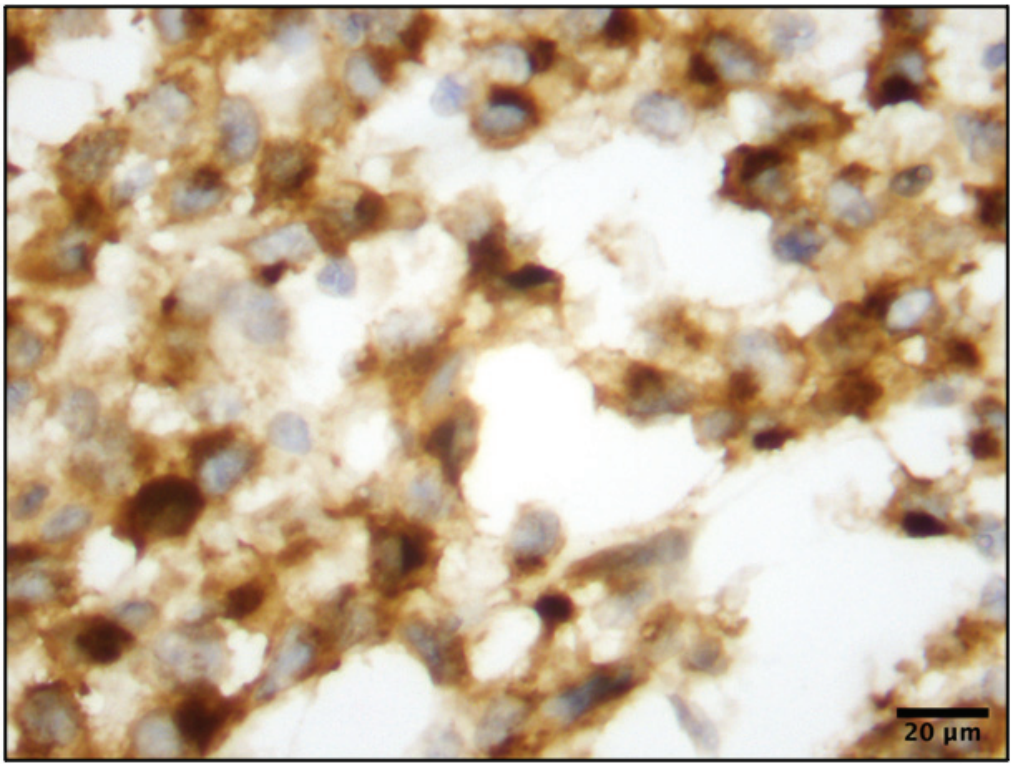

Figure 7. Mast cell tumour (MCT), dog, skin. Micrograph reveals both a loss of perimembrane labelling and perinuclear or stippled cytoplasmic labelling in greater than 10% of neoplastic cells, consistent with a KIT pattern II. 40x magnification.

Table 1. Complete blood count and serum biochemical results.

Parameter	12/12/19 1st Chemo.	23/12/19 2nd Chemo.	06/01/20 3rd Chemo.	13/01/20 4th Chemo.	27/01/20	11/02/20	Reference
WBC (cell/mm^3)	8.9	13.3	22.1	11.8	25.2	9.46	6-17
Monocyte	0.4	0.8	0.4	0.5	1.0	0.5	0.15-1.4
Neutrophil	6.9	11.1	19.7	9.9	21.9	8.1	3.0-11.5
Lymphocyte	1.2	1.2	8	1.3	2.0	0.9	1.0-4.8
Eosinophil	0.4	0.1	1	0.1	0.3	0	0.1-1.3
Basophil	0	0	0	0	0	0	<0.1
Band Neutrophil	0	0	0	0	0	0	0-0.3
Erythrocyte x 10^6	6.2	5.2	5.2	4.9	4.8	3.6	5.0-9.0
Haemoglobin (g/dL)	14.5	12.5	13.4	13.5	13.4	9.2	10.0-18.0
Haematocrit (%)	45.0	38.5	39.7	38.3	38.2	28.0	33.0-35.0
MCV	72.9	74.7	77.0	77.9	78.8	77.4	60.0-77.0
MCH	23.5	24.3	26.0	27.4	27.7	25.4	20.0-25.0
MCHC	32.3	32.5	33.7	35.2	35.1	32.8	32.0-36.0
Platelets x 10^2 (cell/µl)	333	272	278	321	360	34	200-500
RDW	12.7	13.8	14.0	14.0	13.1	12.2	12.0-15.0
Plasma protein	8.0	8.8	8.6	9.0	8.6	8.2	6.0-7.5
Platelets smear	Adequate	Adequate	Adequate	Adequate	Adequate	Decrease	
Morphology	Normal	Normal	Normal	Normal	Normal	Normal	
Alkaline phosphatase (U/L)	-	117	191	134	163	138	23-212
Alanine aminotransferase (U/L)	29	44	122	69	61	29	10-100
Blood urea nitrogen (mg/dL)	-	19	17	20	18	12	7-27
Creatinine (mg/dL)	1.1	1.3	1.4	1.27	1.6	1.4	0.5-1.8

Discussion

The heterogeneity in the behaviour of MCTs stresses the importance of prognostic factors such as grading, staging, location, cell proliferation rate, recurrence after surgery, systemic signs, and c-KIT mutation in the workup (Blackwood et al., 2012; Bostock 1986; London and Thamm 2020; Michels et al., 2002; Patnaik et al., 1984; Warland et al., 2014). MCTs are graded using the three-tiered and the more objective two-tiered grading systems (Bostock 1986; Kiupel et al., 2011; Patnaik et al., 1984). Higher grades tumours show more mitotic figures associated with oedema, ulceration and necrosis (Patnaik et al., 1984). The two-tiered system decreases inter-rater variation by quantitating the histological criteria (Kiupel et al., 2011). In this case, the tumour was graded as poorly differentiated (based on the three-tiered grading system) and high (according to the two-tiered grading system) (Bostock 1986; Patnaik et al., 1984; Kiupel et al., 2011).

The presence of a large MCT without systemic signs is classified as stage III. substage a., according to the World Health Organisation's clinical staging (Owen 1980). Apart from cytological sampling of the mass and lymph nodes, full staging workups include abdominal ultrasound, thoracic radiographs, buffy coat smears and bone marrow examination (Blackwood et al., 2012). Although splenic and hepatic MCT infiltration are associated with reduced survival time, aspirating ultrasonographic normal spleen and liver remains a controversy, as there are no cut-off values differentiating normal, reactive and neoplastic disorders (Finora et al., 2006; Stefanello et al., 2009). Given the absence of ultrasonographic neoplastic features in both organs splenic and hepatic ultrasound FNA were not performed. Pulmonary metastasis is rare; therefore, thoracic radiographs are used to look for other conditions rather than secondary tumours (London and Thamm 2020). Buffy coat evaluation are not routinely performed, as mastocythemia is associated with systemic inflammation rather than mastocytosis (McManus 1999). Similarly, the 2.8% prevalence of bone marrow infiltrations makes bone marrow aspiration low yield in cases without haematological abnormalities (Endicott et al., 2007).

As mitotic count only identifies cells in mitosis, evaluation of Ki-67 and AgNORs are more superior at assessing cellular proliferation, and predicting survival time, metastasis, and disease-free intervals (Bostock et al., 1989; Sledge et al., 2016; Smith et al., 2017; Webster et al., 2007). Ki-67 evaluates cells growth fraction irrespective of their phases, while AgNOR estimates the rate of those cells moving through the cycle (Sledge et al., 2016). Dogs with Ki-67 index more than 23 and AgNOR x Ki-67 score more than 54 are at increased risk of metastasis and mortality with 60% of patients dead within 1 year of presentation (Sledge et al., 2016; Webster et al., 2007). Despite the unknown molecular aetiology of MCTs, c-KIT gene mutations (in exons 8 and 11) are found in 25-30% of intermediate to high grade tumours, and are associated with poorer survival (Downing et al., 2002; Gil da Costa 2015; Letard et al., 2008; Webster et al., 2007). Mutated c-KIT results in receptor tyrosine kinase KIT independent activation leading to MCT proliferation, differentiation and maturation (Sledge et al., 2016; Webster et al., 2007). KIT protein can be localised into three patterns: 1) perimembrane, 2) focal or stippled cytoplasmic, 3) diffuse cytoplasmic pattern (Sledge et al., 2016; Webster et al., 2007). Dogs with c-KIT mutation and aberrant KIT localisation patterns (pattern II and III) are associated with worse prognosis (Sledge et al., 2016; Webster et al., 2007).

Although it has been hypothesised that tyrosine kinase inhibitors are preferred in dogs with c-KIT mutation or aberrant KIT pattern, Weishaar et al. (2018) suggested that no significant differences in progression-free and overall survival were seen between tyrosine kinase inhibitor (toceranib) and traditional chemotherapy (vinblastine). Vinblastine was also associated with less adverse effects than toceranib (89% versus 93%), and may be preferred (Weishaar et al., 2018). Despite better response, the addition of lomustine is associated with increased toxicity (28% hepatotoxicity, 33% severe neutropenia and 54% myelosuppression) compared to protocols without lomustine (6-20% myelosuppression and gastrointestinal toxicity) (Hosoya et al., 2009; Rassnick et al., 1999). Therefore, vinblastine and prednisolone were selected based on the owner's preference to minimise toxicity.

In our case, the total survival time of a dog with high grade, poorly differentiated MCT was 90 days from presentation. This is similar to the median survival time reported in the two-tiered grading system (less than 4 months) (Kiupel et al., 2011), Patnaik et al. (1984) (6% surviving more than 1500 days) and Bostock (1973) (13% surviving past 210 days). However, survival time was shorter than expected given the literature reported dogs with KIT pattern II had an overall survival time of 19 months (Webster et al., 2008). Similarly, the short survival time in this case was incongruent with AgNOR x Ki-67 and Ki-67 indices, as dogs with scores lower than the cut-off points should have survived longer, and 90% of dogs with less than 23 Ki-67 positive cells per grid expected to survive beyond 3 years (Webster et al., 2007). As such, the short survival time in this case study was unexpected given that the histological grading has a strong correlation with Ki-67 and AgNOR (all $P < 0.0001$) (Bergman et al., 2004). The discrepancy in actual survival time in this case may be explained by limitations in the methodology such as preparation, counting technique, fields examined, and microscope field of vision; all of which can confound evaluation of proliferation markers (Sledge et al., 2016). Another limitation was that a necropsy was not performed, it was not possible to deduce whether the dog died from MCT or other causes of death. Additionally, as multiple factors influence patient outcome, it is vital that different prognostic factors are evaluated together to accurately estimate the survival time. For the purpose of this report, we did not focus on other prognostic factors. For future references, more multivariate analysis studies are needed to simultaneously identify the correlation between multiple factors and survival time.

In conclusion, this case report demonstrated that the actual survival time closely aligned with the published estimated survival time based on histological grading. Therefore, histological grading is undeniably an important prognostic factor in determining the clinical outcome and survival of canine MCT. Moreover, it is recommended to perform Ki-67, AgNOR, and immuno-histochemistry labelling for KIT protein localisation pattern to evaluate multiple prognostic variables for survival analysis. Further research into these prognostic tools can provide a better indicator of survival estimate, which could be used to supplement clinical judgment and treatment decisions in canine MCTs.

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Appendix A - Prognostic factors for mast cell tumours in dogs (London and Thamm 2020)

Factor	Comment
Histologic grade	Strongly predictive of outcome. Dogs with undifferentiated tumors typically die of their disease after local therapy alone, whereas those with well-differentiated tumors are usually cured with appropriate local therapy.
Clinical stage	Stages 0 and 1, confined to the skin without local lymph node or distant metastasis, have a better prognosis than higher-stage disease.
Location	Subungual, oral, and other mucous membrane sites are associated with more high-grade tumors and worse prognosis. Preputial and scrotal tumors are also associated with a worse prognosis. Subcutaneous tumors have a better prognosis. Visceral or bone marrow disease usually carries a grave prognosis.
Cell proliferation rate	Mitotic index, relative frequency of AgNORs, and percent PCNA or Ki-67 immunopositivity are predictive of postsurgical outcome.
Growth rate	MCTs that remain localized and are present for prolonged periods of time (months or years) without significant change are usually benign.
Microvessel density	Increased microvessel density is associated with higher grade, a higher degree of invasiveness, and a worse prognosis.
Recurrence	Local recurrence after surgical excision may carry a more guarded prognosis.
Systemic signs	The presence of systemic illness (e.g. hyporexia, vomiting, melena, GI ulceration) may be associated with a higher stage of disease.
Age	Older dogs may have shorter median disease-free intervals when treated with radiation therapy than younger dogs.
Breed	MCTs in boxers (and potentially other brachycephalic breeds) tend to be of low or intermediate grade and are thus associated with a better prognosis.
Sex	Male dogs had a shorter survival time than female dogs when treated with chemotherapy.
Tumour size	Large tumors may be associated with a worse prognosis after surgical removal and/or radiation therapy.
c-kit mutation	The presence of an activating mutation in the c-kit gene is associated with a worse prognosis.
DNA copy number variation	Higher CNVs are observed in tumors of higher grade and those with a worse prognosis.

Appendix B - Grading

Two-tier histologic grading (Kiupel et al., 2011)

Two-tier histologic criteria (High grade has one of the following criteria)	Low grade	High grade
Mitotic figures in 10 HPF	≤ 6	≥ 7
Multinucleated cells in 10 HPF	≤ 3	≥ 3
Bizarre nuclei in 10 HPF	≤ 3	≥ 3
Karyomegaly and anisokaryosis	-	Nuclear diameters of at least 10% of neoplastic cells varying by at least two times

Three-tier histologic classification of MCT (Bostock 1973; London and Thamm 2020; Patnaik et al., 1984)

Grade	Bostock Grading	Patnaik Grading	Microscopic Description
Anaplastic, undifferentiated (high grade)	1	3	Highly cellular, undifferentiated cytoplasmic boundaries, irregular size and shape of nuclei; frequent mitoses, sparse cytoplasmic granules
Intermediate grade	2	2	Cells closely packed with indistinct cytoplasmic boundaries; nucleus-to-cytoplasmic ratio lower than anaplastic; infrequent mitoses; more granules than anaplastic
Well differentiated (low grade)	3	1	Clearly defined cytoplasmic boundaries with regular, spherical or ovoid nuclei, mitoses rare or absent; cytoplasmic granules large, deep staining, and abundant

Appendix C - WHO system of clinical staging of cutaneous and subcutaneous mast cell tumours in dogs (Owen 1980)

Stage	Description
0	One tumour incompletely excised from the dermis, identified histologically, without regional lymph node involvement a. Without systemic signs b. With systemic signs
I	One tumour confined to the dermis, without regional lymph node involvement a. Without systemic signs b. With systemic signs
II	One tumour confined to the dermis, with regional lymph node involvement a. Without systemic signs b. With systemic signs
III	Multiple dermal tumours; large, infiltrating tumours with or without regional lymph node involvement a. Without systemic signs b. With systemic signs
IV	Any tumour with distant metastasis, including bone marrow invasion and the presence of mast cells in the peripheral blood