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RESEARCH ARTICLE

Evaluation of Ki67, Bax, Bcl-2 and KIT in canine cutaneous mast cell tumours and their relationship with histopathology and prognosis

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ABSTRACT

Canine cutaneous mast cell tumours (CCMCTs) are common in dogs and exhibit many unpredictable behaviors. This study aimed to encourage pathology laboratories in developing countries to routinely assess prognosis by applying commonly used histopathological grading systems and immunohistochemistry (IHC) markers. We performed histological grading according to both the Patnaik and Kiupel systems, determined the mitotic count (MC) and carried out IHC for the detection of Ki67, Bax, Bcl-2 and KIT in 54 CCMCT cases. MC was associated with both grading systems in terms of survival following diagnosis and prognostic factors differed among cases categorized by the cut-off value of 5. KIT patterns were associated with grading systems and MC. The cohort with pattern II had a lower survival rate than those with patterns I and III. Ki67 was associated with survival when evaluated over the cut-off value of 0.018. Bax expression was associated with both grading systems. Median survival time was longer in patients with lower Bax expression level. Immunohistochemical detection of KIT, Ki67 and Bax improves histopathology in predicting the prognosis. If IHC is unavailable, reports regarding MC and values from both grading systems are the most effective, convenient and cost-effective way to provide the most reliable prognostic data and guidance for the clinicians.

KEYWORDS

canine cutaneous mast cell tumour, histopathological grading, Ki67, Bax, Bcl-2, KIT

INTRODUCTION

Canine cutaneous mast cell tumours (CCMCTs) are common in dogs and account for 7–21% of all neoplasms and 11–27% of canine malignant skin tumours (Macy, 1985; Preziosi et al., 2004). Two three-grade systems, established by Bostock (1973) and Patnaik et al. (1984) have been widely used for histopathological classification of CCMCTs in the past. However, since the current grading systems include both malignant and benign tumours in grade II (Northrup et al., 2005; Blackwood et al., 2012), a new two-stage approach has been proposed (Kiupel et al., 2011), who stated that these systems should be supported with other prognostic indicators in order to increase the accuracy.

Besides histological grading, the proliferation marker Ki67 is widely used as a prognostic indicator for CCMCTs (Vascellari et al., 2013). Bax expression has been explored as a biomarker in many types of neoplasia, and it is closely associated with tumour malignancy in CCMCTs, but further research is needed to better understand it as a prognostic indicator (Strefezzi et al., 2012). It is known that Bcl-2, an anti-apoptotic protein, is increased in patients with mastocytosis (Hartmann et al., 2003). Vascellari et al. (2013) have reported that there was no data on the possible role of Bcl-2 in CMCT progression and prognosis, and Bcl-2 protein was detected in CMCTs from various anatomical regions, albeit with no

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association between the health status and tumour grade. The KIT protein is a tyrosine kinase receptor produced by the c-kit proto-oncogene. Studies indicate that KIT protein is an important determinant in the prognosis of CMCTs (Preziosi et al., 2004; Webster et al., 2007; Gil da Costa et al., 2007; Zemke et al., 2002; Kiupel et al., 2004). Further research is needed to support the relationship between the cellular location of the KIT protein in CCMCTs, and the two-tier grading system and prognosis (Kiupel et al., 2004).

The mitotic count (MC) is an indirect measure of cell proliferation and is recommended as a strong indicator in various human and canine tumours. Although the prognostic importance of MC has been demonstrated in many studies on CCMCTs, there are still difficulties in adapting this information to the diagnostic setting, such as identifying a standardised cut-off value (Elston et al., 2009; Vascellari et al., 2013).

In the past, there has been proof of enhancement in CCMCT diagnosis and prognosis with the help of auxiliary tests and treatments in developed countries such as the USA and the countries of EU. Yet, there is no routine application of these techniques by most Turkish laboratories. Similarly, no studies have been conducted in Türkiye up to this date in order to investigate the prognostic approaches based on available histopathologic grading systems and other prognostic indicators in CCMTs. However, it would be beneficial to provide standardised reports for clinicians in order to have better insights into the prognosis. This study aims to promote a routine prognostic evaluation approach for pathology laboratories in developing contexts, especially the ones that routinely perform histopathology and can conduct routine immunohistochemistry (IHC) for CCMCTs. Through working on 54 CCMCT cases, the objectives of this study were to evaluate the Ki67, Bax, Bcl-2 and KIT markers in CCMCTs as prognostic indicators. Additionally we planned, to provide additional data on the association between these markers and the two grading systems and on the relation between the cellular location of the KIT protein and grading & prognosis. We also wanted to contribute to the standardisation efforts by evaluating with previously proposed MC values with the help of both grading systems in a different patient group.

MATERIALS AND METHODS

Sampling and data collection

A total of 64 cases of canine mast cell tumours (MCT) were examined from different parts of the skin obtained from dogs. Among these, 59 cases were chosen based on the following criteria: (1) developed for the first time, (2) confirmed as MCT by histological examination, (3) availability of patients' follow-up information and archived tissue samples were fixed in formalin and embedded in paraffin blocks. Subcutaneous MCTs ($n = 4$) and recurrence cases ($n = 1$) were excluded. As a result, 54 CCMCT cases were selected from the tissue archives of the Istanbul University – Cerrahpasa Faculty of Veterinary Medicine ($n = 35$) and private veterinary pathology laboratories in Istanbul ($n = 19$) for the study. Case diagnosis dates varied from January 2011 to February 2017. Approval from the ethics committee was obtained for the research (Istanbul University Animal Experiments Local Ethics Committee's decision dated 04/06/2015 and numbered 2015/55). Tumour biopsies and total extirpations were performed as part of the routine treatment procedure by the responsible veterinary surgeons. Only four patients received chemotherapy while two of them received radiotherapy.

The information (i.e. age, breed, gender, tumour localization, recurrence, death) of each case was gathered using patient files registered in the faculty hospital, interviews with the responsible private veterinarians of the patients and the owners. The patients were also followed up on six-month intervals throughout the study period. The most recent diagnosis was in February 2017, and the most recent follow-up was in June 2019.

Histopathological examination

Tumour tissues were fixed in 10% formalin solution before being embedded in paraffin blocks following routine procedures. Using a rotary microtome, 4–5 µm-thick sections were obtained. The dewaxed sections were then stained with haematoxylin and eosin (HE) and examined under a light microscope. The blocks from each case were examined, and the block that best represented the tumour was chosen. Consecutive sections from the same block were used in both histopathological and IHC studies. Histopathological examination was performed by two pathologists (IAD and AG). The tumours were classified using both the Patnaik's three-grade (Patnaik et al., 1984) and the Kiupel's two-grade (Kiupel et al., 2011) systems. Additionally, MC was also determined and the values were grouped according to the cut-off value of 5, as suggested by Romansik et al. (2007).

Immunohistochemical analyses

IHC was performed on all tissues, using the labelled streptavidin-biotin-peroxidase technique. The tissue sections were taken on positively charged slides, deparaffinised in xylene, gradually rehydrated in alcohol, then washed with phosphate buffered saline (PBS). For antigen retrieval, the slides were boiled in citrate buffer solution in a microwave oven at 800 W for 15 min, then allowed to cool (approximately 20 min), and washed for 2 × 5 min. Finally, they were incubated in 3% hydrogen peroxide for 15 min and pretreatment was completed by washing for 2 × 5 min. A labelled streptavidin-biotin-peroxidase based IHC kit (Millipore, IHC Select® HRP/DAB, USA) was used for the staining procedure according to the manufacturer's manual. The primary antibodies for Bax (polyclonal/rabbit, NB100-5698, Novusbio, dilution: 1:2000, incubation: 90 min/room temperature), Bcl-2 (polyclonal/rabbit, NB100-56098, Novusbio dilution: 1:2000, incubation: 120 min/room temperature), Ki67 (monoclonal/rabbit, NB600-1252, Novusbio, dilution: 1:50, incubation: 120 min/room temperature) and

KIT (polyclonal/rabbit, PA5-16770, ThermoFisher, dilution: 1:200, incubation: 90 min/room temperature) were used in a humid chamber. All antibodies were reactive for canine tissues according to the manufacturer's datasheets.

For the detection of KIT, Bax and Bcl-2, the 3,3′-diaminobenzidine (DAB) procedure was used. After routine haematoxylin staining, the slides were passed through graded alcohols, dehydrated, dried, and kept in xylene for 15 min, then mounted with Entellan (Merck Millipore) and a coverslip. For Ki67, the 3-amino-9-ethylcarbazole (AEC) (Thermo Scientific, UltraVision Detection System) was used as the chromogen. After routine haematoxylin staining, the slides were mounted using Vison Mount (Thermo Scientific). The specificity of the immunolabelling was confirmed by incubating sections with PBS instead of the specified primary antibody. While optimizing the immunostaining processes, canine hyperplastic lymph node tissue for Ki67 and Bcl-2, tumour tissues previously diagnosed as CCMCT for CD117 and mammary carcinoma for Bax were employed as positive control. IHC was not possible for one case as the preparation fell apart during the process. Therefore, IHC studies were evaluated on 53 cases.

After checking the control tissues, the IHC reactions were evaluated by scanning all areas with a light microscope. Evaluation of Bax and Bcl-2 expression was carried out by examining 10 high-power fields (HPFs, 40 × objective) with qualitative and quantitative methods, according to Strefezzi et al. (2012). The Ki67 index was calculated by counting cells in 3–10 HPFs (40× objective) with intense Ki67-positive staining in more than 500 cells, depending on the size of the tumour section, and then by calculating their percentage, according to Ozaki et al. (2007). The KIT staining pattern was evaluated as per Kiupel et al. (2004).

Statistical analysis

The variables were investigated using visual (histograms, probability plots) and analytical methods (Kolmogrov-Simirnov/Shapiro Wilk test) in order to determine whether there was a normal distribution or not. The homogeneous distribution of the variances of the data was checked with the Levene test. Chi-square tests were used for qualitative data. In the case of comparing more than two independent groups in numerical data, one-way variance analysis was used if there was a homogeneous distribution of variances, otherwise, it was examined with the Kruskal-Wallis test. While investigating the association between non-normally distributed and ordinal variables, the correlation coefficients and their significance were calculated using the Spearman test.

Survival analyses were performed using the Kaplan-Meier analysis method, followed by a log-rank test. For the calculation of survival times (survival after diagnosis), the onset of the disease was taken as the date when the tumour was first diagnosed. Survival times of the cases were calculated by considering the time between the diagnosis of the tumour until the death of the patients or the end of the patient follow-up period of the study (last follow-up time: June 30, 2019). Patients that were treated with methods other than surgery (i.e. chemotherapy or radiotherapy) and patients with unavailable survival data were excluded from the survival analysis. Patients that could not be followed up or died from other causes were censored during this calculation. However, these cases were still included in other analyses such as histopathological findings unrelated to survival after diagnosis. Since the evaluation was made according to the available data, the number of groups in the results varied. 5% type-I error level was used to infer statistical significance. Statistical analysis of the data was evaluated with IBM® SPSS® (V. 21, Armonk, NY, USA) program.

RESULTS

Clinical findings

During the follow-up period, 17 patients (31.5%) have died. 26 patients (48.1%) are still alive, while 11 patients (20.4%) could not be followed up due to the loss of communication with the owners. Twelve individuals (71%) died due to MCT or complications related to MCT, while five (29%) died from other causes. In 32 patients (59.3%), there was no recurrence, while the tumour recurred in 12 patients (22.2%). Since 10 patients (18.5%) could not be followed up, no information on recurrence is available for them. Four patients received chemotherapy and two of them received radiotherapy. The mean age of onset was 8.4 years, while the earliest age of onset was 1.5 and 5.5 years in female and male individuals, respectively. The golden retriever was the most common breed (n = 19, 35.2%), followed by cocker (n = 4, 7.4%) and terrier (n = 4, 7.4%). Of the six crossbreeds (11.2%), one was a boxer mix and one was a husky mix. There were 26 females and 28 males in the group. Nine females and seven males were sterilized, while three females and eight males were not. The status of the remaining 14 females and 13 males is unknown. In general, gender or sterilization status were not associated with the survival time (P = 0.29 and P = 0.56 respectively). Tumour localizations were examined in four groups: (a) head-neck (n = 12), (b) extremities (n = 22), (c) trunk (n = 20), (d) perineal-inguinal region (n = 3), with the average survival times of 728, 2077, 1793 and 2292 days, respectively. There was no difference in survival time according to the region (P = 0.18). However, compared to other groups, the average survival time of patients with tumours in the head and neck region was quite low. Data is available on the survival and recurrence of 38 patients. The average survival time of patients with recurrence was significantly lower than that of patients without recurrence (1099 and 2054 days, respectively, P = 0.001, Fig. 1a).

Histopathological findings

According to the Patnaik grading system, 22 cases (41%) were grade I, 26 cases (48%) were grade II. and 6 (11%) cases were grade III CCMCTs. Only grade I and II tumours could

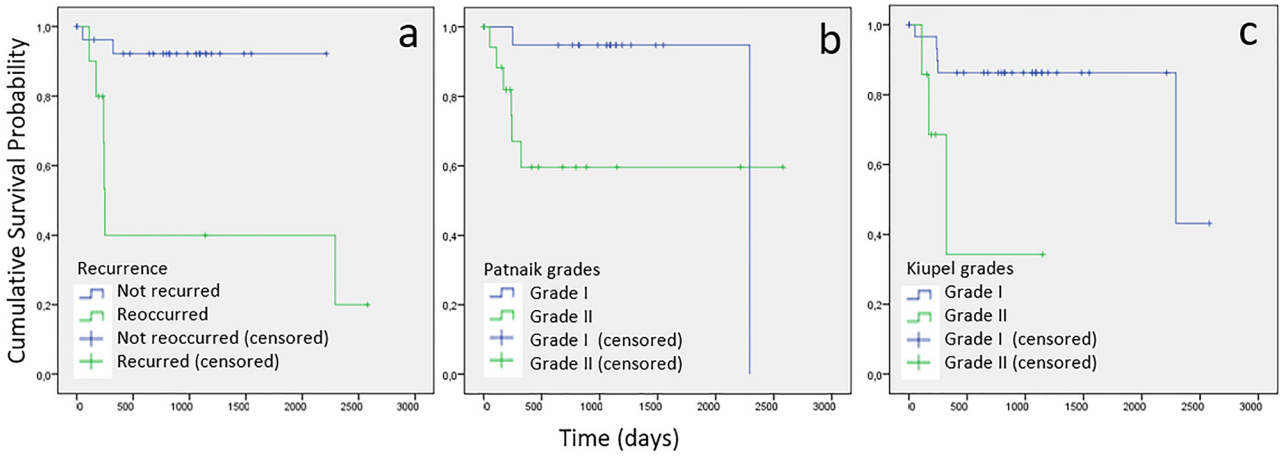

Fig. 1. Survival curves of canine cutaneous mast cell carcinoma cases (Kaplan-Meier analysis followed by a log-rank test) **a:** according to recurrence ($\chi^2 = 11.10$; $P = 0.001$); **b:** according to Patnaik grades I and II ($\chi^2 = 4.08$; $P = 0.043$); **c**: according to the Kiupel grades. ($\chi^2 = 6.68$; $P = 0.01$)

be included in the survival analysis based on the available data of 38 patients who did not receive chemotherapy or radiotherapy. Survival time differed ($P = 0.043$) between grade I (2,184 days) and grade II (1,616 days) tumours (Fig. 1b). According to the Kiupel grading system, 37 cases (69%) were evaluated as low and 17 cases (31%) as high-grade MCT. All Patnaik grade I cases ($n = 22$) were graded low and all Patnaik grade III cases ($n = 6$) were graded high according to the Kiupel grading system. Among Patnaik grade II tumours ($n = 26$), 42.3% ($n = 11$) were graded as high and 57.7% ($n = 15$) as low grade according to the Kiupel system. There was no association between the Kiupel grading and tumour recurrence ($P = 0.135$). There was a significant difference in terms of survival between the Kiupel low-graded (2,126 days) and high-graded (549 days) CCMCT groups ($P = 0.01$) (Fig. 1c). The mean MC for the Patnaik-graded tumours were 0.86 for grade I (in the range of 0–3), 8.7 for grade II (in the range of 2–16), 28.3 for grade III (in the range of 22–40) with a significant difference between the groups ($P < 0.001$). The mean MC values of the tumours, graded by the Kiupel method were 2.1 (range 0–6) for low-grade and 19.7 (range 8–40) for high-grade groups. The mean MC values were associated with high Kiupel grade ($P < 0.001$, $U = 629$).

The MC values were also grouped as MC ≤ 5 ($n = 35$) and MC > 5 ($n = 19$) according to Romansik et al. (2007). Patients with complete survival data and treated only with surgery ($n = 38$) were evaluated. The cut-off value of 5 for MC showed a significant difference in survival between the groups ($P < 0.001$) (Fig. 2a). The mean life expectancy was

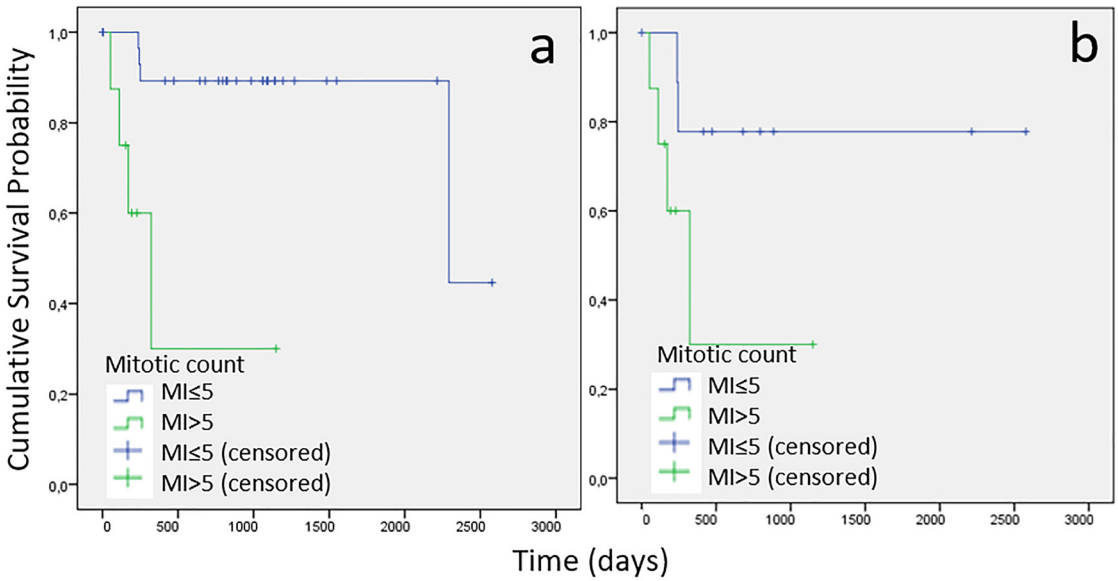

Fig. 2. Survival curves of canine cutaneous mast cell carcinoma cases (Kaplan-Meier analysis followed by a log-rank test) **a:** according to mitotic count (cut-off value of 5) amongst all cases ($\chi^2 = 13.68$; $P < 0.001$); **b:** according to mitotic count (cut-off value of 5) amongst Patnaik grade II cases ($\chi^2 = 3.76$; $P = 0.052$)

73 months in patients with MC ≤ 5 and 16 months in patients with MC > 5, and the median life expectancy was 76 months and 11 months, respectively. Survival in Patnaik grade II cases and MC (based on cut-off value 5) were also statistically associated ($P = 0.052$) (Fig. 2b). The mean life expectancy after diagnosis was 69 months in the MC ≤ 5 group and 16 months in the MC > 5 group. Upon grouping the cases according to the cut-off value 5 with regard to MC, a high association was observed in terms of mortality in general ($\chi^2 = 9.99$; $P = 0.001$). For Patnaik grade II tumours, no significant difference was found between MC groups and in terms of mortality ($\chi^2 = 3.23$; $P = 0.07$). The results of all statistical studies regarding the histopathological findings are summarised in Appendix Table A1.

Immunohistochemical findings

The expression of KIT protein was positive in all 53 cases stained with IHC. Staining pattern I (Fig. 4a) was determined in 17 patients (32%), pattern II (Fig. 4b) in 20 patients (38%), and pattern III in 16 patients (30%). Within the recurred cases ($n = 13$), pattern I was observed in 2 (15.4%), pattern II in 6 (46.2%) and pattern III in 5 (38.5%) cases. There was no significant difference ($P > 0.05$) between the KIT pattern and recurrence. There was a strong association between KIT patterns and both Patnaik ($P = 0.002$) and Kiupel ($P = 0.005$) grading. There was no statistically significant difference between KIT patterns and survival times ($P = 0.22$, Fig. 3a). However, the mean survival times after diagnosis in KIT patterns I and III were 1909 and 2216 days, respectively, while in pattern II was much shorter (640 days). Ki67 IHC was negative in one case. In all positively stained slides, the intranuclear immune reaction was seen in tumour cells. Ki67 index is evaluated over the cut-off value of 0.018 as previously suggested by Scase et al. (2006). Survival and the Ki67 index had a statistical relationship ($P = 0.012$, Fig. 3b). A significant correlation was found between the Ki67 index and both the Patnaik ($P < 0.001$) and the Kiupel ($P < 0.001$) grades. There was no significant association between the expression of Ki67 and the recurrence data ($P = 0.85$).

The Bcl-2 protein expression was found in the cytoplasm of the tumour mast cells. No significant association was seen between the results of the Bcl-2 reaction and the survival time after diagnosis ($P = 0.55$, Fig. 3c), MC ($P = 0.41$), Patnaik ($P = 0.23$) and Kiupel ($P = 0.56$) graded groups or the recurrence ($P = 0.74$).

High Bax expression (Fig. 4c) was seen in 33 (62.3%) cases, while low Bax expression was found in 20 (37.7%) tumours. There was no Patnaik grade III tumour and just one Kiupel high-grade tumour in the group with low Bax expression. There was statistical significance between Bax expression and the Patnaik grading system ($P = 0.005$), the Kiupel grading system ($P = 0.003$) and the average survival time after diagnosis ($P = 0.11$, Fig. 3d). Information summarizing the statistical studies on the IHC findings is presented in Appendix Table A2.

DISCUSSION

There was a consistency between the literature and our study with regard to the breeds with CCMCTs (London, 2010; Leblanc 2016; Romansik et al., 2007). When evaluating the breeds, it should be considered that golden retrievers are extremely popular as companion dogs in Türkiye. In this study, sterilization status was not associated with survival. White et al. (2011) have regarded sterilization to be a risk factor, while Reynolds et al. (2019) have stated that neither gender nor sterilization affected the tumour grade. It will be useful for future prognostic research to collect sterilization data and comment on more cases. Previous studies have reported that the tumour site does not affect the prognosis (Cahalane et al., 2004; Kiupel et al., 2005; Sfiligoi et al., 2005). Reynolds et al. (2019), on the other hand, have claimed that high-grade tumour development is more common in the inguinal and the head regions. According to Kiupel et al. (2005), CCMCTs in the head and neck region present a higher risk for developing new MCTs in distant regions. The majority of these studies have not distinguished between cutaneous and subcutaneous MCT. Our study only focused on CMCTs, and there was no statistical significance of the localization with the survival data in general. However, patients with head and neck tumours had shorter survival times than the others, which supports findings of Reynolds et al. (2019) and Kiupel et al. (2005).

MC for both grading systems and cut-off value of 5 for Patnaik grade II tumours demonstrated a substantial prognostic value that is consistent with the literature. The last consensus document of the Oncology-Pathology Working Group suggests that histopathologic CCMCT reports should contain the results and criteria of both grading systems, as well as standardised MCs at 10 HPFs, since it is easy to count on HE preparations, it does not cause additional expense to the owner of the patient yet has a prognostic value (Berlato et al., 2021). We strongly support this suggestion and encourage all veterinary pathology laboratories to include a short description and information on both grading systems. We recommend the cut-off value of 5 in histopathology reports for the guidance of the clinicians and as a base for the efforts to standardise the prognostic evaluation of CCMCTs.

Reguera et al. (2000) have reported a negative correlation between cellular differentiation and KIT expression. Preziosi et al. (2004) have found a significant statistical correlation between the KIT pattern and the histological grade ($P < 0.001$) stating that the pattern determination is a more useful method than assessing the staining intensity. The diffuse cytoplasmic pattern is closely associated with grade I tumours, the cytoplasmic perinuclear pattern is seen only in grade III tumours whereas the membranous and membranous/perinuclear patterns are seen in grade II tumours. Kiupel et al. (2004) have evaluated MCTs according to the Patnaik system and classified the KIT expression pattern of most tumours as pattern I (42.9%) or pattern II (43.9%). While patterns II and III were associated with decreased survival, this was not the case for pattern I. Webster et al.

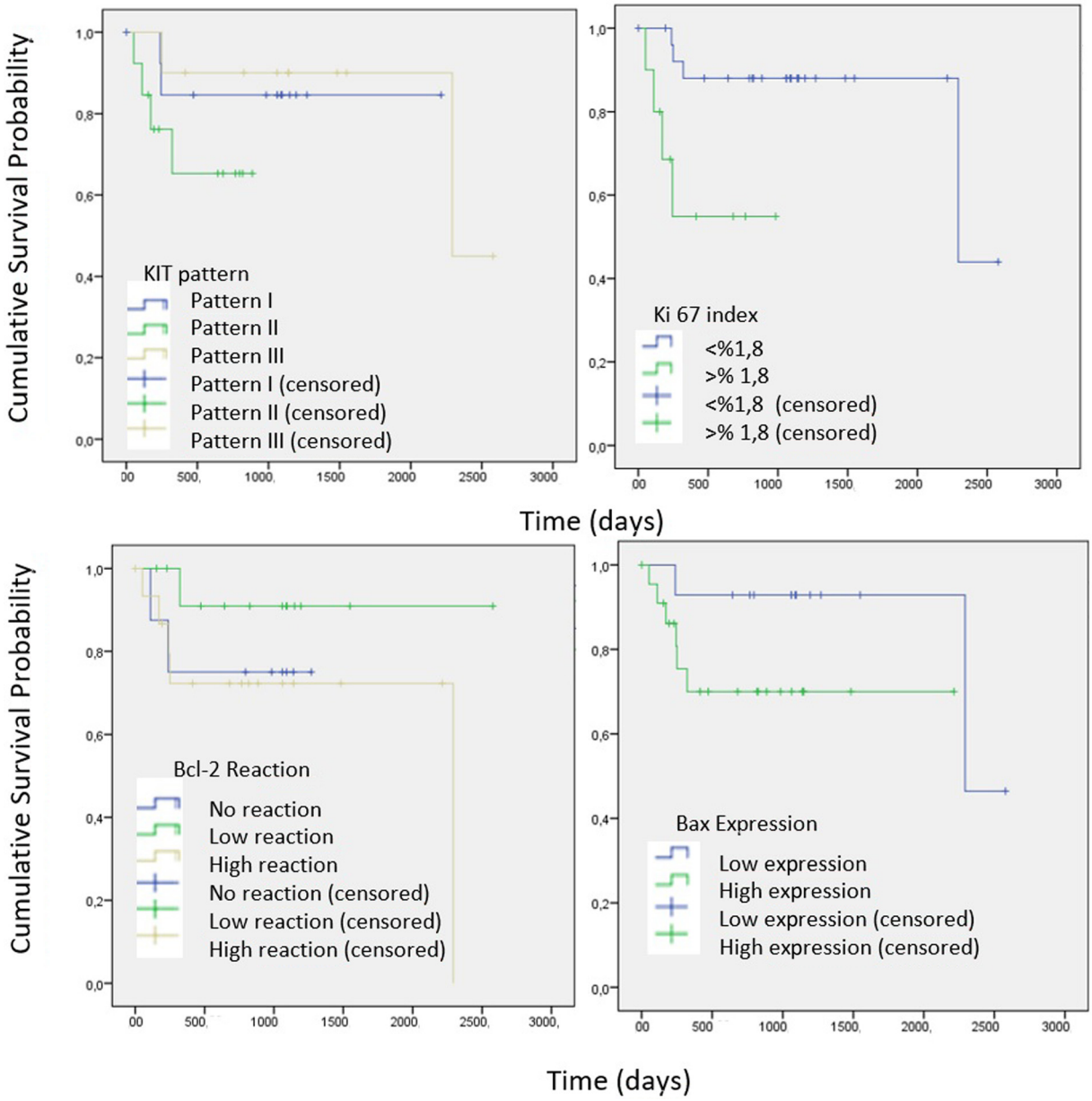

Fig. 3. Survival curves of canine cutaneous mast cell carcinoma cases (Kaplan-Meier analysis followed by a log-rank test) **a:** according to KIT patterns ($\chi^2 = 2.95$; $P = 0.22$); **b:** according to Ki67 index based on cut-off value of 1.8 ($\chi^2 = 6.31$; $P = 0.012$); **c:** according to Bcl-2 reaction ($\chi^2 = 3.41$; $P = 0.55$); **d:** according to Bax expression ($\chi^2 = 2.5$; $P = 0.11$)

(2006) have noted a significant difference between the mutation and increased cytoplasmic localization. According to the Oncology-Pathology Working Group's consensus document, KIT localization may be the most useful marker in most "indeterminate" tumours from a histological perspective (Berlato et al., 2021). The *c-kit* mutation can be used in the detection of histologically low-grade but biologically aggressive tumours, and membrane localization can be used to identify tumours with a low probability of progressive disease (Thamm et al., 2019). Vucicevic et al. (2018), on the other hand, have indicated that all low-grade tumours exhibited membrane-located KIT expression pattern, while the majority of high-grade tumours showed cytoplasmic expression. In our study, there was a significant difference between KIT staining patterns and the Patnaik ($P = 0.002$) and Kiupel grades ($P = 0.005$). The absence of Pattern I in Patnaik grade III tumours and the fact that there was only one Pattern I case in the Kiupel high-grade tumour group support the previous studies.

Previous research has found a link between shortened life expectancy and cytoplasmic-perinuclear KIT pattern (Morini et al., 2004), and increased cytoplasmic KIT staining and increased local recurrence and decreased survival time (Kiupel et al., 2004). Although there was no significant relationship between the KIT expression patterns in tumour cells and survival time after diagnosis in our study, the fact that the patients with cytoplasmic perinuclear pattern (Pattern II) had a very short survival time compared to

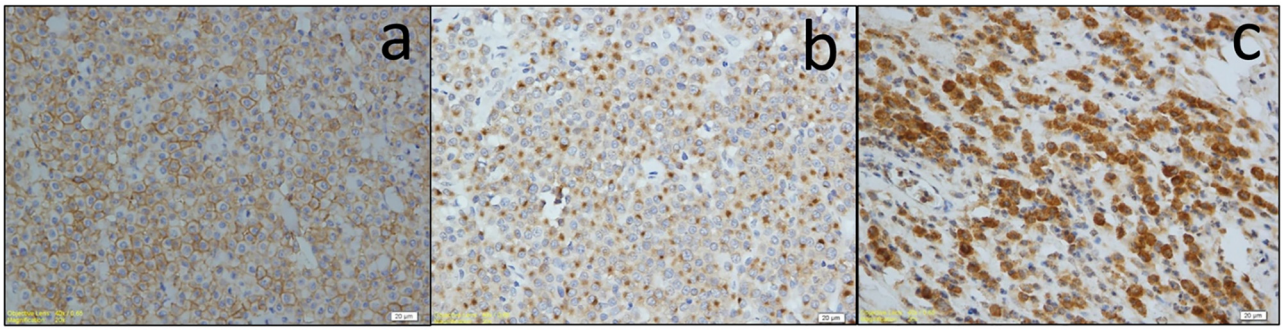

Fig. 4. Immuno-histochemical detection of various tumour markers in tissues from canine cutaneous mast cell carcinoma cases. **a:** Case no. 3, KIT staining pattern I, DAB. 40 ×, Bar: 20 µm; **b:** Case no. 7, KIT staining pattern II, DAB. 40 ×, Bar: 20 µm, **c:** Case no. 27, high Bax expression, DAB, 40 ×, Bar: 20 µm

others supports the previous data. The significant difference between KIT localization patterns and MC data, particularly, Pattern II's high MC in comparison with the other groups, supports the hypothesis of Webster et al. (2007) according to which abnormal KIT localization may be associated with increased cellular proliferation.

Sakai et al. (2002) have reported a significant difference in the Ki67 index between Patnaik grade I and grade II, and Patnaik grade II and III tumour groups. When Patnaik grade I and III tumours ($P = 0.024$), grade I and II tumours ($P = 0.092$), and Kiupel low and high-grade tumours ($P = 0.002$) were examined, Vascellari et al. (2013) have found a strong association between Ki67 expression and grade. Berlato et al. (2013) have not found a significant relationship between the Patnaik grades and the Ki67 index. Kandefer-Gola et al. (2015) have reported no reaction with Ki67 in 14 (23.3%) of 60 MCT cases along with a strong positive correlation between Ki67 expression and the results of Patnaik and Kiupel grading systems ($R = 0.63$ and $R = 0.85$, respectively). Only one of the 53 cases was negative for Ki67 in ICH in our study. Additionally, we found significant differences between the Ki67 index and both the Patnaik ($P < 0.001$) and the Kiupel grades ($P < 0.001$). The Ki67 counts are determined by various methods (Sakai et al., 2002; Scase et al., 2006; Ozaki et al., 2007; Webster et al., 2007; Maglennon et al., 2008; Vascellari et al., 2013; Berlato et al., 2013; Kandefer-Gola et al., 2015; Berlato et al., 2018) and a similar case is true for the determination of the cut-off value of cells expressing Ki67 (Webster, 2007; Vascellari et al., 2013; Scase et al., 2006). In our study, we preferred the method of Ozaki et al. (2007) due to its easy applicability. The methodological differences lead the different cut-off values in various studies and the lack of standardisation makes the comparison and interpretation of the results difficult (Vascellari et al., 2013; Berlato et al., 2013). Due to the small number of cases in each group, we used a previously provided (Scase et al., 2006) and further evaluated (Berlato et al., 2013, Berlato et al., 2018) Ki67 threshold for Patnaik grade II tumours all cases. The results showed that the cut-off value of 0.018 may be used as a prognostic value in veterinary pathology reports concerning life expectancy regardless of the Patnaik or Kiupel grades.

Vascellari et al. (2013) have found no correlation between the detection of Bcl-2 by IHC staining and the results of the grading systems or health status, while lower mRNA levels have been found in the tumour tissues by molecular analyses. According to Barra et al. (2017), high-grade tumours had lower Bcl-2 expression than the dual system, and the death rate from MCT increased six-fold, providing additional prognostic information to the two-grade approach. The three-grade system, on the other hand, revealed no significant difference. There was no significant link between the results of IHC staining Bcl-2 and either grading systems or prognosis in our study.

Strefezzi et al. (2012) have reported a significant correlation between Bax expression and the Patnaik grades. Barra et al. (2017) have observed that expression of Bax was significantly higher in Patnaik grade III tumours than in grade II tumours and Kiupel high-grade tumours compared to low-grade tumours. Similarly, we found a close association between the Bax expression and both grading systems. Previous research has shown that Bax is a good prognostic tool since its high expression is directly related to the death due to MCTs (Strefezzi et al., 2012; Barra et al., 2017). In our study, there was no significant difference between the cases with low and high Bax expression in terms of survival times after diagnosis. Although there are numerous hypotheses, further molecular research is required to understand the increased Bax expression in CCMCTs.

One of the objectives of our study was to provide clinicians with a better prognostic interpretation of CCMCTs under different circumstances. IHC can be applied manually, routinely, and successfully, and the results can be supported by other techniques such as polymerase chain reaction (PCR) in well equipped laboratories. Flores et al. (2016) sought a similar standardisation in Brazil for KIT and Ki67 studies in MCT cases and concluded that, if appropriately optimized, the combination of histopathology and IHC together is easily applicable. In order to be more efficient in terms of patient follow-up and to establish a common database of pathological samples not only for histopathology, but also for PCR to better evaluate the oncological aspects, it is critical for future studies to organize the patient information systems of veterinary faculties for

cancer research in companion animals in Türkiye. Standardisation studies are of great importance in veterinary pathology and inter-institutional standardisation can be accomplished as a first step, followed by the establishment of a national tumour tissue bank network.

The survival time after diagnosis can be a biased indication of the natural course of events, especially when cases are treated with extensive surgical excision. However, because there is no way to leave the animal without sufficient treatment, this is the strategy most researchers utilize. Another bias could be the interpretation of the date of diagnosis as the commencement of the disease, especially when different tumour grades and development rates are considered.

In conclusion, in terms of survival time following diagnosis, our data revealed a substantial link between MC and both the Patnaik and Kiupel grading systems. Furthermore, there were significant differences in the prognostic factors such as survival and mortality among the cases categorized according to the MC cut-off value of 5. There was a significant association between KIT patterns, Patnaik and Kiupel grading systems,and MC values. Although no statistically significant difference in the survival time was detected, life expectancy in the KIT II pattern was found to be lower than in the patterns I and III. When the Ki67 index data were evaluated over a cut-off value of 0.018, it was found to be associated with life expectancy. The Bcl-2 reaction was not associated with survival time after diagnosis. Although not statistically significant, Bax expression was associated with the results of both grading systems, and the average life expectancy after diagnosis was longer in cases with low Bax expression. As a result, the use of KIT, Ki67 and Bax IHC as a complement in predicting poor prognosis was validated. If IHC is not possible, evaluation of MC values standardised with the Patnaik and Kiupel grading systems on histopathology preparations for each tumour will provide the clinicians with the most reliable data in terms of recommending treatment. KIT and Bax IHC can be a valuable support in the prognosis.

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Appendix

Table A1. Summary of the statistical findings on histopathology results

	Patnaik grades	Kiupel grades	Patnaik grade II tumors	MC	MC (based on cut-off value 5)
Recurrence	$P = 0.10$ $\chi^2 = 4.3$	$P = 0.14$ $\chi^2 = 2.1$	–	$P = 0.18$ $t = 1.36$	–
Survival time after diagnosis	$P = 0.043$ $\chi^2 = 4.8$	$P = 0.01$ $\chi^2 = 6.68$	–	–	$P < 0.001$ $\chi^2 = 13.68$
Age	$P = 0.41$ F = 0.9	$P = 0.93$ $t = -0.79$	–	–	–
MC	$P < 0.001$	$P < 0.001$	–	–	–
MC (cut-off value 5)	–	–	$P = 0.052$ $\chi^2 = 3.76$		

Table A2. Summary of the statistical studies on immune-histochemical findings

	KIT Pattern	Ki67 Index	Ki67 Index (based on the cut-off value 0,018)	Bcl-2	Bax
Patnaik grades	$P = 0.002$ $\chi^2 = 15.33$	$P < 0.001$ KW = 19.56	–	$P = 0.23$ $\chi^2 = 5.34$	$P = 0.005$ $\chi^2 = 9.73$
Kiupel grades	$P = 0.005$ $\chi^2 = 10.18$	$P < 0.001$ U = 480	–	$P = 0.56$ $\chi^2 = 1.33$	$P = 0.003$ $\chi^2 = 8.90$
Survival time after diagnosis	$P = 0.22$ $\chi^2 = 2.95$	–	$P = 0.012$ $\chi^2 = 6.31$	$P = 0.55$ $\chi^2 = 3.41$	$P = 0.11$ $\chi^2 = 2.5$
MI	$P = 0.017$ KW = 8.12	$P < 0.001$ R = 0.52	–	$P = 0.41$ $\chi^2 = 0.90$	$P = 0.0001$ U = 512.5
MC (cut-off value 5)	–	–	–	–	–
Recurrence	$P = 0.28$ $\chi^2 = 2.66$	$P = 0.85$ $t = 0.18$	–	$P = 0.74$ $\chi^2 = 0.90$	$P = 0.98$ $\chi^2 = 0.001$