Original Article

Proliferative Index and Microvessel Density as Potential Prognostic Markers of Canine Oral Malignant Melanomas

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Abstract

Several reports have indicated the prognostic significance of the proliferative index (PI) and microvessel density (MVD) in canine oral malignant melanomas (MM). However, a correlation between the PI and MVD of oral MM in dogs has never been elucidated. Here, we evaluated the expression of Ki-67 and CD31 using double immunofluorescence staining to investigate the correlation of PI with MVD in 37 spontaneous canine oral MMs. No correlation was found between the MVD and PI values or between those and any clinicopathologic findings. However, a correlation was found between the PI value and both clinical stage (p = 0.0453) and degree of tumour pigmentation (p = 0.0431). Further, the PI significantly correlated with the survival period of canine oral MM patients (p = 0.0209). The results of our study suggested that no relationship existed between the PI and MVD of canine oral MMs, and that the PI was a more reliable prognostic marker for canine oral MMs than MVD.

Keywords: canine, microvessel density, oral malignant melanoma, proliferative index

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Introduction

Among canine oral neoplasms, oral malignant melanoma (MM) is considered the most common oral malignancy in canine patients (Niemiec, 2008). A higher number of oral melanomas are found to be malignant when compared to the malignancy rates of other canine melanocytic tumours because of their local invasiveness and rapid onset of metastasis, which is similar to human oral or mucosal melanoma (Bergman, 2007). Further, primary oral MMs in canine patients are often found at the gingiva, and less frequently at the lingual, buccal, pharyngeal, tonsilla, and palatine epithelium (Smith et al., 2002).

Although various treatments, including surgical excision, radiation, chemotherapy, and immunotherapy (Bergman, 2007) are available, accurate prognosis and selection of an appropriate treatment regime for canine oral MM are reliant on precise clinical staging. In addition to metastasis to regional lymph nodes and peripheral organs, size of a primary tumour mass is an extremely important factor in clinical staging. However, limitations exist in tumour staging based on tumour size among different dog breeds (e.g. giant versus toy) because of the dissimilar anatomical sizes (Bergman, 2007). Accordingly, several prognostic parameters have been suggested as grading criteria for canine oral MM (Spangler and Kass, 2006). In general, a mitotic index of three or more per high power microscopy field is considered malignant for oral MM. However, this index does not always correlate with a poor outcome (Schultheiss, 2006; Spangler and Kass, 2006). Therefore, numerous markers have been evaluated as potential prognostic indicators of canine oral MM. Among the several parameters studied for possible prognostic indicators for tumour grading in canine melanoma, proliferative activity, which is detected using the Ki-67 monoclonal antibody, was reported to have a correlation with patient survival (Millanta et al., 2002).

In addition to proliferative activity, markers of tumour angiogenesis such as microvessel density (MVD) were recently reported to exhibit potential as prognostic indicators of several human (Weidner, 1995) and canine cancers (Restucci et al., 2003; Wolfesberger et al., 2008). Tumour angiogenesis plays a crucial role in proliferation and metastasis by providing nutrients and oxygen for metabolic tumour tissue (Hicklin and Ellis, 2005). Further, increased proliferative activity of tumour tissue has been found in areas enriched with capillary blood supply (Auerbach and Auerbach, 1982). Although tumour angiogenesis weakly correlated with survival period in canine melanocytic tumours, the MVD was higher in oral MM than in melanocytomas and MM in cutaneous areas (Cuitino et al., 2012). Consequently, tumour neovascularization could play a role in providing nutrition and oxygen to support the proliferative activity, and might be a prognostic indicator of canine oral MM. Nevertheless, there have been no reports on correlations between the proliferative index (PI) and MVD in canine oral MM. The objectives of this study were to examine the correlation between tumour proliferative activity and tumour angiogenesis by evaluating the expression of Ki-67- and CD31-positive vessels on the same tumour tissue area using the double immunofluorescence staining, and to determine the correlation of both parameters with prognostic outcomes in 37 canine patients with spontaneous oral MM.

Materials and Methods

Clinical samples: Forty-two spontaneous canine oral MM specimens were obtained from 37 dogs that underwent a biopsy or surgical resection at the Veterinary Medical Center, University of Tokyo, between May 2004 and November 2012. Patient signalment information, including breed, age, body weight and history of spaying or castration, and clinical information such as tumour location, TMN for clinical staging which was categorized by tumour size, metastatic status of regional lymph nodes and distant organs that examined through diagnostic imaging methods either radiography or computed tomography were recorded. Besides, other prognostic parameters, for example, survival time, disease-free interval and relapsing disease, were obtained from the medical records or from telephone interviews with the owners. Each patient was clinically staged according to the traditional world health organization TNM-based staging scheme for dogs with oral melanoma (Bergman, 2007).

Primary lesions of all canine oral MM obtained following a biopsy or surgical resection were fixed in 10% neutral buffered formalin. Routinely-prepared, 4-µm-thick tissue sections were stained with haematoxylin and eosin (HE) for histopathology. A diagnosis of canine oral MM was made based on tissue histology. Tissue sections were examined microscopically and degree of pigmentation was scored using a scale of 0-3; 0 indicated less than 25%, 1 indicated 25-50%, 2 indicated 50-75%, and 3 indicated more than 75% of melanin pigmentation expressed on the tumour tissue under 10x objective field.

Immunofluorescence staining: To determine the correlation of tumour neovascularization with the PI in canine oral MM tissues, the series of 4-µm-thick sections were double immunofluorescence stained for Ki-67 expression using a mouse monoclonal anti-human Ki-67 antibody (1:100; Dako, Gostrup, Denmark), and for CD31 expression using a rabbit polyclonal anti-human CD31 antibody (1:50; Abcam; Cambridge, MA, USA). After dewaxing and rehydration in graded ethanol, antigen-retrieval for each sample was performed by heat treatment using an autoclave under the condition of 100°C for 10 min 10 mM sodium citrate buffer (pH 6.0). The samples were subsequently treated with 10% normal goat serum, and incubated for 1 h at room temperature. The samples were then incubated with both primary antibodies, mixed in 1.5% blocking serum, and stored at 4°C overnight. The slides were incubated with an appropriate secondary goat anti-rabbit FITC-conjugated antibody (1:200; Invitrogen, CA, USA), and a goat anti-mouse Cy3 conjugated antibody (1:100; Jackson ImmunoResearch, BA, USA). Absence of primary antibody was used as negative control, and canine intestinal and kidney tissues were used as

positive control for Ki-67 and CD31, respectively. To evaluate PI and MVD for each sample, five hot spot areas of CD31-positive vessels were primarily selected under a 200× objective field, and the separate CD31-positive vessels on the designated area were counted. The number of CD31 positive vessel/mm^2 was defined as the MVD value for each sample. Subsequently, an area within each designated hot spot was randomly selected under a high power field (400×), and the number of Ki-67-positive cells were counted. The PI was calculated as the percentage of Ki-67-positive cells per every 1,000 cells.

Statistical Analysis: The PI and MVD values were expressed as mean and standard error of the mean (SEM). Differences in the means corresponding to the PI and MVD values from each tumour tissue sample from the gingiva, mucosa, and palate were analysed by ANOVA and post-tested by Kruskal-Wallis test. Clinicopathological correlation of the PI and MVD values with clinical findings was evaluated using the Fisher's exact test, while the predicted survival periods of dogs with canine oral MM, based on the PI or MVD values, was evaluated using the Kaplan-Meier estimator and compared by the Log-Rank test. In all analyses, $p < 0.05$ was considered statistically significant.

Results

Clinical demographic data: Clinical demographic data from the 37 canine oral MM patients is presented in Table 1. Among the 37 dogs, 17 dogs were male and 20 dogs were female. Among the dogs included in the study, three had one or two tumour recurrences. Clinical stage was based on both the diameter of primary mass and the metastatic conditions. Fourteen dogs exhibited regional lymph node metastasis and nine dogs had metastasis in peripheral tissues. Accordingly, nine dogs were classified as stage I, seven dogs as stage II, twelve dogs as stage III, and nine dogs as stage IV.

Table 1 Clinical Demographics of the Canine Oral Malignant Melanoma Specimens

Clinical feature			Values		
No. of patients			37		
Age (year)			Median	Mean	Range
			12.3	11.7 ± 2.4	7.4–17.1
Sex					
	Female		17		
		Intact	8		
		Spayed	9		
	Male		20		
		Intact	12		
		Castrated	8		
Stage					
	I		9		
	II		7		
	III		12		
	IV		9		
Lymph node metastasis					
	Negative		23		
	Positive		14		
Visceral organ metastasis					
	Negative		28		
	Positive		9		

Forty-two canine oral MM primary mass specimens were collected from the gingiva (n = 25), mucosa (n = 13), and palate (n = 4) of 37 dogs. Pathology of each specimen is detailed in Table 2. Histological patterns included mixed spindle and polygonal cells (n = 16); polygonal cells (n = 9); mixed spindle and round cells (n = 8); spindle cells (n = 4); mixed spindle, polygonal, and polymorphic cells (n = 2); and one sample each of clear cells, mixed polygonal and dendritic cells, and mixed spindle and dendritic cells. Among the 42 samples, 29 samples showed little to no pigmentation (scores 0-1), while 13 specimens revealed moderate to high pigmentation levels (scores 2-3).

PI and MVD values in canine oral MM specimens: The values of PI and MVD, calculated after immunofluorescence staining with Ki-67 and CD31 (Fig 1), are indicated in Table 3. The mean and median values for PI were 28.8 ± 14.2% and 25.5%, respectively (3.6-66.0% range), while the mean and median values for MVD were 33.6 ± 19.5 mm^2 and 27.4 mm^2,

respectively (5.1-95.3 mm^2 range). No significant correlation was found between the PI and MVD values in canine oral MM specimens (p = 0.5377). In addition, the mean and median of MVD tended to be higher in the gingival tumours, while those of PI tended to be higher in the mucosal tumour tissues. However, statistical significance of both correlations could not be detected. (Table 3).

The correlation of the low or high PI and MVD values with clinical findings is presented in Table 4. According to the non-Gaussian distribution of the PI and MVD, the median value was selected as the cut-off value for the low and high values for both parameters. The PI values significantly correlated with clinical stage (p = 0.0453). The PI values were high in higher clinical stage and low in lower clinical stage patients. In contrast to PI, there was no correlation between the MVD values and any clinical findings.

Comparing to the pigmentation score, tissue samples that had lower pigmentation scores (score, 0-1) correlated more strongly with the PI values than the oral MM specimens with higher pigmentation scores (p = 0.0431, Table 4), whereas there were no correlations between the type of primary tissue or pigmentation and the MVD values.

Table 2 Pathologic Features of the Canine Oral Malignant Melanoma (MM) Specimens

Pathologic feature		Values		
Pigment				
	Low	29		
	High	13		
Tissue of primary mass				
	Gingiva	25		
	Mucosa	13		
	Palate	4		
MVD (mm^2)				
		Median	Mean	Range
	Total value	27.4	33.6 ± 19.5	5.1-95.3
	Gingiva	31.3	37.2 ± 22.5	5.1-95.3
	Mucosa	21.4	28.1 ± 10.5	18.0-53.8
	Palate	22.3	29.0 ± 21.3	11.6-60.0
Proliferation (%)		Median	Mean	Range
	Total value	25.5	28.8±14.8	3.6-66.0
	Gingiva	25.0	29.2 ± 15.8	3.6-66.0
	Mucosa	34.8	29.6 ± 12.4	11.2-49.8
	Palate	20.4	23.8 ± 18.5	5.3-49.1

Survival period of canine oral MM: The Kaplan-Meier estimator test was used to evaluate the survival period of dogs with oral MMs related to respective PI and MVD values. The mean and median survival period of all patients were 233.2 ± 239.2 and 143 d, respectively (2-992 days range). The PI significantly correlated with the survival period of canine oral MM patients (p = 0.0209). The patients with a higher PI (n = 21) exhibited a significantly shorter survival period (mean = 152.3 ± 135.8; median, 125 days; 18-558 days range) than the patients with a lower PI (n = 21; mean = 323.0 ± 288.3; median, 218 days; 2-992 days range). In contrast, there was no significant difference between the MVD value and the survival period in canine oral MM patients. The mean survival period of oral MM patients with high MVD values was 252.9 ± 248.1 d (median, 166 days; 2-810 days range), whereas that of oral MM patients with low MVD values was 222.5 ± 234.2 d (median, 131 days; 18-992 days range) (p = 0.4308, Fig 2).

Discussion

The current study examined the correlation between PI and MVD, as well as the correlation of prognostic parameters with clinical outcomes in canine oral MMs. By double immunofluorescence staining (Fig 1), we were able to observe the PI and MVD in the same microscopy field, and in turn investigate the correlation of these markers in tumour tissue. In this study, CD31 or the pan-endothelial marker was selected to detect the tumour endothelial cells due to a previous evidence that CD31 was the most suitable blood vessel marker in canine patients (Baluk and McDonald, 2008; Sleeckx et al., 2013), In addition to CD31, von Willerbrand factor, which is known as the factor VIII related antigen, was reported to be used to detect endothelial cell on canine tumour tissues as seen on the canine classical seminoma (Kim et al., 2010) and canine non-Hodgkin's lymphoma (Ranieri et al., 2005).

Table 3 The Clinicopathological correlation among the clinical findings, Microvessel Density (MVD), and Proliferation Index (PI)

		Number of patients					
		MVD			PI		
Variable	No	Low	High	*p*	Low	High	*p*
No	37	19	18		20	17	
Age (year)				1.0000			0.7307
≤13	24	12	12		12	12	
>13	13	7	6		8	5	
Weight (kg)				0.5171			0.3300
≤8	18	8	10		8	10	
>8	19	11	8		12	7	
Sex				0.3300			0.3248
Female	17	7	10		11	6	
Male	20	12	8		9	11	
Stage				0.7431			0.0453
I-II	16	9	7		12	4	
III-IV	21	10	11		8	13	
Lymph node metastasis				0.5077			0.1014
Negative	23	13	10		15	8	
Positive	14	6	8		5	9	
Distant metastasis				0.447			1.0000
Negative	28	13	15		15	13	
Positive	9	6	3		5	4	

Table 4 The correlation between the Pathologic Features and Microvessel Density (MVD) and Proliferation Index (PI) values

		Number of samples					
		MVD			PI		
Variable	Total	Low	High	*p*	Low	High	*p*
Primary tissue mass	42	21	21		21	21	
				0.8426			0.1831
Gingiva	25	11	14		13	12	
Mucosa	13	7	6		5	8	
Palate, soft	4	2	2		3	1	
Pigment				0.1809			0.0431
Low	29	12	17		11	18	
High	13	9	4		10	3	
CD31 expression							0.5377
Low	21				12	9	
High	21				9	12	

Von Willerbrand factor is a protein that can be found on the endothelial cell organelles. However, comparing to the CD31, von Willerbrand factor is less studied due to its non-uniform appearance on vascular structures (Baluk and McDonald, 2008). Additionally, Ki-67 was selected to recognize a nuclear antigen expressed in late G1, S, G2, and M mitotic phases, but not in the early G1 and G0 phases, which have been shown to correlate with cancer patient survival rates (Brown and Gatter, 1990).

In addition to the comparable clinical stage of canine oral MM patients, the primary oral MM tumours were most frequently found in the gingival area in the current study, which was in accordance with a previous report (Smith et al., 2002). Because of the plentiful blood supply required during tumourigenesis (Takano et al., 2010), the primary oral MM at gingiva might be prone to progression and invasion. In this study, tumours in the gingiva displayed a higher mean and median MVD than those

of the mucosa and palate. In contrast to MVD, the PI of mucosal oral MM revealed higher mean and median values than those of the gingiva or palate. The increase in PI at the mucosa may be the result of the naturally elevated proliferative activity of mucosal tissue. However, owing to the small sample size that could influence the stringency of the statistically analyses, the case control-large sample size of each tissue group will confirm the hypotheses both of MVD and PI expression on each primary tissue types.

Previous studies indicated that a canine oral MM tumour mitotic index greater than three, observed under a high power microscopy field, may not always be a precise prognostic marker (Schultheiss, 2006; Spangler and Kass, 2006). Therefore, the PI, calculated by staining for Ki-67, was selected as a marker to determine the proliferative activity. Our data was in accord with previous reports that a higher PI correlated with survival period (Millanta et al., 2002; Bergin et al., 2011). Although several treatments were used in our patients, there were no significant differences in the survival periods between patients with low or high PI and MVD, regardless of the treatment (data not shown). In addition to survival period, our results also indicated that PI correlated with clinical stage and degree of pigmentation, and confirms that PI could be a useful prognostic marker for canine oral MM.

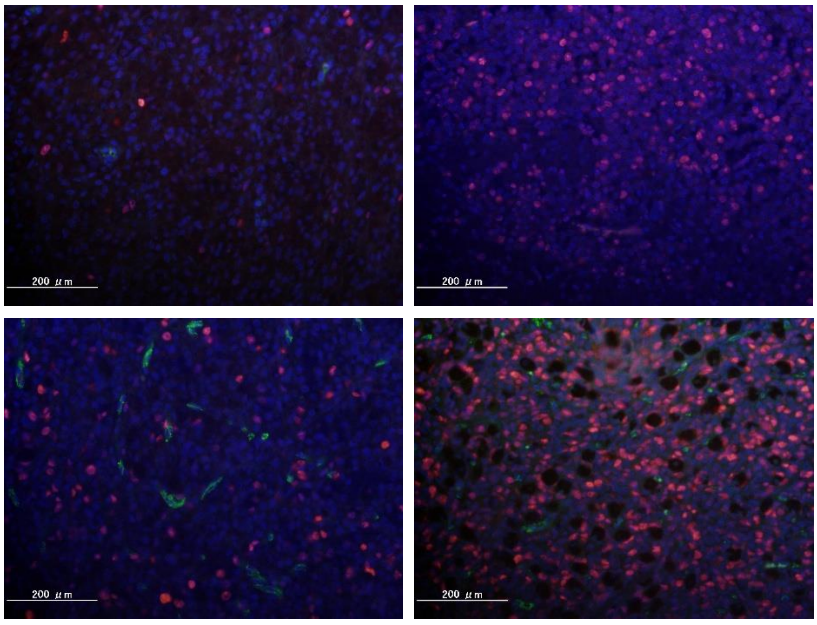

Figure 1 Expression of PI and MVD after staining with Ki-67 and CD31 in canine oral MM tissue sections. Canine oral MM that showed low PI/low MVD (A) and high PI/high MVD (D) were comparable and accounted for 28.5% each, whereas specimens that showed high PI/low MVD (B) and low PI/high MVD (C) were accounted for 21.5% each

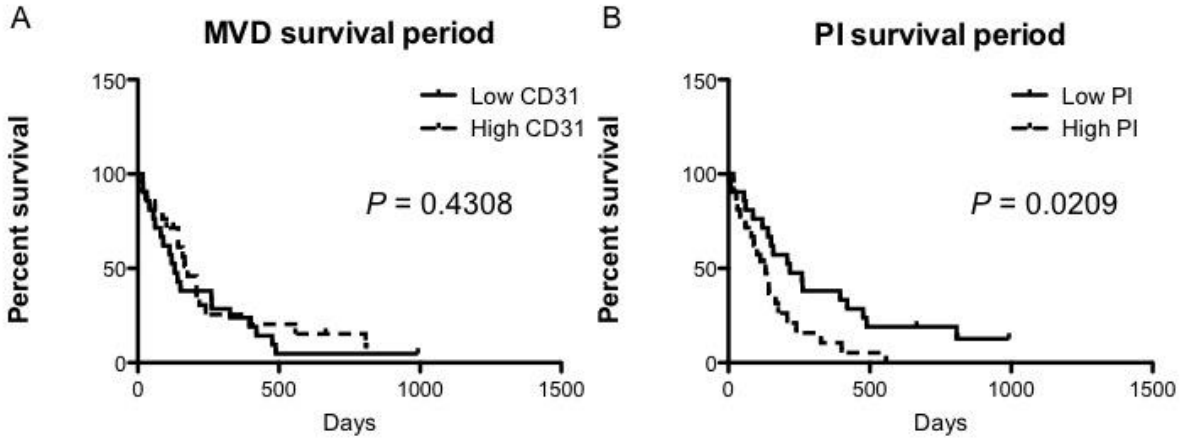

Figure 2 Relationship between survival rates and high or low MVD or PI values in canine oral MMs. The survival periods between the high and low MVD values (A) were not significantly different (p = 0.4308). In contrast, lower PI values (B) correlated with longer survival periods (p = 0.0209)

Previous studies reported that vascular endothelial growth factor (VEGF), one of the well-known tumour angiogenesis proteins, was highly expressed in oral MMs compared to tumours in other locations (Taylor et al., 2007; Cuitino et al., 2012), and that a greater number of microvessels correlated with poor prognostic outcome in canine MM (Mukaratirwa et al., 2006) or correlated with the tumour malignancy

in canine mammary gland tumour (Carvalho et al., 2013), canine classical seminoma (Kim et al., 2010) and canine non-Hodgkin's lymphoma (Ranieri et al., 2005). Data from our study were in accord with results from a previous study (Cuitino et al., 2012) that indicated that no clinicopathological correlation existed in canine oral MM between MVD and other parameters such as clinical findings, survival period, or the PI, although we do report a correlation between PI and survival period. This negative evidence could be caused either by the size of the primary mass and biopsy area from the primary tumour tissue, which might affect the distribution of the tumour vascularization, or by the number of alternative vascular channels that could not be observed through the endothelial markers (Maniotis et al., 1999).

In addition to the alignment of tumour vessels with endothelial cells during tumour angiogenesis, it has been shown that highly malignant tumours could obtain the nutrients and oxygen to survive by the production of separate vascular channels formed by the tumour cells with the basement membrane. This phenomenon was firstly described in human melanoma (Maniotis et al., 1999), and was referred to as vascular mimicry (Zhang et al., 2007; Vartanian et al., 2011). Nevertheless, no evidence of vascular mimicry in canine cancers has been published to date. Further large-scale investigations into the expression of several vascular channels such as MVD and vasculogenic mimicry, compared to the PI and clinical outcome, are warranted to establish prognostic parameters of canine MM.

In conclusion, there was no correlation between the PI and MVD on the same tumour area on the canine oral MM, and the PI value revealed as the superior prognostic marker owing to the correlation between the PI and clinical stage, degree of pigmentation, and survival outcome compared to the MVD observed by the endothelial marker.

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บทคัดย่อ

บทบาทของดัชนีการเพิ่มจำนวนและความหนาแน่นของหลอดเลือดขนาดเล็กในการพยากรณ์โรคมะเร็งเมลาโนม่าในช่องปากของสุนัข

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รายงานทางการแพทย์ได้บ่งชี้ถึงความสำคัญของการพยากรณ์โรคด้วยดัชนีการเพิ่มจำนวนหรือด้วยความหนาแน่นของหลอดเลือดขนาดเล็กในมะเร็งเมลาโนม่าในช่องปากของสุนัข ทว่ายังไม่พบรายงานใดที่รายงานถึงความสัมพันธ์ระหว่างดัชนีการเพิ่มจำนวนและความหนาแน่นของหลอดเลือดในมะเร็งดังกล่าว การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อประเมินความสัมพันธ์ระหว่างดัชนีการเพิ่มจำนวนและความหนาแน่นของหลอดเลือดขนาดเล็ก บนเนื้อเยื่อมะเร็งเมลาโนม่าในช่องปากของสุนัขภายหลังการย้อมสีอิมมูโนฟลูออเรสเซนต์ในสุนัขป่วยจำนวน 37 ราย ภายหลังการย้อมอิมมูโนฟลูออเรสเซนต์ด้วยโปรตีน Ki-67 และ CD31 พบว่าไม่มีความสัมพันธ์ระหว่างดัชนีการเพิ่มจำนวนและความหนาแน่นของหลอดเลือดขนาดเล็กบนเนื้อเยื่อมะเร็งเมลาโนม่าในช่องปากของสุนัข รวมถึงความสัมพันธ์ของพารามิเตอร์ดังกล่าวกับอาการทางคลินิก อย่างไรก็ตาม ค่าดัชนีการเพิ่มจำนวนให้ผลสัมพันธ์กับระดับอาการของโรค (p = 0.0453) และระดับเม็ดสีบนเนื้อเยื่อมะเร็ง (p = 0.0431) นอกจากนั้นระดับการเพิ่มขึ้นของค่าดัชนีการเพิ่มจำนวนให้ผลสัมพันธ์อย่างมีนัยสำคัญกับระยะเวลาการอยู่รอดของสุนัขป่วย (p = 0.0209) ผลการศึกษาครั้งนี้แสดงให้เห็นว่า การเพิ่มขึ้นของดัชนีการเพิ่มจำนวนไม่มีความสัมพันธ์กับความหนาแน่นของหลอดเลือดขนาดเล็กบนเนื้อเยื่อมะเร็งเมลาโนม่าในช่องปากของสุนัข และดัชนีการเพิ่มจำนวนเป็นพารามิเตอร์ที่เหมาะสมสำหรับการพยากรณ์โรคมะเร็งเมลาโนม่าในช่องปากของสุนัขมากกว่าความหนาแน่นของหลอดเลือดขนาดเล็ก

คำสำคัญ: สุนัข ความหนาแน่นของหลอดเลือดขนาดเล็ก มะเร็งเมลาโนม่าในช่องปาก ดัชนีการเพิ่มจำนวน

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