Original Article

KI 67 PROLIFERATION ANTIGEN IN SPONTANEOUS CANINE CUTANEOUS AND SUBCUTANEOUS TUMOURS AND ITS PROGNOSTIC IMPORTANCE

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Abstract: Purpose of the study was to evaluate the prognostic value of Ki 67 proliferation antigen in spontaneous canine cutaneous and subcutaneous tumours by immunohistochemistry. Formalin fixed, paraffin embedded histological sections were immunostained with monoclonal anti-human Ki 67 antibody (GM001). To determine Ki 67 index, approximately 1000 neoplastic cells were counted in 10 representative fields and number of positive cells per 1000 cells was expressed as percentage. Malignant tumours (64) had a mean Ki 67 index of 24.65 ± 1.69 while benign tumours had an index of 11.38 ± 2.61. Statistically significant difference (P < 0.05) was observed between malignant and benign types. The mean Ki 67 expression among the 12 dogs with malignant tumours that died was significantly (P < 0.05) higher (37.37 ± 4.32 per cent) compared to alive group. In 16 dogs with recurrence also the Ki 67 index was significantly (P < 0.05) higher (29.06 ± 2.74 per cent) as compared to alive group. The results of the current study indicated that poor prognosis was associated with higher Ki 67 index and the survival analysis, using Ki 67 index median cut off value indicated the prognostic importance of Ki 67 index in malignant cutaneous and subcutaneous tumours of dogs.

Key words: Cutaneous - subcutaneous tumours, Ki 67 antigen,

INTRODUCTION

Cancer has been reported to be a leading cause of mortality in dogs and cats [1] and second most in humans [2]. Cutaneous and subcutaneous tumours are more common, frequently malignant and have higher tendency of recurrence and metastasis to visceral organs resulting in reduced survival time and rate of affected dogs [3,4]. Histopathology is considered as the referral standard for tumour diagnosis. However, the precise diagnosis, objective evaluation, and assessment of true biological behaviour are difficult to be made by histopathological criteria alone. In recent years,

various cell proliferation assays have been used to predict the growth fraction, biological behaviour and prognosis in canine skin and mammary tumours. The most commonly used histochemical proliferation marker is argyrophilic nucleolar organiser region [5,6] and the most commonly used IHC markers are proliferating cell nuclear antigen (PCNA) [7] and Ki 67 antigen [8,9].

Ki 67 is a proliferation antigen which is expressed in all phases of cycling cell except G0 and early part of G1 [10,11]. According to Zuccari Debora et al. [12] it is a non histone, highly protease sensitive antigen assembled by polypeptide chains having molecular weight of 345 and 395 K Daltons. The expression of Ki 67 in tumour tissue indicates the rate at which the tumour is growing and has a positive correlation with tumour size, metastasis, expression of estrogen nuclear receptors, death due to neoplasia and low survival rate [12,13]. Hence it could be appropriately adopted to determine the prognosis of malignant tumours. Since the expression of Ki 67 in tumours is associated with high degree of proliferation, it has been reported to be immensely valuable in tumour grading and establishing prognosis in a variety of malignancies [14-18], hence present study was carried to evaluate the prognostic value of Ki-67 proliferation antigen in cutaneous and subcutaneous tumours of dog.

MATERIALS AND METHODS

A total of 83 cases of cutaneous and subcutaneous tissue tumours were collected from the dogs presented to Department of Veterinary Surgery, Veterinary College, Hebbal, Bengaluru and private Clinics in Bengaluru which formed the source of material for the present study. All dogs included in the study were followed post surgically for a minimum period of 10 months.

Histopathology: Representative tissue samples obtained after surgical excision were fixed in 10 per cent neutral buffered formalin immediately and processed by routine paraffin embedding technique. Sections of 4-5 µm thickness were cut and stained with hematoxyline and eosin. Tissue sections were examined to record and classify the cutaneous and subcutaneous tumours.

**Immunohistochemistry (IHC) of Ki 67 proliferation**

antigen: For IHC staining, 4µm sections of the tumours were placed on electrostatic slides and deparaffinised in two xylene baths for 5 min and rehydrated in two changes of absolute alcohol followed by distilled water. Slides were subjected to heat induced epitope retrieval by incubation in Tris –EDTA buffer of pH 9 in a microwave oven, followed by 20 min of cooling down and treatment with 3% hydrogen peroxide for 10 mins before antibody application. Then sections were covered with Ki 67 antibody (monoclonal mouse anti-human antibody for Ki-67 antigen clone GM001; ready to use, PathnSitu, Bengaluru) for one hour at room temperature. After washing with PBS the sections were incubated with PolyExel Target binder (Polyclonal goat anti-mouse immunoglobulins; PathnSitu, Bengaluru) for 25 mins at room temperature. Then sections were washed in PBS and incubated with PolyExel HRP (PathnSitu, Bengaluru) for 20 mins followed by washing in two changes of PBS and incubation with StunnDAB for five mins at room temperature. The sections were counterstained with Harris hematoxylin. Ki 67 expression was mainly assessed at the periphery of the tumour where cell proliferation was likely to be higher than in other tumour areas. To determine Ki 67 index per tumour section, approximately 1000 neoplastic cells were counted in 10 representative fields of vision at high magnification (400 x). The number of positive cells per 1000 cells was expressed as percentage.

Statistical analysis: Statistical analysis was performed using the statistical software R version 3.2.4 Revised Copyright (C) 2016. The R Foundation for statistical Computing. Mean values and standard error of mean were calculated and all values were expressed as (Mean ± SE). The data were analyzed by t test-unpaired, ANOVA-Turkey test, one of the multiple comparison tests was used for finding the source of the differences in multiple groups and Kaplan meier survival curve analysis and curve were compared by log rank test. For all statistical analysis P value less than 0.05 was considered significant.

RESULTS

In the present investigation, 83 cutaneous and subcutaneous tumours were classified based on the predominant cell type and histological

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Fig. 9

Fig. 10
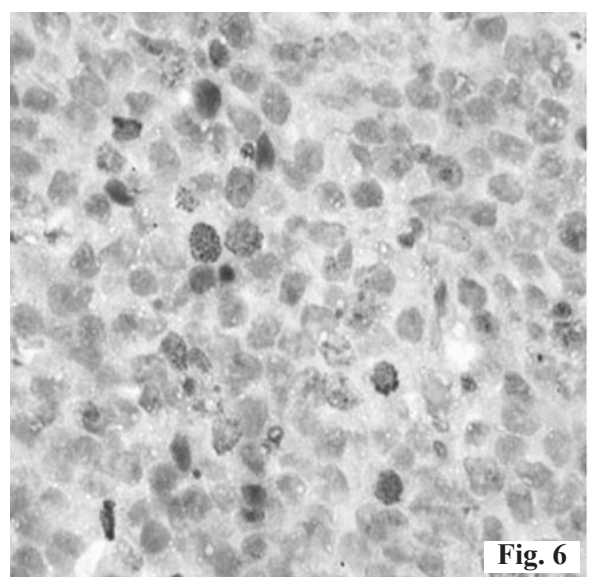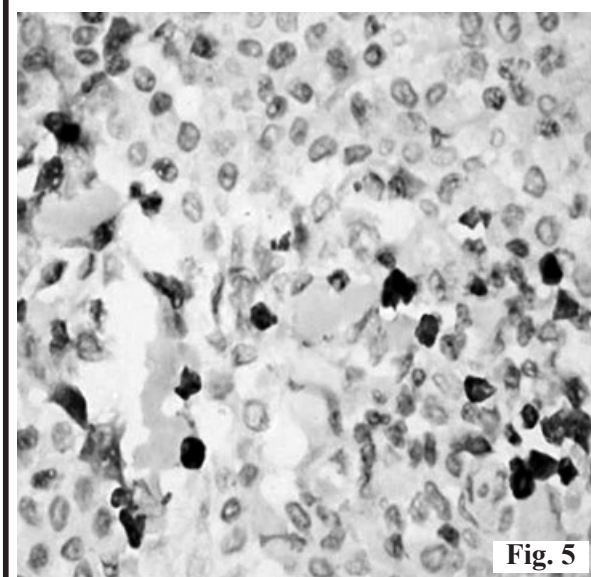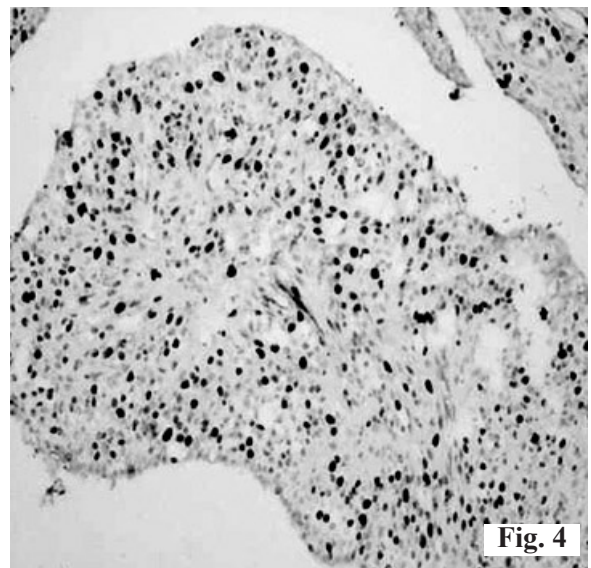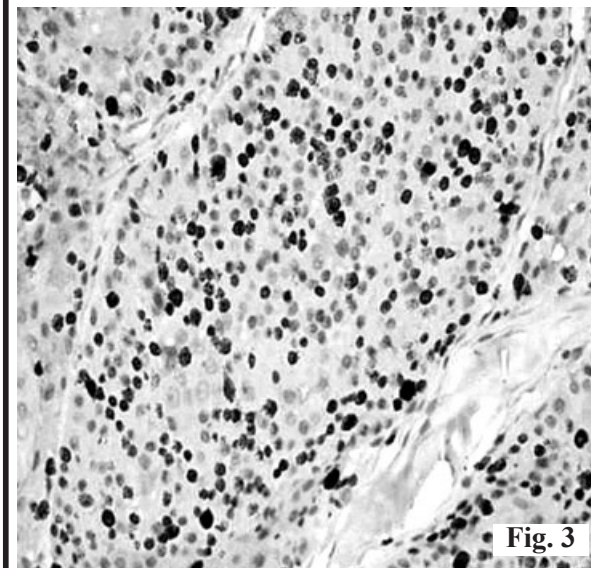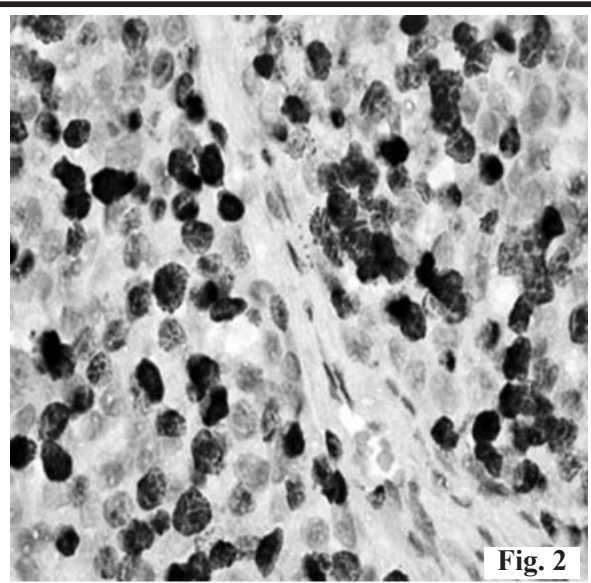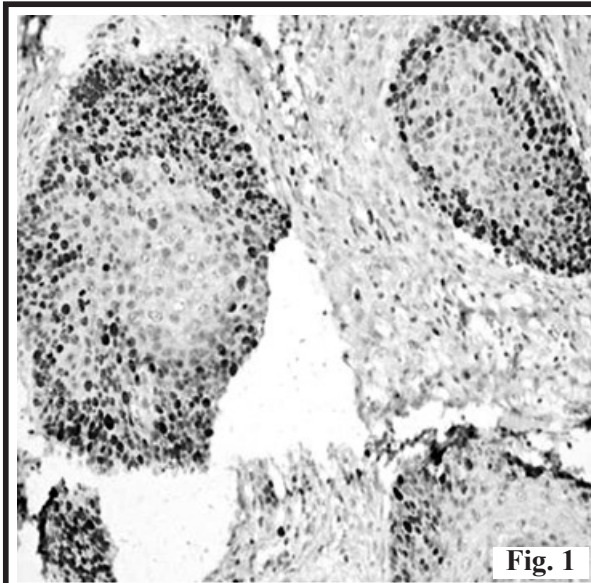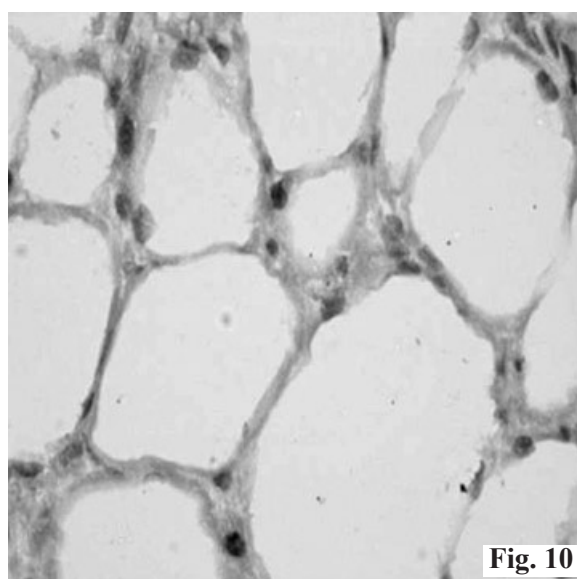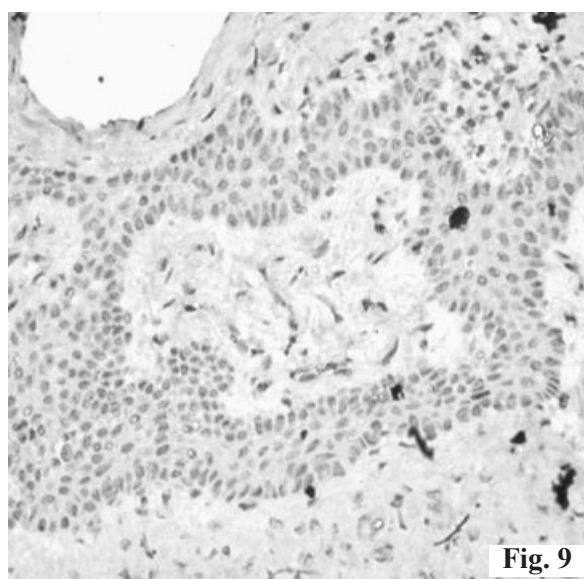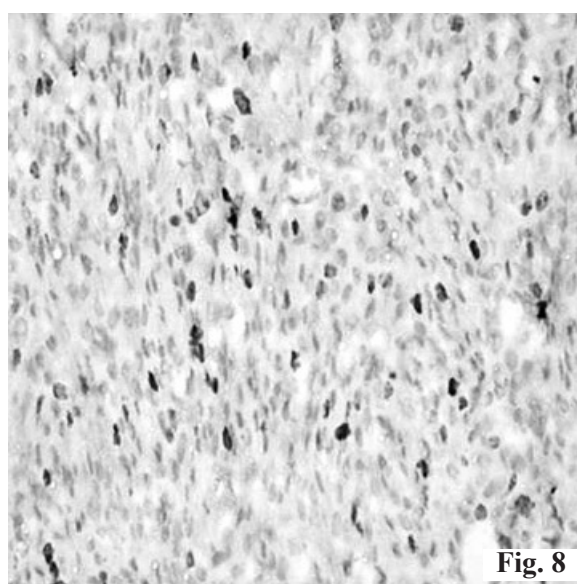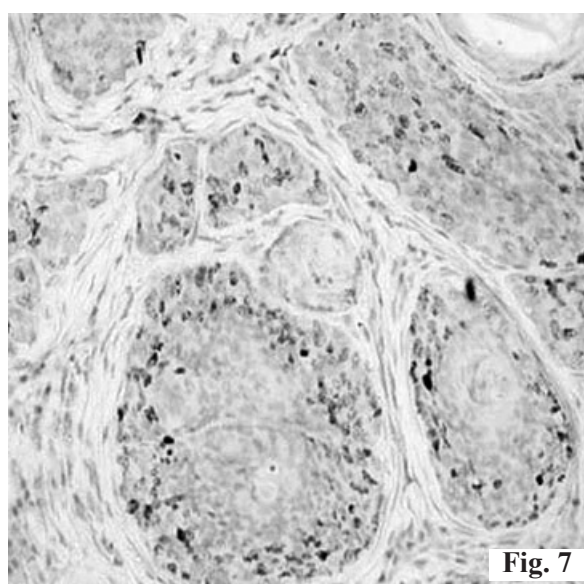
Figures 1 to 4 are sections of various malignant cutaneous and subcutaneous tissue tumours showing higherKi 67 index [IHC].

Fig. 1: Squamous cell carcinoma

Fig. 2: Solid adenocarcinoma of mammary gland

Fig. 3: Hepatoid gland adenocarcinoma

Fig. 4: Sweat gland carcinoma

Figures 5 to 8 are sections of various malignant cutaneous and subcutaneous tissue tumours showing moderate Ki 67 index (IHC)

Fig. 5: Mast cell tumour

Fig. 6: Transmissible venereal tumour

Fig. 7: Malignant Trichoepithelioma

Fig. 8: Fibrosarcoma

Figures 9 to 10 are sections of various benign cutaneous and subcutaneous tissue tumours showing less Ki 67 index [IHC].

Fig. 9: Acanthomatas ameloblastoma (Epulis)

Fig. 10: Lipoma.

Table 1: Mean Ki 67 index values of different cutaneous and subcutaneous tumours in dogs.

Type of tumour	No. of cases	Mean ± SE of Ki 67
Round cell tumours(n=16)		
Malignant type		
Mast cell tumour	9	14.63±1.47
Histiocytoma	4	27.6±0.85
Melanoma	1	10.5
Transmissible venereal tumour	2	16.1±1.6
Mean±SE of Ki 67 in round cell tumours 17.79±1.71		
Epithelial tumours(n=53)		
Benign type (n=13)		
Fibropapilloma	3	20.16±4.26
Squamous papilloma	1	29.5
Benign trichoblastoma	5	12.41±1.4
Ameloblastic odontoma (Epulis)	1	2
Acanthomatas ameloblastoma (Epulis)	1	2.5
Pilomatricoma	2	23±2.5
Mean±SE of Ki 67 in epithelial benign tumours 16.86±2.02		
Malignant type (n=40)		
Squamous cell carcinoma	8	43.1±3.0
Malignant trichoepithelioma	2	29.15±3.35
Hepatoid gland adenocarcinoma	7	23.8±4.89
Sebaceous gland carcinoma	2	14±0.5
Sweat gland carcinoma	2	30±4.5
Solid adenocarcinoma of mammary gland	5	43.8±5.0
Complex adenocarcinoma of mammary gland	5	8.07±1.6
Anaplastic carcinoma	1	12
Simple adenocarcinoma of mammary gland	8	25.99±2.39
Mean±SE of Ki 67 in epithelial malignant tumours 25.55±4.25		
Mesenchymal tumours(n=14)		
Benign type (n=6)		
Lipoma	4	2.24±0.12
Haemangioma	1	1.5
Fibroma	1	3
Mean±SE of Ki 67 in mesenchymal benign tumours 2.26±0.02		
Malignant type (n=8)		
Fibrosarcoma	5	15.85±1.04
Haemangiosarcoma	3	32.16±4.88
Mean±SE of Ki 67 in mesenchymal malignant tumours 21.97±3.44		

characteristics as round cell tumours (16 cases, 19.28%), epithelial tumours-malignant (40 cases, 48.19%) and benign types (13 cases, 15.66%) and mesenchymal tumours-malignant (8 cases, 9.64%) and benign types (6, 7.23%). The malignant tumours (64 cases) predominated over the benign types (19 cases) in the present study and epithelial tumours predominated over other types.

The positive reactivity in the present study for Ki 67 proliferation antigen was observed as dark brown coloured granular material restricted to nucleus. The immunostaining gave mild to strong nuclear labelling which occurred as dense, granular, nucleolar or a mixture of all types with mitotic figures always strongly labelled. The cells that were not proliferative showed no immunoreactivity.

Distribution of Ki 67 antigen positive cells varied between the tumour types (Figs. 1-10). All figures are self explanatory. Mean Ki 67 index in the present study for 83 tumours ranged from 1.5 to 43.8± 5.0 per cent. Malignant tumours (64) had a mean Ki 67 index of 24.65 ± 1.69 while benign tumours (19) had an index of 11.38 ± 2.61. Among all the benign tumours of cutaneous and subcutaneous tissue irrespective of type, mean Ki 67 index was highest in squamous papilloma. Among malignant tumours mean Ki 67 index was more in solid adenocarcinoma of mammary gland and squamous cell carcinoma. In the present study overall mean Ki 67 index was higher in epithelial tumours than the mesenchymal and round cell tumours. Among mesenchymal tumours hemangiosarcoma showed higher index (32.16 ± 4.88). Among the round cell tumours the Ki 67 index was highest in histiocytoma (27.6 ± 0.85). The overall mean Ki 67 index of round cell tumours was 17.79 ± 1.71, 28.93 ± 2.38 for malignant epithelial tumours and 21.97 ± 3.44 for mesenchymal malignant tumours (Table 1). The mean values of Ki 67 index compared by unpaired t-test revealed statistically significant (P < 0.05) difference between malignant and benign tumours of cutaneous and subcutaneous tissue.

Out of 64 dogs with malignant tumours, on follow up 12 dogs were learnt to be dead, 16 cases showed recurrence and 36 were alive with neoplasm free status. The mean Ki 67 index was highest in the dogs which died during follow up (37.37 ± 4.32), followed by dogs that showed recurrence of tumour (29.06 ± 2.74) and lower Ki 67 index was observed in the dogs which were still alive at the end of follow up (19.03 ± 1.63). The mean Ki 67 index was statistically significant (P < 0.05) between alive and dead groups and alive and recurrence groups. However no statistically significant difference was

Table -2: Mean ± SE Ki 67 index value of various post surgical outcome groups.
Note:Means bearing different superscripts are significantly different at P < 0.05

Follow up data	Total Number of cases and percentage	Mean ± SE of Ki 67 expression
Alive	36(56.25%)	19.03±1.63[a]
Recurrence	16(25%)	29.06±2.74[b]
Dead	12(18.75%)	37.37 ± 4.32[b]

Table 3: Subdivision of alive, dead and recurrence groups of dogs using median cut off value of Ki-67 index (25).

Ki-67 index	Alive	Dead	Recurrence
Ki-67 index < 25 (Under expression)	23 (63.89%)	3 (25%)	6 (37.5%)
Ki-67 index ≥ 25 (Overexpression)	13 (36.11%)	9 (75%)	10 (62.5%)

Survival Probability for Ki-67

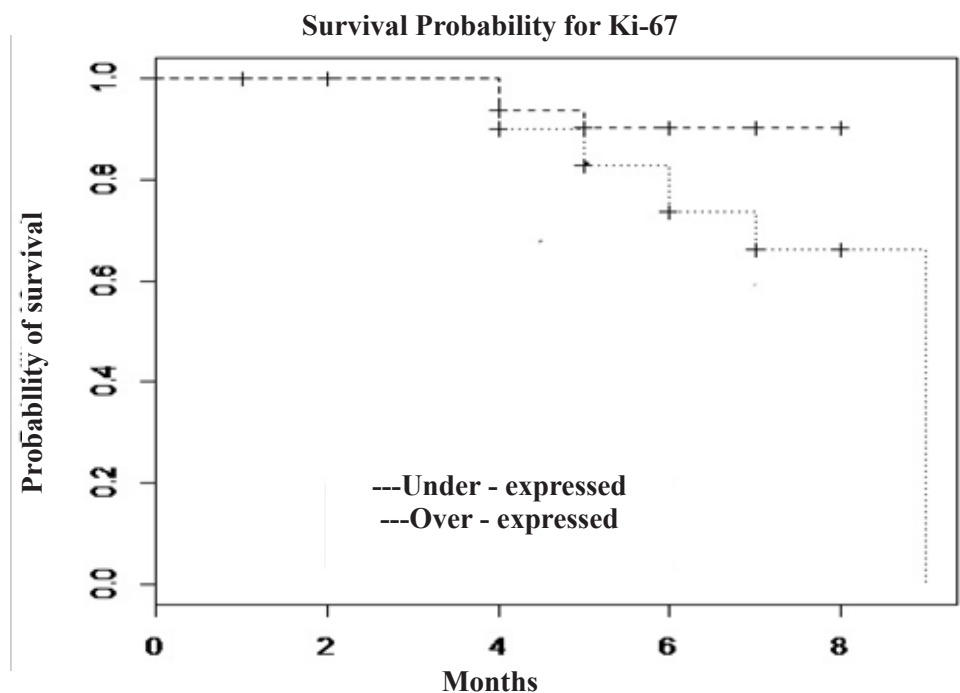---Under - expressed
---Over - expressed

Fig. 11: Kaplan – Meier survival curves for dogs with malignant cutaneous and subcutaneous tumours having Ki 67 more than and less than the calculated median cutoff value of 25 (p<0.05).

observed in mean Ki 67 Index between dogs which showed recurrence of tumours and those that were dead (Table 2).

To determine the prognostic value of Ki 67 index in 64 malignant cutaneous and subcutaneous tumours a median cut off value was calculated, which was 25. It was considered that those animals which had a mean Ki 67 Index lower than median value were under expressed and those with index greater than median value were over expressed. Among 64 dogs with malignant tumours the Ki 67 index was over expressed in 32 cases (50%) and in 32 (50%) cases it was under expressed (Table 3). In assessing long term results of the surgical treatment using Kaplan – Meier survival curves of follow up period of dogs revealed statistically significant (P ≤ 0.05) difference in survival time between dogs having Ki 67 index values above and below the median value. At 0.5 level of probability the mean survival time for dogs with Ki 67 index values more than the median value was found to be nine months and less than the median value was undefined (Fig. 11).

DISCUSSION

The expression of Ki 67 is strongly associated with tumor cell proliferation and growth, and is widely

used in routine pathological investigation as a proliferation marker. In addition Ki 67 expression is signifi¬cantly higher in malignant tumours with poor differentiation compared with benign tumours and normal tissue [15]. The findings of the present study are in concurrence with those of earlier workers who have also reported the applicability of Ki 67 proliferation index in differentiating the benign from the malignant neoplasms in humans and animals [16-21].

In the present study among the benign epithelial tumours the mean Ki 67 index was highest in 16.86 ± 2.02 squamous papilloma which could be probably due to a high rate of turn over observed in the squamous epithelial cells of skin and other locations from which these neoplasms originate [16,17]. In the group of malignant mesenchymal tumours, the mean Ki 67 index was highest in hemangiosarcoma, followed by fibrosarcoma. These findings are in accordance with those reported by several investigators [17,22,23] in human and animal soft tissue tumours. Among the hair follicle tumours highest mean Ki67 index was observed in malignant trichoepithelioma [25].

The prognostic value of Ki 67 has been investigated in a number of studies with its poten¬tial as a reliable marker having been shown in cancers of the breast, soft tissue, lung, prostate, cervix and central nervous system [24,26]. According to its predictive role, Ki 67 expression identifies subpopulations of patients who are more likely to respond to a given therapy. The Ki 67 labeling index is an independent prognostic factor for survival rate, which includes all stages and grade catego¬ries. There is a correlation between the ratio of Ki 67 positive malignant cells and patient survival [27]. In the present study the mean Ki 67 index values were statistically significant (P < 0.05) between alive and dead dogs and alive and recurrence group. However no statistically significant difference was observed in mean Ki 67 index between dogs which showed recurrence of tumours and between dogs which were dead indicating dogs showing recurrence didn't vary much from dead dogs and some of the dogs showing recurrence of tumours may succumb with advancement of tumour. Several earlier workers also have reported the prognostic importance of Ki 67 in various canine malignant neoplasms [14-17,21,28].

Statistical analysis of Kaplan – Meier survival curves of follow up period of dogs was performed considering the median value 25 of Ki 67 expression and were compared with log rank test support the prognostic significance of Ki 97 index, as reported previously [18,29]. A positive correlation between high index values of Ki 67 and metastasis, death from neoplasia, low disease-free survival rates, and low overall survival rates was reported by earlier workers [12,30]. However, Lohr et al. [10] reported that Ki 67 does not have prognostic relevance in canine mammary gland tumours. These contrasting data concerning immunohistochemistry of canine mammary gland tumour might be related to heterogeneity in the prevalence and biological behaviour of the various histological types [29].

It may be concluded that if we set cut off of median Ki 67 index value of 25 it can be predicted that dogs with Ki 67 index value lower than median value will have a more favorable prognosis as only 25% of dogs with Ki 67 index less than 25 died due to malignancy and those dogs with index value greater than 25 had poor prognosis as 75% of dogs which died due to malignancy had Ki 67 index more than 25. Thus Ki 67 index can be used as powerful tool for indicating prognosis of dogs with cutaneous and subcutaneous tumours. Survival analysis, using Ki 67 expression also could be used to predict probable survival period in malignant cutaneous and subcutaneous tumours postsurgically in dogs.

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