BJVP
Brazilian Journal of Veterinary Pathology

Original Full Paper

Prognostic Value of Ki 67 Proliferation Antigen in Canine Malignant Mammary Gland Tumours

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Submitted January 11th 2011, Accepted February 25th 2011

Abstract

Canine malignant mammary gland tumours were surgically resected from 78 dogs to determine the prognostic value of Ki 67 Proliferation antigen. After post surgical follow up for minimum of 1 year, 48 dogs were still alive, while 21 dogs had died as a consequence of malignancy, while remaining nine dogs showed recurrence of tumour. Formalin fixed, paraffin wax embedded histological sections were immunostained with monoclonal antibody Ki 67 (MIB -1). At least 100 cells in eight to 10 representative fields were counted. The Ki 67 index was expressed as the percentage of positive cells. In malignant canine mammary gland tumours, Ki 67 index ranged from 2.23 to 26.34 (14.45 ± 0.51). A statistically significant difference in the Ki 67 index ($P< 0.05$) was found between alive and dead group of dogs. Ki 67 index correlated with histological staging as most tumours in stage II had higher Ki 67 index and showed tumour related deaths. A clear association between the death due to malignancy and Ki 67 index was evident using Ki 67 index median count cut off value of 14.27 Thus Ki 67 index was good indicator of malignancy and dogs having Ki67 index greater than 14.27 have poor prognosis for mammary gland tumours.

Key Words: Ki 67 index, histological staging, mammary gland tumour, post surgical follow up, prognostic value.

Introduction

Among the various tumours of dogs, mammary gland tumours rank second only to skin tumours and are the most common tumours in females accounting for up to 52 per cent of all neoplasms (1, 2). Among mammary gland tumours more than 50 per cent are of malignant type with infiltrative destructive growth pattern leading to recurrence, metastases and death (1, 5, 13). Many studies have focused on the prognostic factor influencing the post surgical time in dogs with mammary gland tumours. Among them, age, diameter of the primary tumour, lymph node metastases, mitotic figures, AgNOR count, oncogene expression especially P53 and proliferation antigen index (Ki 67 or PCNA) along with staging of the tumour (1, 13).

Ki 67 antigen is a non histone, highly protease sensitive nuclear protein assembled by polypeptide chain with an apparent molecular weight of 345 and 395 KDa. This poorly characterized hetrodimer is expressed in all phases continuously in cycling cells except in Go, but it was not present in resting cells entering the cell cycle and Go to the early part of G_1 (6). The aim of present study was to investigate the immunohistochemical expression of Ki 67 antigen in canine malignant mammary gland tumours and to determine whether the Ki 67 index represented a useful prognostic indicator of post surgical time for dogs with mammary gland tumours.

Materials and Methods

Collection of samples and post surgical follow up.

Seventy one malignant canine mammary gland tumours, which had been surgically resected by simple mastectomy from 71 female dogs presented to

Department of Veterinary Surgery, Veterinary College, Hebbal, Bangalore, private clinics, and Government Veterinary Hospitals in Bangalore, India, were studied. Axillary or inguinal lymph nodes were also collected from all dogs depending location tumour. All dogs included in the study were followed post surgically for a minimum period of one year.

Histopathology and Histologic staging of tumours.

Minimum three representative portion of each neoplasm and regional lymph node were fixed in 10 % neutered buffer formalin, routinely processed by paraffin embedding technique and stained with hematoxylin and eosin. Tumour classification was done according to histological differentiation (8). Histological staging of mammary gland tumours was done according to method described by Gilbertson *et al* (7).

Immunocytochemistry and Ki 67 index.

For Ki 67 immunohistochemical detection, 4 µm sections from paraffin tissue blocks were deparaffinised. Before incubation with the primary antibody, tissue sections were subjected to heat induced epitope retrieval by incubation in 0.01M Citrate buffer pH 6 for 10 min in a microwave oven, followed by 20-min cool-down and treatment with 3% hydrogen peroxide before antibody application. After cooling, sections were covered for two hours at 37 C with anti-Ki 67 antibody (monoclonal mouse anti-human antibody for Ki-67 antigen clone MIB-1; ready to use, Dako**,** USA). After washing with PBS the sections were incubated with HRPO conjugated secondary antibody (Polyclonal Goat Anti-Mouse Immunoglobulins - Dako, USA, at 37 C for one hour. Then sections were washed in PBS and incubated with a solution of 0.01 per cent DAB and hydrogen peroxide for five minutes and the sections were lightly counterstained with Harris hematoxylin. Ki 67 expression was mainly assed at the periphery of the tumour where cell proliferation was likely to be higher than in other areas of tumour. To determine the Ki 67 index, ten representative tumour areas were selected and at least 1000 cells was counted, and number of positive cells per 1000 examined was expressed as percentage.

Statistical analysis

Statistical analysis was performed by ANOVA and chi-square test using graph pad prism version 4.01. The Ki 67index count cut off was set according to median value (14.27). Based on the latter, correlation between the tumour related deaths and the Ki67 index was evaluated by Chi-square test. For all Statistical analysis, P Value less than 0.05 was considered significant. Using median value of Ki 67 index, Survival curves were computed using the Kaplan–Meier survival analysis and curves were compared by log rank test.

Results and Discussion

Seventy one mammary gland tumours obtained from 71 female dogs were classified into tubulopapillary adenocarcinoma (30) which was most frequent histological tumour type encountered followed by solid carcinoma (14), malignant myoepithelioma (3), coexisting carcinoma (3), carcinosarcoma (3), lipid rich carcinoma (1) and squamous cell carcinoma (1).

Histological staging of 71 malignant tumours in the present study irrespective of the outcome of post surgical follow up was done has described by Gilbertson *et al* (7), which revealed 34 tumours in stage 0, characterized by no stromal invasion by neoplastic cells, 22 tumours in stage 1 which consisted of highly invasive neoplastic cells with invasion into connective tissue capsule and stroma, and 15 tumours in stage II which showed invasion of neoplastic cells into lymphatic or blood vessel or metastasis to regional lymph nodes. Out of 41 dogs which were alive at end of the post surgical follow-up, 32 were in stage 0 and nine in stage I. Out of nine dogs which showed recurrence during the post surgical follow-up, 2 were in stage 0, 6 were in stage I and one was in stage II. Out of 21 dogs that died at various intervals during post surgical follow up 7 were in stage I and the rest 14 were in stage II.

Immunohistochemical staining for Ki 67 antigen gave a mild to strong nuclear labeling and showed diffuse, granular, nucleolar or mixture of all types of staining pattern (Fig. 1-4). Mitotic figures were always strongly labeled. Ki 67 index in the present investigation ranged from 7.58 to 26.34 for all tumors. Among 71 cases of malignant tumours of mammary gland, highest mean Ki 67 index was observed in squamous cell carcinoma (21.24), followed by coexisting adenocarcinoma and squamous cell carcinoma (19.83), solid carcinoma (18.95), tubulopapillary adenocarcinoma- simple type (15.63), carcinosarcoma (12.77), malignant myoepithelioma (12.38), tubulopapillary adenocarcinoma- complex type (12.30), adenocarcinoma with heterotropic tissue (11.96), papillarycytic adenocarcinoma (9.68) and lipid rich carcinoma (7.58) (Table 1).

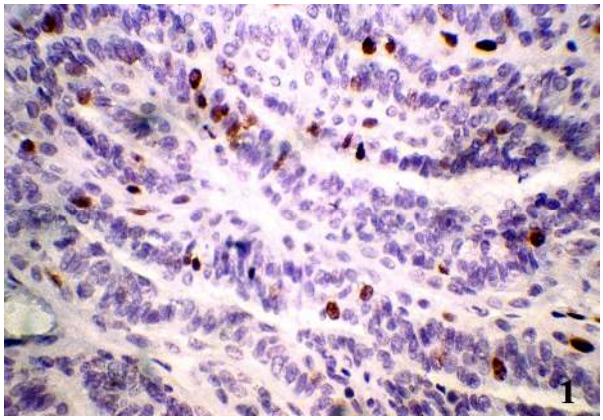

Figure 1: Section of tubulopapillary adenocarcinoma - simple type showing positive brown colored immunostaining for Ki 67 proliferation antigen in cells lining the papillary structures. 400x

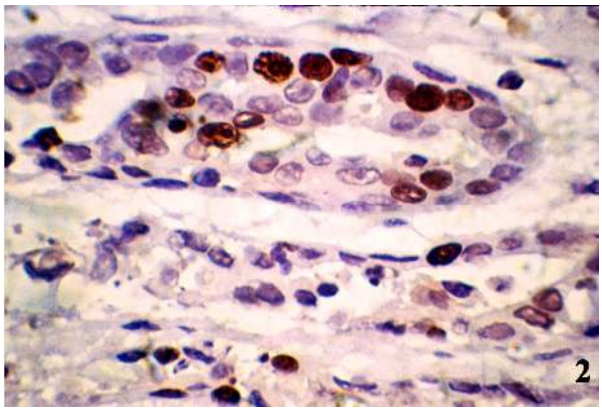

Figure 2: Section of tubulopapillary adenocarcinoma - simple type showing positive immunostaining for Ki 67 antigen in the nucleus of cells lining the tubular structures. 1000x

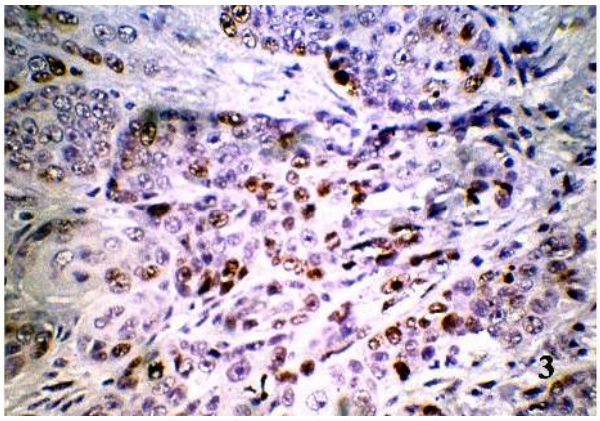

Figure 3: Section of co existing carcinoma showing numerous cells positive for Ki 67 antigen in squamous cell carcinoma component. 400x

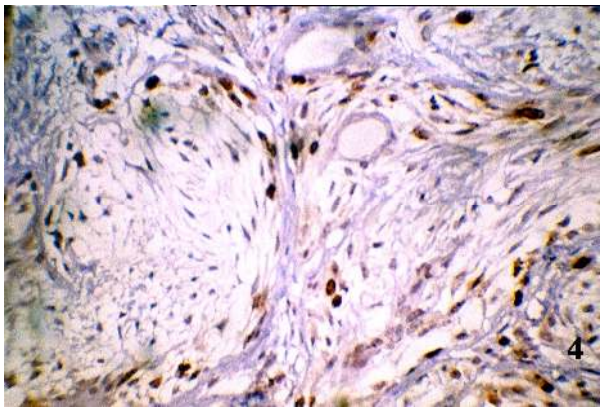

Figure 4: Section of malignant myoepithelioma showing positive immunostaining for Ki 67 in the cells present at the periphery of the solid nests. 400x

Ki 67 Index with respect to histological staging revealed highest count in stage II (17.3 ± 0.905) followed by stage I (14.48 ± 1.019) and stage 0 (13.17 ± 0.659). The mean Ki 67 Index was found to be statistically significant ($P<0.01$) between stage 0 and stage II, but statistically insignificant between stage I and stage II and stage 0 and stage I (Table 2) indicating not much variations in Ki 67 value between these stages (4,11,12). An increased Ki 67 Index in tumours in stage II could be due to more number of cells under proliferation as expression of Ki 67 protein remains immunohistologically detectable throughout the interphase of the cell cycle, reaching its maximal level during mitosis (3, 6).

Table 1: Mean and range of Ki 67 index in different malignant mammary gland tumours in canines.

Type of tumour	No. of dogs	Ki 67 index Mean ± SE	Ki 67 index Range
Tubulopapillary adenocarcinoma - simple type	16	15.63 ± 0.741	10.52 - 23.41
Tubulopapillary adenocarcinoma - complex type	14	12.30 ± 0.594	8.29 - 15.92
Adenocarcinoma with heterotropic tissue	11	11.96 ±0 .792	7.6 - 16.42
Solid carcinoma	14	18.95 ± 0.794	14.13 - 26.34
Papillary cystic adenocarcinoma	5	09.68 ± 0.687	7.88 - 11.43
Coexisting adenocarcinoma and squamous cell carcinoma	3	19.83 ± 2.503	16.58 - 24.75
Carcinosarcoma	3	12.77 ± 2.027	10.26 - 16.78
Malignant myoepithelioma	3	12.38 ± 1.253	10.24 - 14.58
Squamous cell carcinoma	1	21.24	-
Lipid rich carcinoma	1	7.58	-

SE: standard error, No: number

Table 2: Mean Ki 67 index of dogs, which were in different histological stages.

Outcome of follow up	No. of dogs	Ki 67 index (Mean ± SE)
Stage 0	41	13.17 ± 0.66
Stage I	9	14.48 ± 1.01
Stage II	21	17.3 ± 0.91**

** - Statistically highly significant from alive dogs count (P<0.01), SE: standard error, No: number

The mean Ki 67 index among dogs which were dead, alive and had recurrence of tumours during post surgical follow was found to be highest in the dogs which died during follow up (17.75), followed by dogs which showed recurrence of tumour (15.43) and lower Ki 67 index was observed in the dogs which were alive at end follow up (12.54). The mean Ki 67 Index was statistically highly significant (P<0.01) between alive and dead dogs (Table 3). However no statistically significant difference was observed in mean Ki 67 Index between dogs which were alive and showed recurrence of tumours, and between dogs which were dead and showed recurrence of tumour indicating that the tumours in alive dogs and in dogs showing recurrence do not vary much and some of the alive dogs may show recurrence of neoplasms in future, considering only Ki67 index. Similarly 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 (10, 11, 12, 14).

The mean Ki 67 index (17.75 ± 0.80) in dead dogs was statistically significantly higher (P<0.01) when compared with mean Ki 67 Index (12.54 ± 0.59) in dogs which were alive. A clear association (P < 0.05) between the death due to malignancy and Ki 67 index (table 2) was evident using Ki 67 index median count cut off value of 14.27 (Table 4).. 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 Pena *et al.*(11), Zuccari *et al*. (14) and Sarli *et al*. (12). De matos *et al* (3) reported that histologic stage, and Ki 67 index had significant association with poor prognosis in canine mammary gland tumours. However, Lohr *et al* (9) 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 behavior of the various histological types (12).

Table 3: Mean Ki 67 index of dogs, which are Alive, Alive with recurrence and dead

Outcome of follow-up	No. of dogs	Ki 67 index (Mean ± SE)
Alive	41	12.54 ± 0.59
Recurrence	9	15.43 ± 1.09
Dead	21	17.75 ± 0.82**

** - Statistically highly significant from alive dogs count (P<0.01), SE: standard error, No: number

Table 4: Subdivision of alive, dead and recurrence groups of dogs using median value of Ki 67 Index (14.27)

Up to one year survival period	Ki 67 Index ≤ 14.27	Ki 67 Index ≥ 14.27
Alive (%)	30 (73.17%)	11 (26.83%)
Dead (%)	2 (9.52%)	19 (90.48%)
Recurrence (%)	2 (22.23%)	7 (77.77%)

Kaplan–Meier survival curves analysis (figure 5) of dogs grouped into low and high Ki 67 index groups using median value (14.27) revealed a statistically significant difference in survival time between the two groups (P <0.01). Median survival value for dogs with Ki 67 index less than 14.27 was undefined as only few dogs having Ki 67 index less than 14.27 died during follow up period, and for dogs having Ki 67 Index more than 14.27 the median survival time was 10 months. The results of survival and risk analysis support the prognostic significance of Ki 97 index, as reported previously (12).

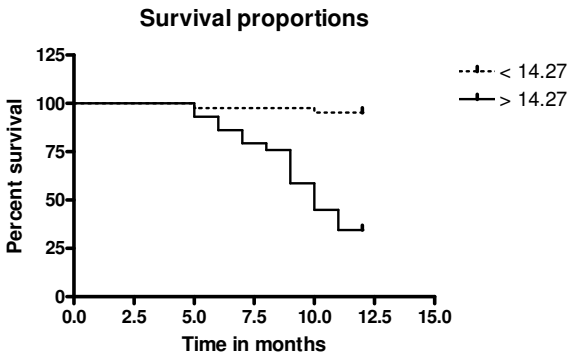

Figure 5: Kaplan–Meier survival curves for dogs with malignant mammary tumours having Ki 67 index more than and less than median value of 14.27. (P<0.01).

The findings in this study indicated that Ki 67 index significantly correlated with post surgical tumour related death in canine mammary gland tumours. It may be concluded that if we set cut off of median Ki 67 index value of 14.27 it can be predicted that dogs with Ki 67 index value equal or lower than median value will have a more favorable prognosis as only 27 per cent of dogs which with Ki 67 index less than 14.27 died due to malignancy and those dogs with index value greater than 14.27 had poor prognosis as 90 per cent of dogs which died due to malignancy had Ki 67 index more than 14.27. Thus Ki 67index can be used as powerful tool for indicating prognosis of dogs with mammary gland tumours.

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