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A study on Ki67 expression in canine mammary tumors

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Abstract
Benign and malignant tumors of the mammary glands occur fairly frequently in female dogs that are not spayed. In fact, mammary gland tumor is the most common type of tumor diagnosed in unaltered female dogs. Canine mammary tumors have been the main focus of research for the veterinarians since past few years. Ki-67 is one of the important proliferation growth markers involved in cancer promotion and spread.

The present study was envisaged to investigate Ki-67 expression in canine mammary tissue and to correlate Ki-67 expression profile with that of different stages of metastasis in canine mammary tumors. Total RNA was isolated from twenty-six tissue samples (sixteen from tumor and ten from normal mammary tissue) using TRIzol. RNA concentration and quality were measured with a Nanodrop and by 2% agarose gel electrophoresis. First-strand cDNA was synthesized from total RNA using the Revert Aid H minus first strand cDNA synthesis kit. The generated cDNA was used as the template for quantitative real-time RT-PCR (qRT-PCR) to check the relative expression levels of Ki-67. HGPRT was used as an endogenous control. The relative gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method. The qPCR data on relative expression level of Ki-67 showed up regulation of 3.52 in Canine mammary tumor. The present study showed consistent up regulation of Ki-67 in all the samples compared suggesting a significant diagnostic role of Ki-67 in canine mammary tumors.

Keywords: Canine mammary tumors, Ki67, PCR expression, HGPRT

Introduction
In female dogs, mammary tumors are the most common neoplasia representing about 50 percent of the tumors affecting this species and 50 percent of mammary tumors in dogs were found to be malignant with most affected age groups being 8 to 14 years (da Costa *et al*., 2019) [1].

With the endless pursuit to understand this phenomenon, the researchers have focused efforts on uncovering how the tumor process unfolds at a molecular level. There are several different markers identified to diagnose the canine mammary tumor which involves proliferation markers, apoptosis markers, extracellular matrix and cell adhesion molecules, angiogenesis and cell cycle regulators. Along with proliferation markers, clinico-pathological factors and histopathological grading remain the most important practical parameters in diagnosing canine mammary tumors. Ki-67 is a nuclear, non-histone protein, which is present during all active phases of the cell cycle, during interphase, and mitosis. It can be detected in the G1 phase of the cell cycle and increases in the S and G2 phases (Nowak *et al.,* 2015 and Tokuda *et al.*, 2017) [4, 8]. In mammary neoplasia, the expression of Ki-67 is strongly associated with tumor cell proliferation and growth which is used in routine pathological investigation as a proliferation marker and prognostic factor (Taoli *et al.*, 2015 and Gjurovski *et al.*, 2019) [9, 3]. The exclusive expression in specific phases of the cell cycle along with its short half-life, made Ki-67 an important and ideal candidate for the development of specific antibodies useful for the diagnosis and prognosis of several tumor types. Considering the role of Ki-67 in tumor progression the present study is aimed to investigate the differential expression of Ki-67 in canine mammary tumors to suggest the plausible use of this gene as potential target to diagnose and treat canine mammary tumors.

Materials and Methods
Canine mammary tumor samples were collected at the time of surgery from Teaching Veterinary Clinical Complex of NTR College of Veterinary Science, Gannavaram, College of Veterinary Science, Rajendranagar, Hyderabad, Veterinary Polyclinic Vishakhapatnam, Bangalore Veterinary college and Private Veterinary Clinics located in Hyderabad. The tumor tissue and surrounding normal tissues were collected from adjoining tissue of the same animals

(with owners' consent) and also from Post Mortem cases. Thus, a total of ten normal and sixteen tumor samples were collected. Pieces of tumor and normal tissues collected were kept in 10% neutral buffered formalin for histopathological examination. The tissues were diagnosed and graded histologically using H & E staining as per World Health Organization (WHO) guidelines for CMTs (Misdorp *et al*., 1999) [5]. Total RNA was extracted for 26 samples using TRIzol method and assessed the quality of the RNA using Nanodrop ™ 2000c (Thermoscientific™). cDNA was synthesized from all the RNA samples using Bio-Rad thermocycler C1000 Touch™ and Himedia Hi-cDNA synthesis Kit. The integrity of cDNA was checked by employing published gene specific primers (forward and reverse) for Ki67 with HGPRT as endogenous control. The specificity of the primers was checked by primer BLAST (http//ncbi.nlm.nih.gov/BLAST/) and Oligo analyzer. The primer details are given in Table 1.

Table 1: Primers used in the study

Gene	Accession no. (NCBI)	Product size	Oligo size	Reference	Primers
Ki-67-FP	ENSCAFT 00000021024	88	20	Vascellari *et al*., 2012	5'CCCACCTGTCCTGAAGAAAA 3'
Ki-67-RP			21		5'TGTGGTCACTTCCAGTTGGTT 3'
HGPRT- FP	NM_001 003357.1	104	20	de Kok *et al*., 2005	5'AGCTTGCTGGTGAAAAGGAC 3'
HGPRT- RP			20		5'TTATAGTCAAGGGCATATCC3'

The expression of Ki-67 mRNA was assayed using QuantStudio3 applied biosystem (Thermo Fisher Scientific, USA) real time PCR system operated by quant studio design and analysis software. The reaction was performed using Bright green qPCR Master Mix-Low ROX (Cat# Master Mix-LR) (abm, canada) as per the manufacturer's instructions. The final 20µl consisted of cDNA Template (100ng), 10pmol/µl of forward and reverse primer, 12.5µl of PCR MasterMix, and 9.5µl of nuclease free water with qPCR cycling conditions as: Initial Denaturation 95^0C / 10 min, Denaturation: 95^0C / 15sec, Annealing: 60^0C-30 sec, Extension: 72 ^{0}C / 40 sec for 40 cycles and Final extension 72 ^{0}C / 10 min and dissociation step: 95° C/1sec. The relative gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008), where in the qPCR data were expressed as fold change in gene expression in the tumor sample, which was normalized to the geometric mean of reference gene relative to the normal mammary gland. To compare the expression of Ki67 in different grades of tumor. A students "t"-test was performed.

Results and Discussion

The qPCR data obtained for Ki67 from all the normal and tumor tissues with HGPRT was subjected to $2^{-\Delta\Delta CT}$. The calculated fold change values with respect to Ki-67 are presented in Table 6. The results revealed a 3.52-fold increase in Ki-67 gene expression in tumor tissues compared to normal, indicating, Ki-67 is upregulated 3.52 times in tumor tissues compared to normal tissues. The calculated fold change values are presented in Table 2.

Table 2: $2^{-\Delta\Delta CT}$ of Ki-67 in Canine mammary tumor tissue

S. No	Tumor no.	Tuor grade	2-ΔΔCT of Ki-67	Type of regulation
1.	T2	GRADE-I	2.17	UP-REGULATED
2.	T4		4.01	
3.	T6		2.99	
4.	T12		3.12	
5.	T7	GRADE-II	3.99	UP-REGULATED
6.	T8		3.29	
7.	T9		3.71	
8.	T13		3.49	
9.	T14		3.79	
10.	T16		3.22	
11.	T1	GRADE-III	3.66	UP-REGULATED
12.	T3		4.11	
13.	T5		3.62	
14.	T10		3.24	
15.	T11		3.67	
16.	T15		4.17	

It was found that the Ki67 was up-regulated in all the grades of tumors. The results of the present study are in concurrence with the findings of Queiroga *et al*., (2011) [6] who reported similar type of Ki-67 expression in benign and malignant tumors in Human Breast Cancer. The Electrophoresis gel run of the qPCR product showing the Ki67 bands is given in the Fig. 1. The amplification plot of Ki67 and HGPRT genes in normal and tumor conditions is given in Fig. 2

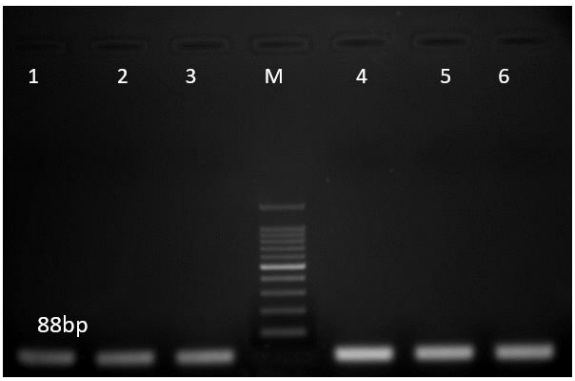

Fig 1: PCR product of KI67 (88bp) from CMT

Lane M: 100bp DNA ladder
Lane 1-3: PCR product of KI67 gene from normal canine mammary tissue
Lane 4-6: PCR product of KI67 gene from canine mammary tumor tissue

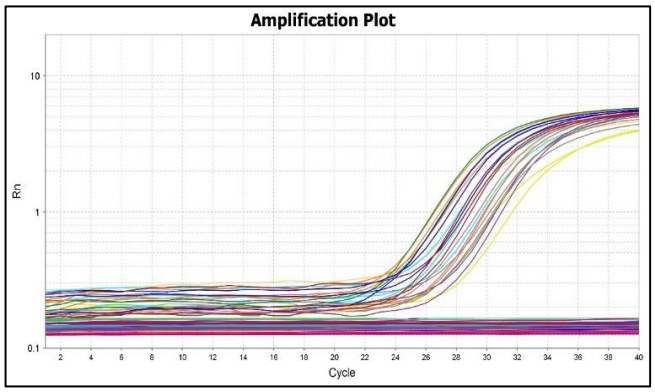

Fig 2: Amplification plot for KI-67 in canine mammary tumor

The presence of single and sharply defined melting curves with narrow peaks obtained was showing specificity of real-time PCR amplification of Ki-67 gene. There was up regulation of Ki-67 in all the grades of tumors that were studied. Several researchers have tried other methods of evaluating expression of Ki-67 such as Immunohistochemistry, Western blotting, and SDS-PAGE. The findings of our study are in accordance with the findings of Yuan *et al*., (2016) [11], who reported that, compared to the controls; Ki-67 expression was significantly increased in the serum and cancer tissue of breast cancer patients when assessed with fluorescent quantitative polymerase chain reaction.

Conclusively, proliferative activity determined by Ki-67 expression may reflect the aggressive behaviour of breast carcinoma. Ki-67 detection provides valuable information about proliferative activity of cells so, it is necessary to combine this with other parameters for better prognosis. The relative gene expression levels of Ki-67 with students- t test in the present study revealed a significant (p<0.05) high (3.52±0.13) expression of Ki-67 gene when compared to that of normal tissue indicating Ki-67 gene is a relatively reliable prognostic marker of canine mammary tumors.

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