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MOLECULAR EXPRESSION OF CYCLIN DEPENDENT KINASE INHIBITOR (*p*21) IN CANINE TUMORS

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ABSTRACT

p21 is a cyclin dependent kinase inhibitor, that plays an important role in cell growth, differentiation and apoptosis. The aim of present study was to analyze the mutation and expression of tumor suppressor gene (*p*21) in canine tumors. A total of 26 tumors (mammary tumors and mast cell tumors) and normal samples from different breed of dogs were analyzed by using direct DNA sequencing and quantitative RT-PCR for mutation and expression profiling of *p*21gene respectively. The coding exon of *p*21 gene (Exon2 and 3) did not show any polymorphism in studied samples while 69% tumors showed up-regulation of gene. Seventy five percent mammary tumors showed up-regulation of gene with highest fold change 8.69, 7.6, 5.63, 5.21 and 60% mast cell tumor showed up-regulation of gene with highest fold change 7.31. These results indicate that altered expression of tumor suppressor gene may be involved in malignant progression of tumors and might be helpful in the diagnosis and prognosis of canine tumors.

K**ey words**: Dog, Mutation, Quantitative RT-PCR, CDKN1A, Carcinomas, Tumor suppressor gene.

INTRODUCTION

Cancer is one of the most important cause of morbidity and mortality in pet animals, upto 41% in canine species (Bonnet *et al.,* 2005).The anatomical and physiological similarities between canine and humans have been the basis of using the canine in oncology research for over a century. Many features make the dog an attractive model for human cancer biology research due to following reasons: long life span, life style, spontaneous cancer development and conventional chemotherapy treatment as compared to other laboratory animals (Kirkness*et al.,* 2003).

Canine mammary tumor is the most common tumor in sexually intact female dog and it represents the second most frequent tumor in dogs and humans after skin and lung cancer respectively (Sorenmo*et al.,* 2003). In 53% of the cases tumor is malignant and more than 95% of these malignant tumors are carcinoma (Misdrop, 2002). Similarly, canine mast cell tumor is the most common neoplasm, representing 7 to 25% and 11 to 27% of all skin tumors and malignant skin tumors in dogs (Bostock*et al.,* 1973). This tumor is 3 to 10 cm in size, arises from dermis and sub cutis, mostly occur on cutaneous sites; 50 to 63% in trunk and perianal region, 33 to 40% on extremities and 10 to 15% on head and neck region (Strefezzi*et al.,* 2009).

The *p21* (tumor suppressor gene) is also known as cyclin dependent kinase inhibitor 1A, located on canine chromosome 12.These cyclin dependent kinase inhibitors(CDKIs), group of inhibitory proteins that control the cyclin dependent kinase activity and cell cycle progression in response to the intracellular and extracellular signals (Vidal and Koff 2000). On the basis of structural similarities and regulation of cell cycle, CDK1s are divided into 2 groups. *CDKN1* or *CIP/KIP* family and *CDKN2* or *INK*-4 family. The *CIP/KIP* family (*CDK* interacting protein) / (kinase inhibitory protein) consist of three members, specifically *p21/CIP1*, *p27/KIP1*, and *p*57*/KIP2*, all of these protein shares common inhibitory domain for the binding of *CDK* complex (Sharpless, 2005). These proteins show structural similarities (60 residue homology) that are important for their inhibitory activity (Xing *et al.,* 1993). The *CIP/KIP* proteins have broad specificity as compared to *INK4* proteins for binding and inhibiting the *cyclin-CDK* complexes. The activity of *cyclin-D-CDK4* is inhibited by these proteins, preventing the Rb phosphorylation during the transition of G_1 to S phase and inhibit the activity of *cyclin-A-CDK2* and *cyclin-E-CDK2* in late G_1 and early S phase respectively (Sharpless, 2005).

The *p21* gene expression is tightly regulated by transcriptional level (*Tp53* dependent and independent mechanism) and posttranscriptional level (ubiquitin

dependent and independent proteasome mediated degradation). The two conserved *p53* binding sites present on *p21* promoter, and one of this site is required for *Tp53* responsiveness after DNA damage (El-Deiry *et al.,* 1995). *p21* interacts with DNA polymerase accessory factor, *PCNA* (proliferation cell nuclear antigen), that regulates the DNA replication and damage repair (Abbas and Dutta, 2009).

The over expression of *p21* gene is positively correlated with the size, grade, invasiveness and aggressiveness of the tumor.

The objectives of the present study are to have an insight of mutation and gene expression analysis of *p21* gene using direct DNA sequencing and RT-qPCR respectively in the two types of canine tumors (mammary tumor & mast cell tumor) and normal tissue samples and to assess the possibility, whether the gene could be a putative target gene for the therapy of these tumors.

MATERIALS AND METHODS

Sample Collection: Total twenty six (n=26) tumors and normal healthy control tissue samples of canine specie were collected from Pet center UVAS and Asim Pet **C**linic Lahore from 2012 to 2015(Table 1). All tissue samples, excisional biopsies, were frozen in liquid nitrogen and stored at -80°C for downstream processing. The study was approved by ethical committee of University of Veterinary and Animal Sciences, Lahore Pakistan.

Histological Examination: Formalinfixed paraffin embedded sections (5µm thickness) from each tumor were stained with hematoxylin and eosin. Tumors were categorized by veterinary pathologist according to WHO classification.

DNA Extraction: Genomic DNA was extracted from tumor and normal healthy control tissues by using DNeasy Blood and Tissue Qaigen kit according to manufacturer's protocol. DNA concentration was measured using Nano Drop 2000 spectrophotometer (Thermo fisher scientific, Pittsburg PA, USA) and visualized by 0.8% agarose gel electrophoresis. All DNA samples were normalized with 50ng/µL concentration for PCR amplification.

Primer designing, Optimization and Amplification: Primers for Exon 2 and 3 were designed from *p*21 gene DNA sequence (Accession no; ENSCAFG00000029853) by primer3 software (http/www.Primer3.com), manufactured by Advance Bioscience International (Table 2). Primers were optimized with wide range of annealing temperature (52-65°C) by gradient PCR and touch down PCR along with variable concentration of $MgCl_2$ (2.5 to 3.0mM), dNTPs and template DNA in thermo cycler (Applied Bio System).

DNA Sequencing: Amplified PCR product**s** were run on 1.5% agarose gel along with 1kb ladder (Fermentas, USA) The gel was stained with ethidium bromide and visualized under UV-trans illuminator (Bio Red). The PCR product**s** were purified by means of ethanol precipitation and sequenced at 3730ABI Genetic Analyzer."BioEdit v7.0.5" was used for sequence analysis.

Total RNA Extraction: Total RNA was extracted from tumor and normal tissues using a Thermo Scientific Gene Jet RNA purification kit (Boom *et al.,* 1990). TriZol reagent was also used for fresh tissue samples (Hummon *et al.,* 2007).Total RNA integrity and concentration was confirmed by agarose gel electrophoresis and Nanodrop Spectrophotometer.

Reverse Transcription: cDNA was synthesized using Revert Aid first strand cDNA synthesis Kit (Themo Fisher Scientific, Pittsburg, PA,USA).Random Hexamer and Oligo(dt) primers were used to synthesized the first strand cDNA according to manufacturer.

Primer Probe for qPCR: Pre-designed primer and probe for *p21*target gene was selected and purchased online by Applied Bio System and for data normalization, Glyceraldehyde-3- phosphate dehydrogenase (*GAPDH*) housekeeping gene was custom designed using ABI primer express software(Table # 3).

Quantitative Real-Time PCR and Data Analysis: The qPCR reaction conditions were determined during initial optimization. The PCR reactions were carried out in 96 well plates that contained cDNA sample for target and housekeeping gene according to the AB1 standard protocol. Data was presented as fold change in gene expression level in the target sample normalized to *GAPDH* housekeeping gene and relative to the control sample. A fold change above 1 was considered as up-regulation and below 1 was considered as down regulation of gene. Relative expression of the target gene (*p*21) was calculated by Livak method (Livak *et al.,* 2000).

RESULTS

The genomic DNA extracted from tumor and normal tissue samples were amplified by using exon2 and 3of *p*21 gene. The amplified product of exon-2(548bp) and exon-3(493bp) were subjected to DNA sequence analysis by Sanger's method which showed no polymorphism in coding exons of *p*21 gene. Real time PCR experiment was conducted on canine tumors and normal samples and all templates DNA(c-DNA) were standardized with concentration 5ng/µL for experiment. Cycle threshold (C_T) values of target and housekeeping gene were retrieved from ABI software and $2^{-\Delta\Delta Ct}$method

was used for calculation of fold change on Microsoft excel 2010. ΔC_T values of test (tumor sample) and calibrator (normal sample) were used for calculating the $\Delta\Delta C_T$ and then these values underwent to the $2^{-\Delta\Delta Ct}$ that represent the efficiency of the experiment. Eight mammary adenocarcinoma samples of *German shepherd* and *Golden retriever* breed of dog were collected in this study; normal mammary tissues were also collected from same dog. *p*21 gene was up-regulated in (6/8) 75 % in mammary adenocarcinoma samples MAC-2 and MAC-6 with highest fold change 8.69 and 7.6 respectively and down regulation of *p*21 gene was measured in MAC-3 and MAC-8 with fold change 0.45 and 0.51 respectively (Figure 1).Similarly, five mast cell tumor samples of *German shepherd*, *Rottweiler* and *Labrador* breed of dog were collected and normal skin tissues also collected from the same dog. *p21* gene was up-regulated in 3/5 (60%) mast cell tumor samples with highest fold change 7.31 in MCT-4 and slightly up regulation was measured in MCT-3 and MCT-5 with fold change 2.65 and 1.81 respectively. MCT-1 and MCT-2 samples showed the down regulation of *p21* gene with fold change 0.42 and 0.6 respectively (Figure 2). Four of the five, grade II mammary adenocarcinomas showed higher *p*21 gene expression with 8.69 highest fold change whereas 2/3 grade III adenocarcinomas showed higher expression with 7.6 highest fold change (Table 4, Fig 1). One of the three, grade I mast cell tumors showed higher *p*21 gene expression where as both tumors of grade II also showed higher expression with 7.31 fold change (Table 4, Fig 2).

Table 1. Canine tumors with histological diagnosis.

Dogs	Sample-ID	Breed	Age	Sex	Tumor Tissues	and Grade
1	MAC-1	German shepherd	11	F	Mammary Tumors	Adenocarcinoma, II
2	MAC-2	German shepherd	9	F	Mammary Tumors	Adenocarcinoma, II
3	MAC-3	Golden retriever	10	F	Mammary Tumors	Adenocarcinoma, II
4	MAC-4	German shepherd	11	F	Mammary Tumors	Adenocarcinoma, II
5	MAC-5	German shepherd	5.5	F	Mammary Tumors	Adenocarcinoma, II
6	MAC-6	German shepherd	7	F	Mammary Tumors	Adenocarcinoma, III
7	MAC-7	German shepherd	5.5	F	Mammary Tumors	Adenocarcinoma, III
8	MAC-8	German shepherd	3.5	F	Mammary Tumors	Adenocarcinoma, III
9	MCT-1	Rottweiler	2	F	Mast Cell tumor	Cutaneous,I
10	MCT-2	German shepherd	3	F	Mast Cell tumor	Cutaneous,I
11	MCT-3	Labrador	5	M	Mast Cell tumor	Cutaneous,I
12	MCT-4	Labrador	12	M	Mast Cell tumor	Cutaneous,II
13	MCT-5	German shepherd	7	M	Mast Cell tumor	Cutaneous,II

Table 2. Primers of *p21 gene*

Primers	Primer Sequences (5' -3')	Product size	Annealing Temperature
Exon -2F	GTGCCATTCCCCAAGTGAC	548bp	56°C
Exon -2R	GCACCACGGATTCTGAGAGT		
Exon -3F	CTTCGGCCCTAGGAGACATC	493bp	59°C
Exon -3R	CAGCCCCACCTTACCTCTG		

Table 3. RT-qPCR primers for *p*21 and housekeeping gene.

Specie	Gene	Assay ID	Assay location/ Exon boundaries	Amplicon Length	Dye
Dog	*p21*	cf-02693025-m1	Exon 2-3	56bp	FAM
Dog	*GAPDH*	cat#4331348custom designed	655bp	59bp	VIC

Table 4: *p*21 gene expression with histological grade of malignancies.

Tumors type	Histology& Grading	No. of Dogs	*p*21 Gene Expression	
			Higher	Lower
Mammary Tumors	Adenocarcinoma, Grade II	5	4(80%)	1(20%)
	Adenocarcinoma, Grade III	3	2(67%)	1(33%)
Mast Cell Tumors	Cutaneous, Grade I	3	1(33%)	2(67%)
	Cutaneous, Grade II	2	2(100%)	-

(grade1=well differentiated, grade II=intermediate differentiated, grade III=poorly differentiated)

Table 4. Tabulated representation of the *p*21 gene expression in canine tumors.

TUMOR SAMPLES				NORMAL SAMPLES					GENE EXPRESSION
SAMPLE ID	*p*21 GENE EXPRESSION (MEAN±S.D)	*GAPDH* EXPRESSION (MEAN±S.D)	ΔC_T	SAMPLE ID	*p*21 GENE EXPRESSION (MEAN±S.D)	*GAPDH* EXPRESSION (MEAN±S.D)	ΔC_T	$\Delta\Delta C_T$	FOLD CHANGE
MAC-1	28.396±0.15	27.392±1.21	1.004	NC-1	31.456±0.24	29.734±0.60	1.722	-0.718	1.64
MAC-2	20.328±0.34	21.722±0.22	-1.394	NC-2	25.387±0.17	23.660±0.40	1.727	-3.121	8.69
MAC-3	26.259±0.68	24.155±0.75	2.104	NC-4	25.889±0.44	24.935±0.20	0.954	1.15	0.45
MAC-4	19.649±0.51	19.504±0.80	0.145	NC-3	23.963±0.11	21.436±0.76	2.527	-2.382	5.21
MAC-5	27.076±0.15	26.732±0.54	0.344	NC-5	32.589±0.63	29.75±0.36	2.839	-2.495	5.63
MAC-6	21.431±0.49	23.171±0.37	-1.74	NC-6	25.632±0.51	24.445±0.54	1.187	-2.927	7.60
MAC-7	28.225±0.19	27.312±0.23	0.913	NC-7	30.514±0.49	28.659±0.33	1.855	-0.942	1.92
MAC-8	29.387±0.70	26.868±0.52	2.519	NC-7	32.596±0.59	31.032±0.80	1.564	0.955	0.51
MCT-1	25.634±0.40	23.694±0.28	1.94	NC-9	28.125±0.75	27.435±0.18	0.69	1.25	0.42
MCT-2	21.747±0.24	18.857±0.50	2.89	NC-10	25.356±0.81	23.181±0.62	2.175	0.715	0.609
MCT-3	31.573±0.73	31.358±0.29	0.215	NC-11	30.57±0.39	28.945±0.50	1.625	-1.41	2.657
MCT-4	32.356±0.20	32.076±0.75	0.28	NC-12	31.212±0.42	28.062±0.40	3.15	-2.87	7.31
MCT-5	24.738±0.18	23.609±0.70	1.129	NC-13	32.165±0.05	30.185±0.38	1.98	-0.851	1.803

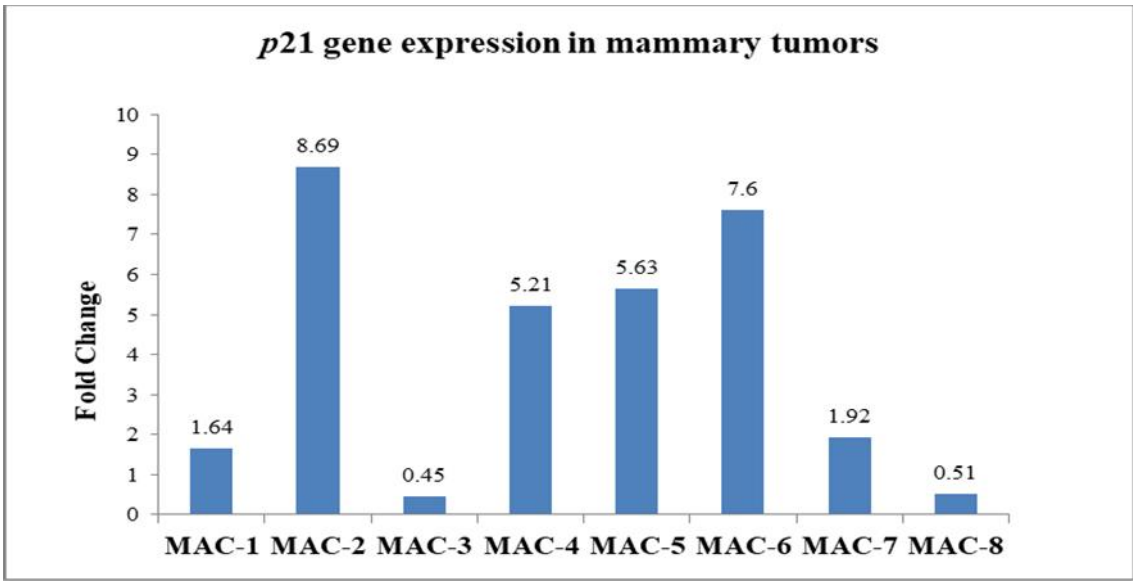

Figure 1: Expression level of *p*21 gene in canine mammary adenocarcinoma (MAC). Values are represented as fold change relative to normal sample (Livak method). Values >1.0 were considered up regulation of gene.

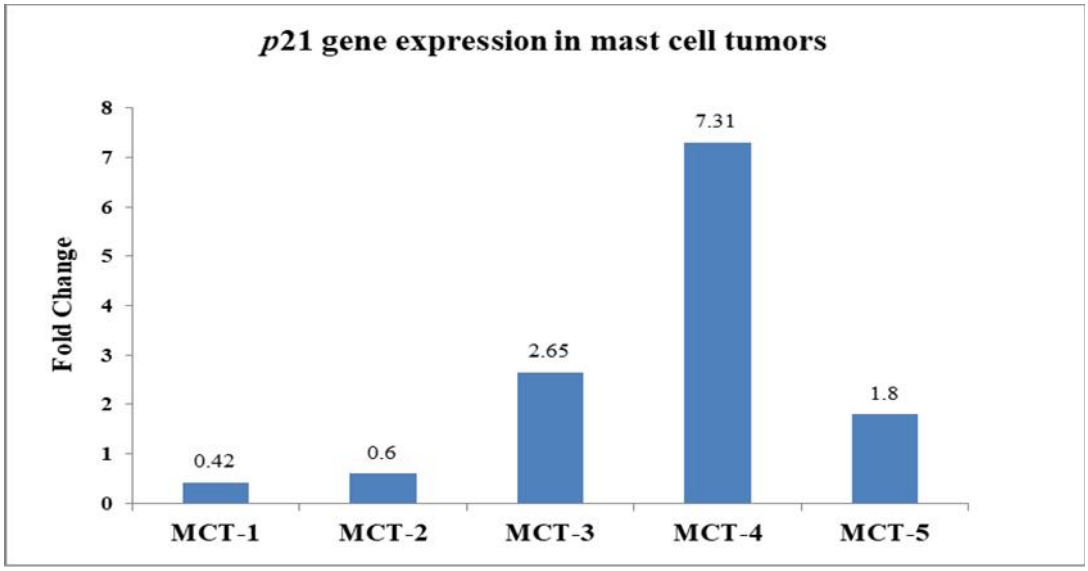

Figure 2: Expression level of *p*21 gene in canine mast cell tumors (MCT). Values are represented as fold change relative to normal sample (Livak method). Values >1.0 were considered up regulation of gene.

DISCUSSION

Cancer is the most common disorder in dogs although some dog breeds have a higher risk of developing certain cancer types. Priester and Mantel (1971) reported that particular breed of pedigree dogs show higher incidence of a particular type of tumor. Boxer and Rottweiler breeds have been reported to be particularly susceptible to cancers (Priester 1967; Peters 1969; Richard *et al.,* 2001). Several studies reported the occurrence of particular tumors in particular breeds such as, mammary tumor in *German shepherd* and *Cocker spaniel* (Frye *et al.,* 1967) and mast cell tumors in *Labrador* and *Golden retriever* (Mcniel*et al*., 2004; Murphy *et al.,* 2004). In the present study, two types of canine tumors, mammary and mast cell tumors were collected from different breed of dogs mainly represented by *German shepherd*, *Golden Retriever*, *Rottweiler* and *Labrador* (Table 1).

Mutation analysis and its associations with changes in levels of expression is a widely explored area. In fact, gene expression studies provide new information regarding the putative role of genes in normal, diseased and other physiological conditions within the body.

Expression profiling information can open new avenues to gain insight into different pathways involved in diseased conditions and how a gene product is influenced by different homeostasis body conditions. A similar hypothesis can be applied to the expression experiments conducted in present work. Precisely, in this study our concern was basically to determine the role of the tumor suppressor gene (*p21*) in canine tumors. Direct DNA sequencing and RT-qPCR techniques were used to find out any change in coding region and in expression of study gene.

p21 is a cyclin dependent kinase inhibitor, that plays an important role in cell growth, differentiation and apoptosis (Xing *et al.,* 1993) and also work as critical downstream effector of *TP53* tumor suppressor gene (Ikeguchi *et al.,* 1999). It was initially thought that somatic mutation in *p21* gene might be involved in tumor development, particularly for cases having wild type *TP53*; however, *p21* gene mutation proved to be extremely rare in breast, lungs and ovarian carcinomas investigated (Marchetti *et al.,* 1995; Mckenzie *et al.,* 1997). The two most common polymorphism, serine to arginine at codon 31 of exon2 and a single nucleotide polymorphism in the 3' UTR of exon 3 have been reported in different human carcinoma (Shiohara *et al.,* 1994). The *p21* codon 31 polymorphism is linked with an increased risk of lung carcinoma (Sjalander *et al.,* 1996), cervical carcinoma (Roh *et al.,* 2009), mammary gland carcinoma (Brenda *et al.,* 2002) and oral squamous cell carcinoma (Lei *et al.,* 2009). However, non-significant correlation was observed between polymorphisms and expression level of *p21*mRNA in gastric tissues (Akama *et al.,* 1996) as well as between the polymorphism and the expression level of *p21* protein in breast cancer (Lukas *et al.,* 1997). In the present study, Sanger's sequencing was performed to obtain exon 2 and 3 sequences of the *p21* gene. No polymorphism was detected in the tumors and normal control tissues samples.

Different applications of gene expression in the field of molecular biology are in vogue for pet animals (dog and cat). In human higher expression of *p21* gene has been studied in different cancers especially, head and neck carcinoma (Kapranos *et al.,* 2001), skin malignancies (Stoyanova *et al.,* 2012), gastric carcinoma (Xiaowen *et al.,* 2014), non-small cell lung cancer (Groeger *et al.,* 2000), hepatocellular carcinomas (Zhang *et al.,* 2009) and lower expression was reported in colorectal carcinomas (Al-Maghrabi *et al.,* 2012) and epithelial ovarian cancer (Yan *et al.,* 2004).

In this study, 69% tumor samples showed the up regulation of *p*21 gene in canine specie. Seventy five percent mammary adenocarcinomas showed moderate to high expression as compared to normal mammary tissues (Fig 1). Over expression of *p21* gene was detected in the canine tumor samples from the *German shepherd* breed with age 3 to 11years. These findings nearly correlate with the Klopfleisch and Gruber (2009) study, where the canine mammary adenocarcinomas showed the overexpression of *p21* genes when compared to adenoma and carcinomas. Chang and colleagues (2015) reported the overexpression of *p21* gene at both mRNA and protein level in breast cancer tissues when compared with noncancerous tissues. Overexpression of *p21* is significantly associated with larger tumor sizes, a poorly differentiated grade and lymph node metastasis. Similarly, in the case of mast cell tumor, 60% of samples showed over expression of *p21* gene with 7.3 highest fold change (Fig 2). Moderate to markedly high expression of *p21* protein in canine cutaneous mast cell tumor was reported by Wu *et al.,* (2004) and in canine hair follicle and epidermal neoplasm by Inoue and coworkers (2006).

In the studied tumors, the incident of *p*21 gene expression increased gradually from lower grade to higher grade and altered expression of *p*21 gene may be involved in malignant progression of studied tumors. These results indicated that *p*21 could be used as an important predictive and prognostic marker in canine tumors to improve therapy and prognosis.

Conclusion: This is first study from this part of world in which we have observed the altered expression of *p*21 gene but absence of polymorphism in coding region of target gene in tumor samples. Altered expression of *p*21 gene may be involved in malignant progression of canine tumors. However, due to limited quantity of canine tumors samples, there is a dire need to study the polymorphism in non- coding region that might be associated with altered expression of gene and larger number of canine tumors is also needed to further verify our results.

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