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Sequence Characterization of Baculoviral Inhibitor of Apoptosis Repeat Containing 5 (*BIRC* 5) Gene from a Case of Canine Mammary Tumour

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ABSTRACT

The *BIRC* 5 (also called survivin), member of the inhibitor of apoptosis protein family is highly over-expressed in human and animal cancers leading to poor prognosis. Still there is limited information about the gene sequence in dogs suffering with canine mammary tumour. Therefore, the present study was undertaken to find out any correlation of *BIRC* 5 gene over-expression with mutation status of the gene. The CMT tissues were confirmed by histopathological examination and included cases of mixed myoepithelioma, complex adenocarcinoma, mixed mammary capillary cystic adenocarcinoma and invasive solid carcinoma etc. Quantitative Real Time PCR (qRT-PCR) revealed 5.6±0.462-60.0±1.476 fold higher *BIRC* 5 gene expression levels in CMT tissues as compared to dog normal mammary gland tissues. The coding region of the gene was amplified, cloned and sequenced from a case of complex mammary carcinoma showing approximately 60.0±1.476 fold amplification of *BIRC* 5 gene. The sequence showed 100% similarity with the mRNA sequences of normal dog *BIRC* 5 present in NCBI. This indicates that *BIRC* 5 gene sequence in healthy dogs is similar to dogs suffering from CMT and showing over-expression of the gene. The multiple sequence alignment of the survivin gene with other species like cat, cow, buffalo, sheep, goat and human etc. revealed more than 90% similarity. The phylogenetic analysis demonstrates that gene is highly conserved across species to maintain its functional integrity. The findings revealed that there is no sequence alteration in *BIRC* 5 gene sequence in the CMT tissue showing more than 60 fold over-expression of the gene.

Key words: *BIRC* 5, survivin, canine mammary tumour, sequence characterization

INTRODUCTION

Incidence of cancer, resulting from complex interactions between environmental and genetic factors has increased alarmingly in the recent years (Pharoah *et al*., 2004; Wang *et al*., 2012). Alterations in many cancer related genes are highly correlated with drastic changes in the gene

expression (Masica and Karchin, 2011). Such type of alterations, affecting gene expression levels can be used to identify driver genes and molecular subtypes of a particular cancer (Verhaak *et al*., 2010; Noushmehr *et al*., 2010).

Canine Mammary Tumours (CMTs) are the most common malignancy of female dogs of more than 5 years of age (Davidson, 2003; Murphy, 2008; Salas *et al*., 2015) accounting for more than 40% of all tumours diagnosed (Sleeckx *et al*., 2011; Beck *et al*., 2013). Majority of canine mammary tumours have poor clinical outcome with thrice higher mortality rates than human breast cancer (Egenvall *et al.,* 2005; Shafiee *et al*., 2013). Chemotherapy, radiation therapy and surgery are the principal therapeutic strategies available to treat malignant CMTs (Queiroga *et al*., 2011). However, not a single chemotherapy protocol has been reported to be effective in management of malignant CMTs (Simon *et al*., 2006; Kumar *et al*., 2010). Further, there is increased risk of recurrence or metastasis, within 2 years following mastectomy (Rutteman and Misdorp, 1993; MacEwen and Withrow, 1996). Thus canine mammary tumour being a severe neoplastic condition of dogs can be considered as a promising candidate for development of strategies for disease management.

Baculoviral inhibitor of apoptosis repeat containing 5 (*BIRC* 5), also called as survivin is a smallest member of Inhibitors of Apoptosis Protein (IAP) family. The gene is present on chromosome 17 q25 in humans (Ambrosini *et al*., 1998). It is highly over-expressed in human and animal cancers leading to poor prognosis. Amplification of the *BIRC* 5 gene has been reported in vast majority of cancers including oesophageal, lungs, ovarian, central nervous system, breast, colorectal, bladder, gastric, prostate, pancreatic, laryngeal, uterine, hepatocellular and renal cancers (Altieri, 2003; El-Magd *et al*., 2012). Over expression of this gene is associated with tumour progression and malignancy (Altieri, 2006) and is considered as an unfavourable prognostic marker of cancer in humans (Tango *et al*., 2010; Bongiovanni *et al*., 2015). The survivin protein contributes to genesis of cancers by acting as negative regulator of apoptosis or programmed cell death. The targeted disruption of survivin induction pathways leads to increased apoptosis and decreased tumour growth (Blanc-Brude *et al*., 2003; Uchida *et al*., 2004; Tao *et al*., 2012). Apart from tumours, the protein is highly expressed in embryonic and foetal tissues, but is completely absent in terminally differentiated adult cells (Chang *et al*., 2004; Lechler *et al*., 2007; Xue *et al*., 2012; Jaiswal *et al*., 2015). The survivin expression in embryonic and foetal development contributes to tissue homeostasis and differentiation (Adida *et al*., 1998; ElSheikh *et al*., 2014). Survivin expression is highly regulated by the cell cycle and the protein is only expressed in the G2-M phase (Altieri, 2008; Jaiswal *et al*., 2015). The aberrations in the developmental pathways results in prominent re-expression of survivin during neoplasia. These data suggest that survivin might provide a new target for cancer therapy that would discriminate between transformed and normal cells. However, the exact mechanism of apoptosis inhibition by survivin is yet poorly understood and still there is limited information about the gene sequence in the canine mammary tumours tissues. Therefore, the present study was taken up to analyze whether survivin gene sequence is altered in dogs suffering from CMT and showing over expression of the survivin gene.

MATERIALS AND METHODS

Ethical statement: The study was carried out after approval from the Institute Animal Ethics Committee (IAEC) of Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, UP, India.

Tumour tissues: The CMT tissues used in the study were obtained after surgical removal of tumours from clinical cases of CMTs presented to the "Referral Veterinary Polyclinic", Indian

Veterinary Research Institute (IVRI), Izatnagar, Bareilly, India. Histological examination and classification of spontaneous mammary tumours from dogs was performed on H and E stained tissue sections according to World Health Organization (WHO) criteria for CMTs (Misdorp *et al*., 1999).

RNA isolation and cDNA synthesis: Total RNA was extracted from tissue samples by RNeasy™ mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The integrity of the isolated RNA was conformed after agarose gel electrophoresis of the sample on 1.5% agarose gel. The purity of RNA was assessed by calculating OD_{260}/OD_{280} using Nanodrop (NanoDrop1000, Thermo Scientific, Singapore). Subsequently, an in solution DNase digestion step (RNase free DNase I, MBI Fermetas USA) was performed to remove genomic DNA contamination. The cDNA was then prepared from total RNA using Revert Aid cDNA synthesis kit (Fermentas, USA) as per the manufacturer's instructions. The cDNA from each tumour sample was synthesized using Oligo (dT) 18 primers and 1 µg total RNA in a total volume of 20 µL and incubated at 65°C for 5 min, 42°C for 60 min followed by final incubation at 70°C for 5 min. The prepared cDNA was stored at -80°C for downstream applications.

Real time quantitative PCR: Survivin mRNA expression was assayed by real time PCR using Applied Biosystems® 7500 fast real time PCR instrument. A set of intron-spanning primer sequences for dog survivin were designed using Premier 5.0 software (National Bioscience) and analyzed using oligoanalyzer 3.1. To normalize the amount of input RNA or cDNA, *RPS* 19 gene was selected as an endogenous control. Reaction was performed using KAPA SYBR FAST qPCR master mix (KAPA Biosystems, Boston, MA, USA) according to the manufacturer's instructions. Briefly, 10 µL of the reaction mixture, consisting of 5 µL SYBR FAST qPCR master mix, 0.4 µL cDNA and 200 nmol L^{-1} of forward and reverse primers was subjected to initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 3 sec and annealing/extension at 60°C for 20 sec. The relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). All mRNA expression levels were normalized to those of normal mammary tissues obtained from dog post mortem cases. Data was expressed as the mean values calculated from experiments performed in triplicate. The specificities of the PCR amplicons were confirmed using melting curve analysis.

Survivin gene amplification, cloning and sequence analysis: Primers for amplification of full length survivin were designed using DNA star laser gene v6 software from the published sequence (NCBI accession number NM_001003348.1) and custom synthesized by Integrated DNA technologies (USA). Sites for restriction enzymes Bam HI and Hind III were incorporated to the 5' ends of forward and reverse primer, respectively. The cDNA from a case of complex mammary carcinoma showing over expression of survivin gene was used as a template for amplification of survivin gene. The cycling conditions for amplification of the gene included, initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 98°C for 20 sec, annealing at 60°C for 15 sec and extension at 72°C for 15 sec with final extension at 72°C for 10 min. The PCR was performed in a 50 µL reaction mixture containing 25 µL KAPA hi-fidelity PCR master mix (2X), 0.5 µL of 20 pM each of forward and reverse primer and 3 µL of template cDNA. The amplified PCR product was purified using Qiagen MinElute® PCR purification kit as per the manufacturer's

instructions. The purified product was subjected to R.E. digestion using BamHI and HindIII restriction endonucleases (NEB, England) at 37°C for 2 h. The pET-32b (+) vector was also subjected to RE digestion with Bam HI and Hind III to generate complementary overhangs. The digested vector and the PCR product were then subjected to overnight ligation at 4°C using T4 DNA ligase (Promega, Madison, USA). The ligated product was subsequently transformed into *E. coli* DH5*a* competent cells. The recombinant clones were screened by colony PCR. The recombinant plasmid was purified from the overnight grown culture using PureYield™ plasmid miniprep system (Promega) and subjected to RE digestion using *Bam* HI and *Hind* III restriction endonucleases for further confirmation of the recombaint clone. The recombinant plasmids were then sent for plasmid DNA sequencing at Eurofins Genomics India Pvt Ltd (Bangalore, India). The gene sequence was analyzed and compared to homology with other survivin gene sequences present in the data bases using DNASTAR's MegAlign sequence alignment software, Blastn (Basic Local Alignment Search Tool) and NCBI. The phylogenetic analysis was done using MEGA6 software.

RESULTS

Gross and histopathological examination of CMT tissues: CMT tissues were collected from clinical cases of dogs which were referred for surgery to "Referral Veterinary polyclinics" Indian Veterinary Research Institute, Izatnagar. The photographs showing gross appearance of some representative tumour masses from CMT cases are shown in Fig. 1. The tumour tissues collected from surgically operated tumour masses were fixed in 10% neutral buffered formalin and H and E staining of the formalin fixed tissue sections was done for histopathological analysis as shown in Fig. 2. Based on the histopathological examination, tumour tissues were classified as shown in Table 1.

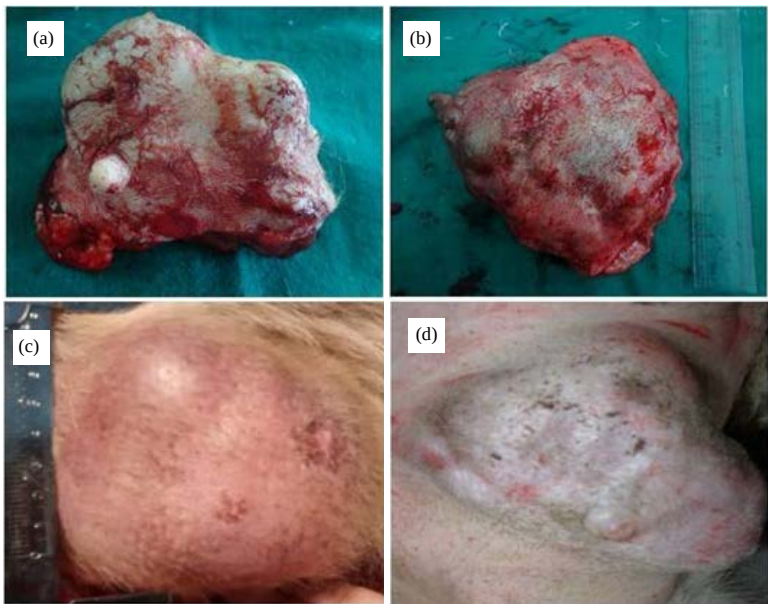

Fig. 1(a-d): Gross appearance of representative canine mammary tumour tissues collected from surgically operated cases of CMTs, (a, b, c and d) represents different CMT cases

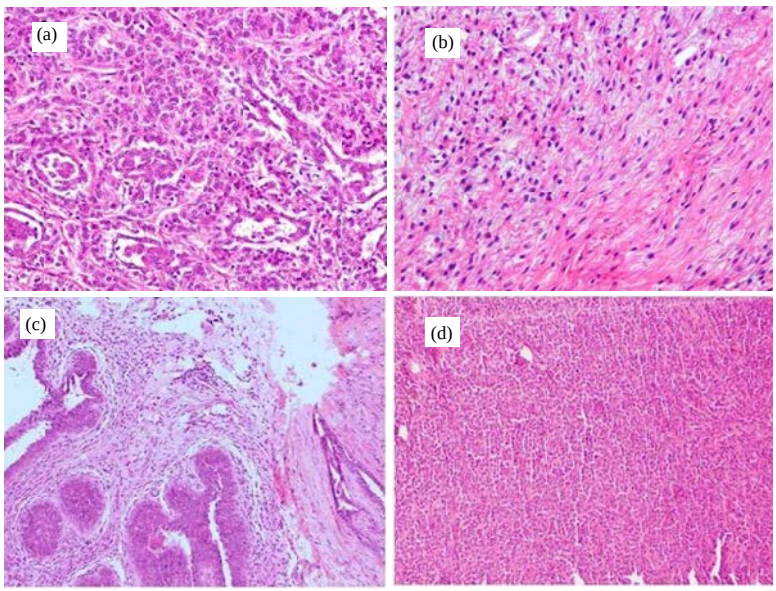

Fig. 2(a-d): Histopathological examination (H and E staining) of some of the representative canine mammary tumour tissues, (a) Intraductal adenocarcinoma, (b) Carcinosarcoma, (c) Complex carcinoma and (d) Invasive solid carcinoma

Table 1: Classification of some of the representative CMT tissues based upon the histopathological findings

Tumour classification
Mixed myoepithelioma
Complex adenocarcinoma
Complex carcinoma
Carcinosarcoma
Mixed mammary capillary cystic adenocarcinoma
Invasive solid carcinoma

Survivin expression in canine mammary tumours vs normal tissues by real time PCR: The concentration of RNA isolated from the mammary tissues ranged from 80-200 ng μL^{-1}. On agarose gel electrophoresis two distinct bands corresponding to 28 S and 18 S rRNA were observed. The $OD_{260/280}$ was found to be~1.9 indicating good purity of the RNA samples. The cDNA's synthesized from the tumour tissues were used for analysis of *BIRC* 5 gene over-expression by quantitative real time PCR (qPCR). The amplification curves for the real time PCR analysis are illustrated in Fig. 3a. Single and sharply defined melting curves with narrow peaks were obtained showing specificity of real-time PCR amplification (Fig. 3b). Overexpression of *survivin* gene was detected in 6 out of 8 (75%) mammary tumour tissues examined. The samples from CMT cases showed 5.6±0.462- 60.0±1.476 fold higher expression levels of the gene as compared to normal mammary gland tissue (Fig. 4).

Survivin gene amplification, cloning and sequence analysis: The PCR amplification of the *BIRC* 5 gene from a case of complex carcinoma showing over-expression of the gene was performed as described earlier. The amplified product showed expected band size of 516 bp on agarose gel electrophoresis (Fig. 5). The amplified product was cloned in pET-32b (+) vector and the recombinants were analyzed by RE digestion and further confirmed by plasmid DNA sequencing. Restriction digestion of the recombinant plasmid pET-32b (+) *survivin* with *Bam* HI and *Hind* III

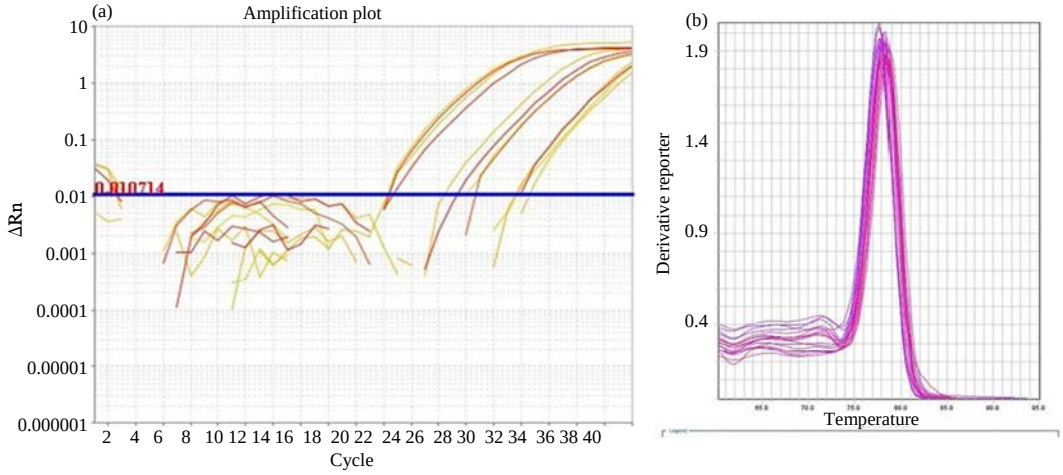

Fig. 3(a-b): Amplification and dissociation curves for Survivin gene (a) Amplification plot and (b) Melt curve (Dissociation curve)

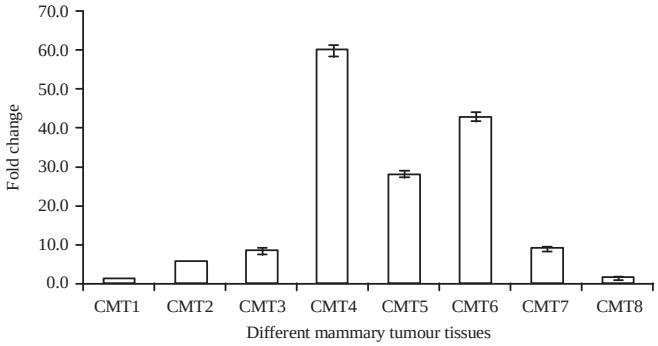

Fig. 4: Relative expression of survivin in different CMT tissues represented as CMT1-CMT8

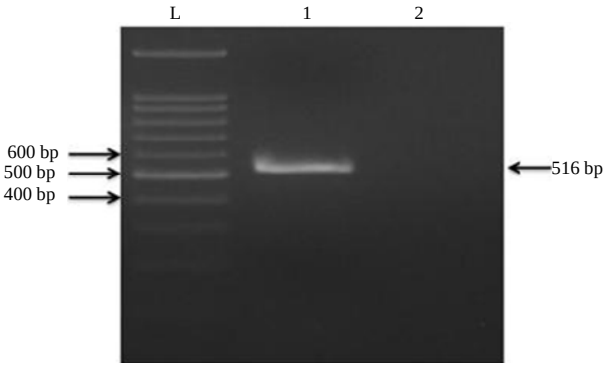

Fig. 5: PCR amplified survivin gene in 1.5% agarose gel. L: 100 bp DNA ladder, Lane 1: Survivin amplified PCR product Lane 2: NTC-No template control

released the insert of 516 bp (Fig. 6). The recombinant plasmid was purified and sent for sequencing. The gene sequence was analyzed and compared for homology with other survivin gene

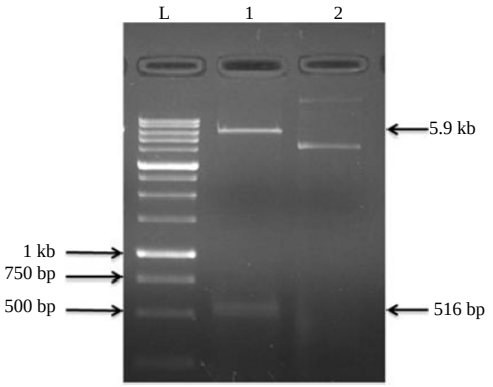

Fig. 6: Restriction analysis for confirmation of recombinant pET 32b (+) *Survivin* plasmid. Restriction digestion of recombinant pET32b (+) *Survivin* plasmid with Bam HI and Hind III showing the insert *(Survivin*) release. L: 1 kb DNA ladder, Lane 1: Insert release (*Survivin*) marked by arrow, Lane 2: Undigested recombinant plasmid

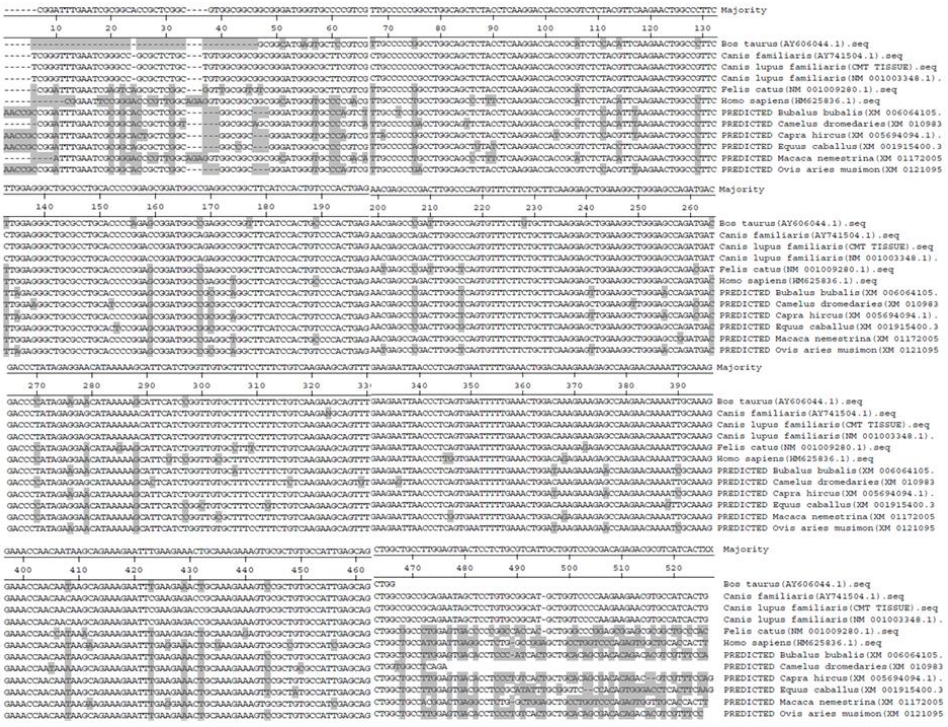

Fig. 7: Multiple sequence alignment of dog survivin gene with the orthologus sequences using MegAlign DNAstar software

sequences present in the databases such as dog (NM_001003348.1), dog (AY741504.1), dog (AB095108.1), cat (NM_001009280.1), horse (XM_001915400.3), cow (AY606044.1), sheep (XM_012109576.1), goat (XM_005694094.1), monkey (XM_011720054.1), buffalo (XM_006064105.1), human (HM625836.1), camel (XM_010983810.1). The comparison of the gene sequences with other species (Fig. 7) revealed that the gene sequence is similar across species with more than 90%

Percent indentity

	1	2	3	4	5	6	7	8	9	10	11	12	
1		92.6	92.9	92.9	93.6	93.8	93.6	93.1	93.3	93.6	94.3	93.6	1
2	7.6		99.8	99.8	88.2	82.6	84.5	80.4	84.7	85.5	84.1	85.1	2
3	7.6	0.0		100.0	88.4	82.8	84.7	80.6	84.9	85.7	84.3	85.3	3
4	7.6	0.0	0.0		88.4	82.8	84.7	80.6	84.9	85.7	84.3	85.3	4
5	6.8	12.6	12.5	12.5		82.6	86.0	80.8	85.9	86.2	83.5	86.4	5
6	6.5	18.4	18.3	18.3	18.7		83.7	80.4	83.3	85.0	97.3	83.9	6
7	6.3	15.9	15.9	15.9	14.1	16.7		82.9	97.9	90.3	84.5	98.4	7
8	7.3	11.0	11.0	11.0	11.0	13.5	7.6		92.0	90.9	89.2	92.5	8
9	6.6	15.5	15.4	15.4	14.2	16.8	1.8	7.9		89.7	84.1	98.4	9
10	6.3	14.4	14.4	14.4	13.6	14.5	10.0	9.1	10.7		85.9	90.1	10
11	6.0	16.8	16.7	16.7	17.8	2.8	15.6	11.1	15.7	13.4		84.5	11
12	6.3	15.2	15.1	15.1	13.6	16.2	1.4	7.4	1.2	10.2	15.4		12
	1	2	3	4	5	6	7	8	9	10	11	12	

Divergence

1 *Bos taurus* (AY606044.1).seq
2 *Canis familiaris* (AY741504.1).seq
3 *Canis lupus familiaris* (CMT TISSUE).seq
4 *Canis lupus familiaris* (NM 00100348.1)
5 *Felis catus* (NM 001009280.1).seq
6 *Homo sapiens* (HM 625836.1).seq
7 Predicted *Bubalus bubalis* (XM 006064105.)
8 Predicted *Camelus dromedaries* (XM 010983)
9 Predicted *Caprahiscus* (XM 005694094.1)
10 Predicted *Equus caballus* (XM 001915400.3)
11 Predicted *Macaca nemestrina* (XM 01172005)
12 Predicted *Ovis aries musimon* (XM 0121095)

Fig. 8: Percentage identity of dog survivin gene with servivin gene sequence from other species

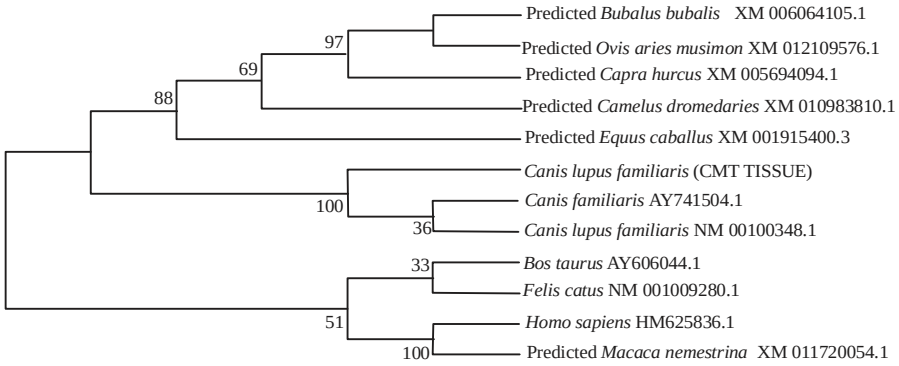

Fig. 9: Phylogram illustrating the evolutionary relationship of survivin gene of dog (*Canis lupus familiaris*) with sheep (*Ovis aries*), cattle (*Bos taurus*) etc. The phylogenetic tree was constructed using Neighbour-Joining analysis. Numbers represent bootstrap values (given as percentages) for a particular node. Thousand replicates were used in bootstrap analysis for good statistical support. The branch lengths are scaled to represent the relative number of substitutions occurring along each branch

similarity (Fig. 8). The phylogram illustrating the evolutionary relationship of dog survivin gene with the gene sequence from different animal species is shown in Fig. 9.

DISCUSSION

Dysregulation of cell death pathways occurs in cancer, autoimmune disorders, immunodeficiency diseases and in neuro-degenerative disorders. Thus, proteins involved in apoptosis regulation are attractive therapeutic targets (Deveraux and Reed, 1999). The IAP family proteins are characterized by a novel domain of ~70 amino acids which is named as Baculoviral Iap Repeat (BIR) because of original discovery of these proteins in baculoviruses (Crook *et al*., 1993; Birnbaum *et al*., 1994). *Survivin*, a unique member of IAPs is highly over expressed in animal and human cancers (Bongiovanni *et al*., 2015) and has thus attracted much attention as a target for new oncotherapies. The gene is thoroughly studied in humans and several polymorphisms have been detected in the promoter region of survivin gene. But most of the mutation studies in humans are restricted within the promoter region of survivn gene and to date, there is a single report of

mutation within the coding region of the gene, i.e., lysine>glutamic acid (K129E) mutation in the exon 4 of the protein (Aljaberi *et al*., 2014). Despite the prevalence of survivin in dog cancers, no studies have been conducted so far in dogs to find out any mutations in the coding region or the promoter region of survivin gene and correlation of such alterations with changes in gene expression in cases of dog cancers. Therefore, the aim of the present study was to analyze the coding region of the survivin gene in cases of CMTs, where the gene expression is highly upregulated. Eight CMT tissues were analyzed for survivin gene over expression and a case of complex mammary carcinoma showing highest (~60 fold) over expression of the gene was selected for sequence analysis of survivin gene coding region. The analysis of the sequence of survivin gene coding region revealed that survivin gene over-expression (in the case under study) is not correlated with any alteration in the gene sequence. Several studies indicate that drastic changes in the expression of cancer related genes are highly correlated with the mutation status of these genes (Masica and Karchin, 2011) but in our study we do not found any correlation in survivin gene over-expression and gene mutation. Further, comparison of the dog survivin gene CDS with other species revealed that the sequence is similar across species with more than 90% similarity. The reasons for similarity in gene sequence could be many, including the conserved nature and abundant expression of the protein in vast majority of cancers. Thus, it can be assumed that the gene is subjected to high selection pressure to maintain its functional conformity. These assumptions are also in line with Behera *et al*. (2015) who reported that the conserved nature of the *hfq* gene of *S. typhimurium* may be due to heavy selection pressure. Other researchers also support that across evolution, the survivin protein is conserved in function as homologues of the protein are found both in vertebrates as well as invertebrates (Tamm *et al*., 1998). The human survivin protein shares 84 and 91.5% sequence identity with mouse (Li and Altieri, 1999) and canine survivin (Uchide *et al*., 2005), respectively, indicative of a conserved function of survivin in mammalian species. The ectopic expression of some baculoviral IAPs blocks apoptosis in mammalian cells also suggests conservation of the cell death program among diverse species and similarities in the pathways used by the IAPs to inhibit apoptosis (Deveraux and Reed, 1999).

CONCLUSION

Survivin gene is over expressed in 75% of canine mammary cancers and real time PCR based assay standardized in this study can be employed for detecting survivin gene over expression in CMT tissues. Further, no correlation was observed between survivin gene over expression and mutation status of the coding region of the gene. The results from the study needs to be further validated in large number of CMT cases.

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