RESEARCH ARTICLE

Expression Pattern of SLIT 2 Genes Discriminates Malignant from Benign Canine Mammary Tumours

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Abstract

The study was conducted to investigate the expression of *SLIT2* gene in representative tissue samples of mammary tumours of female dogs (n=10) with the age between 5.0 and 16.5 years brought to Veterinary Clinical Complex, LUVAS, Hisar. Excisional biopsy was taken after surgery of these cases. Histopathological studies confirmed four cases of adenocarcinoma, three cases of benign mixed tumours and two of myoepithelioma and one case of adenoma. Amplification plots and dissociation curves of RT-qPCR were indicative of specific amplification of the canine *SLIT 2* gene in all the samples. Further, amplification product of a 160 bp *SLIT 2* gene and 120 bp β-actin gene in all the RT-qPCR tubes were visible by 2% agarose gel electrophoresis. Out of 10 tissue samples examined, transcript levels of *SLIT 2* genes in all different samples ranged from 1.90 to 84.98. Conclusively, the expression of *SLIT 2* genes increased in all canine malignant mammary gland tumours, thereby showing its association with tumour malignancy.

Key words: Histopathology; Mammary tumours, Quantitative real-time PCR, *SLIT2* genes.

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Introduction

Spontaneously occurring mammary tumours in female dogs have been demonstrated as useful models for human breast cancer studies owing to their similarities in histochemical features, associated risk factors, clinical progression and response to treatment; biomarkers and molecular targets etc (Queiroga *et al.,* 2011; Hussain *et al.,* 2018). Mammary tumours are one of the most common oncological diseases diagnosed in female dogs (Stan *et al.,* 2020) and account for up to 52% of all tumours in the female dogs (Kumar and Parasar, 2020; Saharan *et al.,* 2022).

Several attempts have been made on the use of differential expression of single gene for an improved prognosis of canine mammary tumours including p53, ERBB1 and BRCA1 (Muthuswamy *et al.,* 2001; Nieto *et al.,* 2003; Klopfleisch *et al.,* 2010). In these studies, a significant correlation was established between protein expression and malignancy. But, none of these markers was able to correctly classify tumour as benign or malignant. Several markers of malignancy have been proposed for canine mammary tumours on the basis of mRNA and protein levels, but no single marker identified to date can be used to reliably predict malignancy for individual tumours. Recently, some genes have been reported to be associated with malignancy of canine mammary tumours (Tanno *et al*., 2006; Klopfleish *et al*., 2011). *SLIT2* gene is involved in angiogenesis of tumours and expression of *SLIT2* mRNA increased in most types of the malignant mammary tumours suggested to be a possible putative marker for malignancy of these tumours (Tanno *et al*., 2006). Therefore, the study was conducted to investigate the expression of *SLIT2* gene in representative tissue samples in mammary tumours in female dogs.

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Materials and Methods

The study was conducted on 10 cases of mammary tumours in intact female dogs brought to the Veterinary Clinical Complex (VCC) of Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, India. The female dogs belonged to the German shepherd (n=5), Mongrel (n=2), Spitz Mixed (n=1), Spitz (n=1) and Rottweiler (n=1) breeds. The age of these animals ranged from 5 years to 16.5 years (median age

of 10.7 years). The ultrasonography and histopathology were conducted as per Saharan *et al.* (2022).

Real-Time Reverse Transcription Quantitative PCR (RT-qPCR) of *SLIT2* Gene Expression:

For RT-qPCR, about 30 mg tissue was collected by biopsy in RNA stabilizer ('RNAlater', Ambion, USA) and stored at -40°C until use for RNA extraction. Total RNA was extracted from 1 mm pieces of 20-30 mg of each canine mammary tissue sample and made DNA-free by DNase I digestion using commercial kit (Qiagen®, Germany) in the RNase-free environment in the Immunotechnology laboratory of the university. The mRNA was quantified in Biophotometer® (Eppendorf, Germany), assessed for purity by A260/A280 ratio, and quality tested by 2.0 % agarose gel electrophoresis in Tris-acetate-EDTA buffer, pH 8.3 using 1 kb DNA size markers.

The mRNA, 500 ng/reaction, was reverse transcribed with oligo $d(T)_{20}$ primer using a commercial kit (Invitrogen®, USA). RT (-) control was kept for each sample. The reaction was carried out in a thermocycler programmed to 25°C for 10 min, at 50°C for 30 min, at 85°C for 5 min and at 4°C for 15 min, and then RNaseH digestion at 37°C for 20 min. The cDNA was stored at -40°C until use. The cDNA was then used to obtain real-time PCR data and Ct values with *SLIT2* specific primers and SYBR Green master mix kit (Invitrogen®, USA). The primers for *SLIT2* and β-actin were those reported by Tanno *et al.* (2006), the details of which are shown in Table 1.

The qPCR was set up in 20 µL reaction tubes by using SLIT2 and *β-actin* forward and reverse primers, and cDNA template dilutions were as per the manufacturer's instructions of the universal kit (Invitrogen®, USA). The reaction was run in a real-time PCR machine (Mx3005P, Stratagene®, USA) for data acquisition and analysis. The thermocycling conditions used in real-time qPCR were in three segments: Segment-I includes single cycle of denaturation at 95°C for 3 min followed by segment-II involving 45 cycles of denaturation (95°C for 20 s), annealing (60°C for 22 s) and extension (72°C for 5 s.) and lastly segment-III involving single cycle of dissociation curve (95°C for 1min, 55°C for 30s and 95°C for 30s). The Ct data of *SLIT2* gene was normalized against the reference/ house-keeping β-actin gene and the quantification was done by $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001). The results were displayed as fold-change in the transcript levels in different tissue samples. The *SLIT 2* amplicon was resolved by 2% agarose gel electrophoresis, extracted from the gel and subjected to DNA sequencing. The DNA sequence was determined in an automatic Sanger's dideoxy chain termination DNA sequencing facility in the Department of Animal Biotechnology of the University.

Results and Discussion

Real-Time RT-qPCR of *SLIT2* Gene Expression:

Quantity of total RNA extracted from different samples was in the range of 20 µg/mL to 100 µg/ mL from 20-30 mg of different tissues. The total RNA showed two major fragments of ribosomal RNA at approximately 0.9 kb and 1.25 kb in agarose gel electrophoresis, indicating good quality of total RNA. Amplification plots and dissociation curves of RT-qPCR were indicative of specific amplification of the canine *SLIT2* gene in all the samples (Fig. 1).

Table 1: Primers used in RT-qPCR

Canine Gene-specific primer designation	Sequence (5′-3′)	Tm value °C	Amplicon size (bp)
SLIT2-F	ACTGCCAGGATCACAAGTGTAAAA	65.5	160
SLIT2-R	GTTCTGACAGTCGAAGTTGTCACAG	66.1	
β-actin-F	GCCATCTCTTGCTCGAAGTC	64.0	120
β-actin-R	TACAGCTTCACCACCACAGC	64.0	

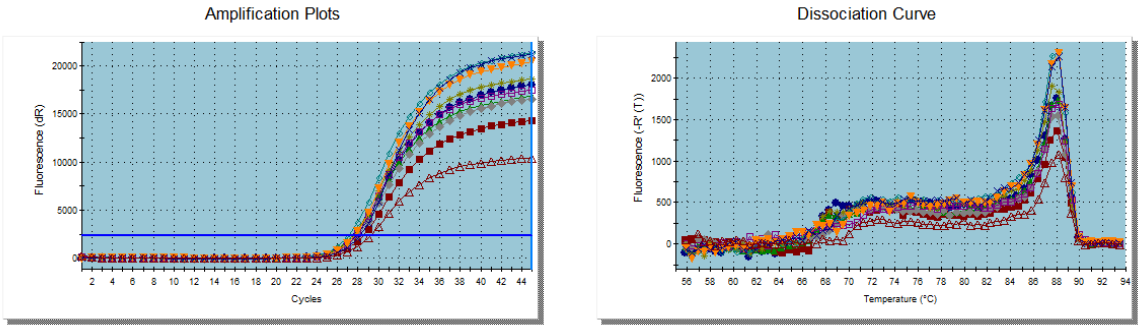

Fig. 1: Amplification plots and dissociation curves in real-time PCR of *SLIT2* RT-qPCR products of different samples of canine mammary tumours

Further, amplification product of 160 bp *SLIT2* gene and 120 bp β-actin gene in all the reaction tubes was visible by 2% agarose gel electrophoresis (Fig. 2). Partial DNA sequence of a 96 nucleotide long segment of *SLIT2* gene further confirmed specific amplification of the gene in the present study (Table 2).

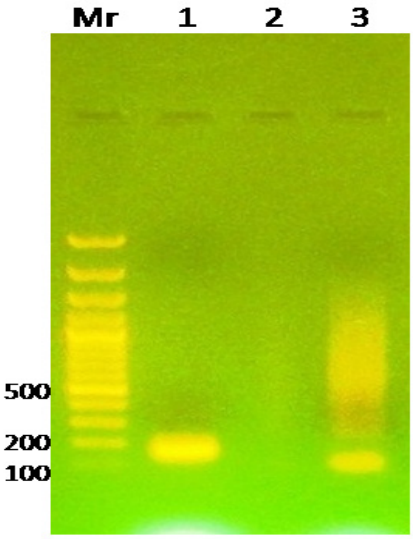

Fig. 2: 2 % Agarose gel electrophoresis resolved canine *SLIT 2* gene and *β* -actin gene amplified by RT-qPCR in real-time PCR machine [lane Mr: 100 bp plus DNA ladder, 1: slit 2 RT-qPCR product, 2: empty, 3: *β* -actin RT-qPCR product]

Table 2: DNA sequence of *SLIT 2* gene segment

5'-	GGCTACACGTGCACCTGCCCCGAAGGCTACAGCG-GCTTGGTCTGTGAATTCTCCCCGAACATGGTCCTCC-CACGCACCAGCCCCTGTGACAACTTC	- 3'

Canine *SLIT2* Gene Expression Levels:

Transcript levels of *SLIT2* gene in canine mammary tumours as determined by real-time RT-qPCR are in agreement with its previous histopathological findings (Saharan *et al.,* 2022) as shown in Table 3. Out of 10 tissue samples studied, *SLIT2* transcript levels in all different samples ranged from 1.90 x-fold to 84.98 x-fold higher than the mean level of the normal tissues .

The present study compared histology and *SLIT2* transcript levels for diagnosis of malignancy of canine mammary tumours. Our findings confirmed those of Tanno *et al*. (2006) that *SLIT2* transcript levels were elevated in malignant tumours. There was unanimity in favour of using histology to distinguish between malignant and benign tumours. Interestingly, the malignant tumours showed several folds higher *SLIT2* transcript levels than the tissues from the benign cases. However, one case each of myoepithelioma and adenoma also showed 32x and 24x fold higher transcript levels than those of the normal, which could be probably for the reason that these cases were turning into malignant ones (Table 3). The aim of present study was to test the association of expression levels of *SLIT 2* gene with malignancy of these canine mammary tumours as suggested by Tanno *et al*. (2006). Four samples (*i.e.,* XIII, XIV, XV and VII) which shared signs of malignancy by histopathology as well as ultrasonography had *SLIT 2* transcript levels respectively 60x, 85x, 52x and 38x fold higher than those of normal tissue. However one tissue, *i.e.,* sample XII also had 32x fold higher levels than normal, but was classified as benign on the basis of ultrasonography and histopathology. The *SLIT 2* mRNAs were strongly expressed in canine mammary malignancies which agreed with those of Tanno *et al*. (2006). In the *SLIT 2* analyses, 5 of 6 cases of complex carcinoma showed increased expression in study of Tanno *et al*. (2006). In the present study, the expression of *SLIT 2* genes increased in all malignant mammary gland tumours, thereby showing its association with tumour malignancy. One sample, *i.e.,* XII which was suggested as benign by histopathological examination did show 32x fold higher levels. Identification of other tumour markers has also been reported. The role of mutation in p53 tumour suppression gene in tumourogenesis of human and canine breast and mammary cancers has been suggested in carcinomas prognosis (Lee and Kweon, 2002). However, the diagnostic and prognostic value of *SLIT2* gene expression in

Table 3: Relative transcript levels of *SLIT2* gene in canine normal tissues and mammary tumours determined by real-time PCR

Sr. No.	Tissue sample	x Fold level ±SD	Previous histopathological findings (Saharan *et al.,* 2022)	*SLIT2* RT-qPCR showing X fold increase above normal
1	XIII	59.88±16.19	Adenocarcinoma	60x
2	XIV	84.98±17.19	Adenocarcinoma	85x
3	XV	51.77±5.74	Adenocarcinoma	52x
4	XII	32.31±3.93	Myoepithelioma	32x
5	I	4.27±0.39	Myoepithelioma	4x
6	VII	38.16±10.32	Adenocarcinoma	38x
7	III	1.90±0.41	Benign mixed tumour	2x
8	IX	13.92±7.1	Benign mixed tumour	14x
9	IV	23.74±8.29	Adenoma	24x
10	II	12.94±2.77	Myxoma	13x

canine mammary malignant tumours needs to be established in a large study.

Conclusions

The genes responsible for the aggressive behaviour of mammary tumours are not very clear, and poor prognosis associated with malignant mammary tumours emphasize the necessity to unravel the underlying pathways and genes which could act as targets for future therapy. In the current investigation, the level of *SLIT 2* gene expression was up-regulated in all canine mammary tumours tissue by RT-qPCR. The study further provided evidence of association of *SLIT 2* up-regulations in canine mammary malignancies.

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