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Differential expression of *Tissue inhibitor of metalloproteinase* 2 (TIMP2) gene in canine mammary tumour

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Abstract
Mammary tumour is the second most common cancer among dogs, just after skin cancer, accounting about fifty per cent of all tumours among dogs. Certain breeds like English Springer Spaniel, Boxer, Poodle, Bull Mastiff, German Shepherd, Cocker Spaniel, Dachshund and Fox Terrier dogs showed higher risk of incidence of mammary tumour, whereas certain other breeds like Collie, Shetland Sheep dog and Bernese Mountain Dog were considered to be at low risk. This breed predilection suggested that genetic factors play a role in canine mammary neoplasms. The role of numerous genes in canine mammary tumour (CMT) development and progression have been investigated, but the importance of the microenvironment is frequently disregarded as far as CMT is considered. *Tissue inhibitor of metalloproteinase* 2, commonly referred to as *TIMP2*, is one of several genes that influence the microenvironment of tumours. The interaction between *Matrix Metalloproteinase 2* (*MMP2*) and *TIMP2* is crucial for the invasion, development, and metastasis of tumours. The relative mRNA expression level of *TIMP2* was examined in the current investigation in both CMT-affected and normal glands. A ten - fold reduced expression of *TIMP2* was found in tumour samples than normal glands, which was statistically significant.

Keywords: Tissue inhibitor of metalloproteinase 2, canine mammary tumour, expression study

1. Introduction
Dogs have served man as a guard, a courier in war, a life saver, a true worker pulling a sledge, and a loyal companion throughout history. Burgeoning urbanisation, the rise of the nuclear family, and a shift in society's attitude toward pets have all contributed to India's growing pet population. Currently, India is home to millions of pet dogs, both exotic and indigenous breeds. The increase in dog population coincided with an increase in reports of canine diseases in the country, both infectious and non-infectious. It has been found that there is a steady peaking of lifestyle diseases in pet dogs. Cancer, epilepsy, autoimmune illnesses, blindness, cataract, and heart diseases were recognised as the top six lifestyle diseases affecting dogs (Sutter and Ostrander, 2004) [1]. Rowell *et al*. (2011) [2] reported that canine cancers were more aggressive and could be considered as a leading cause of death especially in elderly dogs. Mammary tumour is the second most common cancer among dogs, just after skin cancer, accounting about fifty per cent of all tumours among dogs (Kelsey *et al*., 1998; Egenvall *et al*., 2005; Zuccari *et al*., 2011) [3, 4, 5]. According to Borge *et al*. (2011) [6] there exist a breed predisposition in CMT. Borge *et al*. (2013) [7] reported that certain breeds like English Springer Spaniel, Boxer, Poodle, Bull Mastiff, German Shepherd, Cocker Spaniel, Dachshund and Fox Terrier dogs showed higher risk of incidence of mammary tumour, whereas certain other breeds like Collie, Shetland Sheep dog and Bernese Mountain Dog were considered to be at low risk. Such a breed predisposition pointed towards the genetic involvement in canine mammary neoplasms. Intensive inbreeding with limited founder stock to maintain the purebred characteristics resulted in accumulation of risk alleles within CMT predisposed breeds of dogs. Mammary tumour is polygenic and several genes have been studied in association with CMT. The genetic role of several genes in association with CMT has been studied but the role of micro environment in cancer development and progression is often ignored as far as CMT is considered.

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Abnormal microenvironment plays a crucial role in tumour cell proliferation, invasion, and metastasis. Understanding the specific interplay between tumour cells and their surroundings can even aid in tumour treatment. Fortunately, the tumour microenvironment, as well as its cellular and molecular makeup involved in tumour growth, are attracting more scientific attention in case of human breast cancer (HBC) which is often lacking in CMT. Matrix metalloproteinase and their inhibitors are a collection of genes that have a significant impact on the microenvironment of tumours. *Tissue inhibitor of metalloproteinase 2* also known as *tissue inhibitor of metallopeptidase 2* (*TIMP2*) is one among several genes which affect the tumour micro environment. The interaction of *Matrix Metalloproteinase 2* (*MMP2*) and *TIMP2* was crucial in tumour invasion, progression, and metastasis (Kawai *et al.*, 2006) [8]. In present study, the relative mRNA expression level of *TIMP2* was analysed in both CMT affected and normal glands.

2. Materials and Methods
In current investigation, thirteen CMT-affected glands and six mammary glands from healthy animals were included. Tissue samples were taken and later delivered in RNase-free tubes (Sigma-Aldrich). The samples were transferred to the lab and kept at -80°C until the RNA was isolated. Total RNA Isolation Kit (Origin) was used to extract RNA from tissue samples. Isolation of RNA was carried out in a laminar airflow cabinet (Labline, India) as per protocol provided by the company. The presence of genomic DNA in RNA samples may affect the results of downstream applications. To overcome this, extracted RNA samples were treated with DNase enzyme (Sigma Aldrich's DNase I kit) to breakdown any DNA present.

The NanodropTM 1000 spectrophotometer was used to determine the yield and purity of extracted RNA samples (Thermo Scientific, USA). The ratio between the OD measurements at 260 nm and 280 nm was used to calculate the purity of the RNA samples. For further research, those samples with OD values of 260/280 around 1.8 to 2 and 260/230 around 2 to 2.2 were chosen. The quality and integrity of the RNA were assessed electrophoretically using one per cent agarose gel prepared in 1X Tris Acetate EDTA (TAE) buffer. Those samples with good quality and integrity were further used for downstream process. Thermo Scientific Verso cDNA Synthesis Kit (Thermo Scientific, #AB-1453/A) was used to make complementary DNA from extracted RNA. Further the relative expression of *TIMP2* was evaluated in normal and tumour affected mammary tissues. The Illumina Eco® Q- RT PCR system was used to perform relative gene expression quantification using SYBR green chemistry. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was chosen as the internal control gene for this investigation based on the literature, and primers were designed for it. Primers for amplification of a small fragment of *TIMP2* were constructed using anticipated canine *TIMP2* mRNA sequence (GenBank accession number NM 001003082.1). Primers were synthesised commercially (Sigma). The *TIMP2* and *GAPDH* primer sequences used in Q-PCR are listed below (Table.1).

Table 1: Sequences and properties of primers designed for qPCR

Primer	Sequence (5'-3')	Product size (bp)
TIMP2_F	GCAACGCGGACGTAGTGATTAG	82
TIMP2_R	ATGGGGTTGCCGTAGATGTC	
GAPDH_F	ATTCCACGCCACAGTCAAG	117
GAPDH_R	TACTCAGCACCAGCATCACC	

Reaction conditions were standardised using conventional PCR for different gradients of annealing temperatures in a gradient thermalcycler (BioRad, USA). The optimum conditions for real time PCR analysis were obtained (Table. 2).

Table 2: Quantitative PCR conditions to amplify *TIMP2* and *GAPDH* genes

Sl. No.	Step		Temperature (°C)	Time
1	Initial denaturation		94	3 min
2	Denaturation		94	30 sec
3	Annealing	*GAPDH*	64	30 sec
		TIMP2		
4	Extension		72	30 sec
Steps 2 to 4 repeated 40 times. Data acquisition was performed during extension step of each cycle.				

Maxima SYBR Green qPCR Master Mix was used to set up Q-PCR experiments for *TIMP2* and *GAPDH* genes (Thermo Scientific, USA). Three technical replicates were used to amplify thirteen samples of tumour-affected dogs and six samples of normal canines. Reactions were carried out in an Illumina Eco Real-Time PCR System using Eco 48-well plates sealed with Eco adhesive sealing.
Relative quantification was performed by $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) [9]. The RT-PCR was normalized to a reference gene (*GAPDH*). The RQ value was compared statistically between normal and mammary tumour samples using an independent sample t-test (SPSS V.24).

3. Results
Quantitative PCR was performed by using the synthesised cDNA from mammary tissues of tumour affected and normal dogs. The analysis was done using Illumina Eco® Q- RT PCR machine. The amplification plots and melt curves are shown in Fig. 1 to 5.
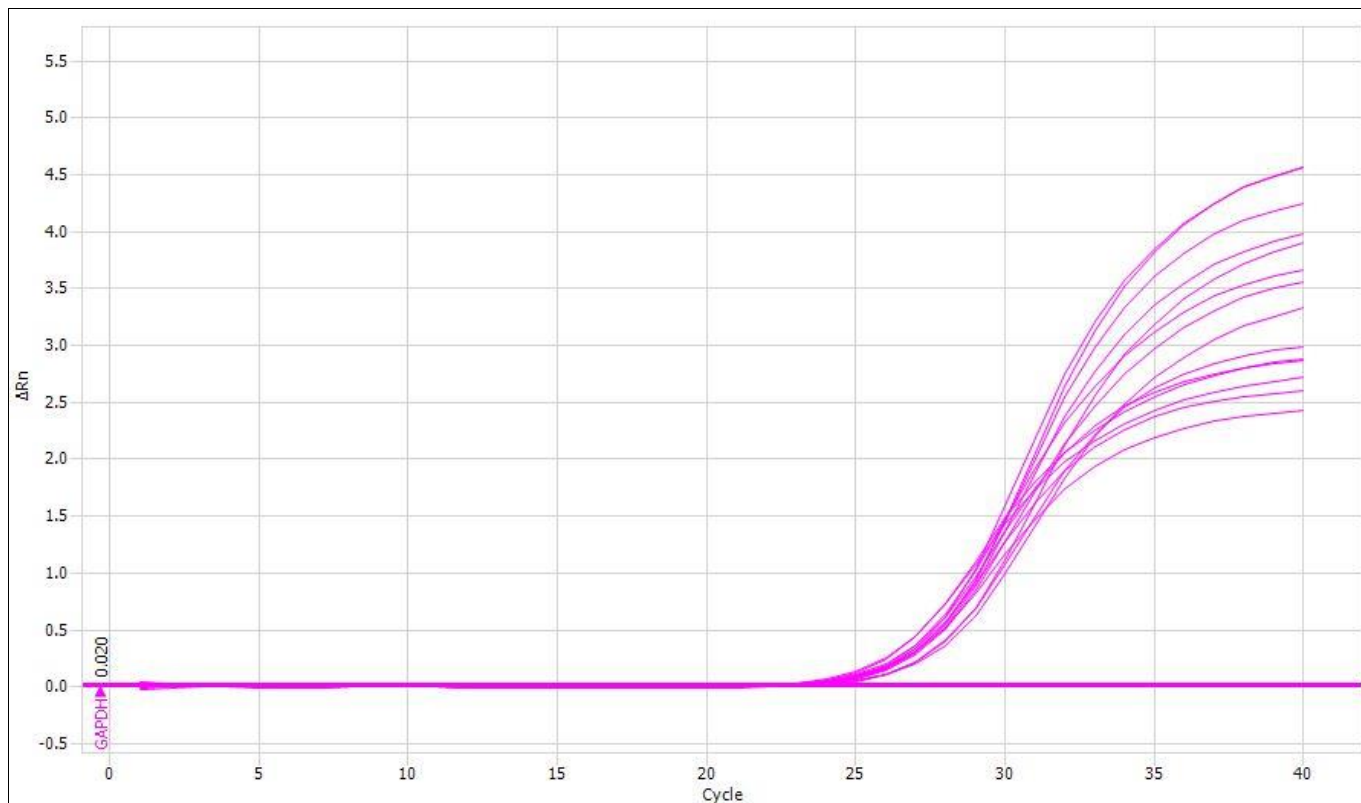
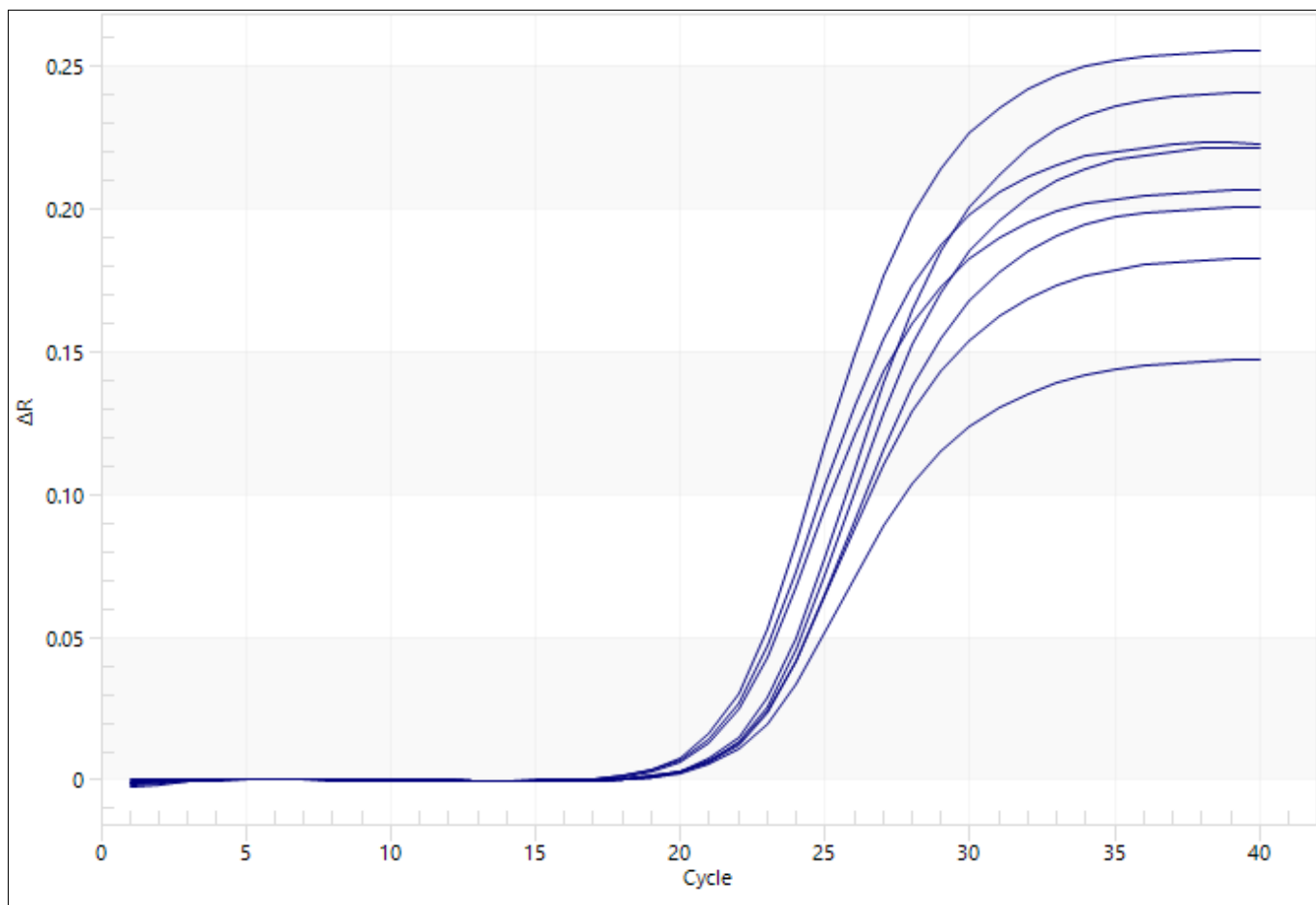**Fig 1:** Amplification plot generated during quantitative real-time PCR assay of *GAPDH*

Fig 2: Amplification plot generated during quantitative real-time PCR assay of *TIMP2* in normal samples
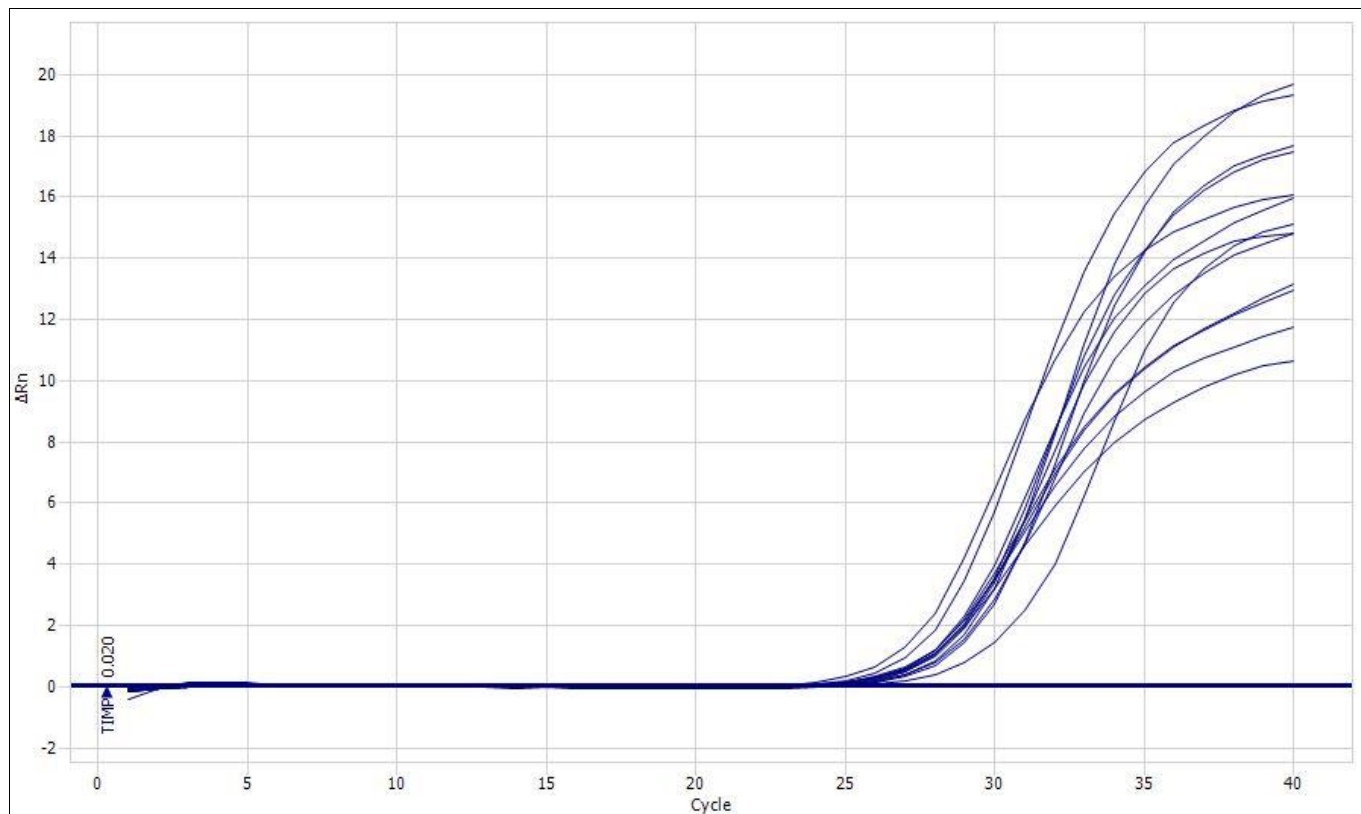
Fig 3: Amplification plot generated during quantitative real-time PCR assay of *TIMP2* in tumour samples
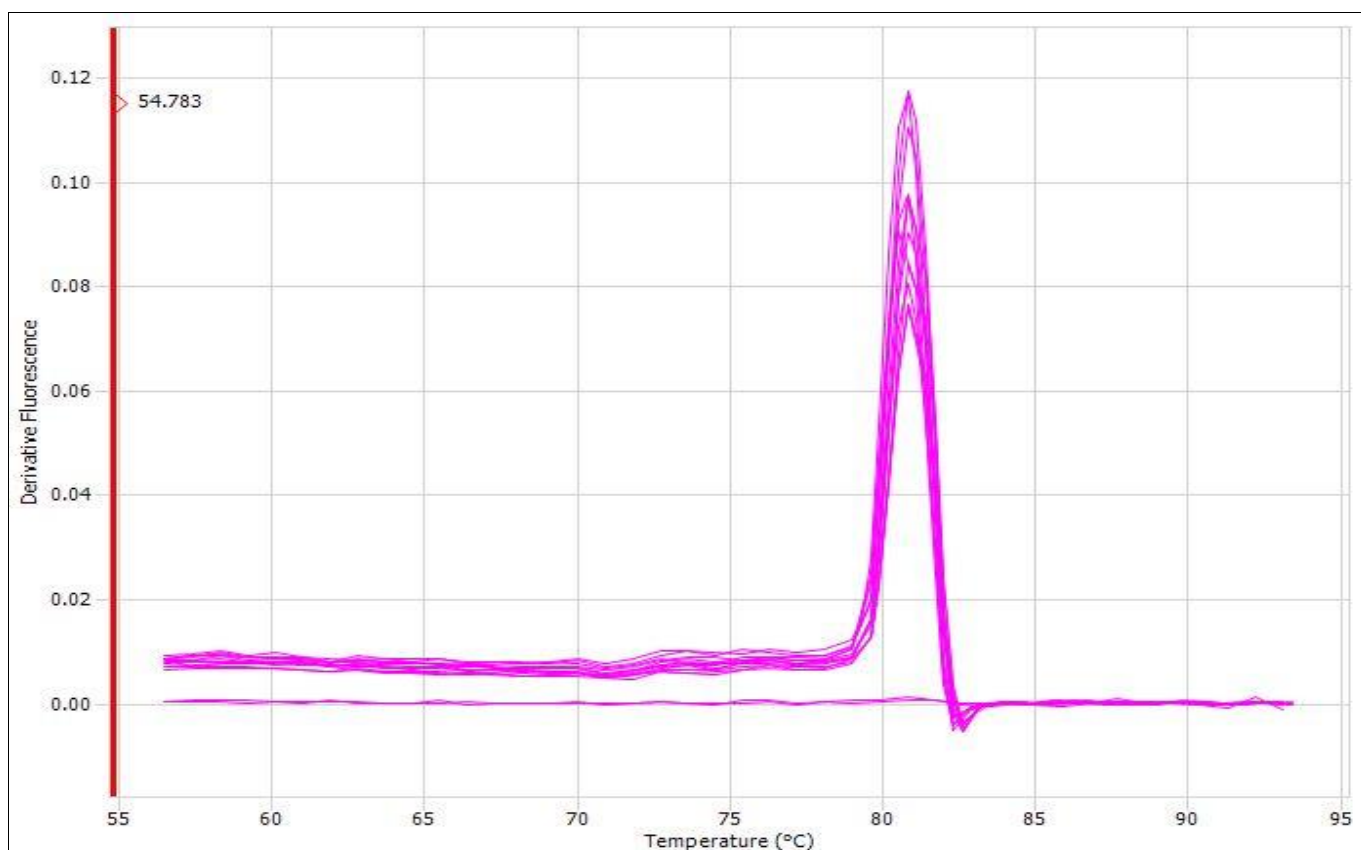

Fig 4: Melt curve generated during quantitative real-time PCR assay for *GAPDH* gene
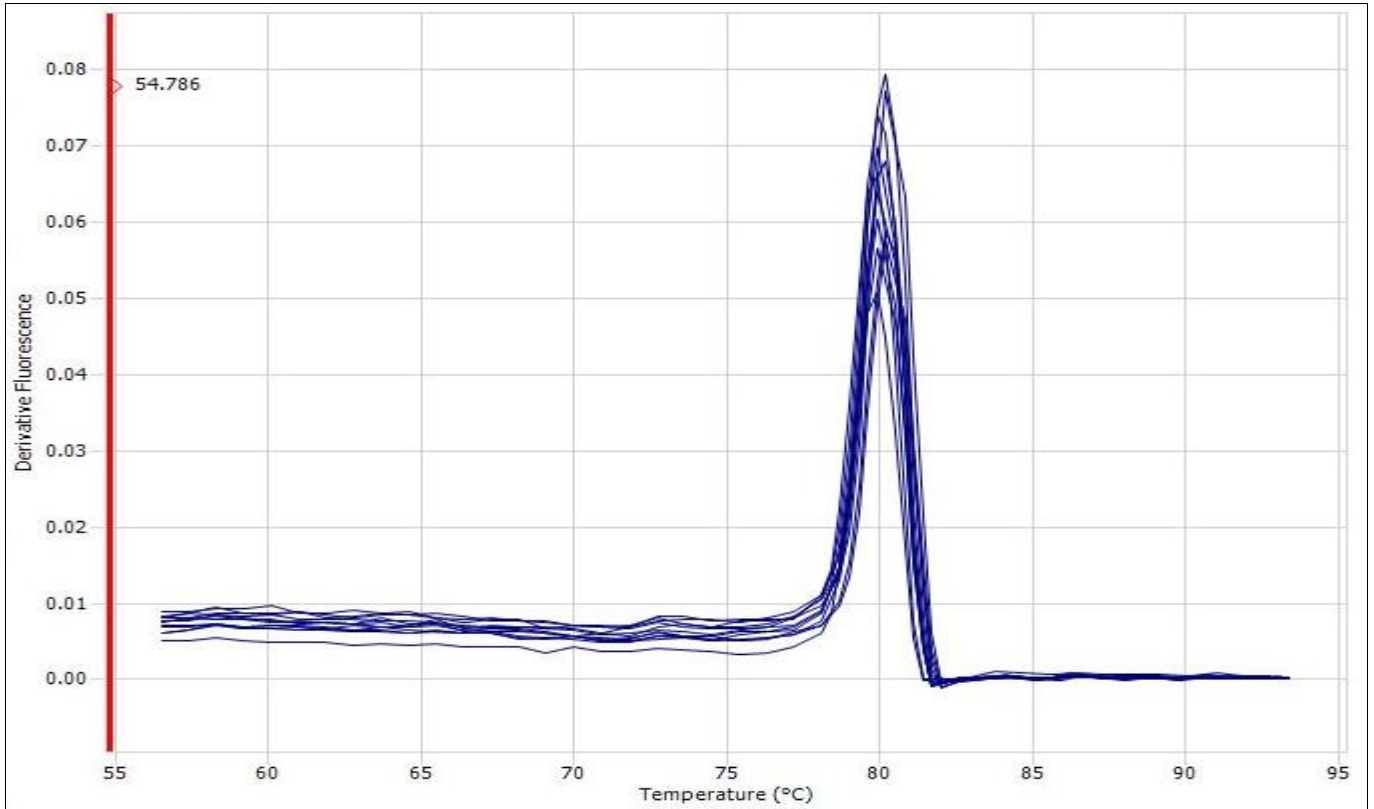
Fig 5: Melt curve generated during quantitative real-time PCR assay for *TIMP2* gene

Table.3 shows the mean C_T value for *TIMP2* and *GAPDH*, as well as the, ΔC_T, $\Delta\Delta C_T$, and fold change of control ($2^{-\Delta\Delta C_T}$). Figure. 6 shows the relative tissue expressions of *TIMP2* mRNA in tumour-affected and normal mammary glands. *TIMP2* expression was considerably (p0.05) lower in tumour-affected glands than in normal mammary glands (control).

Table 3: Relative expression profile of *TIMP2*

Mammary Tissue	Mean C_T ±SE		ΔC_T ± SE	$\Delta\Delta C_T$ ± SE	RQ ($2^{-\Delta\Delta C_T}$)
	TIMP2	*GAPDH*			
Normal (Control)	22.15±0.13	23.04±0.05	-0.89±0.14	0±0.26	1[a]
Tumour	24.8915±0.08	23.0175±0.07	1.874±0.08	2.77±0.08	0.147[b]

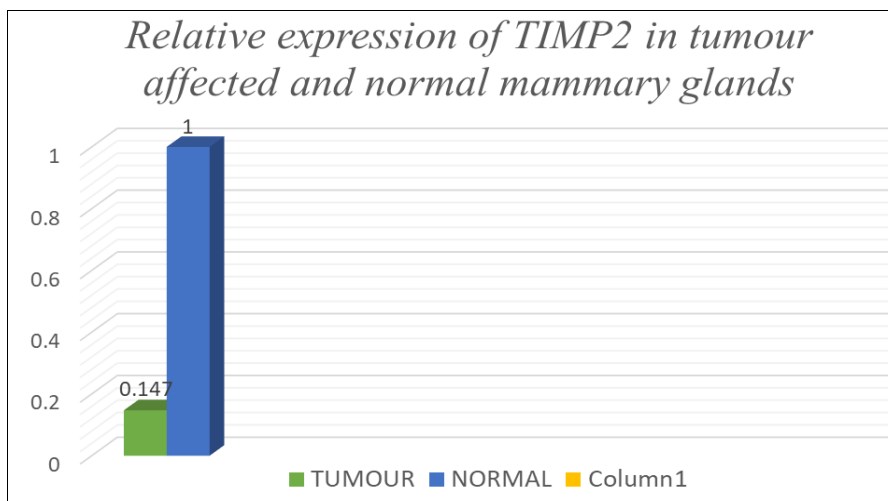Values with different superscripts differs significantly ($p \leq 0.05$)

Fig 6: Relative expression of *TIMP2* in tumour affected and normal mammary glands

4. Discussion

Being loved members of the family, dogs are usually kept until old-age and CMT is one among the top lifestyle diseases in dogs which develop mainly from middle age to old-age (McVean *et al*., 1978; Chang *et al*., 2005; Rowell *et al*., 2011; Petrov *et al*., 2014) [10, 11, 2, 12]. Most studies in canine oncology are generally focused on the tumour cells whereas the impact of tumour microenvironment is often ignored.

Investigations have demonstrated that tumour cells also trigger changes in their microenvironment. *Matrix metalloproteinase* and their inhibitors are a group of genes which greatly affect the tumour micro environment. The MMPs especially *MMP2* mainly involve in tumour induced angiogenesis, apoptosis and cell proliferation, whereas as the inhibitory activity of *TIMP2* over *MMP2* suppresses cell proliferation and tumour associated angiogenesis. The balance

between *MMP2* and *TIMP2* played a major role in tumour invasion, progression and metastasis (Kawai *et al.,* 2006) [8]. Conflicting evidences were reported by different authors regarding the expression and prognostic role of *TIMP2* in different types of cancer, such as breast, lung, cervical, ovarian and bladder cancer (Remacle *et al.,* 2000; Davidson *et al.*, 2002; Vasala *et al.*, 2008; Zhu *et al.*, 2015) [13, 14, 15, 16]. In present study, the relative mRNA expression level of *TIMP2* was analysed in both CMT affected and normal glands.

Total RNA was isolated from mammary gland samples of 13 CMT affected and six normal female dogs using ORIGIN KIT. Good quality cDNA was synthesised from uniform concentrations of RNA from all the samples using Thermo Scientific Verso cDNA Synthesis Kit. The *GAPDH* was reported as common housekeeping gene in cancer research (Mocellin *et al.*, 2003) [17]. Hence in current study *GAPDH* was selected for analysing relative expression of *TIMP2*. Quantitative PCR was standardized for *TIMP2* and *GAPDH* genes. The melt curve analysis showed single peak for *GAPDH* and *TIMP2* representing only the desired product. A ten -fold reduced expression of *TIMP2* was found in tumour samples than normal glands, which was statistically significant.

The findings in present study were in accordance with Vinothini *et al.* (2009) [18], where the authors evaluated the molecular markers in CMT and their correlation with histological grades of tumour. They obtained a reduced expression of *TIMP2* in tumour samples and the expression was comparatively reduced from grade one to grade two mammary tumour. Wang *et al.* (2019) [19] evaluated the role of *TIMP2* in metastasis and prognosis of human colorectal cancer, and they observed a reduced expression of *TIMP2* in cancer cells when compared to normal samples. They reported correlation between under expression of *TIMP2* with poor survival rate of the cancer patient.

The role of *TIMP2* in canine cancer is one among the least explored area especially within CMT. The findings from present study clearly indicates the role of *TIMP2* in invasion of tumour cells however, a protein expression analysis would enable better understanding of role as well as the involvement of *TIMP2* in CMT. The findings from the same can even be extended to human oncology.

5. Acknowledgement

6. Declaration of interest statement
The authors report no conflict of interest

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