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Hareesha DM
Masters Scholar, Department of Veterinary Pathology, Veterinary College, Bengaluru, Karnataka, India

Jayaramu GM
Professor and Head of Department, Department of Veterinary Pathology, Veterinary College, Bengaluru, Karnataka, India

KR Anjan Kumar
Associate Professor, Department of Veterinary Pathology, Veterinary College, Bengaluru, Karnataka, India

Sanganagouda K
Associate Professor, Department of Veterinary Microbiology, Veterinary College, Gadag, Karnataka, India

Roopa Devi YS
Assistant Professor, Department of Veterinary Pathology, Veterinary College, Bengaluru, Karnataka, India

Santhosh CR
Associate Professor, Department of Veterinary Pharmacology and Toxicology, Veterinary College, Bengaluru, Karnataka, India

Corresponding Author:
Hareesha DM
Masters Scholar, Department of Veterinary Pathology, Veterinary College, Bengaluru, Karnataka, India

Expression and prognostic significance of CTLA-4 in canine mammary tumors

Hareesha DM, Jayaramu GM, KR Anjan Kumar, Sanganagouda K, Roopa Devi YS and Santhosh CR

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Abstract
The present study investigated the expression pattern and prognostic significance of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) in spontaneous canine mammary gland tumors (CMGTs) using immunohistochemistry (IHC) and quantitative real-time PCR (qPCR). A total of 50 CMGT samples, comprising both benign and malignant forms, were analyzed to assess the relationship between CTLA-4 expression and tumor behavior. Immunohistochemical evaluation revealed variable CTLA-4 immunopositivity among tumor subtypes, with notably higher expression in aggressive variants such as carcinosarcoma and comedocarcinoma. qPCR analysis further confirmed a significant upregulation of CTLA-4 mRNA in malignant tumors compared to benign counterparts (p<0.05). Statistical analysis demonstrated that elevated CTLA-4 expression was strongly correlated with shorter overall survival (mean=174 days) and earlier tumor recurrence (mean=178 days), suggesting that CTLA-4 contributes to tumor progression through mechanisms of immune evasion. Moreover, CTLA-4 expression levels exhibited a positive association with higher histological grade and lymphatic invasion, emphasizing its role in tumor aggressiveness. Collectively, these results indicate that CTLA-4 serves not only as a marker of malignancy but also as a prognostic indicator in canine mammary tumors. The study provides foundational evidence supporting the potential use of CTLA-4 as a therapeutic target in canine oncology. Considering its parallel function in human breast cancer, these findings further underline the translational relevance of canine models for comparative oncology research. Targeting CTLA-4 through immune checkpoint blockade may represent a promising strategy for managing advanced or recurrent mammary tumors in dogs.

Keywords: CTLA-4, qPCR, canine mammary gland tumor, immunohistochemistry, prognostic biomarker, immunotherapy

Introduction
Cancer remains one of the most significant health concerns in veterinary medicine, representing a major cause of morbidity and mortality among dogs. Mammary gland tumors are the most frequently diagnosed neoplasms in intact female dogs, accounting for nearly half of all malignancies in this population (Sorenmo, 2003; Goldschmidt *et al*., 2011) [12, 6]. These tumors are clinically and histologically diverse, making accurate prognostication and therapeutic planning challenging (Nieto *et al*., 2000) [11]. Due to the close similarities between canine mammary gland tumors (CMGTs) and human breast cancer in terms of histopathology, molecular behavior, and hormonal influence, spontaneous CMGTs have been recognized as a valuable model for studying human oncology and evaluating novel cancer therapies. Recent advances in tumor immunology have underscored the critical role of immune system modulation in cancer development and progression. The host immune system normally performs immunosurveillance by recognizing and eliminating aberrant or transformed cells (DeNardo and Coussens, 2007) [5]. However, tumor cells can escape immune detection by exploiting inhibitory pathways known as immune checkpoints, which suppress T-cell activation and effector function (Greenwald *et al*., 2005; Keir *et al*., 2008) [7, 9]. Among these, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death ligand-1 (PD-L1) are well-characterized inhibitory molecules implicated in tumor immune evasion and poor clinical outcomes in several human cancers (Wülfing *et al*., 2014; Topalian *et al*., 2015) [16, 15].

CTLA-4 (CD152) is a type I transmembrane glycoprotein belonging to the immunoglobulin superfamily, first described by Brunet *et al*. (1987). Structurally, it functions as a covalent homodimer that binds to B7 ligands (CD80 and CD86) expressed on antigen-presenting cells, thereby competing with the co-stimulatory receptor CD28 and inhibiting T-cell activation (Teft *et al*., 2006) [14]. Through this mechanism, CTLA-4 acts as a negative regulator of immune responses, promoting immune tolerance and preventing excessive immune activation (Alegre *et al*., 2001) [1]. The essential regulatory function of CTLA-4 is evident from studies in knockout mice, where its absence leads to fatal lymphoproliferative disease and multi-organ inflammation (Teft *et al*., 2006) [14]. In addition to its expression on activated T cells and regulatory T cells (Tregs), CTLA-4 mRNA and protein have been detected in several non-T-cell types, including monocytes, B cells, and stem cells (Teft *et al*., 2006) [14]. Alternative splicing of the CTLA-4 gene also generates soluble isoforms (sCTLA-4), which retain ligand-binding activity and may contribute to peripheral immune regulation. In dogs, similar splicing mechanisms have been identified, producing a soluble form of CTLA-4 structurally analogous to that in humans (Tagawa *et al*., 2017) [13], suggesting a conserved role across species. The immunosuppressive activity of CTLA-4 has made it an attractive therapeutic target in oncology. In human medicine, anti-CTLA-4 monoclonal antibodies such as ipilimumab and tremelimumab have demonstrated efficacy against metastatic melanoma and other malignancies, significantly improving patient survival (Hodi *et al*., 2010; Arce-Vargas *et al*., 2018) [8, 3]. These treatments work by blocking CTLA-4-mediated inhibition, thereby enhancing T-cell activation against tumor cells. However, such interventions are also associated with immune-related adverse effects, reflecting CTLA-4's physiological role in maintaining self-tolerance (Khoja *et al*., 2017) [10].

In veterinary oncology, emerging evidence indicates that CTLA-4 plays a prognostic and potentially therapeutic role in canine mammary gland tumors. Studies have demonstrated that CTLA-4 expression, both at the protein and transcript levels, is elevated in malignant CMGTs compared to benign lesions (Ariyarathna *et al*., 2020) [2]. Furthermore, increased CTLA-4 expression has been significantly correlated with metastatic behavior and reduced survival time in affected dogs, suggesting that neoplastic cells may exploit this pathway to evade anti-tumor immunity. The overexpression of CTLA-4 within malignant mammary tissues likely facilitates immune suppression, leading to more aggressive tumor phenotypes and poorer clinical outcomes. These findings collectively indicate that CTLA-4 serves not only as a marker of immune evasion and malignancy but also as a potential prognostic biomarker in canine mammary tumors. The therapeutic blockade of CTLA-4, alone or in combination with other immune checkpoint inhibitors such as PD-L1 antagonists, may therefore represent a promising strategy for the management of advanced or recurrent CMGTs. Further exploration of CTLA-4 expression patterns and their clinical significance could aid in refining prognostic assessment and developing targeted immunotherapies in veterinary oncology.

Materials and Methods

A prospective study work on the title "Expression and Prognostic Significance of CTLA-4 in Canine mammary Tumors" was carried out at the Department of Veterinary Pathology, Veterinary College, Hebbal, Bengaluru. A total of 50 suspected cases of spontaneously occurring mammary gland tumors submitted to Department of Veterinary Pathology over a period of nine months from Veterinary College Hospital, Bengaluru and from constituent hospitals of KVAFSU for diagnosis were collected.

Immunohistochemistry

The primary antibody used for detecting CTLA-4 was a mouse monoclonal IgG1κ anti-CTLA-4 antibody (Clone F-8, sc-376016; M/s Santa Cruz Biotechnology, USA), and the corresponding secondary antibody was a ready-to-use HRP-conjugated anti-mouse IgGκ binding protein (Clone sc-516102; M/s Santa Cruz Biotechnology, USA). Visualization was performed using a DAB Substrate Kit containing a 10× DAB solution (25 mL) and a stable peroxide substrate buffer (250 mL) (Thermo Scientific®, USA). Formalin-fixed, paraffin-embedded tissue sections mounted on poly-L-lysine-coated slides were deparaffinized, rehydrated, and subjected to antigen retrieval in citrate buffer (pH 6.0) by microwave heating. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, followed by incubation with normal blocking serum. Sections were then incubated overnight at 4 °C with the primary antibody validated for canine tissue. Detection was carried out using a polymer-based HRP detection system, and visualization was achieved with DAB chromogen followed by hematoxylin counterstaining. The expression of CTLA-4 protein was analyzed qualitatively based on the proportion of positive cells and staining intensity according to Ariyarathna *et al.* (2020) [2]. Immunostaining intensity in neoplastic cells under a high-power (400×) microscopic field was scored on a scale of 0 to 3: 0=absence of staining in both membrane and cytoplasm; 1=incomplete membrane staining with or without mild to moderate cytoplasmic staining; 2=moderately intense and complete membrane staining with or without moderate cytoplasmic staining; and 3=intense, complete membrane staining possibly accompanied by moderate to intense cytoplasmic staining.

RNA Extraction and RT-PCR

Real-time polymerase chain reaction (RT-PCR) was performed to determine the relative expression of the CTLA-4 gene in canine mammary gland tumors. Tumor samples were collected in RNAlater and stored at-80 °C until use. Total RNA was extracted using the TRIzol™ method (M/s Thermo Fisher Scientific) following the manufacturer's protocol, and RNA purity was confirmed using a NanoDrop spectrophotometer at 260/280 nm, with absorbance ratios of approximately 2.0. Complementary DNA (cDNA) was synthesized from 4 µg of total RNA using the RevertAid™ First Strand cDNA Synthesis Kit (M/s Thermo Fisher Scientific) as per the manufacturer's instructions. Gene-specific primers for CTLA-4 were selected from published literature (Ariyarathna *et al.*, 2020) [2], while primers for the housekeeping gene GAPDH were designed using the canine reference sequence available in the NCBI database (Accession No. BI817044.1) (Table 1). Real-time PCR amplification was carried out using the Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix (M/s Thermo Fisher Scientific) in a CFX96 Real-Time

System (M/s BIO-RAD, USA). The 20 µL reaction mixture contained SYBR Green master mix, forward and reverse primers, nuclease-free water, and cDNA temFig, with each sample run in duplicate. Thermal cycling conditions for CTLA-4 amplification included UDG activation at 50 °C for 2 minutes, initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds, and extension at 70 °C for 20 seconds. Relative gene expression levels were quantified using the comparative Ct (2^-ΔΔCt) method, with normal canine mammary gland tissue serving as the calibrator and GAPDH as the internal control, to determine the fold change in CTLA-4 expression between tumor and normal tissues.

Statistical Analysis

To determine the prognostic value of CTLA-4 expression, the follow up details including post-surgical disease-free survival, recurrence, metastasis/ death were taken into the consideration for a period of 9 months. Further the values above and below the median expression value of CTLA-4 are considered as over and under expressed respectively. Data were analyzed with GraphPad Prism v8.4.3. Differences between tumor grades were assessed using Kruskal-Wallis and Mann-Whitney U tests. Survival outcomes were evaluated with Kaplan-Meier curves.

Ethical approval

The study protocol was reviewed and approved by Institutional Animal Ethics Committee (IAEC), Veterinary College, Hebbal, Bengaluru. All of the investigated samples were obtained for diagnostic purposes as part of routine and standard care. Procedures were designed to avoid or minimise discomfort, distress and pain. IAEC approval number: VCH/IAEC/2025/37.

Results

Out of 50 canine mammary tumor cases, 30 were selected for immunohistochemical evaluation of CTLA-4 expression, representing both benign and malignant tumors with prominent lymphocytic infiltration. Immunostaining with CTLA-4 showed cytoplasmic localization, with brown to dark brown coloration (Fig 1 & 2). CTLA-4 expression was observed in both tumor-infiltrating lymphocytes and tumor cells and was qualitatively graded as 0+, 1+, 2+, and 3+ based on staining intensity, where 0+ indicated absence of cytoplasmic staining (Fig 3), 1+ denoted mild cytoplasmic staining (Fig 4), 2+ represented moderate cytoplasmic staining (Fig 5), and 3+ corresponded to intense cytoplasmic staining (Fig 6). Due to the relatively low number of positive cells in several samples, a qualitative evaluation approach was adopted, focusing on the overall staining pattern and intensity rather than quantitative counts. This qualitative method enabled accurate assessment of CTLA-4 expression, particularly in tumors with limited positive cell populations, and categorized staining intensity from 0+ (absent) to 3+ (strong), reflecting a range from no detectable expression to intense cytoplasmic localization (Table 2). Among the 30 evaluated cases, 10 showed 0+ intensity, including myoepithelioma (N=1), adenoma (N=2), complex adenoma (N=2), benign mixed tumor (N=1), complex carcinoma (N=1), malignant myoepithelioma (N=1), tubular carcinoma (N=1), and cystic papillary carcinoma (N=1). Nine cases exhibited 1+ intensity, comprising complex carcinoma (N=4), malignant myoepithelioma (N=2), cystic papillary carcinoma (N=1), tubulo-papillary carcinoma (N=1), and comedo carcinoma (N=1). Another nine cases displayed 2+ intensity, including tubular carcinoma (N=4), squamous cell carcinoma (N=2), tubulo-papillary carcinoma (N=2), and complex carcinoma (N=1), while two cases of carcinosarcoma demonstrated 3+ intensity. Canine tonsil tissue served as the positive control (Fig 7).

Real-Time PCR Expression

In the present study, real-time polymerase chain reaction (RT-PCR) was successfully performed in duplicate on 30 out of 50 canine mammary gland tumor cases (Table 3), while the remaining samples were excluded due to low RNA purity and yield. Normal mammary gland tissue served as a negative control, with Cq values of 34.76 and 34.54 showing no detectable CTLA-4 expression, whereas canine spleen, used as a positive control, showed Cq values of 28.23 and 28.13. CTLA-4 mRNA expression in malignant mammary gland tumors (N=23) varied widely from 0.04-to 73.77-fold, with a mean (±SE) of 7.30±3.22-fold. Among the malignant tumor types, the mean (±SE) fold expression was highest in carcinosarcoma (45.03±28.74; N=2), followed by tubulo-papillary carcinoma (6.73±6.45; N=3), tubular carcinoma (5.45±2.12; N=5), complex carcinoma (4.63±2.48; N=3), squamous cell carcinoma (3.60±0.02; N=2), comedo carcinoma (2.89; N=1), cystic papillary carcinoma (1.25±0.54; N=4), and lowest in malignant myoepithelioma (0.46±0.12; N=3) (Table 4). In contrast, benign mammary gland tumors (N=7) showed minimal to no CTLA-4 expression, ranging from 0 to 0.08-fold, with a mean (±SE) of 0.018±0.011-fold (Table 4). Statistical analysis using the Mann-Whitney test revealed a significantly higher CTLA-4 expression in malignant tumors compared to benign tumors ($p \leq 0.05$), (Tables 5 and 5a).

Determination of prognostic value of CTLA-4 Expression

The prognostic significance of CTLA-4 mRNA expression was evaluated by correlating expression profiles with post-surgical outcomes in malignant mammary gland tumors. The median fold expression among the 23 malignant tumors was 2.63, which served as the cut-off to categorize cases as CTLA-4 overexpressed or underexpressed. Overexpression was observed in 12 cases (52.17%), while underexpression was found in 11 cases (47.83%) (Table 6). Follow-up data revealed tumor recurrence in 3 cases, death in 5 cases reason for death being metastasis in 3 cases as confirmed by radiography (Fig 8) and systemic illness in 2 cases and 15 dogs alive and disease-free. Among the deceased dogs, 4 exhibited CTLA-4 overexpression (mean±SE, 6.47±2.90), whereas 1 underexpressed case died due to systemic illness. These cases were histologically classified as comedo carcinoma (N=1), carcinosarcoma (N=1), tubulo-papillary carcinoma (N=1), tubular carcinoma (N=1), and squamous cell carcinoma (N=1) (Table 8). All three recurrence cases also showed CTLA-4 overexpression (mean±SE, 32.24±21.22), comprising carcinosarcoma (N=1), tubulo-papillary carcinoma (N=1), and squamous cell carcinoma (N=1), (Table 9). Among the 15 disease-free dogs, 10 showed underexpression and 5 showed overexpression, with a mean (±SE) CTLA-4 level of 2.44±0.88 (below median). These included complex carcinoma (N=3), cystic papillary

carcinoma (N=4), malignant myoepithelioma (N=3), tubular carcinoma (N=4), and tubulo-papillary carcinoma (N=1) (Table 10). The Kruskal-Wallis test showed statistically significant differences in mean CTLA-4 fold change between the recurrence and alive groups, as well as between the deceased and alive groups ($p \leq 0.05$, *P=0.0233) (Tables 11 and 11a).

Kaplan-MEIER survival and recurrence curves for CTLA-4

Kaplan-Meier survival analysis, based on CTLA-4 overexpression and underexpression (cut-off=2.63), demonstrated a statistically significant difference ($p \leq 0.05$) in post-surgical survival times between the two groups. Dogs with CTLA-4 overexpression had a markedly reduced median survival period of 174 days, whereas the median survival for the underexpression group was undefined (Graph. 2). Similarly, recurrence-free survival analysis revealed a significant difference ($p<0.05$) between groups, with the overexpression group showing a median recurrence period of 178 days compared to an undefined median in the underexpression group (Graph. 3), indicating that elevated CTLA-4 expression was associated with decreased survival and increased recurrence risk in canine mammary gland tumors.

Table 1: Primers used for real time Pcr

Primer code	Sequence (5-3)	Primer length (bp)
CTLA-4-F	CCCCGTCTTCTCCAAAGGGAT	174 bp
CTLA-4-R	TATGTCGCGGCACAGACTTC	
GAPDH-F	GCTCCTCTAGCCAAAGTCATC	159 bp
GAPDH-R	GGAAGCAGGGATGATGTTCT	

Table 2: Qualitative classification of CTLA-4 immunostaining

CTLA-4 Intensity	Number of Cases (n)	Tumor Types
0+ (Absent)	10	Myoepithelioma (n=1), Adenoma (n=2), Complex Adenoma (n=2), Benign Mixed Tumor (n=1), Complex Carcinoma (n=1), Malignant Myoepithelioma (n=1), Tubular Carcinoma (n=1), Cystic Papillary Carcinoma (n=1)
1+(mild)	9	Complex Carcinoma (n=4), Malignant Myoepithelioma (n=2), Cystic Papillary Carcinoma (n=1), Tubulo-papillary Carcinoma (n=1), Comedo Carcinoma (n=1),
2+(moderate)	9	Tubular Carcinoma (n=4), Squamous Cell Carcinoma (n=2), Tubulo-papillary Carcinoma (n=2), Complex Carcinoma (n=1)
3+(intense)	2	Carcinosarcoma(n=2)

Table 3: Expression value of CTLA-4 canine mammary gland tumors of dog by real time PCR (N= 30)

Sl. No.	Histological type	Fold expression value of CTLA-4
1	Carcinosarcoma	73.77
2	Tubulo-papillary carcinoma	19.63
3	Carcinosarcoma	16.28
4	Tubular carcinoma	11.35
5	Tubular carcinoma	9.61
6	Complex carcinoma	9.58
7	Squamous cell carcinoma	3.62
8	Squamous cell carcinoma	3.57
9	Tubular carcinoma	3.22
10	Comedo carcinoma	2.89
11	Cystic papillary carcinoma	2.87
12	Tubular carcinoma	2.63
13	Complex carcinoma	2.51
14	Complex carcinoma	1.81
15	Cystic papillary carcinoma	0.90
16	Cystic papillary carcinoma	0.71
17	Malignant myoepithelioma	0.66
18	Tubulo-papillary carcinoma	0.52
19	Cystic papillary carcinoma	0.52
20	Carcinosarcoma	0.50
21	Tubular carcinoma	0.43
22	Malignant myoepithelioma	0.22
23	Tubulo-papillary carcinoma	0.04
24	Myoepithelioma	0
25	Myoepithelioma	0.03
26	Simple adenoma	0
27	Simple adenoma	0
28	Complex adenoma	0.01
29	Complex adenoma	0.08
30	Benign mixed tumor	0.01

Table 4: Mean (±SE) of CTLA-4-fold expression values of RT-PCR in various types of mammary gland tumors in dogs

Sl. No.	Histological type	No of tumors (n)	Mean (±SE) of CTLA-4 in RT PCR (fold expression)
I. Malignant neoplasms			
1	Carcinosarcoma	2	45.03 ± 28.74
2	Comedo carcinoma	1	2.89
3	Complex carcinoma	3	4.63 ± 2.48
4	Cystic papillary carcinoma	4	1.25 ± 0.54
5	Malignant myoepithelioma	3	0.46 ± 0.12
6	Squamous cell carcinoma	2	3.60 ± 0.02
7	Tubular carcinoma	5	5.45 ± 2.12
8	Tubulo-papillary carcinoma	3	6.73 ± 6.45
II. Benign Neoplasms			
1	Myoepithelioma	2	0.02 ± 0.02
2	Simple adenoma	2	0
3	Complex adenoma	2	0.05 ± 0.04
4	Benign mixed tumor	1	0.01
Total		30	

Table 5: Mean (±SE) expression value of CTLA-4 in benign and malignant tumors

Type of tumor	Number of cases	Mean (±SE)
Malignant neoplasms	23	7.30±3.22
Benign Neoplasms	7	0.018±0.011

Table 5a: Mann-Whitney test for comparison of mean (±SE) CTLA-4 values between benign and malignant tumors

Type of tumor	Number of cases	Mean (±SE)
Malignant neoplasms	23	7.30±3.22
Benign Neoplasms	7	0.018±0.011
Significance		***

Note: Mann-Whitney test: *-Significant at $p \leq 0.05$; **-Significant at $p \leq 0.01$; ***-Highly significant $p < 0.001$, NS-Non-Significant.

Table 6: Various malignant tumors showing CTLA-4 under expression and overexpression

CTLA-4 overexpressed (≥ 2.63)				CTLA-4 under expressed (< 2.63)			
SL. No.	**Type of tumor**	**N**	**Mean (±SE)**	**SL. No**	**Type of tumor**	**N**	**Mean (±SE)**
1	Comedo carcinoma	1	2.89	1	Malignant myoepithelioma	3	0.46±0.12
2	Complex carcinoma	1	9.58	2	Complex carcinoma	2	2.16±0.35
3	Cystic papillary carcinoma	1	2.87	3	Cystic papillary carcinoma	3	0.71±0.11
4	Carcinosarcoma	2	45.03±28.74	4	Tubular carcinoma	1	0.43
5	Tubular carcinoma	4	6.70±2.21	5	Tubulo-papillary carcinoma	2	0.28±0.24
6	Tubulo-papillary carcinoma	1	19.63				
7	Squamous cell carcinoma	2	3.60±.025				
Total		12				11	

Table 7: Details of CTLA-4 expression in relation to postsurgical outcome in malignant tumors

CTLA-4	Number	Postsurgical outcome			
		Alive	**Recurrence**	**Dead**	**Total**
Overexpression	12	5	3	4	12
Under expression	11	10	0	1	11
Total	23	15	3	5	23

Table 8: Histological types, period of postsurgical follow up and values of CTLA-4 in dead group

SL. No.	Histological type	Days	CTLA-4-fold expression
1	Comedo carcinoma	121	2.89
2	Carcinosarcoma	135	16.28
3	Tubular carcinoma	16	9.61
4	Tubulo-papillary carcinoma	215	0.04
5	Squamous cell carcinoma	216	3.57

Table 9: Histological types, period of postsurgical follow up and values of CTLA-4 in recurrence group

SL. No.	Histological type	Days	CTLA-4-fold expression
1	Carcinosarcoma	159	73.77
2	Squamous cell carcinoma	178	3.62
3	Tubulo-papillary carcinoma	138	19.63

Table 10: Histological types, period of postsurgical follow up and values of CTLA-4 in alive group

SL. No.	Histological type	Days	CTLA-4-fold expression
1	Complex carcinoma	181	2.51
2	Cystic papillary carcinoma	68	0.52
3	Cystic papillary carcinoma	98	0.71
4	Cystic papillary carcinoma	153	0.90
5	malignant myoepithelioma	210	0.22
6	malignant myoepithelioma	183	0.50
7	malignant myoepithelioma	159	0.66
8	Tubular carcinoma	211	0.43
9	Complex carcinoma	150	1.81
10	Tubulo-papillary carcinoma	200	0.52
11	Complex carcinoma	206	9.58
12	Cystic papillary carcinoma	110	2.87
13	Tubular carcinoma	173	2.63
14	Tubular carcinoma	67	3.22
15	Tubular carcinoma	74	11.35

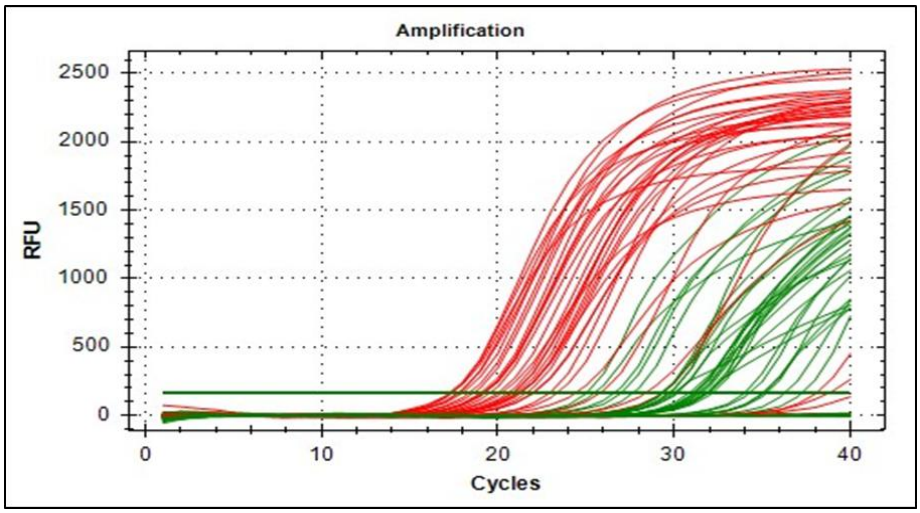
Table 11: Mean (±SE) CTLA-4 RT-PCR values of various post-surgical outcome groups

Follow-up data (index)	Total number of cases	Mean (±SE) of CTLA-4 expression RT PCR (fold expression)
Alive	15	2.44±0.88
Recurrence	3	32.24±21.22
Dead	5	6.47±2.90

Table 11a: Kruskal Walli's test for comparison of mean CTLA-4 values between various postsurgical outcome groups

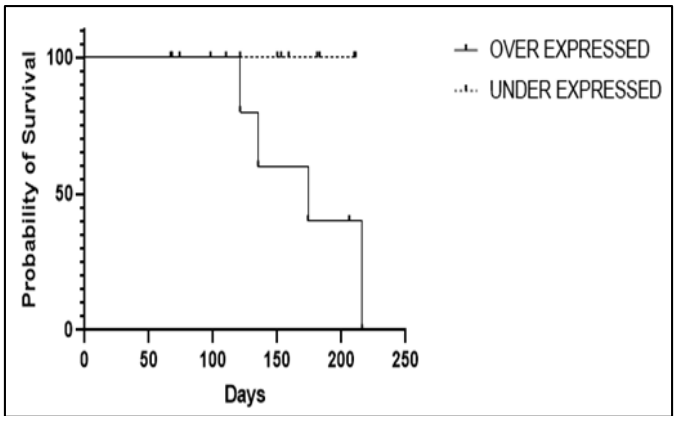
Post-surgical outcome	Total number of cases	Mean (±SE) of CTLA-4 expression RT PCR (fold expression)
Alive	15	2.44±0.88[a]
Recurrence	3	32.24±21.22[b]
Dead	5	6.47±2.90[c]

Note: Kruskal-Walli's test: Mean (±SE) bearing different superscripts within a column are significant $p \leq 0.05$ (P =0.0233) *.

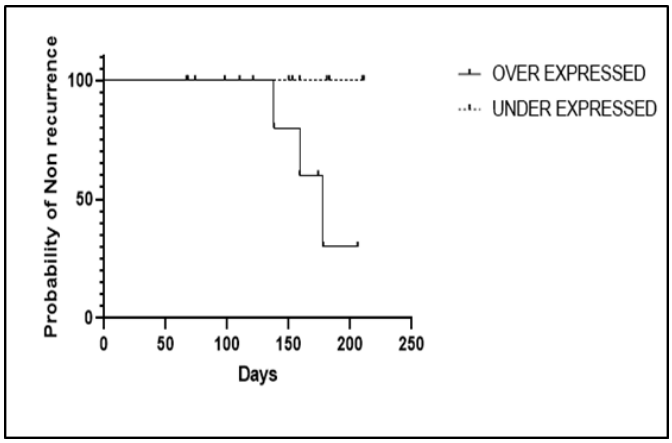


Note: Red: GAPDH, Green: CTLA-4, Blue: NTC (no template control)

Graph 1: CTLA-4 and GAPDH gene mRNA expression amplification plot for canine mammary gland tumors



Graph 2: Kaplan Meier curves for alive vs dead in dogs with mammary gland tumors grouped into overexpressed and under expressed CTLA-4 profile ($p \leq 0.05$)



Graph 3: Kaplan Meier curves for recurrence in dogs with mammary gland tumors grouped into overexpressed and under expressed CTLA-4 profile ($p \leq 0.05$)

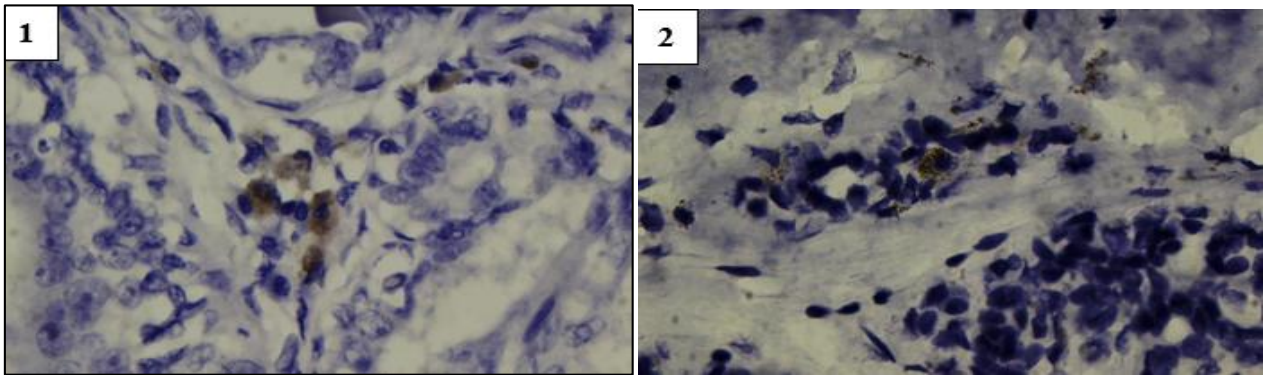


Fig 1 and 2: CTLA-4 immunostaining showed cytoplasmic localizations and appeared brown to dark brown in colour. (IHCX1000)

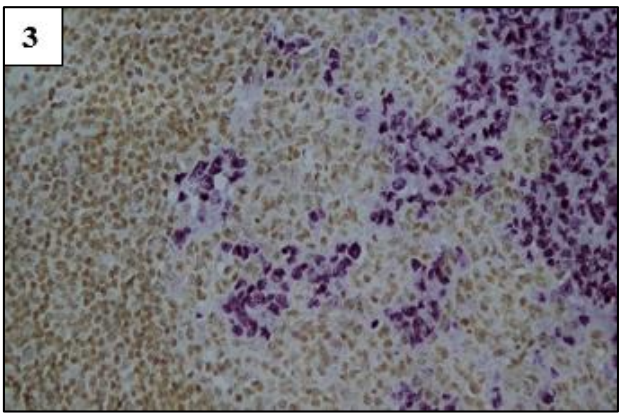


Fig 3: CTLA-4 immunostaining of canine tonsils served as positive control. (IHC X400)

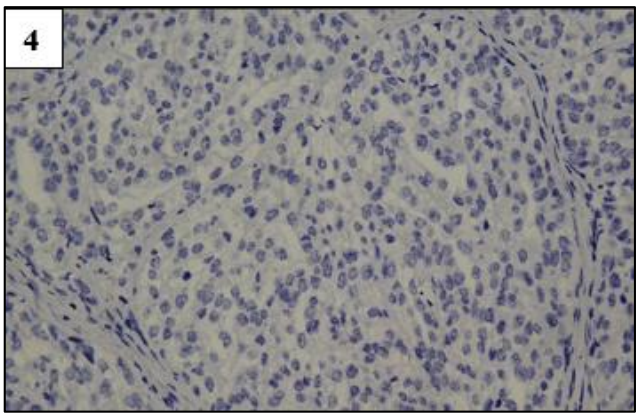


Fig 4: CTLA-4 immunostaining of Adenoma showed 0+ cytoplasmic staining. (IHC X400)

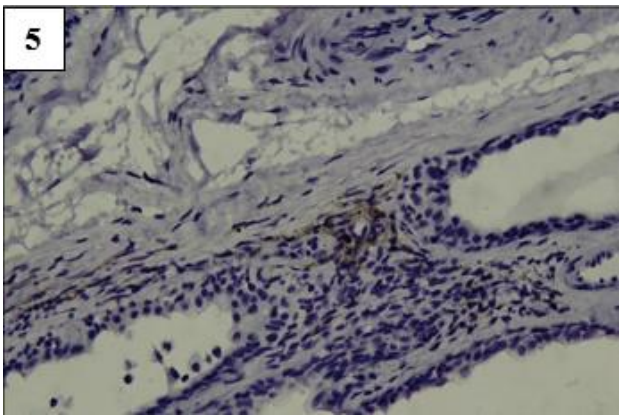


Fig 5: CTLA-4 immunostaining of Cystic papillary carcinoma showed 1+ cytoplasmic intensity. (IHC X400)

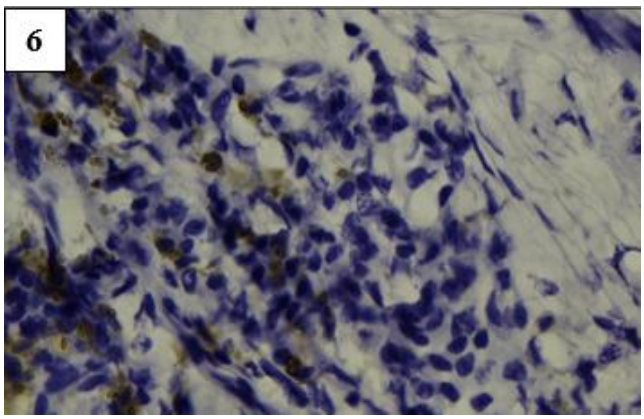


Fig 6: CTLA-4 immunostaining of Complex carcinoma showed 2+ cytoplasmic intensity. (IHC X400)

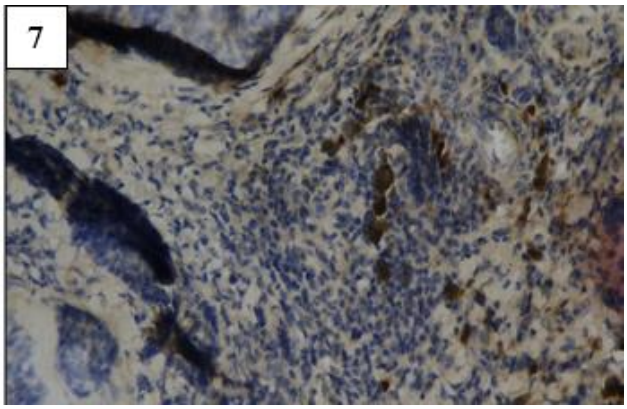


Fig 7: CTLA-4 immunostaining of carcinosarcoma showed 3+ cytoplasmic intensity (IHC X400)

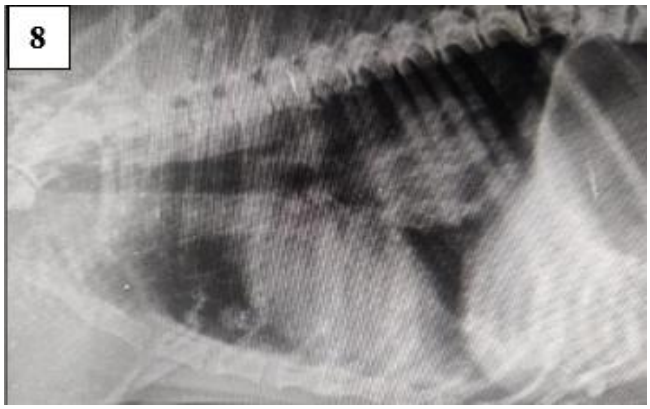


Fig 8: Radiographic picture of pulmonary metastasis

Discussion

CTLA-4 has emerged as a central immune checkpoint molecule influencing carcinogenesis, progression, and prognosis in canine mammary gland tumors (CMGTs). The present study reinforces the immunosuppressive role of CTLA-4, observing pronounced cytoplasmic localization and graded expression in both tumor-infiltrating lymphocytes and tumor cells, with malignant tumors displaying significantly higher levels of CTLA-4 compared to benign lesions. Such findings align with Ariyarathna *et al*. (2020) [2], who reported elevated CTLA-4 expression in malignant CMGTs and linked it to metastatic behavior and poorer survival outcomes, reinforcing the marker's diagnostic and prognostic potential in veterinary oncology.

The immunological context of CMGTs further underpins the relevance of CTLA-4. Tumor cells utilize immune checkpoints, most notably CTLA-4 and PD-L1, to evade host immunosurveillance and suppress T-cell effector functions, fostering a permissive microenvironment for tumor growth and recurrence (Greenwald *et al*., 2005; Keir *et al*., 2008) [7, 9]. The present study's association between elevated CTLA-4 expression and decreased post-surgical survival or recurrence-free intervals corresponds with clinical observations in both canine and human settings, where immune checkpoint upregulation is frequently associated with aggressive phenotypes and adverse patient outcomes (Wülfing *et al*., 2014; Topalian *et al*., 2015) [16, 15]. Structurally and functionally, CTLA-4 serves as a negative regulator of immune responses by competitively binding to B7 ligands and inhibiting T-cell activation, as described by Brunet *et al*. (1987) and further elucidated in knockout mouse models that demonstrate fatal lymphoproliferation in the absence of CTLA-4 (Teft *et al*., 2006) [14]. The detection of CTLA-4 mRNA and protein not only in T cells but also in other immune cell types, including monocytes and B cells, highlights its broad immunoregulatory capacity (Teft *et al*., 2006) [14]. Moreover, the production of soluble CTLA-4 forms via alternative splicing in dogs parallels mechanisms observed in humans, suggesting a conserved pathway with implications for peripheral immune regulation and escape (Tagawa *et al*., 2017) [13].

Given its pivotal role in immune modulation, CTLA-4 has become a target for cancer therapy, with monoclonal antibodies such as ipilimumab showing improved survival in metastatic melanomas (Hodi *et al*., 2010; Arce-Vargas *et al*., 2018) [8, 3]. These agents enhance anti-tumor immunity by blocking CTLA-4, but they come with a risk of immune-related adverse events, emphasizing the regulatory importance of CTLA-4 in maintaining self-tolerance (Khoja *et al*., 2017) [10]. Translationally, similar therapeutic strategies could be adapted for veterinary oncology, especially for advanced or recurrent CMGTs, providing a path for future research into immune checkpoint blockade and combination therapies to refine prognostic assessment and improve clinical outcomes.

Conclusion

This study demonstrates that CTLA-4 expression in canine mammary gland tumors (CMGTs) is significantly associated with tumor histological type, clinical outcome, and overall prognosis. Immunohistochemical analysis revealed that 60% of malignant tumors (N=18/30) exhibited moderate to strong CTLA-4 staining (2+ and 3+), whereas 100% of benign tumors (N=7/7) showed absent or mild staining (0+ and 1+). Correspondingly, RT-PCR analysis showed CTLA-4 mRNA fold-change expression ranging widely from 0.04 to 73.77, with malignant tumors exhibiting a significantly higher mean expression (7.30±3.22) compared to benign tumors (0.018±0.011, $p \leq 0.05$). Within malignant subtypes, the highest mean CTLA-4 expression was observed in carcinosarcomas (45.03±28.74), followed by tubulo-papillary carcinoma (6.73±6.45) and tubular carcinoma (5.45±2.12). Kaplan-Meier analysis confirmed that CTLA-4 overexpression (fold-change > 2.63) significantly correlated with decreased post-surgical survival (median 174 days vs. undefined for underexpression, $p \leq 0.05$) and shorter recurrence-free intervals (median 178 days vs. undefined, $p < 0.05$). These findings identify CTLA-4 as a robust prognostic biomarker for canine mammary tumors, reflecting tumor aggressiveness and immune evasion, and underscore the translational potential for targeting CTLA-4 in future veterinary immunotherapeutic strategies, especially as canine-specific monoclonal antibodies emerge to facilitate clinical interventions. Longer-term, multi-institutional cohort studies will be essential to validate these results and further refine immunotherapy approaches in veterinary oncology.

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