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mRNA and protein expression of programmed cell death-ligand-1 on canine mammary gland tumour in dogs of Chiang Mai, Thailand

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ABSTRACT

Metastasis-related disease is a major cause of death in canine mammary tumours (CMTs). Immunotherapy has been investigated due to the less successful outcomes of systemic therapy. This study aims to examine the expression of Programmed Cell Death Ligand-1 (PD-L1) in canine mammary tumours in dogs of Chiang Mai, Thailand, and determine the relationship between the level of mRNA expression and clinicopathologic characteristics. A total of 28 CMT samples were collected at the Small Animal Hospital, Chiang Mai University. Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) and western blot assays were performed. The results revealed that all CMTs in this study expressed PD-L1 mRNA and PD-L1 protein. The mean relative mRNA expression showed no significant differences between groups categorized by age, tumour size, or histopathological findings. However, the mean relative mRNA expression in tumours with a TNM stage >3 was significantly lower compared to those with TNM stage ≤2. In conclusion, this study investigates the expression of PD-L1 mRNA and PD-L1 protein, particularly in malignant CMTs. The findings strongly support the potential for developing effective immunotherapy methods targeting the PD-1/PD-L1 pathway for advanced CMTs in the future. For further conclusive assessment, future studies should focus on refining immunotherapy strategies for CMT cases expressing PD-L1.

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1. Background

Immunotherapy has been widely studied as a therapeutic approach for advanced forms of cancer [1]. Programmed cell death-1 (PD-1), an inhibitory signalling receptor expressed on the surface of activated T and B cells, is considered one of the multiple immune escape mechanisms of cancers [2–4]. Its ligand, programmed cell death-ligand-1 (PD-L1), has been observed to be expressed on tumour cells and stromal tumour-infiltrating lymphocytes (TILs) [3].

The interaction between PD-L1 and the PD-1 receptor, expressed on the membrane surface of activated cytotoxic T cells, acts as a regulatory checkpoint to prevent excessive immune responses to antigens and autoimmunity [5,6]. Previous studies suggest that the PD-1/PD-L1 pathway functions as an active immune checkpoint in tumours [1,7]. Thus, targeting the PD-1/PD-L1 pathway may inhibit the suppression of T-cell signalling and reactivate T cells to mediate tumour cell killing [4].

The expression of PD-L1 has been reported in various human cancers, including melanoma, non-small cell lung cancer, pancreatic cancer, oesophageal cancer, and breast cancer [8,9]. PD-1 and PD-L1 inhibitors, which bind to their targets and suppress the tumour escape pathway, have demonstrated effectiveness in various treatments and are now considered a promising option for advanced stages of PD-L1-expressing cancers [10–13]. Importantly, studies investigating the expression of these molecules in canine cancer tissues remain limited [14].

Canine mammary tumours (CMTs) are common tumours in intact female dogs, with approximately 50% of cases reported as malignant [15,16]. Metastasis to distant organs is the leading cause of CMT-related deaths [17]. Traditional treatments, such as wide-margin surgery, have limitations due to factors like the dog's age, the number and size of tumours, and the stage of tumour growth-particularly in advanced CMT cases [18]. Recurrence rates after surgery are as high as 58%, contributing to poor prognosis and mortality in cases of high-grade malignant CMTs [19–21].

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Chemotherapy has also shown limited success in controlling tumours and is associated with significant side effects [22–25]. However, a recent clinical study discovered that a canine monoclonal antibody targeting PD-L1 may have therapeutic effects on canine oral melanoma and undifferentiated sarcoma [26]. This study aims to explore potential treatment opportunities for dogs by detecting the mRNA expression of the canine PD-L1 gene and investigating PD-L1 protein expression in CMTs in dogs of Chiang Mai, Thailand.

2. Materials and methods

2.1. Tissue samples

Twenty-eight fresh tumour tissue samples from dogs diagnosed with canine mammary gland tumours at Chiang Mai University Small Animal Teaching Hospital were collected between November 2017 and December 2018. In addition, five normal mammary tissues were also collected and used as control expression. The age groups were defined into three groups as following, young to middle age (<8 years), middle age (8–11 years), or old age (≥12 years). CMT samples were classified according to the histological classification method as previously described [27]. The samples included four benign neoplasms, 22 malignant epithelial neoplasms, and two malignant mesenchymal neoplasms. Non-cancerous tissue samples were obtained from cadaveric dogs. All samples were immediately frozen with liquid nitrogen and stored at −70°C for total RNA extraction and protein extraction.

2.2. RNA extraction and cDNA synthesis

Liquid nitrogen was poured over the frozen tissue samples, which were then ground using an RNAse-free mortar and pestle until they became powder-like. RNA extraction was performed using the TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's instructions. Extracted RNA samples were quantified using a UV/Vis Spectrophotometer (Beckman Coulter, California, USA), and the absorbance ratio of 260/280 was measured to assess RNA purity. RNA samples were then analysed by electrophoresis to check the sample integrity. Degraded RNA specimens were excluded from the analysis. cDNA synthesis was performed using the reverse transcription enzyme according to the manufacturer's instructions (Invitrogen). Briefly, 4.5 µg of total RNAs was used to initiate a 20 µl of the cDNA synthesis reaction. Total RNA was mixed with 50 µM oligo (dT) 20 primer, 10 mm dNTP mix, and DEPC treated water, incubated at 65°C for 5 min, and chilled on ice immediately. Then, a mixture of 10× RT buffer, 25 mm $MgCl_2$, 0.1 M DTT, 40 U RNase inhibitor, and 200 U reverse transcriptase enzyme was added. The reaction was incubated at 50°C for 50 min, followed by incubation at 85°C for 5 min, and chill on ice immediately. RNase inhibitors were then added to the reaction and incubated at 37°C for 20 min. The cDNA samples were stored at −20°C until use.

2.3. Primer verified by polymerase chain reaction (PCR) and sequencing analysis

Primer sequences were designed using Oligo 7 Primer Analysis Software (Molecular Biology Insights, Colorado, USA). The primer was selected to specifically target the coding region of the gene. Primers located on two exons and the relevant parts of the gene were used to amplify the region of the final mature mRNA produced by the gene after the introns removal through RNA splicing (Table 1). Each primer pair was verified to have no homology to other canine sequences using the Primer-BLAST tool available at https://www.ncbi.nlm.nih.gov/tools/primer-blast/.
For the PCR reactions, 25 µl of reaction mixture was prepared using an RBC *taq* DNA polymerase kit (RBC Life Sciences Inc., Texas, USA) according to the manufacturer's instruction. Each amplification reaction contained 50 ng of the total RNA sample, which had already been reverse transcribed into cDNA. The cycling conditions were as follows: initial denaturing at 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 57°C of c*PD-L1* primers and 55°C of c*GAPDH* primers for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 10 min. The PCR products were stored at 4°C for the next step.

2.4. Sequencing analysis

The PCR products were electrophoresed on 2% agarose gels stained with Maestrosafe nucleic acid loading dye (Maestrogen, Hsinchu, Taiwan). Electrophoresis was performed for 40 min at 100 V to check the product size by comparison to a DNA molecular size

Table 1. Primer pair sequences of canine *GAPDH* and *PD-L1* genes.

Gene	Forward and reverse primers (5″–3″)	Product (bp)	Location of primer	NCBI/Ensemble Database[a,b]
cGAPDH	AGAAAGCTGCCAAATATGACGAC	195	between exon 8–9	NM_001003142/
	CATACCAGGAAATGAGCTTGACA		between exon 9–10	ENSCAFG00000015077
cPD-L1	CGCTGAACATCAATGCAAC	168	exon 5	NM_001231972.1/
	CACCAAGAAGCAACAGGAAAG		exon 6	ENSCAFG00000002120

[a]National Center for Biotechnology Information (NCBI) database. http://www.ncbi.nlm.nih.gov. (Accessed 31 May 2024).
[b]Ensemble genome database. http://asia.ensembl.org.index.html. (Accessed 31 May 2024).

marker. The bands were visualized under UV (UVP Benchtop UV Transilluminators; Fisher Scientific, California, USA). A single amplified fragment was purified using a PCR purification kit (Invitrogen) according to the manufacturer's protocol. Direct sequencing analysis was performed to confirm the homology of the RT-PCR products. Sequence identification and multiple alignment analysis were carried out using the BLAST program (http://blast.ncbi.nlm.nih.gov) of the National Center for Biotechnology information (NCBI) and multiple sequence comparison was performed using the log-expectation (MUSCLE), respectively.

2.5. Nucleotide sequence accession numbers

The nucleotide sequences for the *PD-L1* gene of the canine mammary tumours reported here have been deposited in the GenBank database under accession number: MN416128.

2.6. Real-time polymerase chain reaction (qPCR)

The qPCR reaction was performed in triplicate for each sample using the SensiFast™ SYBR® Hi-Rox kit (Bioline Meridian Bioscience, London, UK) according to the manufacturer's recommendations. Briefly, 100 ng of cDNA was used as the starting material in a 20 µl reaction. The cycling conditions were performed using a two-step protocol as follows: initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 10 s, and annealing/extension at 60°C for 30 s. Dissociation curves were analysed between 60°C and 90°C. The efficiency (E) of the qPCR reaction was calculated using the formula: $E = 10^{(-1/slope)} - 1$. The relative mRNA expression of the target gene was compared to the housekeeping gene and calculated as: $2^{-(Ct\ cPD\text{-}L1\ -\ Ct\ cGAPDH)}$

2.7. Primary antibody selection

The peptide translation of the target *PD-L1* RT-PCR product sequences was predicted using the BLAST program (Figure 1). The predicted canine PD-L1 peptide sequence LNINATANEIFYCTFLRSGPEENNTAEL VIPERLPVPASERTHFMILGPFLLLLG (accession number: MN416128) was compared with the full length of human PD-L1 immunogenic peptide sequence (Figure 1). A commercial antibody, generated using an immunogenic peptide that aligned with the canine PD-$L1_{1-42}$ peptide, was then selected (Figure 2).

2.8. Protein extraction and Western blot analysis

Protein extraction was performed using 1 ml of RIPA lysis buffer (50 mm Tris, pH 8.0, 150 mm NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) mixed with 1 µL of cocktail protease inhibitor (cOmplete™, MilliporeSigma, Massachusetts, USA). The lysates were incubated on ice for 2 h. The protein fraction was collected by centrifugation, and the protein concentration was measured using the BCA assay kits

a

Canine PD-L1 mRNA Sequences (MN416128)

```
 cg ctg aac atc aat gca aca gct aat gag att ttc tac tgc act ttt ctg aga tca ggt
 -  L  N  I  N  A  T  A  N  E  I  F  Y  C  T  F  L  R  S  G
cct gag gaa aac aat act gcc gag ttg gtc atc cca gaa cga ctg ccc gtt cca gca agt
 P  E  E  N  N  T  A  E  L  V  I  P  E  R  L  P  V  P  A  S
gag agg act cat ttc atg att ctg gga cct ttc ctg ttg ctt ctt ggt
 E  R  T  H  F  M  I  L  G  P  F  L  L  L  L  G
```

b

Species	Sequences	identity
Dog(query)	LNINATANEI FYCTFL-RSG PEE--NNTAE LVIPE--RLP VPASERTHFM ILGPFLLLLG	
Dog(NP_001278901.1)	LNINATANEI FYCTFQ-RSG PEEN--NTAE LVIPE--RLP VPASERTHFM ILGPFLLLLG	98.18
Red fox(XP_025857411.1)	LNINATANEI FYCTFQ-RSG PEE--NNTAE LVIPE--LLP VPTSKRTHFV ILGPFLLLLG	90.00
Ferret(XP_012918820.1)	LRINATANEI FYCTFR-RSG PEEDRNNTAK LVIPE--PLP VPSNERTHFM VLGAVLVFLG	77.19
Cat(XP_006939101.1)	LRINTTANEI FYCTFLQRSS PEG--NSTAE LVIPE--PFL VPANERTHFM ILGAILLFLV	76.36
Canada lynx(XP_030149121)	LRINTTANEI FYCTFLQRSS PEG--NSTAE LVIPE--PFL VPANERTHFM ILGAILLFLV	76.36
Puma(XP_025787442.1)	LRINTTANEI FYCTFLQRSS PEG--NSTAE LVIPE--PFL VPANERTHFM ILGAILLFLV	76.36
Cheetah(XP_014919968.1)	LRINTTANEI FYCTFLQRSS PEG--NSTAE LVIPE--PFL VPANERTHFM ILGAILLFLV	76.36
Human(NP_054862.1)	LRINTTTNEI FYCTFR-RLD PEE--NHTAE LVIPE-LPLA HPPNERTHLV ILGAILLCLG	68.42
Human(NP_001254635.1)	LRINTTTNEI FYCTFR-RLD PEE--NHTAE LVIPE-LPLA HPPNERTHLV ILGAILLCLG	68.42

Figure 1. PD-L1 mRNA sequencing results of CMTs. a) Sequencing results of the PD-L1 mRNA gene in CMT samples. b) Alignment of PD-L1 peptide sequences from dogs, humans, and other species.

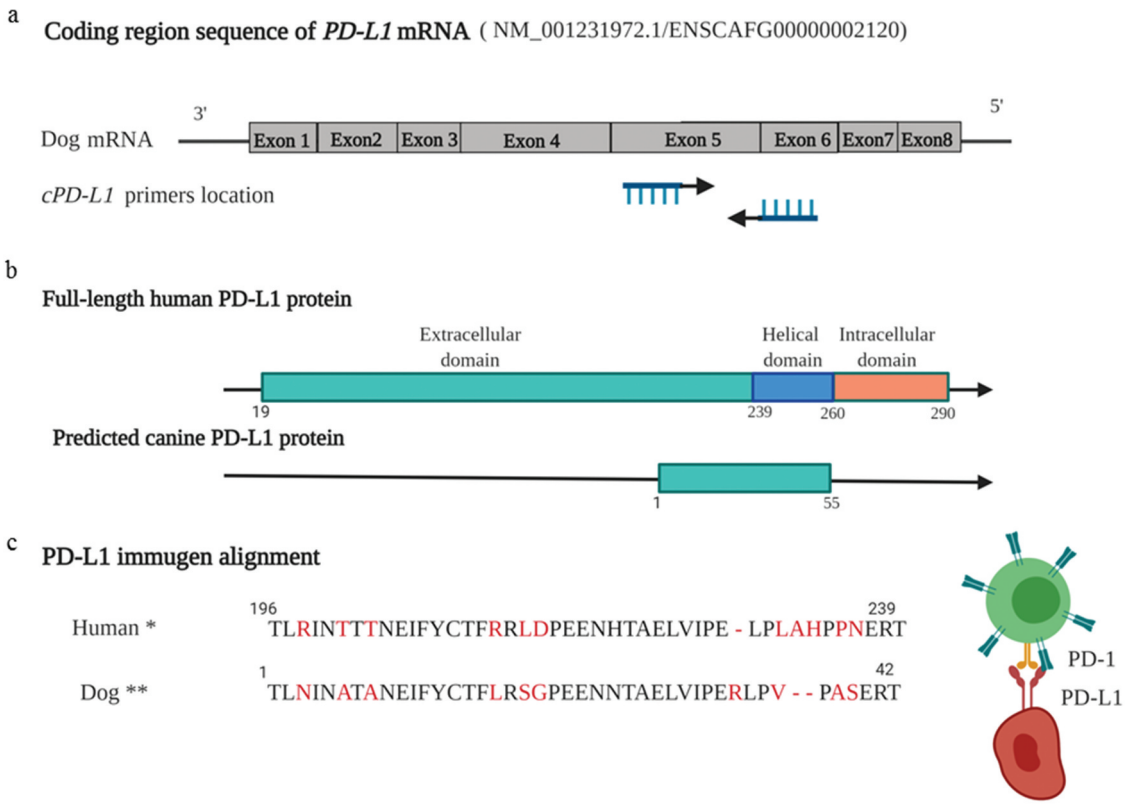

Figure 2. Predicted canine PD-L1 peptide. a) Location of primer pairs targeting exon 5 and exon 6 within the coding sequence of canine PD-L1 mRNA. b) The predicted canine PD-L11-55 peptide, translated from the RT-PCR product, aligns with the extracellular and transmembrane (helical) domains of the full-length human PD-L1 protein. c) Alignment of the canine PD-L11-42 peptide with the human PD-L1 extracellular domain (immunogen of rabbit anti-human PD-L1 MAb clone 28–8), showing 70.45% identity. * The partial protein sequence of the human extracellular domain immunogen of the rabbit anti-human PD-L1 MAb clone 28–8 at amino acids 196–239. ** The partial predicted protein sequence of canine PD-L1 based on the RT-PCR product.

(Thermo Scientific, Massachusetts, USA) according to the manufacturer's instructions. For each sample, 25 µg of protein was mixed with an equal volume of 2× Laemmli buffer containing 5% 2-mercaptoethanol, and boiled at 95°C for 7 min [28]. The protein samples were separated by the 12% sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into the nitrocellulose membranes using the electro-blotting technique [29]. The membranes were incubated with the blocking buffer [5% skim milk powder (MilliporeSigma, New Jersey, USA) in Phosphate-buffered saline solution (PBS), pH 7.4] at room temperature for 1 hr and washed with PBST (0.1% of Tween 20 in PBS, pH 7.4) 3 times for 5 min each. The membranes were then incubated with the rabbit anti-human PD-L1 MAb clone 28–8 (Abcam, Cambridge, UK) at a dilution of 1:2,000 in the blocking buffer at 4°C overnight and washed 3 times with PBST for 5 min each. To visualize the bands, the membranes were incubated with Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Massachusetts, USA) at a dilution of 1:2,000 in blocking buffer at room temperature for 2 h and then washed 3 times with PBST for 5 min each. The 0.05% DAB and 0.02% H_2O_2 solutions in PBS were poured on membranes for 5–10 min. Brown-coloured bands indicated positive results. Chicken anti-human GAPDH polyclonal antibody (AB2302, MilliporeSigma, New Jersey, USA) at a dilution of 1:2,000 in the blocking buffer and HRP conjugated goat anti-chicken IgG (Seracare Life Sciences Inc., Massachusetts, USA) at a dilution of 1:2,000 were used as an internal loading control for all samples. Human colon cancer cell line HT-29 (ATCC No. HTB-38) and RIPA lysis buffer were used as the positive and negative controls, respectively.

2.9. Data analysis

The mean and standard error of the mean (S.E.M.) were used to analyse the age of the patients in this study. The Student's t-test was used to compare the mean relative fold change in mRNA expression of PD-L1 between the groups of interest. Descriptive analysis was performed to explain the results of mRNA and protein expression. A significance level was set at a P-value ≤0.05.

2.10. Institutional review board statement

The study was conducted according to the guidelines of the Institute of Animals for Scientific Purposes Development (IAD), National Research Council of Thailand (NRCT), and approved by the Animal Care and Use Committee (FVM-ACUC), Faculty of Veterinary Medicine, Chiang Mai University (protocol code: S30/2560 and date of approval: 19 January 2018). Chiang Mai University Institutional Biosafety Committee (CMU-IBC) had reviewed and approved that the project is compiled with biosafety guideline for modern biotechnology, pathogen and toxic act (approval number CMUIBC A-0763014 and date of approval: 28 October 2020).

3. Results

3.1. Tumor histopathological classification

In this study, CMT specimens were classified as 4 benign neoplasms, 22 malignant epithelial neoplasms, and 2 malignant mesenchymal neoplasms (Table 2). In addition, five normal mammary tissues were used as control expression. All CMT-diagnosed patients in this study were female. The mean age of CMT-diagnosed patients was 10.39 ± 0.47 years. The mean ages of patients with benign and malignant growths were 10.67 ± 1.92 years and 10.21 ± 2.15 years, respectively. Primary tumours with a size ≥3 cm at diagnosis were observed more frequently than those <3 cm. Similarly, advanced disease (regional-distant) with TNM stage >3 was more frequently detected than localized disease with TNM stage ≤2 (Table 2).

3.2. Sequencing analysis of cPD-L1

The expected sizes of the RT-PCR products for canine PD-L1 and GAPDH were 168 and 195 base pairs, respectively (Figure 3). DNA sequencing confirmed 98.18% and 100% homology with the original canine nucleotide database for PD-L1 and GAPDH, respectively (Figure 1).

Table 2. Clinical and pathological characteristics of the CMT diagnosed patients.

Animals	Number (%)	Mean of the relative fold change *cPD-L1* mRNA expression
Total cases	28 (100%)	0.054 ± 0.012
Age at diagnosis (years)		
<8	3 (10.71%)	0.101 ± 0.050
8–11	16 (57.14%)	0.044 ± 0.012
≥12	9 (32.14%)	0.058 ± 0.027
Primary tumour size (cm)		
<3	3 (10.71%)	0.062 ± 0.013
≥3	25 (89.29%)	0.054 ± 0.014
TNM stage		
≤2	6 (21.43%)	0.098 ± 0.038[a]
>2	22 (78.57%)	0.040 ± 0.010[b]
Histopathological classification [27]		
Benign	4 (14.28%)	0.040 ± 0.014
Malignant	24 (85.71%)	0.057 ± 0.013
Epithelial malignant tumour	22 (78.57%)	0.057 ± 0.015
Epithelial simple type	12 (42.86%)	0.051 ± 0.009[a]
Epithelial complex type	7 (25%)	0.092 ± 0.008 [a]
Epithelial mixed type	3 (10.72%)	0.003 ± 0.002[b]
Mesenchymal malignant tumour	2 (7.14%)	0.051 ± 0.051

The values with different superscript letters in column are significantly different ($p < 0.05$).

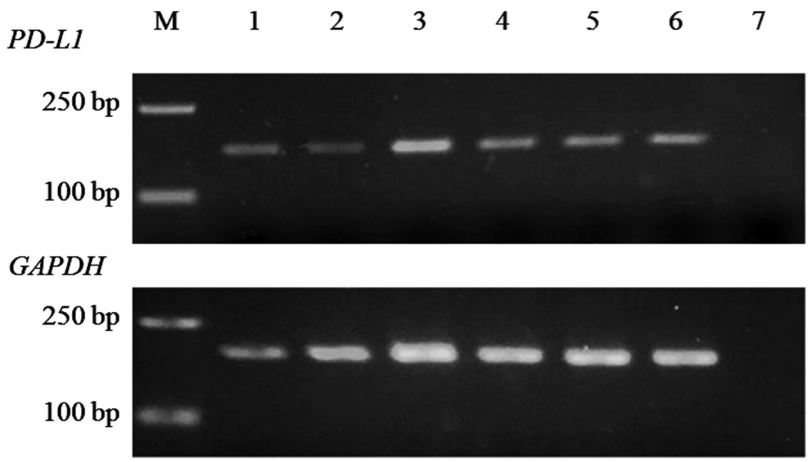

Figure 3. RT-PCR amplification of mRNA expression of the canine PD-L1 gene (168 bp) and GAPDH (195 bp) in canine mammary glands. M: Marker, 1–6: Total RNA from each CMT sample, 7: PBS (negative control).

3.3. Relative level of cPD-L1 mRNA expression in CMTs

mRNA expression was determined using RT-qPCR. The mean relative fold change of cPD-L1 mRNA to cGAPDH mRNA is presented in Table 2. There was no significant difference in the mean fold-change relative expression between age groups. In contrast, localized disease with TNM stage ≤2 exhibited higher mean cPD-L1 mRNA expression levels than advanced disease with TNM stage >3. Moreover, tumours ≤3 cm tended to have higher cPD-L1 mRNA expression levels than those >3 cm, although the difference was not statistically significant. Based on histopathological characteristics, malignant tumours tended to have higher mean cPD-L1 mRNA levels than benign tumours, particularly in epithelial complex type tumours (Figure 4a,b). This tumour type exhibited the highest mRNA levels compared to other types. However, the individual mRNA expression values (Figure 4c) revealed one outlier with the highest value in the complex carcinoma group. The two mesenchymal tumour specimens showed different mRNA expression levels, with the higher value observed in osteosarcoma and the lower in fibrosarcoma (Figure 4b).

3.4. Western blotting analysis

The results revealed 100% cross-reactivity of approximately 51 kDa, corresponding to suspected canine PD-L1, with anti-PD-L1 MAb clone 28–2 in all CMT specimens (Figure 5a). Additionally, colon cancer cells exhibited immunopositivity with anti-PD-L1 MAb at approximately 55 kDa (Figure 5b). In contrast, the RIPA lysis buffer, used as a negative control, showed no immunoreactivity with anti-PD-L1 MAb. Furthermore, all tissues expressed GAPDH, which served as an internal control, at approximately 37 kDa.

4. Discussion

The success of advancements in canine mammary tumour (CMT) treatment has been limited due to the immune evasion mechanisms of tumour cells.

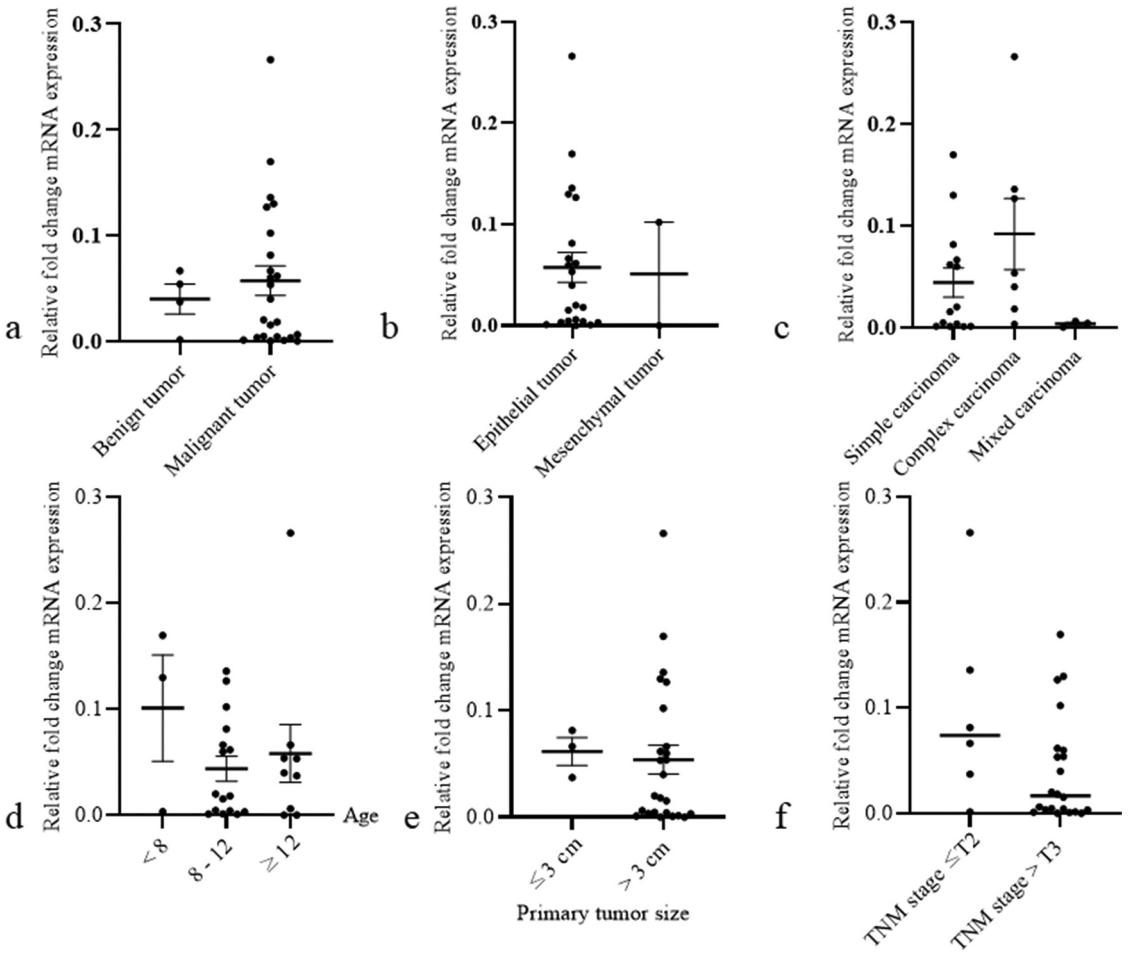

Figure 4. Graphs showing the mean relative fold change in PD-L1 mRNA expression. (a – c) Mean relative fold change in mRNA expression classified by tumour type: benign vs. malignant tumours, epithelial vs. mesenchymal malignant tumours, and epithelial tumour subtypes (simple carcinoma, complex carcinoma, and mixed carcinoma), respectively. d) Mean relative fold change in mRNA expression classified by age: <8 years, 8–11 years, and ≥12 years. e) Mean relative fold change in mRNA expression classified by primary tumour size: ≤3 cm and >3 cm. f) Mean relative fold change in mRNA expression classified by TNM score: ≤2 and >2.

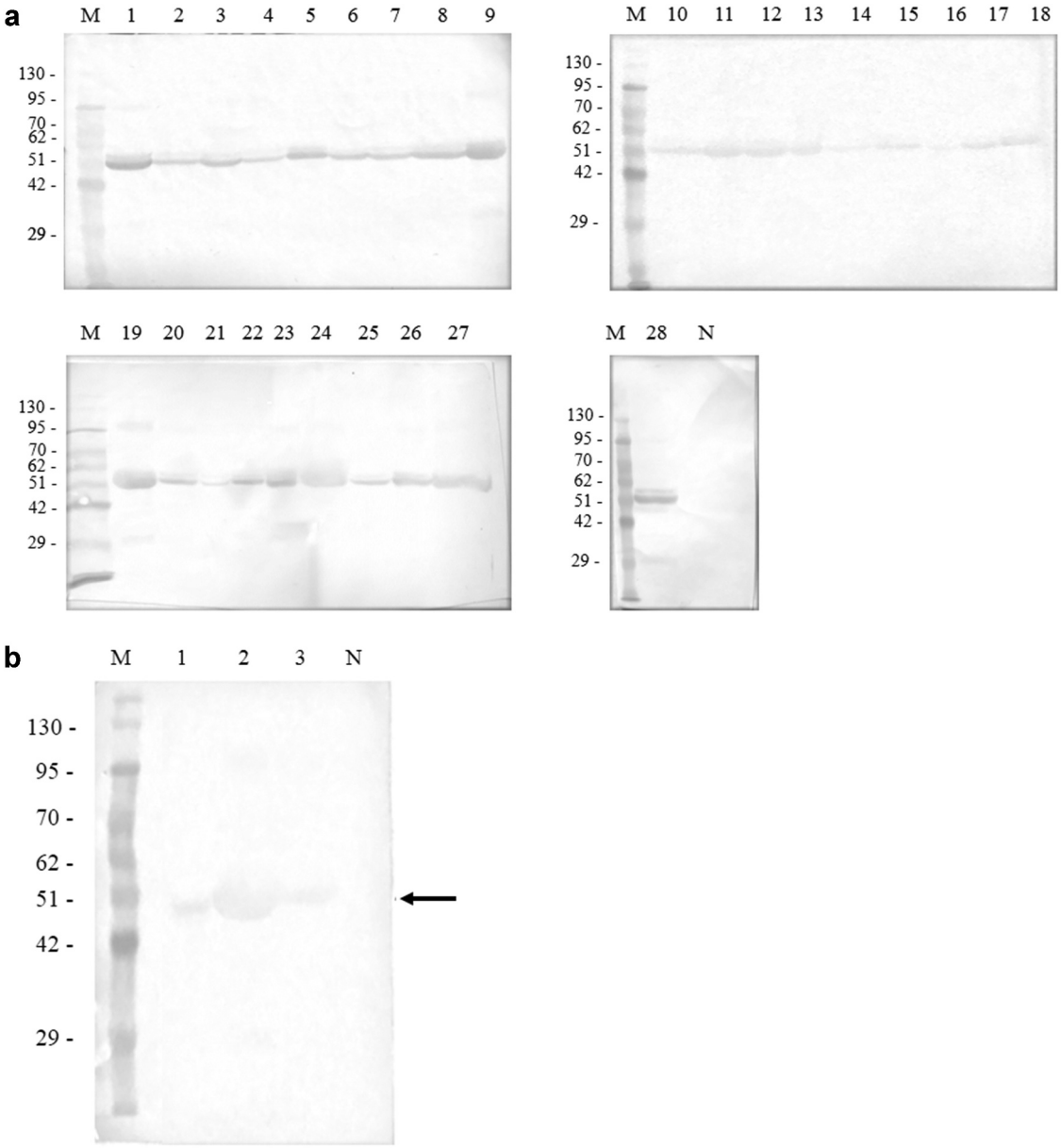

Figure 5. Western blot analysis results of CMTs. a) Results showing cross-reactivity of anti-PD-L1 MAb clone 28–8 with CMT tissues. M: Marker, 1: Human colon cancer cells (positive control), 2–3: CMTs, 4: RIPA lysis buffer (negative control). Each well contained 25 µg of crude protein. b) Results showing cross-reactivity of rabbit anti-human PD-L1 MAb clone 28–8 in CMT tissues. M: Marker, 1–28: CMTs, 29: RIPA lysis buffer (negative control).

Programmed death-ligand 1 (PD-L1), a ligand of the immunoinhibitory molecule PD-1, is inducibly expressed on activated T, B, and myeloid cells [6,30]. The expression of PD-L1 in human tumours enables escape from the host immune system, leading to recurrence after surgery, metastasis to distant organs, and resistance to chemotherapy [31]. However, studies investigating this tumour escape mechanism in dogs remain scarce [14,32]. This study developed detection assays for canine PD-L1 (cPD-L1) in CMTs using RT-qPCR and western blot analysis, aiming to provide a foundation for potential immunotherapeutic interventions in CMT treatment.

DNA stores the information necessary to encode cellular proteins, while RNA acts as the intermediary, translating this code into polypeptides. Specifically, mRNA carries the protein blueprint from DNA to ribosomes, where protein synthesis occurs [33]. In malignant CMTs, which typically have a poorer prognosis than benign tumours, relative fold changes in PD-L1 mRNA expression tended to be higher than in benign CMTs, though the difference was not statistically significant. These findings align with studies in human breast cancer tissues, where PD-L1 mRNA expression levels showed no correlation with age, tumour size, lymph node status, or histologic grade [34]. Limited research exists on cPD-L1 mRNA expression in CMTs. However, several studies have reported cPD-L1 protein expression using western blot and immunohistochemistry methods. This study detected PD-L1 protein expression in 100% of the samples using western blot analysis, consistent with

prior findings showing 100% immunopositivity in six canine mammary gland tumour cell lines [35]. Immunohistochemistry studies have reported PD-L1 protein expression in 80% (4/5) of high-grade CMTs [36].

Interestingly, while the predicted molecular weight of canine PD-L1 protein was approximately 33 kDa (based on the NCBI database, accession number: NM_001291972.1), western blot analysis revealed bands around 51 kDa. Similar findings have been reported, with PD-L1 extracted from CMT cell lines showing a size of approximately 47 kDa [35]. Post-translational modifications, such as glycosylation, could explain these discrepancies. In human studies, non-glycosylated PD-L1 proteins appear at 33 kDa, while glycosylated forms range from 45 to 55 kDa on western blots [37]. Glycosylation enhances protein stability, prolongs half-life, and upregulates PD-L1 expression in cancer stem cells, promoting immune evasion [37,38]. In contrast, the size of canine GAPDH protein remained stable, consistent with the human colon cancer positive control at approximately 37 kDa.

This study confirmed that RT-qPCR assays specifically detected the extracellular and transmembrane domains of cPD-L1 mRNA (Figure 2). However, mRNA levels do not always correlate with protein expression levels. Some CMT samples with low PD-L1 mRNA expression exhibited strong immunopositivity for PD-L1 protein. This discrepancy might be due to factors such as mRNA splice variants, which could result in soluble PD-L1 (sPD-L1) without a transmembrane domain. sPD-L1 has been identified in human melanoma, renal cell carcinoma, and the blood of multiple myeloma patients [39–41] including it has been described in the peripheral blood of dogs with different oncological diseases, as well [42]. Additionally, differences in mRNA and protein half-lives, extraction yields, and translation processes could have contributed to the observed variability [14,36,43,44].

PD-L1 has emerged as a promising therapeutic target in the treatment of canine mammary tumours (CMTs), reflecting its established role in human oncology [14,26,36,45]. PD-L1 expression in canine mammary tumours is associated with immune evasion and tumour progression, making it a critical checkpoint for therapeutic intervention [45–47]. Immunotherapy, particularly using anti-PD-L1 monoclonal antibodies (mAbs), has shown the potential to restore T-cell activity and enhance anti-tumour immune responses, thereby inhibiting tumour growth [48–51]. Early studies in dogs have yielded encouraging results, suggesting that PD-L1 blockade could complement traditional treatments like surgery and chemotherapy to improve clinical outcomes [41]. However, further clinical trials are necessary to refine therapeutic protocols, assess long-term efficacy, and address potential immune-related adverse effects.

Recent advancements in the development of canine PD-1/PD-L1 antibodies have focused on addressing the lack of commercially available immunotherapeutic options targeting canine immune checkpoint molecules. Several cPD-L1-specific mAbs have been developed [26,48–51] and have demonstrated specific binding to both recombinant and native PD-L1 molecules in dogs. Among these, mouse anti-canine PD-L1 mAbs have shown effective binding to various canine cancers, including cutaneous T-cell lymphomas, mammary carcinomas, soft tissue sarcomas, squamous cell carcinomas, and malignant melanomas [50]. While these findings are promising, functional assays are needed to validate the therapeutic efficacy of anti-cPD-L1 antibodies and their potential use as prognostic markers [52]. Larger-scale clinical studies across diverse canine cancer populations will be critical to establishing their clinical utility.

Additionally, the therapeutic efficacy of cPD-L1 antibodies has been evaluated in mouse models as part of initial safety and pharmacological studies in dogs [51]. These studies demonstrated the potential of cPD-L1 antibodies to act as effective immune checkpoint inhibitors, paving the way for translational research in naturally occurring canine cancers. This research not only advances veterinary oncology but also provides valuable insights for the development of immunotherapy in human cancers. Nonetheless, further investigations are essential to confirm the clinical applications and therapeutic potential of PD-1/PD-L1 immunotherapy in both veterinary and human medicine.

5. Conclusion

Immunotherapy is increasingly recognized as an effective treatment for several canine cancers. Despite its promise, challenges remain regarding access to checkpoint inhibitor therapy for dogs. The cost-effectiveness of such treatments and their feasibility within the pet cancer industry require further investigation. Additionally, breed variety and heterogeneous tumour biology complicate standardization and pharmaceutical development. To advance the understanding of tumour immune escape in dogs, further studies on the regulation of the PD-L1 gene and protein are essential. Large-scale analyses may clarify the role of post-translational modifications in CMTs, aiding in the development of more effective therapies.

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Disclosure statement

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WS, PK, AM, KS and TV: investigation, formal analysis, and resources.
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Data availability statement

Data is available on request due to privacy/ethical restrictions.

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