Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2026.063

RESEARCH ARTICLE

Stage-Dependent Expression Of AFP And PDE4D As Complementary Biomarkers In Canine Mammary Tumors: A Correlative Analysis With Ki-67

Mai Thi Kieu Trinh[1], Sekkarin Ploypetch[2], Saritvich Panyaboriban[3], Promporn Raksaseri[3], Kongkiat Srisuwatanasagul[3] and Sayamon Srisuwatanasagul[3]*

[1]Graduate Program in Veterinary Science and Technology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand; [2]Department of Clinical Sciences and Public Health, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand; [3]Department of Anatomy, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
*Corresponding author: Sayamon.s@chula.ac.th

ARTICLE HISTORY (25-1086)

Received: November 15, 2025
Revised: March 13, 2026
Accepted: March 16, 2026
Published online: March 18, 2026

Key words:
Alpha-fetoprotein
Biomarkers
Canine mammary tumors
Ki-67 proliferation index
Phosphodiesterase 4D

A B S T R A C T

Canine mammary tumors (CMTs) are the most prevalent neoplasms in female dogs; however, the diagnosis and prognosis of CMTs show a significant challenge in veterinary oncology. This study evaluates alpha-fetoprotein (AFP) and phosphodiesterase 4D (PDE4D) as possible biomarkers for the progression of CMTs, in correlation with the proliferation marker Ki-67. The protein expression was studied across the CMTs spectrum which were normal, adjacent, benign, and malignant tissues by using immunohistochemistry (IHC) and western blotting (WB). Quantitative IHC demonstrated that both AFP and PDE4D levels increased in a stage-dependent manner. AFP levels increased gradually from normal to benign and highest in malignant CMTs, indicating that it is an early and persistent marker of neoplastic transformation. PDE4D exhibited a comparable pattern, indicating its role in sustaining cancer cell viability. WB demonstrated that both proteins exhibited similar higher expression in benign and malignant CMTs, revealing differences between total protein and *in situ* localization. Correlation analyses showed a significant positive relationship between AFP and Ki-67 only in benign cases, while it disappeared in malignant CMTs. This uncoupling mechanism in malignant CMTs suggested that growth pathways beyond initial cell proliferation were involved. In conclusion, this study demonstrates a new perspective on the novel biomarkers for CMTs. While AFP acts as an early sensitive marker of dedifferentiation of mammary tissue, PDE4D serves as a definitive protein that flips during the transition to aggressive malignant CMTs. These findings establish an integration of AFP- and PDE4D into future precision diagnosis for the development of targeted therapy in veterinary oncology.

To Cite This Article: Trinh MTK, Ploypetch S, Panyaboriban S, Raksaseri P, Srisuwatanasagul K and Srisuwatanasagul S, 2026. Stage-dependent expression of afp and pde4d as complementary biomarkers in canine mammary tumors: A correlative analysis with Ki-67. Pak Vet J, 46(4): 952-960. http://dx.doi.org/10.29261/pakvetj/2026.063

INTRODUCTION

In veterinary medicine, canine mammary tumors (CMTs) are a major concern, as it accounts for more than half of all tumor cases in female dogs. CMTs are a significant health and quality-of-life issue globally, as 50% to 70% of these tumors are malignant (Goldschmidt *et al*., 2011; Burrai *et al*., 2020). Hormones, age, and late spaying significantly increase the risk of CMTs (Varallo *et al*., 2019; Srisawat *et al*., 2024). The metastasis of malignant CMTs to vital organs such as the lungs, liver, or bones complicates treatment and worsens the prognosis (Kim *et al*., 2021). Identifying reliable and helpful biomarkers for accurate diagnosis and prognosis of this disease remains a priority in veterinary oncology. In addition, the oncology medicine tends to rely on the digital diagnostic techniques, the integration of reliable biomarkers becomes extremely important. The anatomical and protein biomarker mapping provides the crucial data needed for the digital models. These data may offer the comprehensive biological frameworks required for upcoming AI- driven diagnostic platforms (Choudhary *et al*., 2025), i.e., the data on the related biomarker proteins.

In the search for effective biomarkers, proteins associated with human carcinogenesis are of interest. Alpha-fetoprotein (AFP) is a glycoprotein that is generally produced and responsible for fetal development. However, it is reported that AFP re-expressed in many adult malignant neoplasms (Moro *et al*., 2012; Liu *et al*., 2024). AFP is recognized for facilitating tumor growth by stimulating cell proliferation, assisting immune evasion, and enabling metastasis, often via the activation of pathways such as PI3K/AKT and JAK/STAT (Głowska-Ciemny *et al*., 2023).

Phosphodiesterase 4D (PDE4D) is one of the significant markers that is connected to human cancers, such as lung, breast, and prostate cancers (Rahrmann *et al*., 2009; Lin *et al*., 2013; Liu *et al*., 2019). Its primary function is to modulate the concentration of cyclic AMP (cAMP), a signaling molecule essential for several biological processes (Lusardi *et al*., 2024). PDE4D is an important signaling molecule that modifies proteins, growth factors, and regulatory genes involving parts of the cell cycle. PDE4D's dysregulation is linked to activities like increasing cell proliferation, decreasing apoptosis, and permitting invasiveness and metastasis by modulating intracellular signaling (Lin *et al*., 2013). From these reasons, PDE4D is an important pharmaceutical target, as selective PDE4D inhibitors may have the potential in the treatment of cancer, inflammatory diseases, and some neurological conditions (Lusardi *et al*., 2024). Besides these tumor proteins, Ki-67, a classical biomarker of cell proliferation, is commonly used to assess the aggressiveness of cancers (Brunetti *et al*., 2021; Uxa *et al*., 2021). A high Ki-67 index is a well-known marker of poor prognosis, as it signifies that the tumor grows rapidly and is associated with unfavorable clinical outcomes (Kilickap *et al*., 2014; Davey *et al*., 2021).

In general, the animal models persist a basis for the progress of new surgical techniques, medical implants, and targeted drug delivery systems (Choudhary, 2025). Because of the physiological similarities between species, canine mammary tissues can be served as a vital model for human oncology and medical innovation. Collectively, these proteins of interest are acknowledged to significantly contribute to human cancer; thus, their mechanisms in canine mammary tumors (CMTs) remain unidentified. Consequently, the changes in these expression patterns throughout the shift from benign to malignant CMTs have not been comprehensively examined. Furthermore, characterizing stage-dependent biomarkers like AFP and PDE4D not only enhances veterinary diagnostics but also strengthens the role of the canine model in advancing broader medical research and surgical therapies. Therefore, this study aims to evaluate the expression patterns of AFP and PDE4D in different canine mammary tissues, including normal, adjacent, benign, and malignant mammary tissues and to elucidate their biological roles in CMTS. By advancing the understanding of their biological significance, the results from this study may contribute to the development of more effective prognosis methods, offering improved outcomes for affected dogs and human oncology medicine.

MATERIALS AND METHODS

Animals and tissue specimens: This study was conducted at the Faculty of Veterinary Science, Chulalongkorn University, with approval from the Institutional Animal Care and Use Committee (IACUC) and adherence to institutional regulations and national guidelines for the ethical use of animals in scientific research (Animal Use Protocol No. 2531026). This study comprises 40 female dogs diagnosed with mammary tumors, including 20 with benign tumors and 20 with at least one malignant mammary tumor, as well as 10 female dogs exhibiting histologically normal mammary tissues as control samples. The categorization of tumor types employed in this study adhered to the framework established by Goldschmidt *et al*. (2011) and is shown in Table 1.

Table 1: Histopathological classification of the 40 canine mammary tumors analyzed in this study, detailing the distribution of benign and malignant tumor types and their respective subtypes

Tumor types	Subtypes	Number of subtypes
Benign (n=20)	Simple adenoma	2
	Complex adenoma	4
	Benign mixed tumor	12
	Tubulopapillary adenoma	1
	Complex papillary adenoma	1
Malignant (n=20)	Mixed adenocarcinoma	7
	Complex adenocarcinoma	2
	Simple adenocarcinoma	3
	Cystic papillary adenocarcinoma	3
	Tubulopapillary adenocarcinoma	1
	Sarcoma	2
	Special carcinoma	2

In dogs with mammary tumors, both tumor tissue and adjacent non-tumorous mammary tissue were collected to study as an internal control. These samples were taken from histologically normal areas of the mammary gland close to the tumor margin, usually 1–2cm away from the main tumor mass.

Immunohistochemistry (IHC): 4µm-thick tissue sections will be placed on gelatin-coated slides and processed histologically. The antigen retrieval was done by heating in the microwave using citrate buffer (pH 6.0) for AFP (two cycles at 750 W for 5 minutes) and Ki-67 (five cycles at 400W and one at 800W), and Tris-EDTA (pH 9.0) for PDE4D (three cycles at 600W for 5 minutes). 3% hydrogen peroxide was applied to stop endogenous peroxidase. The unspecific blocking was done by using normal goat serum AFP and PDE4D, and normal horse serum. The antibodies used were mouse anti-Ki-67 (clone 31A3, Bio-Rad, USA; 1:100) primary antibody, with horse anti-mouse secondary; rabbit anti-PDE4D (MBS9614299, Mybiosource, USA; 1:200) with goat anti-rabbit secondary; and HRP-conjugated rabbit anti-AFP (MBS2038900, Mybiosource, USA; 1:100) without secondary. In the final step, 3,3′-diaminobenzidine (DAB) was applied as the chromogen, and all tissue sections were counterstained with hematoxylin. The omission of primary antibodies was served as the negative controls.

All stained sections were imaged by the Panoramic Digital Scanner (3DHISTECH Ltd., Budapest, Hungary). The QuantCenter software (3DHISTECH Ltd., Hungary) was used to measure protein immunoreactivity, as explained by Thatsanabunjong *et al*. (2026). The staining intensity was rated on a scale of 1 (weak), 2 (moderate), or 3 (strong), with 0 being the lowest score and 300 being the highest. The H-score was then calculated as follows:

H-score = [(0×% negative) + (1×% weak) + (2×% moderate) + (3×% strong)].

Western blotting: Cold RIPA buffer (Tris-HCl pH 7.4, NaCl 2.5 M, EDTA 100 mM, Triton X-100, NaF, and 10μM protease inhibitor) was used to lyse powdered tissue samples. We used 10% SDS-PAGE to separate the proteins, then transferred them to Hybond-ECL nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with 5% non-fat milk in TBST for one hour at room temperature. They were then incubated overnight at 4°C with rabbit anti-AFP HRP-linked (MBS2038900, Mybiosource, USA; 1:1000), rabbit anti-PDE4D (MBS9614299, Mybiosource, USA; 1:1000), and β-actin (rabbit monoclonal anti-β-actin 4967, Cell Signaling Technology, EUA, 1:2000). Goat anti-rabbit IgG (Vector Laboratories, USA; 1:4000) was applied as a second antibody for PDE4D. After washing the membranes in TBST (0.1% Tween-20), a Western Blot Lightening Plus-ECL Detection Kit (Revvity, USA) and a Chemidoc Imaging System (Bio-Rad, USA) was applied to detect the protein bands. In the final step, ImageJ software was used to evaluate the grayscale intensity of Western blotting images.

Statistical analysis: One-way ANOVA, Kruskal-Wallis and the Tukey's HSD test was used for statistical analyses. The correlation between AFP, PDE4D, and Ki-67 was evaluated by using Spearman's correlation coefficient. GraphPad Prism 10.6.0 and SAS 9.4 (SAS Institute, Cary, NC, USA) were applied to perform all statistical analyses. The results are presented as mean±standard error of the mean (SEM), with a P-value of less than 0.05 was designed as significant.

RESULTS

Immunohistochemistry: The IHC analysis revealed that AFP and PDE4D were primarily localized in the cytoplasm of all examined tissues, which were malignant, benign, adjacent malignant, adjacent benign, and normal mammary tissues (Fig. 1 and Fig. 2, respectively). On the other hand, Ki-67 expression was limited to the nuclei. The quantitative evaluation of AFP and PDE4D immunoreactivity represented as H-scores and the Ki-67 proliferation index was shown in Fig. 3.

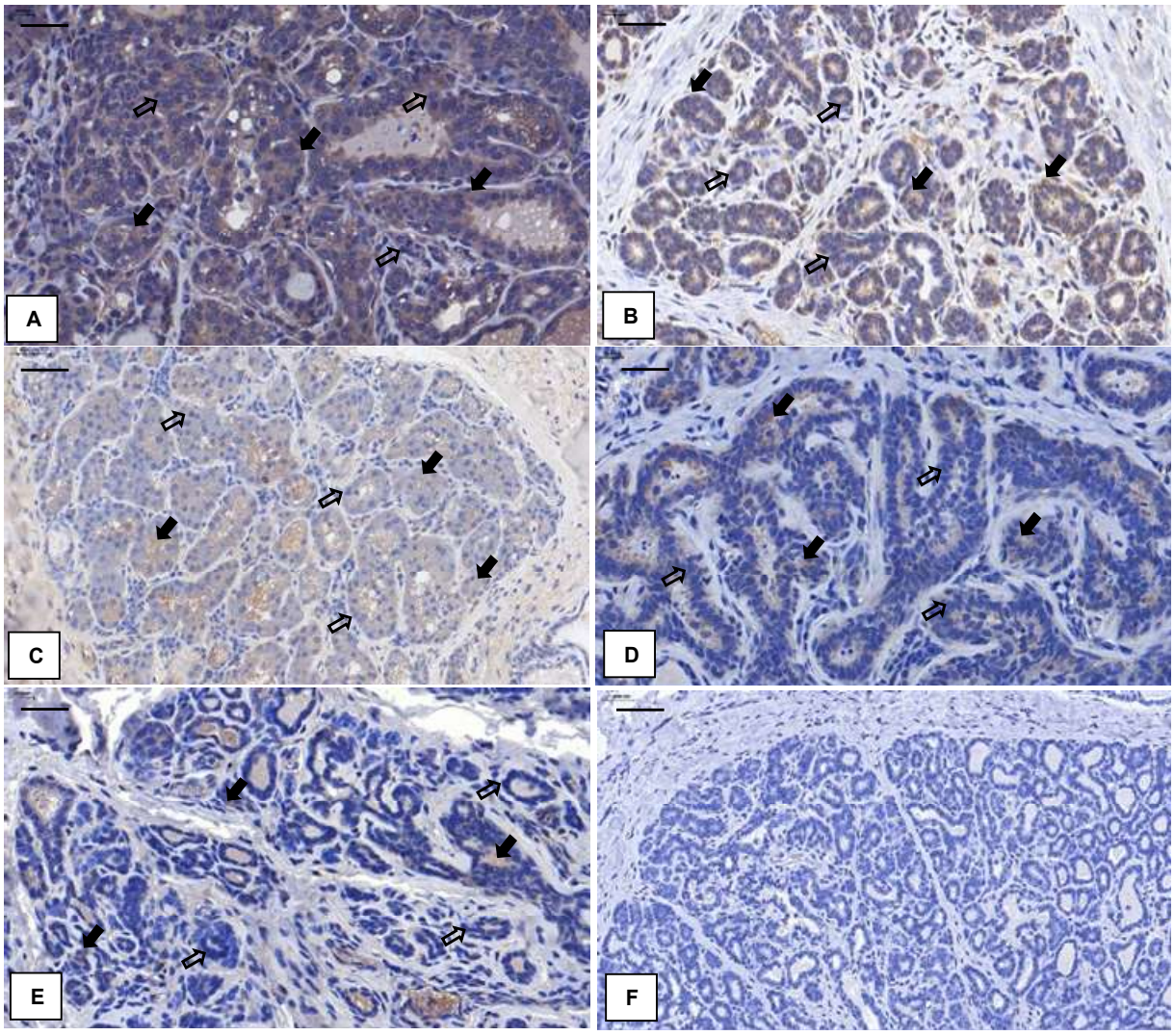

Fig. 1: Immunohistochemical staining of AFP in canine mammary tissues. Solid arrows denote hematoxylin counterstaining (blue), highlighting cell nuclei, while open arrows indicate DAB chromogenic staining (brown), marking AFP-positive immunoreactivity. Panels represent: (A) malignant tumor tissue, (B) benign tumor tissue, (C) tissue adjacent to malignant tumors, (D) tissue adjacent to benign tumors, (E) normal mammary tissue, and (F) negative control processed without primary antibody. Scale bar=20μm.

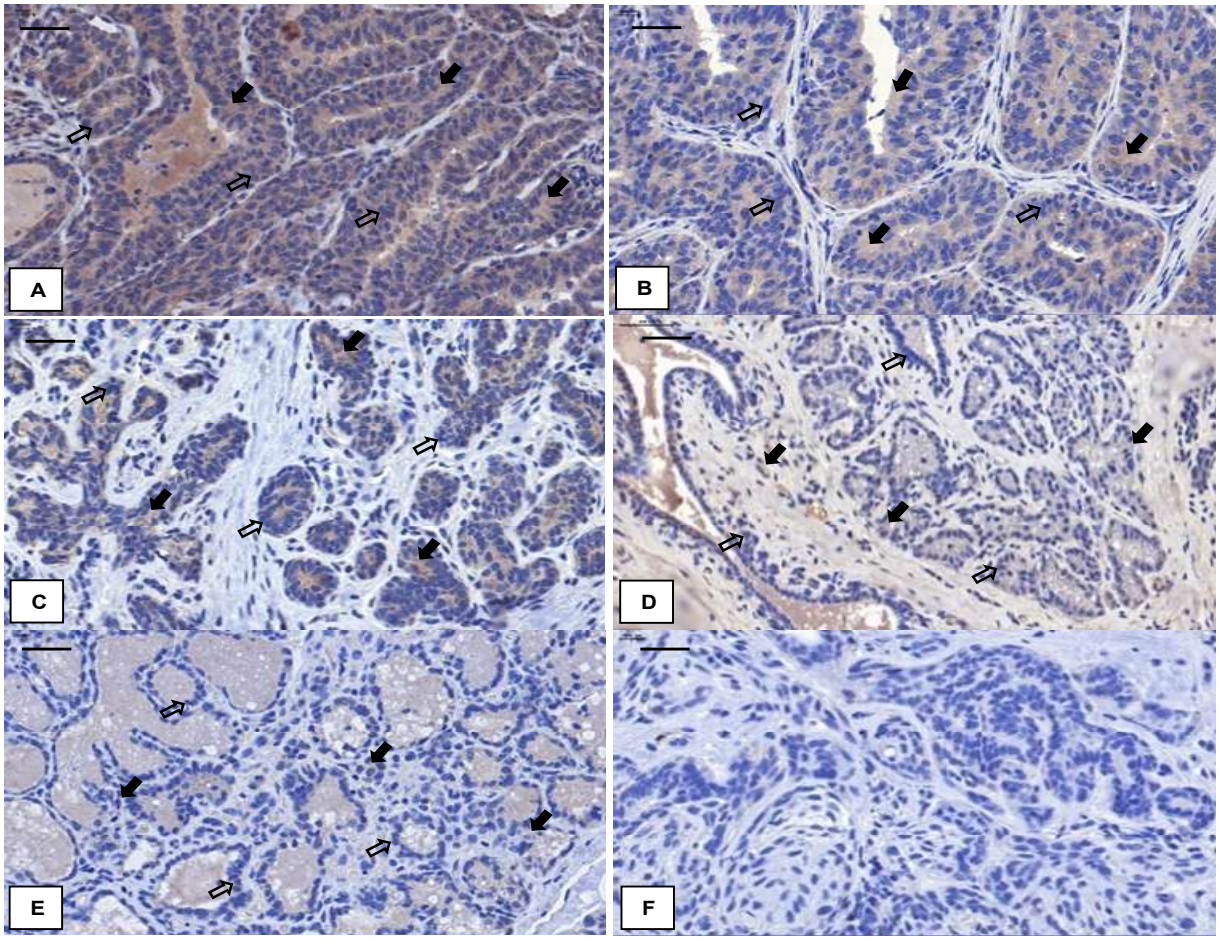

Fig. 2: Immunohistochemical staining of PDE4D in canine mammary tissues. Solid arrows denote hematoxylin counterstaining (blue), highlighting cell nuclei, while open arrows indicate DAB chromogenic staining (brown), marking PDE4D-positive immunoreactivity. Panels represent: (A) malignant tumor tissue, (B) benign tumor tissue, (C) tissue adjacent to malignant tumors, (D) tissue adjacent to benign tumors, (E) normal mammary tissue, and (F) negative control processed without primary antibody. Scale bar=20μm.

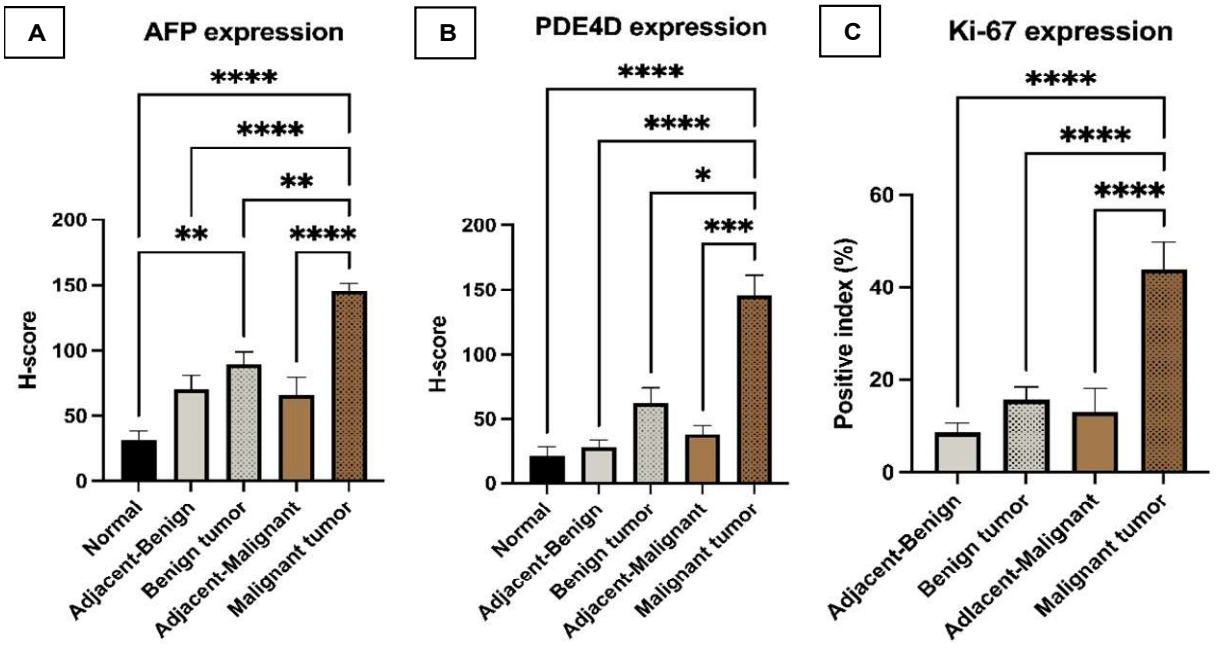

Fig. 3: Immunohistochemical expression of target proteins across the experimental groups of canine mammary tumors. (A) AFP expression, (B) PDE4D expression, and (C) Ki-67 expression. Statistical comparisons were performed using one-way ANOVA for AFP and the Kruskal–Wallis test for PDE4D and Ki-67. Significance levels: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Quantitative analysis showed significant differences in AFP expression among the groups (Fig. 3A). Malignant CMTs displayed the highest immunoreactivity, indicated by H-score of 145.4±5.96, significantly higher than all other groups (P<0.01). Benign tumors also exhibited considerable H-score expression (88.92±10.22), markedly higher than normal mammary tissue (P<0.01). The adjacent-benign (69.96±10.62) and adjacent-malignant (65.57±13.63) tissues exhibited similar intermediate H-score expression levels, whereas normal mammary tissue demonstrated the lowest basal H-score expression (33.25±8.49) which was significantly lower than CMT groups (P<0.01 for malignant CMTs and P<0.05 for benign CMT).

A similar trend was observed for PDE4D, with highest expression levels found in malignant CMTs (Fig. 3B). The highest H-score (145.4±16.18) was significantly different when compared with benign CMTs (62.37±11.49), adjacent tissues as well as normal mammary tissue (P<0.01 for adjacent-benign tissue and P<0.05 for adjacent-malignant tissue). Normal mammary tissues showed the lowest H-score (20.72±7.13), which was similar to the H-scores of adjacent-malignant (38.23±6.84) and adjacent-benign tissues (27.40±5.69) (P>0.05).

The Ki-67 proliferation index analysis showed differences among groups (Fig. 3C). Malignant tumors exhibited a significantly elevated proliferative activity, with a mean positive index of 43.99±5.74. This value was markedly higher than those of all other non-malignant and adjacent tissue types analyzed. The proliferation index in malignant tumors was significantly elevated compared to adjacent benign tissue (8.5±2.25) and adjacent malignant tissue (13.06±5.09) (P<0.01). On the other hand, the moderate proliferation index observed in benign tumors was not statistically different from that of the adjacent-malignant or adjacent-benign tissues (P>0.05).

Analysis of western blotting: Western blotting revealed separate bands for AFP (70kDa; Fig. 4A) and PDE4D (50-55kDa; Fig. 4B) derived from canine mammary tissues. Fig. 4C-D shows the normalized band intensities for each protein, with β-actin as the reference. Densitometric analysis of AFP indicated that the malignant tumor group demonstrated the highest expression levels. This upregulation was statistically significant relative to the expression level in the normal control tissue (P<0.05, Fig. 4C). The benign tumor, adjacent-malignant, and adjacent-benign tissues tended to increase compared to the normal control tissue; however, these differences did not reach statistical significance (P>0.05).

A unique and more pronounced pattern was also observed for PDE4D expression (Fig. 4B, 4D). The benign tumor, adjacent-malignant, and malignant tumor groups showed much higher PDE4D expression than the normal control tissue (P<0.05). Expression levels in these three groups were all high, with no significant differences among them. Expression in the adjacent benign tissue was elevated compared to the control, yet this difference was not statistically significant (P>0.05).

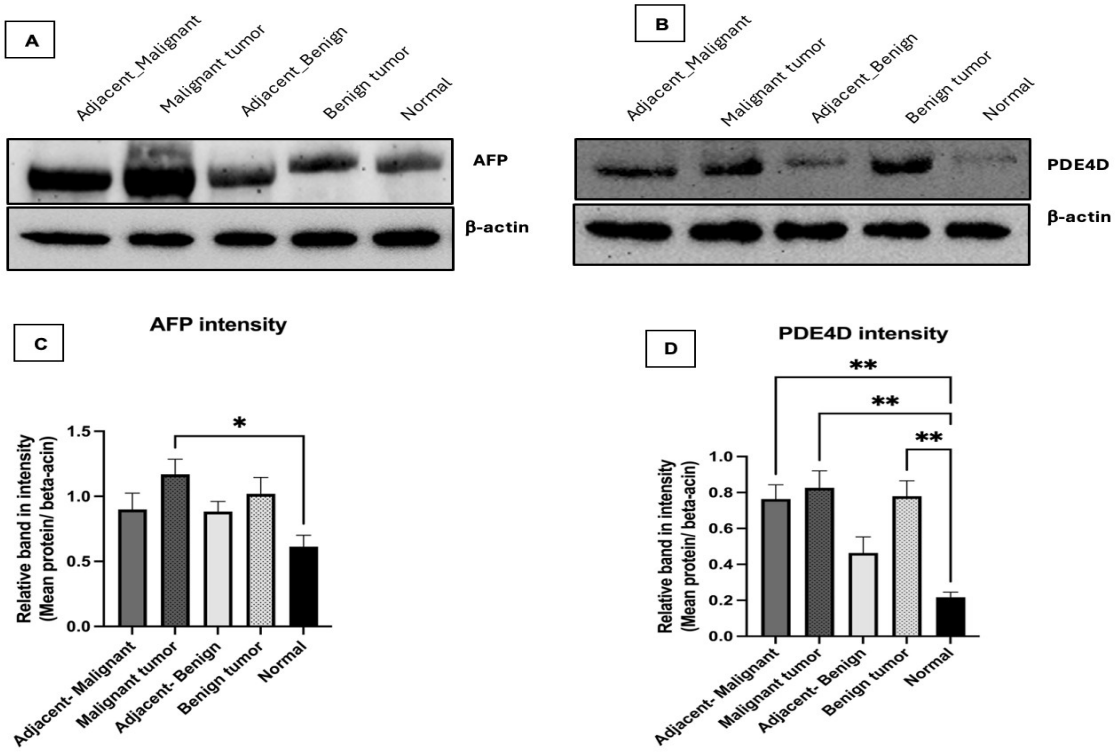

Fig. 4: Western blot analysis of target proteins across the experimental groups of canine mammary tumors. (A) Representative blot showing AFP (~70 kDa) and β-actin (~45 kDa). (B) Representative blot showing PDE4D (~50–55 kDa) and β-actin (~45 kDa). (C) Densitometric quantification of AFP expression normalized to β-actin. (D) Densitometric quantification of PDE4D expression normalized to β-actin. Statistical comparisons were performed, *P<0.05.

The correlation between the biomarkers AFP, PDE4D, and Ki-67 in CMTs: The correlation analyses of the expression of AFP, PDE4D, and the Ki-67 proliferation index in canine mammary neoplasms were performed to investigate the possible interactions among the biomarkers (Fig. 5). The analysis of the entire neoplasm cohort revealed two significant positive correlations. A moderate positive correlation was observed between AFP expression and the Ki-67 index (Fig. 5A; r=0.5, P=0.001). There was a significant positive correlation between AFP and PDE4D expressions (Fig. 5G; r=0.33, P=0.03). The overall correlation between PDE4D and the Ki-67 proliferation index showed a positive trend, but it was not statistically significant (Fig. 5D; r=0.3, P=0.058). The significant correlation between AFP and Ki-67 remained in the benign tumor subgroup when the analysis was categorized by tumor type (Fig. 5B; r=0.5, P=0.02). However, this association was not present in the malignant tumor subgroup (Fig. 5C; r=0.13, P=0.57. Furthermore, no significant correlation was identified within the separate benign or malignant classifications (Fig. 5E, 5F, 5H, 5I).

DISCUSSION

In the present study, alpha-fetoprotein (AFP) and phosphodiesterase 4D (PDE4D) were studied in order to evaluate these proteins as new biomarkers for canine mammary tumors (CMTs). In addition, these proteins were correlated with the proliferative marker, Ki-67, for the diagnostic and prognostic value and their role in cell cycle progression in CMTs (Sun & Kaufman, 2018; Wongmaneerung *et al*., 2025). In general, the immunohistochemical results confirmed that AFP and PDE4D expressions were mainly cytoplasmic, while Ki-67 expression was exclusively nuclear (Uxa *et al*., 2021). These results validate the antibody specificity and confirm the usage of these markers in CMT studies.

Regarding the quantitative IHC results, AFP exhibited a distinct, gradual rise from normal mammary tissue to benign tumors, peaking in malignant CMTs. This graded, progressive pattern is consistent with earlier studies in other tumors (Chen *et al*., 2020; Głowska-Ciemny *et al*., 2023), highlighting AFP as a sensitive and potential marker of tumor transformation in CMTs. Further, it indicates an ongoing process of cellular dedifferentiation rather than a clear change between benign and malignant conditions and it supports the earlier study showing that higher AFP expression correlates with more aggressive tumors (Kim *et al*., 2025). Similarly, PDE4D expression was significantly higher in malignant tumors compared to benign tumors and normal tissues (Böttcher *et al*., 2016; Ren *et al*., 2022). This expression pattern suggested that PDE4D likely plays a role in the molecular pathways driving cancer progression. PDE4D, like AFP exhibited an association with histological severity, indicating their potential utility as practical biomarkers for diagnosis and prognosis in CMTs (Varallo *et al*., 2019; Burrai *et al*., 2020). However, PDE4D results from IHC demonstrated a distinct pattern to AFP, and no significant difference was observed

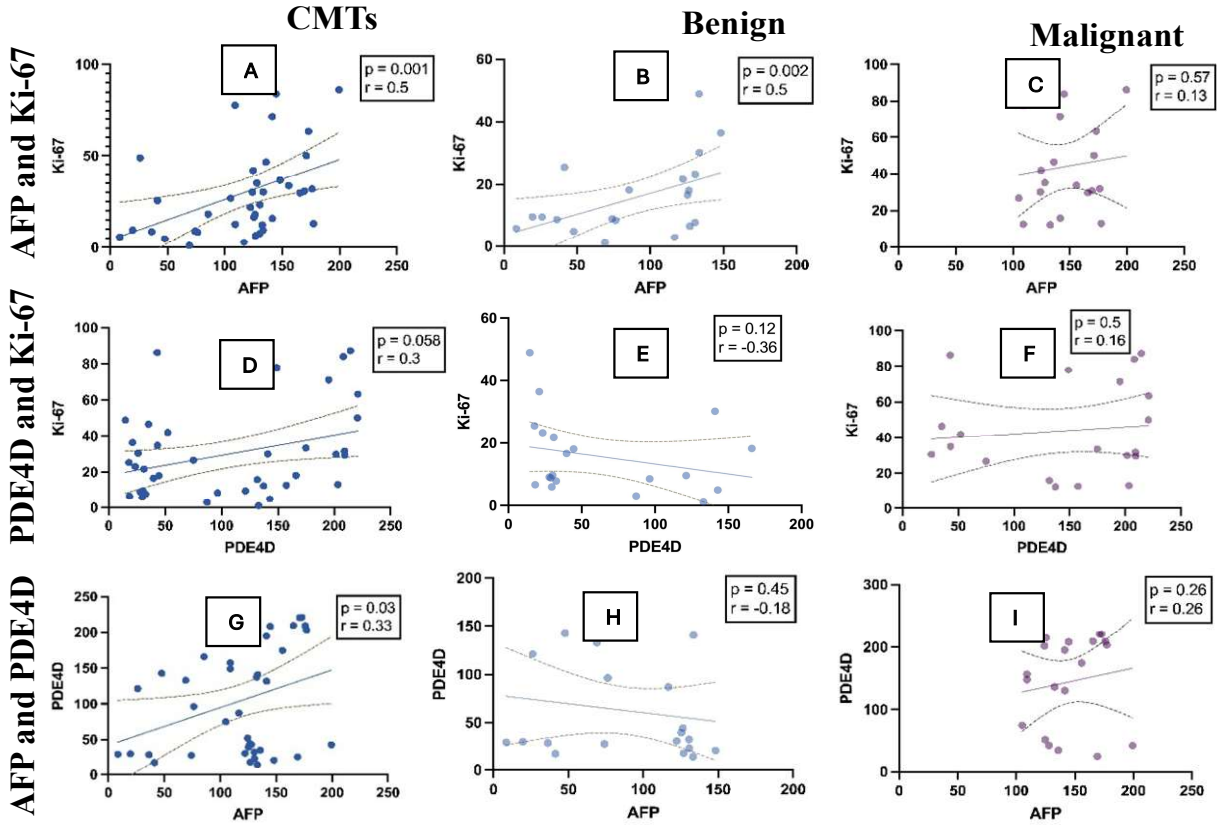

Fig. 5: Correlation analysis of target protein expression in canine mammary tumors based on immunohistochemistry. (A) Correlation between AFP and Ki-67, (B) correlation between PDE4D and Ki-67, and (C) correlation between AFP and PDE4D. Statistical significance was assessed using the Spearman correlation test.

between benign CMT and normal tissues. The lack of the PDE4D difference between normal and benign CMTs suggested that PDE4D could be served as a valuable differential diagnostic tool. The upregulation of PDE4D may be occurred at a later stage of tumorigenesis and is specifically associated with the transition to malignancy rather than common tissue hyperplasia (Ren *et al*., 2025). Since PDE4D is known to regulate cAMP which involved with cell cycle arrest and apoptosis, the similar expression of PDE4D in benign CMTs and normal mammary tissue may indicate that the cAMP-mediated signaling pathways might still be normal even in the benign CMTs.

In the present study, the Ki-67 was used as the indicator for cell proliferative activity (Chidananda, 2023). It was shown that malignant CMTs showed a significant higher proliferative index (43.99%) when compared to benign tumors (15.66%) and adjacent tissue (13.6 and 8.5% for malignant adjacent and benign adjacent tissue respectively), indicating the aggressiveness and rapid growth in malignant CMT (Kilickap *et al*., 2014; Wongmaneerung *et al*., 2025). On the other hand, the Ki-67 index was comparable among benign tumors and adjacent mammary tissues, suggesting that benign CMTs involve structural and morphological changes without the profound cell-cycle disruptions that cause malignancy. In addition, while the adjacent tissue may be exposed to the same environmental factors or any paracrine signals from the tumor, the cell cycle disruption still appears only in the malignant lesion.

Regarding the methods used in the present study, some differences could be observed. Our IHC result on PDE4D clearly revealed a higher expression in malignant CMTs compared to benign CMT and normal mammary tissue, while WB showed similarly high levels in all CMTs and adjacent tissue compared to normal mammary group. This indicated that PDE4D protein rose early and remained high in both benign and malignant CMTs as well as adjacent tissues. This suggests that the increase of total PDE4D protein (as measured by WB) may imply an initial event in canine mammary cancer, occurring in the benign phase and persisting during malignant transformation (Pullamsetti *et al*., 2013). On the other hand, the intensity and cellular concentration of PDE4D as evaluated by IHC only reach its peak during malignant progression. when the tumor becomes more aggressive. The IHC data may indicate that despite a comparable total protein level, the distribution and intensity (as represented by H-score) within tumor cells are more pronounced in the malignant stage when compared with benign CMTs.

In the present study, tumor or tissue analyses were preferred over serum evaluation to overcome several critical diagnostic burdens. As systemic proteins concentrations are often diluted in the bloodstream, and that it is difficult to identify the tissue of origin (Nakamura *et al*., 2017). This is more pronounced for AFP level which is naturally produced and circulated in a serum samples, thus the distinguishing between baseline physiological levels and tumor-derived is impracticable (Sell, 2008), especially in CMTs. On the other hand, the *in situ* method like immunohistochemistry could demonstrate the localization of AFP and PDE4D within the neoplastic tissues. This application revealed a complex biological mechanism that AFP and Ki-67 were correlated during early stage of the tumorigenesis, but they decoupled as the tumor became malignant. These important findings may be lost in the average analyses using the blood test. Therefore, the tissue-based evaluation not only demonstrates the localization of these proteins, but it also provides the essential biological data necessary to transform or modify these marker proteins into reliable and effective serum diagnostic and prognostic tools in the future (Duffy *et al*., 2018).

In the present study, significant positive correlations were observed between AFP expression and Ki-67 in the tumor cohort. However, as CMTs transformed from benign to malignant, the decoupling between AFP and Ki-67 was observed. In benign CMTs, the positive correlation between AFP and Ki-67 suggested that AFP is involved in the mechanism of early cell proliferation onset (Li *et al*., 2011; Qiang *et al*., 2021). However, in malignant CMTs, the malignant cells may be under several growth pathways besides its initial expansion and no longer relies on the increase of cell proliferation alone. This independence may lead to the hallmark of high-grade malignancy and cause the no return point of malignant biology of CMTs. Despite the lack of correlation between AFP and Ki-67 in malignant CMTs, the expression of AFP remained higher in malignant tumors compared to other groups studied. These may indicate a saturation effect that, while AFP expression reached that malignant level, the cell proliferative index, Ki-67 may be sustained by other more aggressive mechanisms, resulted in high expression of AFP regardless of the proliferation rate. Further, the correlation between AFP and PDE4D indicates the co-regulation of malignancy pattern. Since high PDE4D expression can deplete intracellular cAMP, the inhibitor of the cell cycle, its co-regulator with AFP may cause a priming event that prepared mammary tissue for tumor growth and expansion.

The marked increases in PDE4D and AFP levels in malignant tumors suggested the possibility of developing targeted therapies, in addition to their application as diagnostic and prognostic tools. It is imperative that cancer patients maintain elevated levels of PDE4D expression over an extended duration. PDE4D is known to help tumors develop by breaking down cAMP, which lowers the amount of this critical second messenger in cells (Feng *et al*., 2023). cAMP typically inhibits cellular proliferation (Pullamsetti *et al*., 2013). In many cancer models, PDE4D-selective inhibitors have been demonstrated to arrest the cell cycle and cause cell death by restoring cAMP activity. This makes it a prospective target for treatment (Donders *et al*., 2024). AFP also seems like an excellent choice for targeted intervention. AFP is a tumor-associated antigen (TAA), which suggests that it can be employed in new ways to treat cancer. It also plays a biological role in the growth of tumors. Antibody-drug conjugates (ADCs) that direct cytotoxic agents to AFP-expressing cells and CAR-T cell platforms that identify AFP are two promising techniques now under investigation in human oncology (Zhu *et al*., 2021). Our findings, which demonstrate increased activity of both PDE4D and AFP in canine mammary cancers, offer a persuasive rationale for the investigation of these specific therapeutic approaches in veterinary and human medicine. This could make it easier to find more specific and mechanism-based ways to treat mammary tumors in the future.

Conclusions: In conclusion, this study demonstrates a new perspective on the novel biomarkers for CMTs by highlighting AFP and PDE4D as reliable, stage-specific biomarkers from normal to malignancy. While AFP acts as an early sensitive marker of dedifferentiation of mammary tissue, PDE4D serves as a definitive protein that switches during the transition to aggressive malignant CMTs. These findings establish an integration of AFP and PDE4D into future precision diagnosis for the development of targeted therapy not only in veterinary oncology but also in human medical research in the future.

Authors contribution: MTKT: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing. SK: Methodology, Validation, Formal analysis, Investigation. PR: Methodology, Validation, Formal analysis, Investigation, Supervision. SP: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing, Supervision. KK: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing, Supervision. SS: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Funding acquisition.

Generative AI Declaration: During the preparation of this manuscript, the author(s) used QuillBot version 39.3.3 for the purpose of language editing. The author(s) subsequently reviewed and edited the content as necessary and assume full responsibility for the accuracy and integrity of the published work.

Acknowledgements: This work was supported by the 100th Anniversary Chulalongkorn University fund for Doctoral Scholarship, the 90th Anniversary of Chulalongkorn University, Rachadapisek Sompote Fund (CGUGR1125691041M) and Thailand Science research and Innovation Fund Chulalongkorn University, (FF_68_032_3100_004).

REFERENCES

Böttcher R, Dulla K, van Strijp D, *et al*., 2016. Human PDE4D isoform composition is deregulated in primary prostate cancer and indicative for disease progression and development of distant metastases. Oncotarget 7(43):70669-684.

Brunetti B, Bacci B, Angeli C, *et al*., 2021. p53, ER, and Ki67 expression in canine mammary carcinomas and correlation with pathological variables and prognosis. Veterinary Pathology 58:325-31.

Burrai GP, Gabrieli A, Moccia V, *et al*., 2020. A statistical analysis of risk factors and biological behavior in canine mammary tumors: a multicenter study. Animals 10:9.

Chen W, Peng J, Ye J, *et al*., 2020. Aberrant AFP expression characterizes a subset of hepatocellular carcinoma with distinct gene expression patterns and inferior prognosis. Journal of Cancer 11:403-13.

Chidananda MG, 2023. Ki-67 index and its correlation with clinical and pathological variables in breast cancer. Indian Journal of Surgical Oncology 14(4):943–48.

Choudhary OP, 2025. Animal models for surgeries and implants: a vital tool in medical research and development. Annals of Medicine and Surgery 87(7):4090-95.

Choudhary OP, Infant SS, As V, *et al*., 2025. Exploring the potential and limitations of artificial intelligence in animal anatomy. Annals of Anatomy – Anatomischer Anzeiger 258:152366.

Davey MG, Hynes SO, Kerin MJ, *et al*., 2021. Ki-67 as a prognostic biomarker in invasive breast cancer. Cancers 13:17.

Donders Z, Skorupska IJ, Willems E, *et al*., 2024. Beyond PDE4 inhibition: a comprehensive review on downstream cAMP signaling in the central nervous system. Biomedicine & Pharmacotherapy 177:117009.

Duffy MJ, McDermott EW and Crown J, 2018. Blood-based biomarkers in breast cancer: From proteins to circulating tumor cells to circulating tumor DNA. Tumor Biology 40(5):101042831877616 9.

Feng B, Pan B, Huang J *et al*., 2023. PDE4D/cAMP/IL-23 axis determines the immunotherapy efficacy of lung adenocarcinoma via activating the IL-9 autocrine loop of cytotoxic T lymphocytes. Cancer Letters 565:216224.

Thatsanabunjong F, Kanthawat S, Raksaseri P, *et al*., 2026. Ovarian stage-dependent expression of GDF9, BMP15, and AMH in domestic cats. Theriogenology 249:117662.

Głowska-Ciemny J, Szymański M, Kuszerska A, *et al*., 2023. The role of alpha-fetoprotein (AFP) in contemporary oncology: the path from a diagnostic biomarker to an anticancer drug. International Journal of Molecular Sciences 24(3):2539.

Goldschmidt MH, Peña L, Rasotto R, *et al*., 2011. Classification and grading of canine mammary tumors. Veterinary Pathology 48(1):117–31.

Kilickap S, Kaya Y, Yucel B, *et al*., 2014. Higher Ki67 expression is associated with unfavorable prognostic factors and shorter survival in breast cancer. Asian Pacific Journal of Cancer Prevention 15:1381–5.

Kim H, Jang M and Kim E, 2025. Exploring the multifunctional role of alpha-fetoprotein in cancer progression: implications for targeted therapy in hepatocellular carcinoma and beyond. International Journal of Molecular Sciences 26(10): 3390.

Kim S, Bok E, Lee S, *et al*., 2021. Metastasis prognostic factors and cancer stem cell-related transcription factors associated with metastasis induction in canine metastatic mammary gland tumors. Journal of Veterinary Science 22:e62.

Li M, Li H, Li C, *et al*., 2011. Alpha-fetoprotein: a new member of intracellular signal molecules in regulation of the PI3K/AKT signaling in human hepatoma cell lines. International Journal of Cancer128:524-32.

Lin DC, Xu L, Ding LW, *et al*., 2013. Genomic and functional characterizations of phosphodiesterase subtype 4D in human cancers. Proceedings of the National Academy of Sciences of the United States of America 110(15):6109–14.

Liu F, Ma J, Wang K, *et al*., 2019. High expression of PDE4D correlates with poor prognosis and clinical progression in pancreatic ductal adenocarcinoma. Journal of Cancer 10:6252-60.

Liu K, Wu C, Zhu M, *et al*., 2024. Structural characteristics of alpha-fetoprotein, including N-glycosylation, metal ion and fatty acid binding sites. Communications Biology 7:505.

Lusardi M, Rapetti F, Spallarossa A, *et al*., 2024. PDE4D: a multipurpose pharmacological target. International Journal of Molecular Sciences 25(15):8052.

Moro R, Gulyaeva-Tcherkassova J and Stieber P, 2012. Increased alpha-fetoprotein receptor in the serum of patients with early-stage breast cancer. Current Oncology19: e1-e8.

Nakamura M, Horiuchi S, Kagawa F, *et al*., 2017. Shift current photovoltaic effect in a ferroelectric charge-transfer complex. Nature Communications 8:281.

Pullamsetti SS, Banat GA, Schmall A, *et al*., 2013. Phosphodiesterase-4 promotes proliferation and angiogenesis of lung cancer by crosstalk with HIF. Oncogene 32:1121-34.

Qiang Z, Zhang W, Jin S, *et al*., 2021. Carcinoembryonic antigen, α-fetoprotein, and Ki67 as biomarkers and prognostic factors in intrahepatic cholangiocarcinoma: A retrospective cohort study. Annals of Hepatology 20:100242.

Rahrmann EP, Collier LS, Knutson TP, *et al*., 2009. Identification of PDE4D as a proliferation promoting factor in prostate cancer using a sleeping beauty transposon-based somatic mutagenesis screen. Cancer Research 69:4388-97.

Ren H, Chen Y, Ao Z, *et al*., 2022. PDE4D binds and interacts with YAP to cooperatively promote HCC progression. Cancer Letters 541:215749.

Ren H, Zhang S and Li P, 2025. The multifaceted role of phosphodiesterase 4 in tumor: from tumorigenesis to immunotherapy. Frontiers in Immunology 16:1528932.

Sell S, 2008. Alpha-fetoprotein, stem cells and cancer: how study of the production of alpha-fetoprotein during chemical hepatocarcinogenesis led to reaffirmation of the stem cell theory of cancer. Tumor Biology 29(3):161-80.

Srisawat W, Pringproa K, Prachasilchai W, *et al*., 2024. Epidemiology and classification for canine and feline mammary gland tumors: a histopathological survey of 437 mammary gland tumor biopsies performed in a secondary care hospital in Chiang Mai, Thailand from 2012 to 2019. PeerJ 12:e17077.

Sun X and Kaufman PD, 2018. Ki-67: more than a proliferation marker. Chromosoma 127(2):175-86.

Uxa S, Castillo-Binder P, Kohler R, *et al*., 2021. Ki-67 gene expression. Cell Death & Differentiation 28:3357-70.

Varallo GR, Gelaleti GB, Maschio-Signorini LB, *et al*., 2019. Prognostic phenotypic classification for canine mammary tumors. Oncology Letters 18:6545-53.

Wongmaneerung P, Chitapanarux I, Traisathit P, *et al*., 2025. The association between Ki-67 expression and survival in breast cancer subtypes: a cross-sectional study of Ki-67 cut-point in northern Thailand. BMC Cancer 25:346.

Zhu S, Zhang T, Zheng L, *et al*., 2021. Combination strategies to maximize the benefits of cancer immunotherapy. Journal of Hematology & Oncology 14(1):156.