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Research Article

Prognostic Significance of MTDH and Ki-67 Expression in Canine Mammary Tumors: An Immunohistochemical and Survival Study

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Abstract

Biomarkers play critical roles in understanding tumor biology and evaluating prognosis in canine mammary tumor (CMTs) research. MTDH and Ki-67 are crucial factors and markers in the carcinogenesis of multiple organs and tissues in human oncology. However, the role of MTDH in CMTs and its relationship with Ki-67 are not well characterized. This study investigated MTDH and Ki-67 expression and their correlation in 64 benign and malignant CMT tissues using immunohistochemistry (IHC). The association of MTDH and Ki-67 expression with clinicopathological features was also evaluated, followed by assessing their potential prognostic value in a prospective survival study. IHC analysis revealed MTDH expression in both the cytoplasm and nucleus of tumor cells. In contrast, Ki-67 was predominantly in the nucleus. MTDH expression significantly correlated with tumor malignancy grade (P=0.035), tumor size (P<0.0001), Ki-67 index (P<0.0001), and metastasis (P<0.0001). High MTDH expression was significantly associated with reduced disease-free survival (P=0.0042) and overall survival (P=0.0113) in malignant CMTs. These results indicate that the expression levels of MTDH and Ki-67 are positively correlated with adverse clinicopathological parameters and jointly signify aggressive tumor behavior and poor prognosis. MTDH and Ki-67 are thus potential prognostic biomarkers for CMTs.

Keywords: Dogs, Immunohistochemistry, KI-67, Mammary Tumors, MTDH

Introduction

Canine mammary tumors (CMTs) are the most prevalent neoplasms in female dogs, accounting for 70% of all tumors in intact females [1]. They exhibit bimodal distribution and are either benign or malignant. Notably, malignant tumors constitute approximately 50% of CMTs [2]. CMTs exhibit hormone dependency and carry a significant risk of local recurrence post-resection or metastasis, particularly to the lymph nodes and lungs [3]. Mitigating cancer-related morbidity and mortality requires accurate diagnosis and prognostication. Prognostic factors for CMTs, including the histological type, tumor grade, invasiveness, growth rate, lymph node status, and tumor size [4], are crucial for assessing and determining prognosis, as well as predicting tumor molecular behavior.

MTDH (also known as AEG-1 or LYRIC) is a multifunctional oncoprotein strongly associated with initiating breast cancer, metastasis, drug resistance, and immune evasion [5]. It is located within the 8q22 chromosomal region, a frequent site of genomic amplification. Aberrant amplification or transcription at this locus drives MTDH overexpression [6]. This overexpression enhances malignant cell adhesion to circulating blood cells, facilitating tumor metastasis [7]. Noteworthy, MTDH is highly expressed across diverse malignancies [8] but exhibits low expression in non-neoplastic tissues, including normal breast epithelium [9]. It modulates vital signaling pathways such as PI3K/Akt, NF-κB, Wnt/β-catenin, and MAPK [10]. MTDH overexpression in multiple cancer types correlates with critical oncogenic processes including tumorigenesis, proliferation, invasion, metastasis, and chemoresistance [11]. For instance, it promotes tumor growth and proliferation in human breast cancer [12] and further drives invasion, metastasis, and therapeutic resistance [13]. Notably, MTDH has been

identified as a metastasis gene in phage display libraries of metastatic breast cancer, where it binds lung vasculature-associated proteins, mechanistically explaining its role in pulmonary metastasis [14].

Ki-67 is a high-molecular-weight nuclear protein expressed in proliferating cells. It primarily exists as 320 kDa and 359 kDa isoforms [15] and localizes predominantly throughout the nucleoplasm or at the nuclear membrane, serving as a well-established crucial marker of cellular proliferative activity [16]. Ki-67 is expressed during all active phases of the cell cycle: G1, S, G2, and M. However, it is absent in quiescent (G0) cells [17]. The intensity and proportion of nuclear immunoreactivity reflect cellular proliferative activity and aid in evaluating the malignancy potential of neoplasms [18]. Determining Ki-67 protein expression levels in tissues objectively measures the cellular proliferation rate and growth fraction in both tumor and normal tissues. This determination is usually via immunohistochemistry (IHC), and the expression level is typically quantified as the Ki-67 index or proliferation index [19]. In clinicopathological diagnosis, the Ki-67 index is a crucial indicator for tumor grading, aggressiveness assessment, prognostic prediction, and treatment response evaluation in various malignancies, including breast cancer, lymphoma, and neuroendocrine tumors [20]. A high Ki-67 index is generally associated with increased tumor aggressiveness, rapid growth kinetics, and poorer prognosis [21].

This study employed immunohistochemistry (IHC) to detect the expression of MTDH and Ki-67 in canine mammary tumors (CMTs) in dogs. The expression levels were further analyzed to evaluate their relationship and correlate them with clinicopathological features to explore the potential prognostic value of MTDH and Ki-67.

Material and Methods

Ethical Statement

The research protocol used was reviewed and approved by the Research Ethics Committee of Henan Institute of Science and Technology (Approval No: 202009023).

Tissue Samples

A total of 64 surgically resected canine mammary tumor samples and adjacent non-neoplastic tissues were collected from various animal hospitals in Xinxiang City, Henan Province, and the surrounding regions between 2019 and 2023. Histopathological examination confirmed that 30 of the 64 were benign while 34 were malignant neoplasms. All sample collection and usage procedures were performed with informed consent from the pet owners.

This study exclusively included cases with histologically confirmed primary mammary neoplasms following surgical resection. The CMT tissue samples were fixed in 10% neutral-buffered formalin at room temperature for 48 h and subsequently embedded in paraffin blocks. The tissues were then cut into 4 μm-thick sections and stained with hematoxylin and eosin (H&E) for definitive pathological diagnosis. The H&E-stained sections were subsequently mounted on slides and evaluated microscopically. Tumors were classified according to the criteria established by Goldschmidt et al.[22] and histologically graded using the system proposed by Peña et al.[23]. The Ki-67 proliferation index, categorized as ≤15% or >15% positive tumor cells and tumor size categorized as ≤3 cm, 3-5 cm, or >5 cm, were also assessed. The study included cases with solitary and multiple mammary tumors. The tumor exhibiting the most aggressive clinicopathological features was selected for analysis in dogs having multiple malignant tumors [1].

Immunohistochemistry

Paraffin-embedded tissues were cut into sections (4 μm thick) using a rotary microtome (Yidi Medical Equipment, Jinhua, China). The sections were dried at 60°C for 1-2 h, dewaxed by dipping in xylene twice (5 min each), and then rehydrated through a graded ethanol series (100% twice, 95%, 90%, 80%, and 70%; 3 min each). Antigen retrieval was carried out under pressure in citrate-EDTA buffer (pH 6.0; Beyotime Biotechnology, 40xP0086, China) using a DGS-280C pressure cooker (Lichen Technology, China) for 20 min. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide at room temperature for 30 min. Non-specific binding sites were blocked with normal horse serum (Beijing YITA Biotechnology, YT2515, China) for 20 min. The sections were subsequently incubated with primary antibodies overnight at 4°C. The primary antibodies used were goat anti-MTDH (1:300; Jiangsu Qinke Biotechnology, DF13437, China) and rabbit anti-Ki-67 (1:500; Jiangsu Qinke Biotechnology, AF0198, China). The sections were then rinsed with PBS to remove the excess primary antibodies and subsequently incubated for 1 h at room temperature with species-specific HRP-conjugated secondary antibodies. The secondary antibodies used were goat anti-IgG (SOLARBIO Biotechnology, I5256, China) and rabbit anti-IgG (SOLARBIO Biotechnology, SA13, China). The sections were visualized using a DAB chromogen kit (Zhongshan Jinqiao Biotechnology, ZLI-9017, China) for 90 seconds, with the reaction stopped by immersion in distilled water. The sections were counterstained with hematoxylin, dehydrated through graded ethanol, cleared in xylene, and mounted on slides for imaging using a DS-Ri1 microscope (Nikon Corporation, Japan). The sections were rinsed using phosphate-buffered saline (PBS) between all major steps.

Quantitation of IHC Staining

Immunohistochemistry (IHC) results were determined using the immunoreactive score (IRS) method. The IRS method involved the calculation of the immunoreactive score as the product of staining intensity (SI) and the percentage of positive cells (PP). Five random high-power fields (HPFs) were examined under light microscopy in each case, with counts of 100 cells per field. PP was determined as: (the number of positively stained cells/100 cells counted) x 100%. Positive staining for both MTDH and Ki-67 was achieved by the presence of yellow-brown granules within the nucleus or cytoplasm of tumor cells. For mixed histotypes, immunoreactivity was evaluated in the neoplastic epithelial compartment as the primary readout; stromal/mesenchymal staining was documented separately when present. Five representative HPFs (x400) were assessed per case under pathologist guidance.

MTDH expression was immunohistochemically assessed using the Aperio Cytoplasm V2 algorithm, adapted from established human breast cancer criteria [24,25]. Five representative high-power fields (400× magnification) per specimen were analyzed to generate a composite score based on cytoplasmic staining intensity (SI) and percentage of positive tumor cells (PP) [26]. The scoring thresholds were defined as: PP - 0:0%; 1:1-20%; 2: 21-50%; 3: 51-70%; and 4: >70% and SI - 0: no staining; 1: weak (light yellow); 2: moderate (brownish yellow); and 3: strong (dark brown). The immunoreactive score (IRS = PP × SI) categorized the expression as low (IRS<5) or high (IRS≥5). MTDH intensity was manually scored (0-3 scale) [24]. +2 and +3 scores denoted overexpression, while 0 and +1 scores denoted low expression.

Ki-67 immunohistochemical expression was characterized by nuclear-localized brown staining and was quantified using the Aperio Nuclear V9 algorithm [27,28]. This algorithm measured the nuclear reactivity index by calculating the proportion of the positively stained cells as (positive cells/1000 total cells) x 100%. A Ki-67 index ≥15% denoted high proliferative activity regardless of the staining intensity [29]. The CMT cases were stratified into low-risk (<15%) and high-risk (≥15%) prognostic groups through standardized statistical modeling based on this threshold.

Follow-up Data

All dogs with CMTs had follow-up assessments as follows: at least one preoperative visit, every 3 weeks postoperatively for the first 3 months, and quarterly thereafter for ≥2 years. Pet owners were advised to contact the hospital immediately if any abnormalities, including non-CMT-related signs, were observed. All evaluations including physical examinations, thoracic radiography (three views), abdominal ultrasonography, fine-needle aspiration (FNA), biopsy, necropsy (when applicable), and/or computed tomography (CT) when clinically indicated were performed at the Teaching Animal Hospital of Henan Institute of Science and Technology or referred to partner facilities. Newly detected mammary lesions, clinically abnormal lymph nodes, or suspicious lesions in other organs prompted further diagnostic procedures, such as FNA, excisional biopsy, and CT, to exclude secondary tumors or confirm local recurrence or metastasis. All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA).

Statistical Analysis

Case data and diagnostic results from affected dogs were systematically collated. Statistical correlations between MTDH expression and clinicopathological parameters were analyzed using Fisher's exact test, chi-square test, and Pearson/Spearman correlation analysis. Survival outcomes were evaluated by generating Kaplan-Meier curves. Between-group comparisons were assessed via log-rank testing.

Disease-free survival (DFS) was defined as the duration in months from initial surgery to first detection of local recurrence or metastasis. In contrast, overall survival (OS) spanned from surgery to cancer-specific death. The exclusion criteria for OS analysis comprised dogs dying of non-mammary tumor-related causes, dogs lost to follow-up, or those alive at the 24-month endpoint. The exclusion criteria for DFS analysis comprised cases lost to follow-up, dogs that died without metastatic evidence from non-tumor causes, or those that were metastasis-free at 24 months postoperatively. The exclusion timelines on survival curves were denoted by the censored date points. The level of statistical significance was at $P<0.05$.

Results

Data Characteristics

This study comprised 64 histologically confirmed canine mammary tumor (CMT) cases. *Table 1* details the clinicopathological characteristics of all the cases. The mean age of dogs with benign CMTs was 11.00 years (range: 6-16), while that of dogs with malignant cases was 11.94 years (range: 6-15). The cohort included 47 intact and 17 spayed females. The most predominant breeds were Toy Poodles (n=17) and Bichon Frises (n=14). Benign histopathological classifications comprised complex adenomas (n=13), simple adenomas (n=8), and mixed tumors (n=9). Malignant subtypes were identified as mucinous carcinomas (n=14), carcinomas (n=10), carcinosarcomas (n=8), and tubular carcinomas (n=2). Metastatic lesions were confirmed in 4 malignant cases.

Immunolocalization

Immunohistochemical analysis of MTDH and Ki-67 expression across canine mammary tumor subtypes *(Table 2)* revealed distinct localization patterns. MTDH exhibited cytoplasmic and nuclear expression (yellow-brown granules) in neoplastic cells. In contrast, Ki-67 was predominantly in the nuclear (brown granules). Both markers demonstrated significantly higher expression in malignant CMTs compared to benign CMTs. Beyond predominant perinuclear/cytoplasmic staining in neoplastic epithelial cells, clustered stromal/mesenchymal positivity was occasionally observed in mixed malignant subtypes (e.g., carcinosarcoma), consistent with their biphasic composition. Notably, MTDH exhibited heterogeneous stromal distribution in carcinosarcomas and tubular carcinomas. Clustered stromal cells exhibited intensified MTDH immunoreactivity compared to adjacent non-neoplastic tissues *(Fig. 1)*. MTDH positivity rate reached 81%, while high Ki-67 expression (index ≥15%) was observed in 71% of cases among the 64 cases.

Relationship Between MTDH Expression and Tumor Grade or Pathological Factor

There was high MTDH expression in 76.5% of malignant canine mammary tumors (CMTs). High expression rates were observed in 50.0% of grade II and 42.9% of grade III malignancies based on stratification by histological. Elevated MTDH expression occurred in 12.5% of simple adenomas, 15.4% of complex adenomas, and 22.2% of mixed tumors among the benign tumors. Malignant subtypes demonstrated variable expression: 64.3% in mucinous carcinomas, 70.0% in carcinomas, 75.0% in carcinosarcomas, and 50.0% in tubular carcinomas. Notably, MTDH expression levels were significantly correlated with tumor grade (P=0.049), size (P<0.0001), metastatic status (P<0.0001), and Ki-67 index (P<0.0001). However, their levels were not correlated with other clinicopathological parameters, including histological subtype (*Table 3*).

Correlation Between MTDH Overexpression and Clinical Outcome

Kaplan-Meier analysis of the prognostic significance of MTDH overexpression in malignant canine mammary

Table 1. *Comparison of signalment data (age, sex, breed and histologic diagnosis) of benign and malignant mammary gland tumors in 64 dogs*

Characteristic	Benign Tumors (n=30)	Malignant Tumors (n=34)
Median age (range)	11.00 (6-16)	11.94 (6-15)
Sex (n)	Intact female (21) Spayed female (9)	Intact female (26) Spayed female (8)
Breed (n)	Teddy bear dog (10) Bichon Frise (6) Poodle (3) Golden Retriever (3) Cocker spaniel (2) Schnauzer (2) Chow Chow (2) Chihuahua (1) Pekingese (1) Bichon Frise (1)	Bichon Frise (8) Teddy bear dog (7) Poodle (5) Golden Retriever (4) Schnauzer (4) Cocker spaniel (2) Alaskan malamute (2) Pekingese (1) Chihuahua (1) Bichon Frise (1)
Histologic type (n)	Complex adenoma (13) Adenoma simplex (8) Mixed adenoma (9)	Myxoid fibroma (14) Adenofibroma (10) Carcinosarcoma (8) Tube-like tumor (2)

Table 2. *Statistical results of immunohistochemical examination of canine mammary tumors*

Type of Tumor	Classification	The Expression Status of MTDH	The Expression Status of Ki-67	P
Benign tumor	Adenoma simplex	Weakly positive (+)	Weakly positive (+)	<0.001
	Complex adenoma	Weakly positive (+)	Weakly positive (+)	
	Mixed adenoma	Weakly positive (+)	Weakly positive (+)	
Malignant tumor	Myxoid fibroma	Positive (++)	Weakly positive (+)	<0.001
	Adenofibroma	Positive (++)	Weakly positive (+)	
	Carcinosarcoma	Positive (++)	Positive (++)	
	Tube-like tumor	Strongly positive (+++)	Strongly positive (+++)	

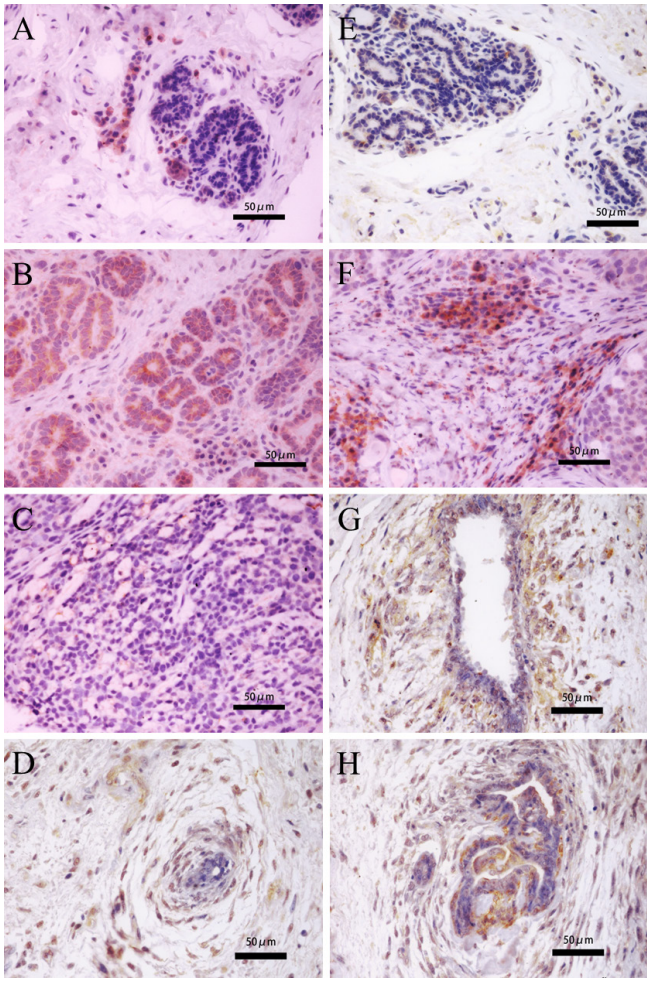

Fig 1. Immunohistochemical localization of MTDH and Ki-67 in canine mammary tissues. MTDH shows predominant perinuclear/cytoplasmic staining in neoplastic epithelial cells, whereas Ki-67 displays nuclear labeling. MTDH panels: (A) non-neoplastic mammary tissue, negative (–); (B) benign mammary tumor, weak (+); (C) carcinosarcoma, moderate (++); (D) tubular carcinoma, strong (+++). Ki-67 panels: (E) non-neoplastic mammary tissue, negative (–); (F) benign mammary tumor, weak (+); (G) carcinosarcoma, moderate (++); (H) tubular carcinoma, strong (+++). All sections are counterstained with hematoxylin; objective magnifications and scale bars are indicated

Table 3. *Expression of MTDH and KI-67 in canine mammary tumors and analysis of their relationship with histological grading, clinical staging and characteristics*

Variable		MTDH Expression			P
		Number of Tumors	Low	High	
Benign tumor	Adenoma simplex	8	7	1	0.854
	Complex adenoma	13	11	2	
	Mixed adenoma	9	7	2	
Malignant tumor	Myxoid fibroma	14	5	9	0.991
	Adenofibroma	10	3	7	
	Carcinosarcoma	8	2	6	
	Tube-like tumor	2	1	1	
Histological grade	I	8	4	4	0.049
	II	12	5	7	
	III	14	1	13	
Tumor size	<3 cm	8	8	0	<0.0001
	3~5 cm	8	0	8	
	>5 cm	48	16	32	
Metastases	Absent	14	14	0	<0.0001
	Present	20	0	20	
KI-67 labelling index	≤15%	14	12	2	<0.0001
	>15%	50	12	38	

tumors (CMTs) revealed that there were 21 tumor-related deaths with 8 censored observations among the 34 malignant cases. Survival curves demonstrated significantly reduced disease-free survival (DFS; median=18 months) and overall survival (OS; median=24 months) in the high-expression (n=26) cohort (log-rank P<0.05) compared to the low-expression (n=8) cohort. Noteworthy, dogs with MTDH overexpression exhibited worse prognoses than their low-expression counterparts across both survival endpoints.

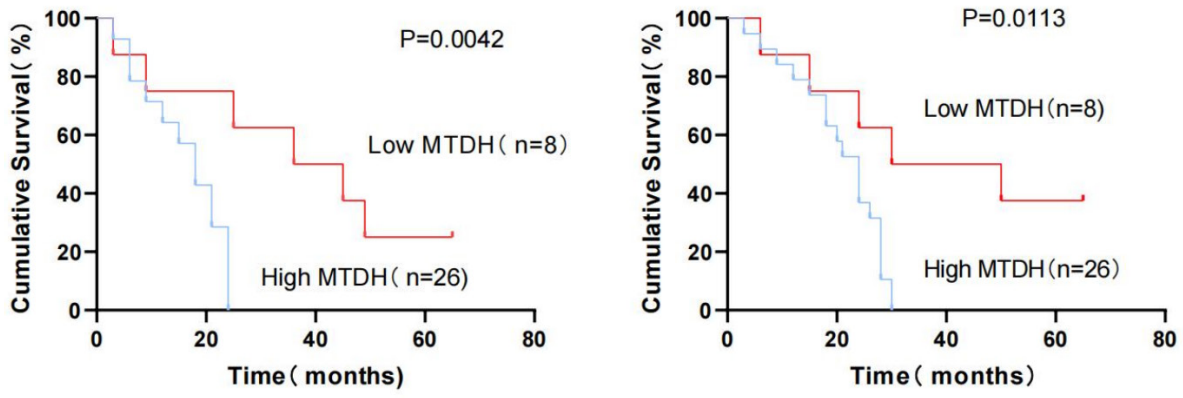

Fig 2. Kaplan-Meier survival curves of 34 dogs with malignant CMTs based on MTDH expression status for A, disease-free survival (median: 18 months) and B, overall survival (median 24 months)

Discussion

This study investigated MTDH and Ki-67 expression in canine mammary tumors (CMTs). MTDH demonstrates negligible expression in normal tissues. However, it is overexpressed in diverse malignancies, including mammary carcinoma. Mechanistically, MTDH enhances tumor-endothelial adhesion, facilitating vascular invasion and distant colonization [30]. Human oncology studies [31] postulate that elevated MTDH levels correlate with advanced tumor stage, lymph node metastasis, and poor prognosis in CMTs. This correlation is potentially through pro-proliferative and anti-apoptotic pathway activation [32]. Notably, MTDH exhibits preferential expression at tumor invasion fronts, corroborating its prometastatic role. MTDH overexpression significantly reduces the overall survival (OS) and disease-free survival (DFS) in human cancers, including breast, ovarian, and pancreatic cancers [33]. The findings of this study confirmed parallel prognostic implications in CMTs, highlighting MTDH as a critical regulator of cell cycle progression and proliferation. Functionally, MTDH drives metastasis, mediates therapeutic resistance, and maintains cancer stemness, positioning it as a molecular linchpin in CMT malignant progression [34]. Herein, MTDH expression was significantly associated with the pathological grade, metastatic risk, and survival outcomes, conferring dual utility as a diagnostic biomarker and therapeutic target [35]. Emerging human-targeted agents, such as C26-A6 inhibitors and MitoQ, offer translational potential for canine oncology. However, direct veterinary clinical evidence remains limited [36].

The associations between MTDH expression and clinicopathological parameters, including tumor size, histologic subtype, grade, metastasis, and Ki-67 index, highlighted MTDH expression as a prognostic indicator in canine mammary tumors (CMTs) [37]. The higher MTDH overexpression observed in 'connective tissue–associated' tumors reflect the biology of mixed malignant histotypes (e.g., carcinosarcoma) rather than contradicting the overall increase seen in malignancies. These entities contain variable epithelial and mesenchymal proportions that can modulate apparent immunoreactivity on IHC. While our primary objective was to evaluate overall prognostic associations, we acknowledge that compartment-level heterogeneity may confound pooled comparisons. Future studies using dual-marker IHC (e.g., cytokeratin/vimentin) and compartment-aware digital quantification are warranted to delineate cell-type-specific MTDH expression and refine prognostic modeling. MTDH overexpression significantly correlated with advanced tumor grade, metastatic dissemination, and elevated Ki-67 expression. Ki-67 levels were significantly higher in malignant CMTs than in benign CMTs, consistent with human oncology paradigms. Notably, MTDH exhibited a strong positive correlation to Ki-67, highlighting MTDH's involvement in proliferation-driven tumor progression. Mechanistically, MTDH activates epidermal growth factor receptor (EGFR), triggering MAPK/ERK pathway signaling that upregulates cyclin D1 expression. The upregulation of cyclin D1 accelerates G1-S phase transition and enhances proliferative capacity [18]. MTDH also inhibits apoptosis by activating PI3K/AKT, further augmenting tumor cell accumulation [18]. These synergistic pathways functionally converge with Ki-67 overexpression to potentiate neoplastic growth [38]. Clinically, dual assessment of MTDH/Ki-67 provides superior prognostic stratification compared to single-marker evaluation. CMTs exhibiting co-expression of both markers exhibit higher invasiveness and recurrence risk, warranting intensified adjuvant therapy [39]. However, these biomarkers should be validated against standardized clinical endpoints, including tumor burden, nodal status, and survival, to establish evidence-based implementation protocols using multi-institutional studies.

This study had several methodological limitations. A limitation of this study is reliance on manual, light-microscopy-based semiquantitative scoring (IRS), which may introduce observer subjectivity in densely stained regions. Future studies will prospectively incorporate standardized digital image analysis in an independent cohort to validate and refine these IRS-based estimates. While the sample size of 64 cases is generally acceptable for a veterinary pathology study, the unbalanced distribution of benign (n=30) and malignant (n=34) cases may limit the statistical power and generalizability of our findings. The unequal distribution could introduce biases, particularly when evaluating prognostic factors that may vary between tumor subtypes. Moreover, the limited incidence of MTDH overexpression in benign tumors (≤22.2% across subtypes) hindered robust correlation assessments between histopathological classifications and clinicopathological features. Additionally, the study did not include translational validation of MTDH expression through quantitative methods, such as mRNA quantification (RT-qPCR) or protein immunoblotting (Western blot). Future studies with larger, more balanced cohorts and molecular profiling to elucidate MTDH's regulatory dynamics in both physiological and neoplastic contexts are necessary to confirm the applicability of these results to a broader population of canine mammary tumors and to ensure more reliable prognostic interpretations.

The findings of this study collectively establish foundational evidence for evaluating MTDH expression and its association with Ki-67 in canine mammary tumors. The dual-marker assessment paradigm demonstrates significant potential as a cornerstone for precision

oncology in CMTs management. The findings herein substantiate the prognostic utility of MTDH and Ki-67 and advocate for further investigation into their dual functionality as diagnostic biomarkers and therapeutic targets in translational veterinary oncology.

Declaration

Availability of Data and Materials: The data and materials used in this study are available upon request from the corresponding author (X. Xia).

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Ethical Statement: The research protocol used was reviewed and approved by the Research Ethics Committee of Henan Institute of Science and Technology.

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Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted Technologies.

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