Immunohistochemical assessment of TTF-1, EGFR, Napsin-A, p53, and proliferation biomarkers in canine pulmonary adenomas

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Abstract

Primary lung tumors are sporadic in dogs, and connective tissue-caused benign pulmonary adenomas and primary pulmonary neoplasms are infrequently reported in the genera Canis and Felis. This study aimed to examine the pathological and immunohistochemical features of canine pulmonary adenomas diagnosed over 10 years. The dogs with lung adenoma (n = 6) underwent necropsy and were investigated for hematological changes, histopathology (H&E, Masson's trichrome, and Perl's Prussian blue staining), and immunohistochemistry. Out of 882 dogs of various breeds with clinical respiratory signs, pulmonary adenomas were detected in six (0.68%), mostly large, 8-12-year-old dogs. Hematological assessments evidenced remarkable neutrophilia in the tumor-beading group ($P<$ 0.05), and all tumors were identified as papillary adenomas. Interestingly, two tumor-affected dogs had concurrent chronic heart failure manifested in pulmonary hemosiderophages, which is being reported for the first time. The tumors' immunophenotype was TTF-1$^-$, EGFR$^-$, Napsin-A$^-$, CEA$^-$, EMA$^-$, CK$^+$, Vim$^-$, p53$^-$, and Ki67 negative for cell proliferation, indicating the lack of tumor malignancy potential. Whilst EGFR expression was not detected in tumorous lungs, the positive expression profiles were observed for EGFR, TTF-1, and Napsin-A in, respectively, around 30%, 30%-40%, and 40% of normal pulmonary cells. The expression profiles for these markers match in dogs' normal pulmonary cells, with variations in TTF-1 and Napsin-A expressions acting as a potent source of EGFR mutation and its altered expression profile in malignant lung tumors such as adenocarcinomas. The results suggest that canine pulmonary adenomas are completely benign and have no malignant potential, and immunohistochemical assessments can aid in disease prognosis and inform the development of tailored therapy protocols.

Keywords: Adenoma, Canine lung pathology, EGFR, Napsin-A, TTF-1

1. Introduction

Primary lung tumors (PLTs) are infrequently ascended but serve as a key contributor to lung disease in dogs, accounting for roughly 1% of all tumors in the genus Canis. PLTs are characteristically malignant and rarely correlated to benign lesions such as adenoma, papilloma, hemangioma, and granuloma. In the veterinary literature, other tumor types are localized histiocytic sarcoma, fibrosarcoma, chondrosarcoma, osteosarcoma, hemangiopericytoma, mast cell tumor, and neuroendocrine tumors (1). With a reported prevalence of 4.17 per 100,000, PLTs rarely occur in dogs. However, the incidence rate has followed an ascending trend over a two-decade period, terminating in 1989, presumably due to the growing age of dogs (2). Despite digital radiography revealing more countable bronchi than analog radiography, this increased visibility should not automatically lead to a diagnosis of airway disease in veterinary patients (3). Therefore, pathology will be more appropriate diagnostic method for pulmonary disorders. Since the solid pattern of PLTs is scarce in domestic animals, it remains paramount to assess morphological features and conduct immunohistochemical essays for the precise diagnosis of primary pulmonary neoplasms (4). Some of the IHC markers could not be considered as independent and prognostic factors, and therefore it seems these markers are more reliable when used with others as an antibody panel (5,6). A pulmonary papillary adenoma is a benign epithelial tumor that frequently occurs in the periphery of the lung. Since the first case in humans reported by Spencer et al. in 1980, 41 cases of this tumor have been reported worldwide (7). In precision medicine, immunohistochemistry possesses marked versatility in classifying tumors as subtypes and assessing biomarkers for systemic therapy and precise decision-making (8). Though TTF-1 is a specific and sensitive marker for canine PTL, it is further expressed in thyroid carcinomas, which frequently metastasize to the lung. Napsin A and surfactant protein A (SP-A) are key markers in the histological diagnosis of human non-small-cell lung cancer. Nonetheless, they have not been entirely studied in canine neoplasms (9). Of all the markers specific to pulmonary epithelium, TTF-1 is the subject of numerous research. In normal lung tissues, TTF-1 is predominant in the nuclei of alveolar cells, particularly in type II pneumocytes, non-ciliated bronchiolar cells (Clara cells), and basal cells. Of all the non-small cell lung tumors, TTF-1 expression has been reported in up to 94% of adenocarcinomas (10). Similarly, Napsin A is a potent marker in differentiating type 2 pneumocytes (11). Taking note of the foregoing, this research characterizes and evaluates the pathological pattern of canine PLTs and compares variations in the expression profiles for immunohistochemical markers TTF1, CK, EMA, CEA, Ki67, Vimentin, p53, Napsin-A, and EGFR.

2. Materials and Methods

2.1. Animals

The current study was conducted and approved under the supervision of the Research Ethics Committee of Islamic Azad University, Urmia Branch, with approval number IR.IAU.URMIA.REC.1403.152, on September 8, 2024.

1,831 dogs of various breeds were studied at the Veterinary Clinic of Islamic Azad University, Urmia Branch, and four private veterinary clinics of Urmia County between December 22, 2014, and December 21, 2024, within 10 years. Among them, 882 dogs demonstrated clinical respiratory signs,

and a total of 6 necropsied dogs demonstrated pulmonary masses. The studied were dogs submitted to necropsy due to various reasons such as failure to respond to routine anti-inflammatory and antibiotic therapy for pulmonary diseases, deteriorating patient conditions, metastatic tumors, road traffic accidents, and upon the owner's request. Suspicious tissue masses of various lung lobes were carefully examined for color, tissue consistency, size, location, and local lymph nodes, i.e., bronchial and mediastinal lymph nodes, and the findings were recorded. Naturally, encountering tumors, particularly in older dogs, was expected due to the high index of pet health and longevity. As a few of the referred dogs showing respiratory signs were from petrochemical or chemical manufacturing plants, neoplastic masses existence in the respiratory system was likely.

2.2. Hematology

Blood samples containing EDTA were drawn from the jugular veins of six healthy dogs and six dogs with pulmonary adenoma tumors utilizing a 21-gauge needle and evaluated using the Abbott Cell Dyn 3500 automatic hematology analyzer.

2.3. Pathology

Tissue samples of lung mass were fixed in 10% formalin for at least 48 hours, then processed using standard methods. Dehydration, clearing, and impregnation were done with a tissue processor before making paraffin blocks. Six-micrometer sections were prepared and stained with hematoxylin and eosin (12). Masson's trichrome and Perl's Prussian blue stains were used to identify connective tissue and iron deposits respectively.

2.4. Immunohistochemistry

After confirming pulmonary adenoma through light microscopy and determining its pathological type and pattern, 6 normal and 6 adenoma lung tissue sections were immunohistochemically stained using the EnVision + Dual Link System-HRP method.

Paraffin-removed sections were rehydrated with ethanol and treated with 3% hydrogen peroxide for 30 minutes to stop peroxidase activities. Antigen retrieval used 0.01 molar citrate buffer at pH 6.0 for 25 minutes. Non-specific staining was blocked by incubating slides in 5% bovine serum albumin in TBS for 30 minutes. Slides were then washed, incubated overnight at 4°C with various primary antibodies including TTF-1 (Dako, USA; 1:200), Ki67 (Dako, Denmark, 1:100), cytokeratin (Dako, Glostrup, Denmark, clone AE1/AE3; 1:50), VIM (Dako; clone 3B4; 1:100), EGFR (Clone 31G7, Zymed Laboratories, San Francisco, California, USA, 1:50), EMA (Dako, USA; 1:100), CEA (Dako, USA; 1:300), p53 (Dako, Glostrup, Denmark, 1:100) and Napsin-A (Biocare, Concord, CA, 1:100), washed again with PBS, treated with streptavidin-horseradish peroxidase, and counterstained with Harris hematoxylin (13). The positive cells (with brown immunoreactivity) were counted in 5 random fields (each spanning 2.37 mm^2) using an X40 microscope lens to grade the expression level and intensity of immunohistochemical markers for each normal and pathological sample. The percentage of total positive cells of any intensity relative to total cells was calculated. The following formula was employed to calculate the H-Score (Histochemical scoring assessment), which is a superior indicator of marker expression intensity and distribution of different expression intensities across each sample:

$$\text{H-score} = (0 \times P_0) + (1 \times P_1) + (2 \times P_2) + (3 \times P_3)$$

In this context, P represents the percentage of positive cells (+, ++, and +++) in the tissue, displayed as P1, P2, and P3, respectively. The numerical H-Score scale ranges between 300-0 (14,15).

2.5. Statistical Analysis

Statistical analysis of the obtained data was carried out using GraphPad Prism version 9. Normality was assessed through the Shapiro-Wilk test in the quantitative data. Then, an independent t-test was used to examine differences in mean blood factors between study groups due to the normal distribution of blood factor data. Additionally, the mean difference in histochemical factor scoring (% and H-Score) in healthy and adenoma tumor dogs was investigated using an independent t-test. $P \leq 0.05$ was significant.

3. Results

3.1. Clinical Diagnosis and Necropsy

Out of 882 dogs that had respiratory signs, six dogs had lung adenoma tumors, with tissue samples obtained by necropsy and confirmed by light microscopic observation. It must be noted that a necropsied dog with a lung adenoma tumor had died during a road accident. The tumor was discovered by complete accident during a necropsy inspection. Dogs with respiratory signs were from various breeds, including German Shepherd, Shih Tzu, Dachshund, Rottweiler, Terrier, Iraqi, Sarabi, and mixed native Iranian breeds. Out of the six dogs that had adenoma tumors, two were of mixed Iranian breeds (both male, 8-10 years old), one was a Shih Tzu (female, 12 years old), two were German Shepherds (both male, 8-12 years old), and one was a Rottweiler (female, 10 years old). The tumors appeared as single, separate masses with distinct boundaries, were white to gray, and had a relatively soft to firm consistency. Tumor shapes were mostly circular or oval, with diameters ranging from 4-10 centimeters (Figure 1). The dogs with tumors had shown clinical signs for three to eight weeks, including cough, anxiety, halitosis, increased heart rate, and lethargy, and had a history of non-response to treatments with conventional antibiotics such as gentamicin and cephalexin. During the necropsy, the tumor mass was found in the right lung's caudal lobe in four animals and in the middle lobe in two animals. In addition to the tumor, numerous gray to brown-colored spots were diffusely observed in various lung lobes during the necropsy on two dogs (one mixed Iranian breed and a German Shepherd.

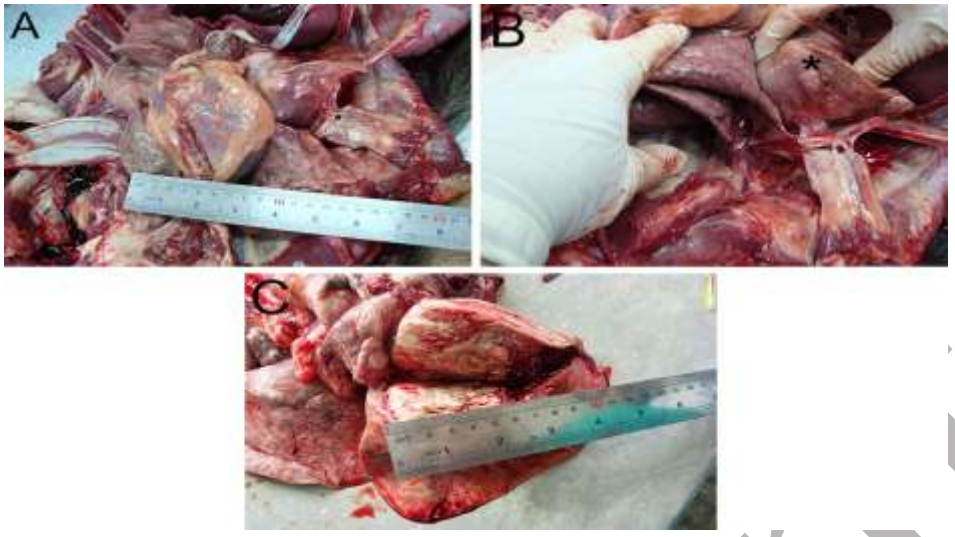

Figure 1: Gross appearance of lung mass (adenoma) in a 10-year-old male German Shepherd during necropsy. (A). Location of lung and heart with adenoma (scalpel). (B). Adenoma (asterisk) in the lung with gray-brown spots within the lung parenchyma. (C). Cross-section of the adenoma with an approximate diameter of 8 centimeters, white color, firm consistency, and heterogeneous composition of tissue.

3.2. Hematology

The quantitative data of blood factors in the studied dogs, categorized as normal and with pulmonary adenoma, are presented in Table 1 as mean ± standard deviation. Accordingly, the neutrophil count significantly increased in the tumor group compared to the normal group ($P \leq 0.05$). No significant increase or decrease was observed in other blood parameters between the two groups ($P > 0.05$).

Table 1: Comparison of the hematological variables between normal dogs and dogs with lung adenoma. Data are presented as Mean ± SD.

Variable	Dog selection (n= 12)		*P* value
	Normal (n= 6)	With lung adenoma (n= 6)	
HCT (%) (Reference: 37-55)	44.33 ± 4.45	42.17 ± 5.19	0.456
Hb (g/l) (Reference: 140-190)	163.30 ± 9.52	161.80 ± 9.06	0.785
RBC (10^{12}/l) (Reference: 5.8-8.50)	6.80 ± 0.83	6.62 ± 0.68	0.687
MCV (*f*l) (Reference: 66-75)	69.50 ± 1.87	69.83 ± 2.04	0.774
MCHC (g/l) (Reference: 32-36)	33.17 ± 1.42	33.00 ± 1.26	0.837
PLT (10^9/l) (Reference: 150-400)	250.20 ± 52.53	328.50 ± 61.95	0.731
WBC (10^9/l) (Reference: 6-13)	9.20 ± 1.31	11.05 ± 1.98	0.086
Neut (10^9/l) (Reference: 3-10.50)	6.60 ± 1.13	10.72 ± 1.29	0.001
Lym (10^9/l) (Reference:1-4)	2.47 ± 0.66	2.60 ± 0.52	0.706
Mon (10^9/l) (Reference: 0.15-1.2)	0.55 ± 0.26	0.65 ± 0.33	0.582
Eos (10^9/l) (Reference:0-0.1.3)	0.45 ± 0.21	0.47 ± 0.23	0.901

Note: $P < 0.05$ is significant.

3.3. Pathology

Microscopically, pulmonary masses found in six necropsied dogs were diagnosed as adenomas. Glandular structures with papillary patterns and thick scaffolding with limited connective tissue surrounding glandular units were observed in pulmonary adenomas. Uniform glands were completely differentiated without pleomorphism or anisocytosis, and mitotic figures without hemorrhage or necrosis in the lung parenchyma, all of which indicated tumor benignity (Figure 2A, D). However, a limited number (1-4) of cysts with varying sizes were present (cystadenoma) in the parenchyma of two adenoma tumors. Meanwhile, there was abundant smooth muscle tissue in thick parenchymal scaffolding with minimal connective tissue (Masson's trichrome) (Figure 2B, D). Alveolar macrophages were observed in (H&E)-stained sections, which were the same gray-brown spots (pigments) in the lung parenchyma involved with the tumor. The pigments turned blue after being stained with the Prussian Blue method, confirming their iron nature (Figure 2E, F). It was definitively determined that the existence of alveolar macrophages containing hemosiderin in the lung parenchyma of two animals with adenomas indicated left-sided heart failures.

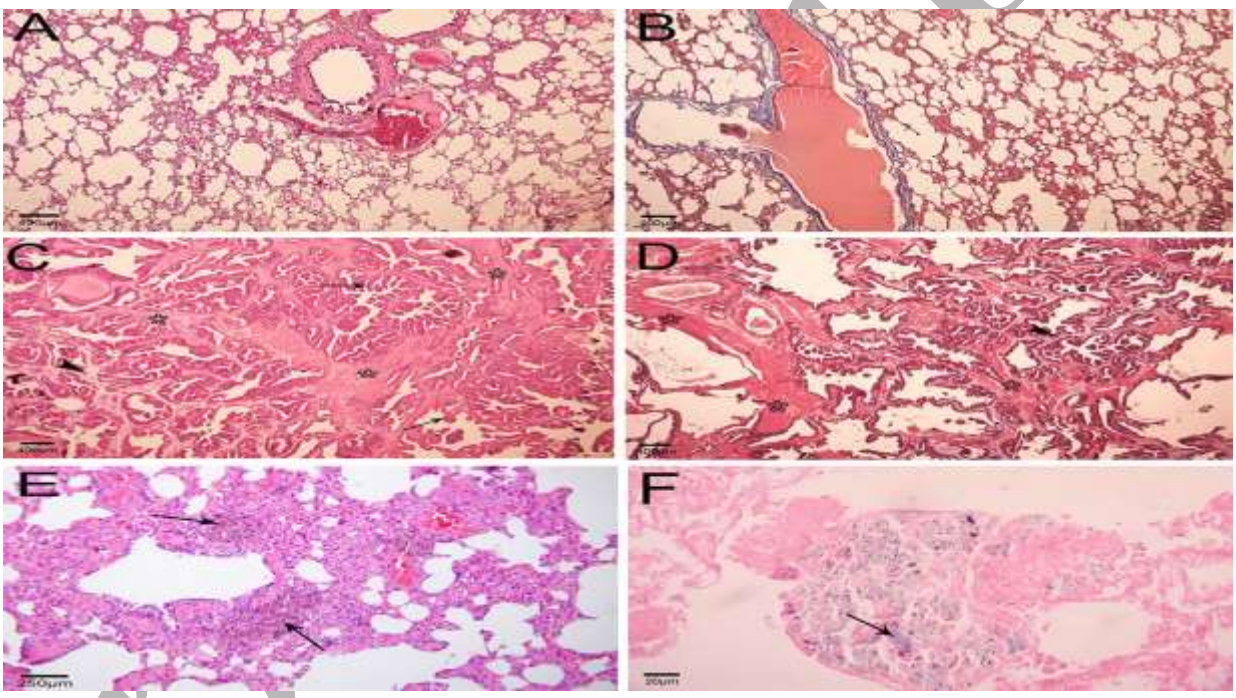

Figure 2: Light microscopic photomicrograph of a canine normal lung tissue and lung adenoma. (A). Normal lung with regular structures (H&E). (B). Normal lung without fibrosis in the parenchyma and minimal connective tissue around blood vessels in blue (Masson's trichrome). (C). Lung adenoma with papillary glandular pattern (arrow), thick smooth muscle scaffolds (asterisk) and less connective tissue (arrowhead) surrounding glandular units without necrosis or hemorrhage in the parenchyma. Glandular cells are fully differentiated without anisocytosis, pleomorphism and mitotic figures (H&E). (D). Adenoma with the presence of extensive smooth muscle tissues (asterisk) in pink-red and minimal connective tissue (arrowhead) in blue among glandular units (Masson's trichrome). (E). Existence of intra-alveolar macrophages as heart failure cells (arrow) in lung parenchyma with brown

pigments within them (H&E). (F). Iron deposit inside hemosiderophages in blue, confirming left-sided heart failure in a dog with lung adenoma (Perl's Prussian Blue).

3.4. Immunohistochemistry

The study compared immunohistochemical marker scores between dogs with pulmonary adenoma and normal dogs using an independent t-test (Figure 3A, B). Results showed significantly higher levels of TTF1, EGFR, and Napsin-A in healthy lungs compared to tumor lungs ($P< 0.001$). The expression percentage and H-Score for EMA and CEA markers were low in both groups, but statistically different ($P< 0.001$). CK showed higher expression in the pulmonary tumor group, indicating epithelial origin. Vim expression in normal lung cells was minimal. However, the expression percentage and H-Score in stromal cells located in interstitial spaces of glandular units (smooth muscle and connective cells) demonstrated a significant increase ($P< 0.001$), indicating the mesenchymal origin of tumor stromal cells. Cellular expression percentage and H-Score were negligible in both groups for the p53 marker. The average cellular expression in normal lung and tumor tissue was lower than 1% and 5%, respectively. Ultimately, cellular expression of the Ki67 marker across both groups was minimal and under 1%. Moreover, the H-Score in the tumor group was much lower compared to the normal group. The findings of this marker and the pathological pattern indicate that the cellular proliferation rate in the tumor group was minimal. Thus, all pulmonary adenoma tumors among the examined dogs were benign and lacked malignancy potential. Finally, immunohistochemical expression changes for TTF-1, EGFR, Napsin-A, EMA, CEA, CK, Vim, p53, and Ki-67 markers in the normal lung group and adenoma group were compared in Figures 4 and 5.

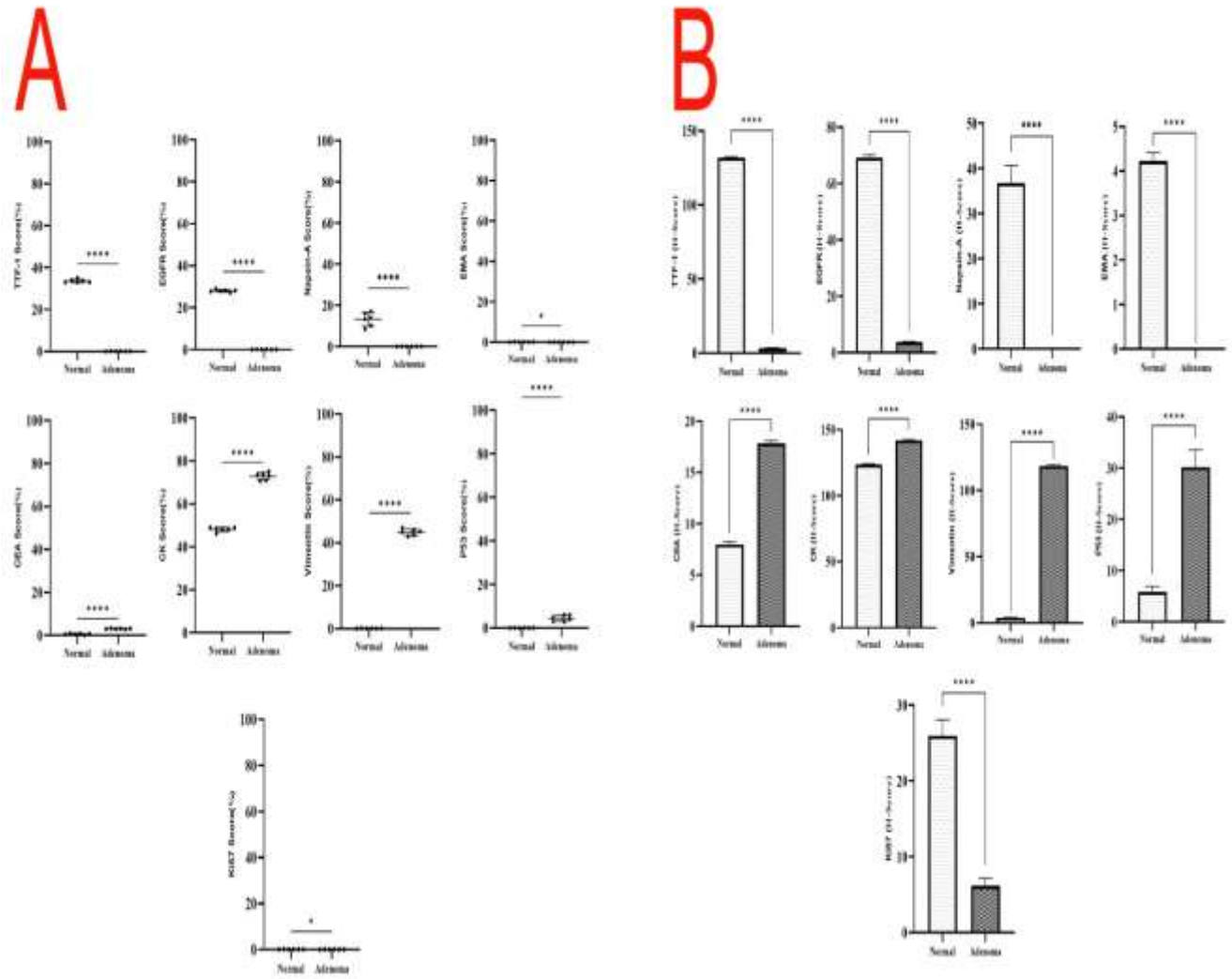

Figure 3: (A): The results of the independent t-test for the percentage of cellular expression of various immunohistochemical markers in normal and adenoma-involved lungs. Data are presented as Mean ± SD. *: Significant value ($P< 0.05$). ns: non-significant. (B): The results of the independent t-test for the H-Score of the different immunohistochemical markers in normal and adenoma-involved lungs. Data are presented as Mean ± SD. *: Significant value ($P< 0.05$). ns: non-significant.

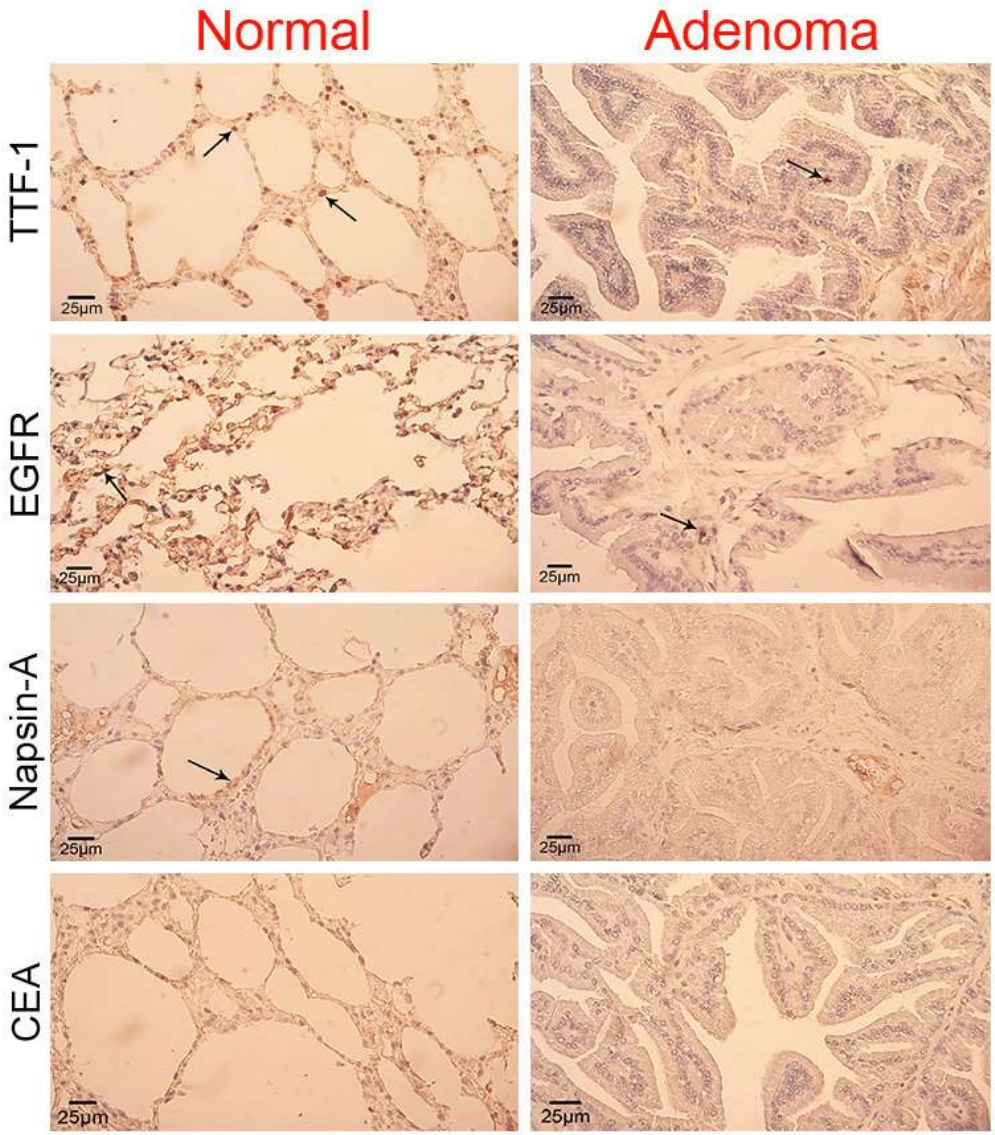

Figure 4: Immunohistochemical photomicrograph of tumor and normal lung tissues. Relatively strong nuclear TTF-1 expression in type II pneumocytes (arrow) in the normal lung and weak expressions in the tumor cells (arrow). Cytoplasmic expressions of Napsin-A and EGFR in the normal lung (arrow) and lack of expression or weak expressions (arrow) in tumor tissue. The absence of CEA expression in both tumor and normal lung tissue is observed as well (IHC).

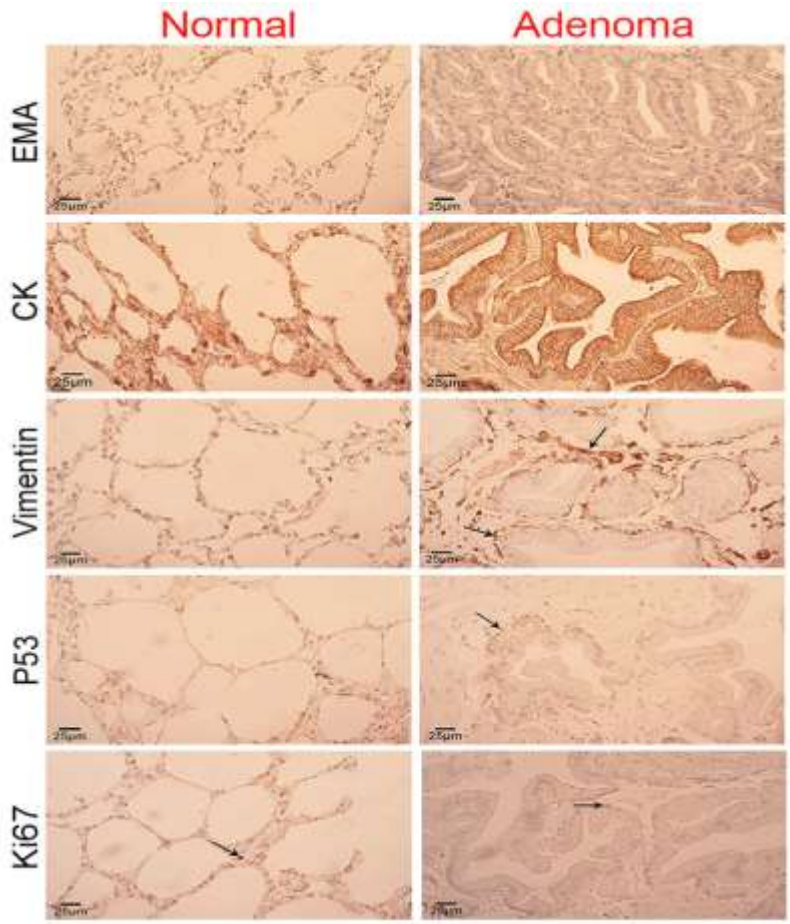

Figure 5: Continuation of immunohistochemical staining in the lungs with tumor and the normal ones. Absence of EMA expressions in tumor and normal lungs, weak nuclear P53 expressions in tumor cells (arrow), and strong, diffuse cytoplasmic CK expressions in normal lungs, which is even higher in the tumor cells. Absence of Vimentin marker expressions in normal lung cells and strong expressions in the stromal scaffold tissue (muscle and connective tissue cells) surrounding glandular units of the tumor tissue (arrow). Weak nuclear Ki67 expression (arrow) in normal and tumor lung cells, which indicates the absence of potential for cell proliferation and benign tumor without malignancy tendency (IHC).

4. Discussion

This research conducted over 10 years in Urmia studied 882 dogs aged 8-12 years showing respiratory issues. It found that six dogs (0.68%) had pulmonary adenoma tumors, mostly in larger breeds, but the rarity of the tumors makes it hard to draw firm conclusions about their links to age, sex, and breed. Previous studies noted possible associations, including a higher occurrence in male dogs (16) and specific breeds like Boxers, Australian Shepherd, Irish, Doberman Pinscher, and Mountain dog breeds (2,17). This issue may be related to the popularity of various dog breeds in different geographical locations (17). In the current research, we found some confined cysts whose walls were lined with cuboidal neoplastic cells. According to the tumors' benign nature, necrotic and desquamated epithelial cells didn't exist in the mentioned tumors, as seen in cyst adenocarcinoma tumors (18). Most research has focused on malignant tumors, but this study looked at the benign

adenoma's characteristics and potential for malignancy. A significant finding was the occurrence of pulmonary adenoma alongside chronic heart failure in two dogs, noted through specific cells in the lungs. There was no clear cause for the coexistence of these conditions, and no similar cases have been reported. Necropsy showed no other tumors or vascular damage, although some dogs exhibited left ventricular hypertrophy.

This study found that neutrophilia in cases of pulmonary adenoma might be due to inflammation in the respiratory system, as the animals had shown respiratory signs like coughing for about a month before their necropsy. The present study evaluated the TTF-1 marker levels in normal and adenoma-affected lungs. In normal lungs, 20-40% nuclear expression and high intensity (H-Score) of TTF-1 was observed, while no positive reaction was found in lung cells with adenoma. TTF-1 is a key marker for canine lung tumors and is also seen in thyroid cancers that spread to the lungs. Other markers like Napsin A and SP-A, important for human lung cancer, are gaining attention for canine neoplasms (9). TTF-1 negativity is associated with an unfavorable cancer phenotype, including a lack of genomic functional changes, higher tumor load, and poorer performance status (19). The marker should be used alongside other diagnostic indicators to differentiate tumor types, with tumor characteristics also playing a vital role in treatment decisions.

The findings of the current study regarding the Napsin-A marker indicate both expression and intensity (H-Score) in the normal group, with no marker expression in the adenoma group. These are similar to the work of Ramos-Vara et al. (2016), who reported the immunopositivity for this marker in both normal canine lung tissue samples (20). Our results showed that normal dog lung alveolar cells exhibited approximately 30% positive cytoplasmic expression of EGFR, with an H-Score of 60-80, whereas tumor cells had nearly negative expression. Unlike humans, EGFR mutations are rare in canine lung adenocarcinoma. Increased EGFR was also seen in lungs affected by anthracosis, suggesting a link to air pollution and cancer risk (17). The present study found no EGFR expression in adenoma lungs, while about 30% of normal lung cells showed EGFR positivity. TTF-1 and Napsin-A expressions were similar in normal lung tissue, and changes in these may affect EGFR in malignant lung tumors like adenocarcinoma (21). It should be noted that in the current study, the expressions of the mentioned three markers in tumor parenchymal cells were negative due to the benign nature of lung adenoma. Moreover, the expressions might undergo significant changes with tumor malignancy. The IHC results for EMA and CEA markers showed weak expression in tumors and normal lungs. Negative results were expected for benign adenoma tumors, ruling out neuroendocrine tumor origin (22). Regarding IHC results for CK in the current study, the origin of tumor cells was definitely epithelial. Previous studies on humans and canines have revealed that CK expression increases in lung epithelial tumors, particularly malignant tumors like adenocarcinomas (4,7). IHC results showed no Vimentin expression in normal lung cells. In the adenoma group, 40-60% of mesenchymal cells between the glands had positive expression, but glandular cells were negative for the Vim marker due to their epithelial origin. Although a study showed that a combined cytokeratin and vimentin expression in canine lung tumors is similar to humans (16). Vimentin is a type III intermediate filament strongly expressed in lung cancer, associated with metastatic tissue and shorter survival time in patients with non-small cell lung cancers. Vimentin's role in the metastatic process has been extensively studied in experimental conditions (23). Concerning the immunohistochemical expressions of the p53 marker in the current study, results showed no expression in the normal lungs and weak expressions of less than 10% in the tumor cells. Previous studies have indicated that dog

p53 family proteins have biological activities similar to human counterparts. These similarities make dogs a decant tumor model for human studies (24). Ultimately, IHC results for the Ki67 marker in the current study indicated no expression in tumor and normal tissues. This marker is employed to evaluate cellular proliferation and is often employed as a biomarker for malignant tumor prognosis. The negative status of adenoma tumor cells in the current study indicates their benign nature and lack of malignancy potential. Evaluating the cell proliferation index might be valuable in assessing prognosis following surgical treatments of canine lung cancers (25). High Ki67 expressions in malignant lung tumors typically indicate a poor prognosis. The percentage of Ki67-positive cells in lung adenomas in humans has been cited at 2-6% (7,11). In malignant canine lung tumors like adenocarcinoma, the percentages of Ki67-positive cells have been noted at 20-50% (26).

The study conducted in Urmia County over 10 years involved 882 dogs showing respiratory symptoms. Only six dogs (0.68%) were diagnosed with lung papillary adenoma. In two cases, the dogs also had chronic heart failure. Tumors showed specific immunophenotypes as TTF-1$^-$, EGFR$^-$, Napsin-A$^-$, CEA$^-$, EMA$^-$, CK$^+$, Vim$^-$, p53$^-$, and Ki67$^-$ indicating a low chance of malignancy. The findings suggest that canine lung adenoma tumors are benign. The results from the immunohistochemical analysis could help in creating treatment plans and assessing disease outcomes.

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Author's Contribution

Study concept and design: N. H, A. A

Acquisition of data: N. H, A. A

Analysis and interpretation of data: A. A

Drafting of the manuscript: N. H, A. A

Critical revision of the manuscript for important intellectual content: A. A

Statistical analysis: A. A

Administrative, technical, and material support: N. H, A. A

Study supervision: A. A

Ethics

This study was approved by the Research Ethics Committees of the Islamic Azad University, Urmia Branch, with the approval number of IR.IAU.URMIA.REC.1403.152 on September 8, 2024.

Conflict of Interest

There is no conflict of interest between the authors.

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Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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