Opis przypadku *Case report*

Immunohistochemical and histopathological evaluation of malignant pheochromocytoma: a case study

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Summary

Malignant pheochromocytoma is an uncommon neoplasm in dogs. This study evaluated the histopathological and immunohistochemical profile of malignant pheochromocytoma. The report presents a case of bilateral adrenal gland tumor in a 6-year-old female golden retriever, diagnosed during necropsy. The necropsy also showed changes in other organs, i.e. heart (left ventricular hypertrophic cardiomyopathy, right ventricular dilated cardiomyopathy and infarction foci), lungs (multifocal alveolar emphysema and atelectasis). Metastases were not present. A histological examination showed that neoplastic cells were round, oval, polygonal and spindle with a high degree of anisocytosis and anisokaryosis. The nuclei were small, round, oval and some were divided. The nucleoli were faintly visible, and numbered from 2 to 3. The number of mitotic figures per 400 high power fields ranged from 0 to 2. Immunohistochemical staining using an antibody panel revealed that the tumor cells were strongly positive for chromogranin A, S100 protein and neuron specific enolase, but were negative for Ki67. PCNA expression was observed in some neoplastic cells, especially in those located close to the connective tissue stroma. Malignant pheochromocytoma was diagnosed on the basis of a immunohistochemical examination and tumor morphology.

Keywords: adrenal gland tumor, S100 protein, chromogranin A (CGA), neuron specific enolase (NSE), proliferating cell nuclear antigen (PCNA), Ki67

Pheochromocytoma is an endocrine neoplasm derived from epinephrine and norepinephrine-producing chromaffin cells localized in the adrenal medulla (2, 13, 14) and can be benign or malignant. Chromaffin cells are modified post-ganglionic sympathetic neurons derived from the same sympathoadrenal (SA) progenitor cell as sympathetic neurons. The major difference between chromaffin cells and sympathetic neurons is the ability of the chromaffin cells to secrete epinephrine and phenylethanolamine N – methylotransferase (PNMT) (14). Pheochromocytomas are rare tumors in all animal species except rats, in which they are common. Dogs and cattle are more susceptible to pheochromocytoma than other animals (13, 14). In humans, the pheochromocytoma is a more frequent neoplasm, especially in the case of the familiar predisposition for this kind of tumor (14). Benign phoechromocytomas occur as solitary, mostly unilateral neoplasms of the adrenal gland medulla. Bilateral phoechromocytomas are frequently seen in rats. The tumor size is variable. Benign adrenal gland tumor is unencapsulated, with low-to-medium cellularity, composed of round-to--polyhedral secretory epithelial cells with pale eosinophilic, finely granular cytoplasm and round-to-ovoid hyperchromatic nuceli (5). Nuclear pleomorphism is mild-to-moderate (15). Malignant pheochromocytoma is composed of a mixture of small cells resembling chromaffin cells and larger, more pleomorphic (often spindle) cells. Cells are organized in lobules, solid sheets and palisades, usually localized along sinusoids. In over 50% cases of malignant pheochromocytoma in animal metastases into the vena cava, tissues surrounding the adrenal gland, lymph nodes and distant organs such as liver, lung, kidney, spleen and bone were noted (3-5). Barthez et al. (1) found that in 61 cases of pheochromocytoma, tumors were locally invasive in 39% of affected animals and metastases were found in 13% of cases. Massive retroperitoneal hemorrhages due to

Tab. 1. Primary antibodies used with the particular methods of antigen retrieval and visualization

Primary Antibody	Clone	Optimal dilution	Source	Antigen retrieval	Visualization system	Chromogen
CGA	polyclonal rabbit anti – human chromogranin A	Ready-to-Use	DAKO, Denmark	2 × 3 min* Tris EDTA buffer pH = 9	DAKO, Denmark[a]	DAB[a]
NSE	monoclonal mouse anti – human, clone BBS/NC/VI – H14	1 : 100	DAKO, Denmark	2 × 3 min* Tris EDTA buffer pH = 9	DAKO, Denmark[a]	DAB[a]
S100 protein	polyclonal rabbit anti – S100 antibody	1 : 50	DAKO, Denmark	2 × 3 min* Tris EDTA buffer pH = 9	DAKO, Denmark[a]	DAB[a]
Ki67	monoclonal mouse anti – human, clone MIB-1	1 : 75	DAKO, Denmark	2 × 3 min* Tris EDTA buffer pH = 9	DAKO, Denmark[a]	DAB[a]
PCNA	monoclonal mouse anti – PCNA, clone PC10	1 : 200	DAKO, Denmark	2 × 3 min* Tris EDTA buffer pH = 9	DAKO, Denmark[a]	DAB[a]

Explanations: CGA = chromogranin A; NSE = neuron specific enolase; PCNA = proliferating cell nuclear antigen; *antigen retrieval was conducted in a microwave oven, 650 W; [a]Dako EnVision + System-HRP (DAB), DakoCytomation, Denmark

the high blood pressure and injury to the blood vessel walls were also observed in affected animals (4). The accompanying clinical signs in dogs are different and depend on the secondary involved organs (8).

Histopathological diagnosis of pheochromocytoma is difficult due to the variable morphology of tumor cells. Therefore, the final diagnosis should be based on the immunohistochemical identification of cells. Pheochromocytoma cells show cytoplasmic expression of chromogranin A (CGA). Chromogranins are the major proteins of adrenal medulla and play an important role in binding and aggregating intracellular calcium. They also play a role as prohormones, molecular chaperones and gene expression modulators (10). In malignant pheochromocytoma there is a population of spindle cells which are positive for CGA. Those cells are absent in the benign form of the tumor (12). In immunohistochemical evaluation, chromaffin neoplastic cells also show moderate reaction with leu- and met-enkephalin (ME) (5), which depends on the tumor origin. If the tumor is forming extramedullary, the concentration of leu- and met-enkephalin in cell cytoplasm is lower than in the medullary tumor (16). Other antibodies which react with neoplastic cells are substance P (SP), synaptophysin (SYN), vasoactive intestinal peptide (VIP), neuropeptide Y, calcitonin gene-related polypeptide (CGRP), calcitonin, protein gene product 9.5 (PGP 9.5), Leu 7, S100 protein and galanin (GAL) (10, 13).

The cell proliferation markers seem to be an important prognostic factor, but so far there is no single histological or immunohistochemical marker which may predict the malignant behavior of the tumor. Therefore, it is justified to undertake research on determining a group of features and markers that may have a diagnostic value in distinguishing benign from malignant pheochromocytoma (15).

The aim of this study was to determine the histopathological and immunohistochemical profile of a malignant pheochromocytoma in a dog.

Material and methods

A 6-year-old female golden retriever was euthanized at the university clinic. The clinical signs and history of treatment were unknown. A necropsy examination was performed. During the necropsy, left ventricular hypertrophic cardiomyopathy, right ventricular dilated cardiomyopathy and infarction foci in a heart muscle were observed. In the lungs, multifocal alveolar emphysema and atelectasis were seen. Hydrothorax was also diagnosed. The kidneys were swollen and infarction foci in the cortex were present. Both adrenal glands were enlarged. Two reddish, 3-cm-in-diameter tumors were found in the left and right adrenal glands. Metastases were not found.

The tumor samples were collected, fixed in 10% buffered formalin and embedded in paraffin. The paraffin sections (5 μm) were routinely stained with hematoxilin and eosin (HE). Reticulin fibers were detected by using the modified method according to Gordon and Sweet (Bio-Optica, Italy) and collagen fibers were shown in Mallory trichrome staining (Bio-Optica, Italy).

The immunohistochemical examination was performed using primary antibodies, a visualization system and chromogen as summarized in Table 1. The specimens were counterstained with Mayer's hematoxylin. The slides were scanned and evaluated using 3DHISTECH (Hungary) software. The PCNA expression was evaluated in nine randomly-chosen areas of the slide (40 × magnification). The PCNA index was expressed as the percentage of PCNA-positive cells.

Results and discussion

Macroscopically, the tumor was well-demarcated from the surrounding tissues, but the capsule was not present. In the tumor stroma, numerous foci of necrosis were present. Histopathological examination revealed the presence of mixed cell populations of neoplastic cells arising from adrenal gland medulla. The cells were round, oval, spindle and polygonal with a high degree of anisocytosis and anisokaryosis. Smaller cells were mostly round with small, round nuclei and scant, eosinophilic cytoplasm. The medium and large cells were oval, polygonal or spindle and had medium or large, round-to-oval nuclei and abundant, eosinophilic

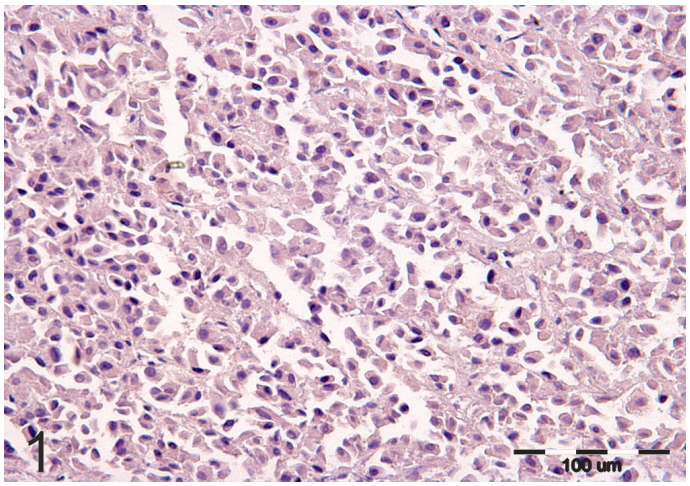

Fig. 1. Adrenal gland, dog. Pleomorphic neoplastic cells. Nucleoli are faintly visible. In single cells 2-3 nucleoli are seen. HE

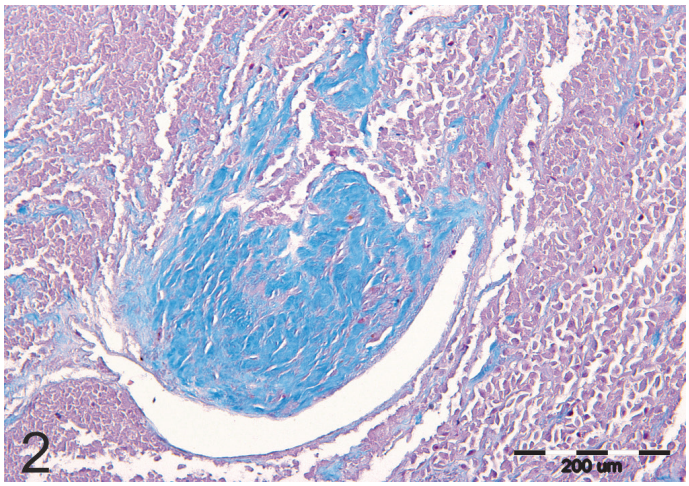

Fig. 2. Adrenal gland, dog. Small clusters of collagen fibers inside the tumor mass. Collagen structures are surrounded by neoplastic cells. Mallory trichrome

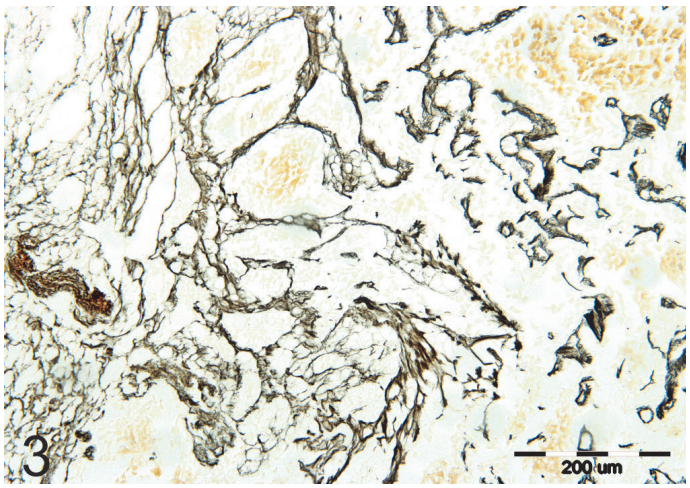

Fig. 3. Adrenal gland, dog. Reticulin fibers which are dividing neoplastic cells into Zellballen pattern. Silver impregnation

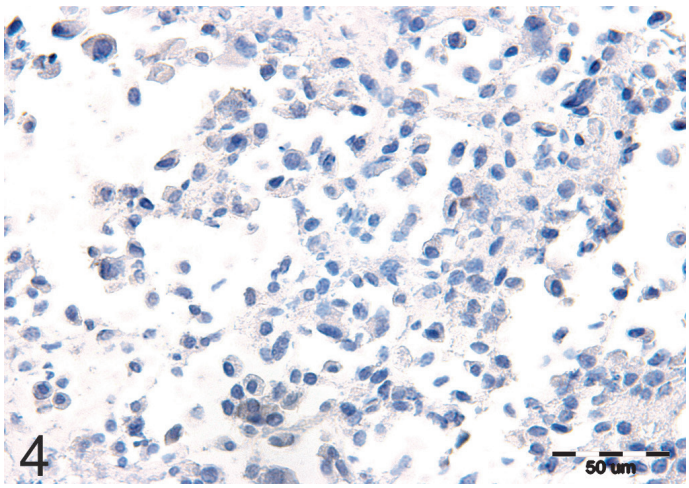

Fig. 4. Adrenal gland, dog. Neoplastic cells Ki67 negative. Ki67 immunostaining

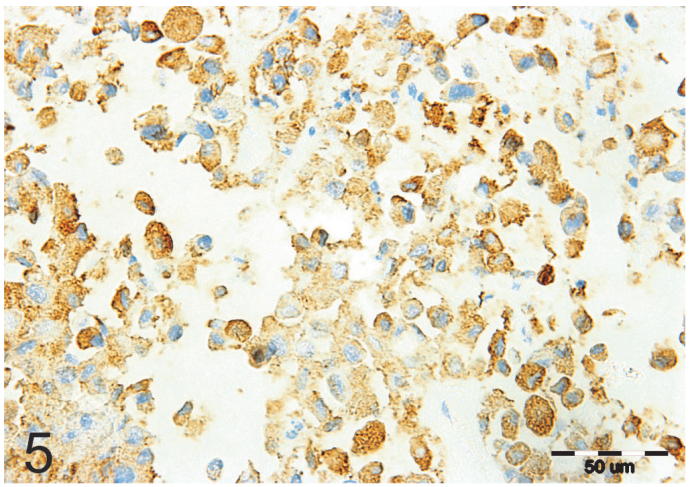

Fig. 5. Adrenal gland, dog. Positive neoplastic cells show cytoplasmic granular staining pattern. CGA immunostaining

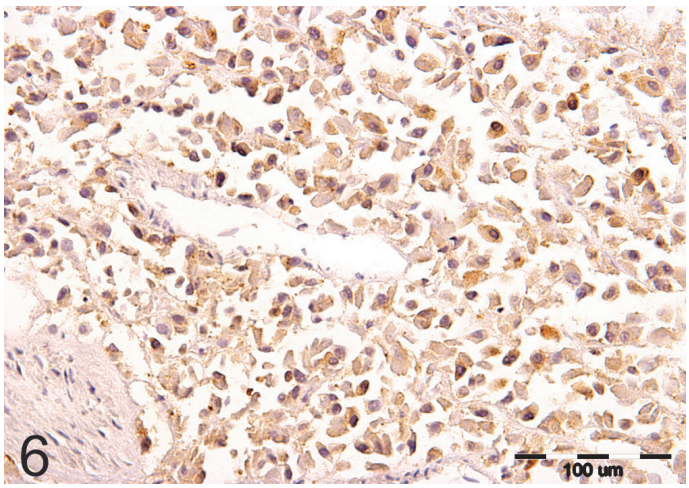

Fig. 6. Adrenal gland, dog. Neoplastic cells show positive moderate to strong cytoplasmic expression. NSE immunostaining

cytoplasm and 2-3 indistinct nucleoli. Some nuclei were divided, resembling "coffee beans", which gave an image of a double nucleus. The chromatin in the nuclei was condensed or stippled. Mitotic figures were not frequent and ranged from 0 to 2 per 400 × magnification (Fig. 1).

The mallory trichrome method showed the presence of collagen fibers surrounding neoplastic cells (Fig. 2). They were also seen in tumor parenchyma as small clusters. Silver impregnation showed abundant reticulin fibers which divided neoplastic cells into medium-sized nests (Fig. 3).

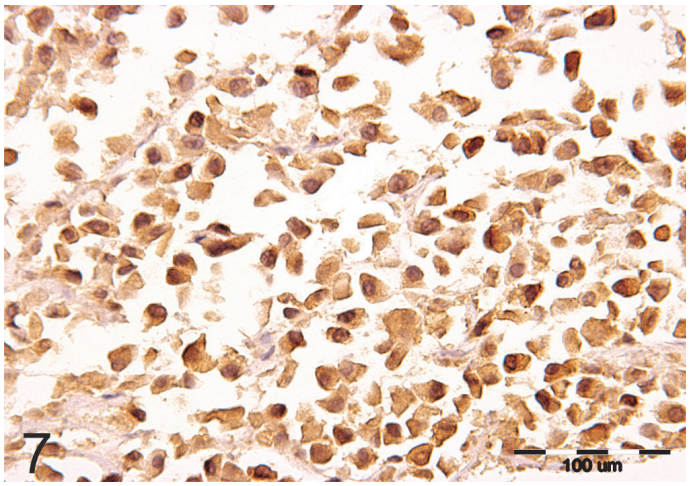

Fig. 7. Adrenal gland, dog. Neoplastic cells show strong reaction. S100 protein immunostaining

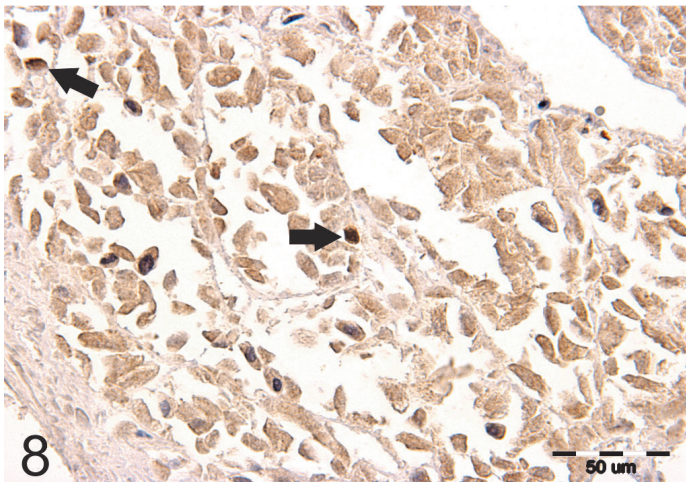

Fig. 8. Adrenal gland, dog. A small percentage of the tumor nucleus showed PCNA expression. PCNA immunostaining

Immunohistochemical examination using NSE (Fig. 6) and S100 protein (Fig. 7) revealed a positive reaction in the cytoplasm of the tumor cells. Immunohistochemical reaction with CGA was also positive and was seen as a granular staining pattern in the cytoplasm of all populations of neoplastic cells (Fig. 5). PCNA positive cells were located near fibrous tissue (Fig. 8). The PCNA index was 17%. The neoplastic cells were negative for Ki67 (Fig. 4). Malignant pheochromocytoma was diagnosed on the basis of the histological and immunohistochemical examination findings.

Pheochromocytoma is a tumor of chromaffin cells of the adrenal gland medulla (7, 9). The diagnosis of the tumor during life is difficult due to a variety of clinical symptoms (6, 8). Some authors suggest that clinical signs such as weakness, episodic collapse, tachypnoea, panting, tachycardia, pacing, polyuria, polydipsia, hypertension, vomiting and diarrhea have prognostic value (6). However, these symptoms are non-specific and therefore cannot be indicators of the disease. Glison et al. (3) found that in 24 out of 50 dogs (48%), none developed any clinical signs during pheochromocytoma and tumors were usually diagnosed accidentally during necropsy or surgery. When the clinical signs occurred, they were related to nervous system (27%), circulatory and respiratory distress (17%) or were linked to many systems, which was probably connected with the relatively long period of the tumor presence/growth. The current diagnosis was based mostly on the histopathology of adrenal tissue after adrenalectomy or necropsy. In humans, detection of increased concentrations of catecholamins and their breakdown products, such as metanephrine (MN) and normetanephrine (NMN) in urine or plasma, is a standard test for pheochromocytoma diagnosis. Unfortunately, this test is rarely performed in animals. Measurement of the urinary NMN-to-creatinine ratio has been shown to be significantly increased in dogs with pheochromocytoma compared to dogs with hyperadrenocorticism or healthy animals (4). Symptoms found during clinical examination are non-specific and cannot be used to diagnose adrenal gland tumor. Pheochromocytomas may be associated with clinical signs as a result of continuous or episodic secretion of the catecholamines. Evaluation of blood pressure induced by the sudden release of catecholamines can precipitate acute congestive heart failure, pulmonary edema, myocardial infarction, ventricular fibrillation and cerebral hemorrhage (13).

Glison et al. (3) also showed that malignant pheochromocytoma with local invasion was present in 26 out of 50 dogs (52%), with metastasis to a regional lymph node in 6 out of 50 dogs (12%) and distant metastasis in 12 out of 50 dogs (24%). The tumor was the main cause of death in 19 out of 50 dogs (38%) (3). In our study no local or distant metastases were diagnosed.

Morphology of pheochromocytoma cells is not very characteristic. According to the WHO classification, cellular and nuclear pleomorphism of neoplastic cells and diffuse areas of necrosis are criteria of tumor malignancy (5), as was confirmed in our study. An immunohistochemistry examination, particularly the presence of a positive CGA reaction in the cytoplasm of neoplastic cells, confirmed the chromaffin cell origin, which was consistent with the results of other studies (1, 5, 9, 10, 13). Some authors also suggest that the presence of spindle CGA-positive cells is linked to malignant behavior (12, 15). In our case, spindle cells were CGA-positive. No metastases were noted in the presented case, which may indicate benign tumor behavior. However, many studies have confirmed that malignant pheochromocytoma can grow at the origin site without any signs of the metastases (1, 3). On the other hand, some authors have accepted the thesis that the only absolute criterion of tumor malignancy is the presence of distant metastases (9). The Ki67 index is not a reliable indicator of malignancy in malignant pheochromocytoma. In the presented case it was absent, which was also confirmed by other authors (12, 15). The PCNA index in malignant pheochromocytoma

varies. Pace et al. have shown that the proliferation index in rat malignant tumors differ from 11% to 61% (11).

Silver impregnation showed plenty of reticulin fibers, which divided the neoplastic cells into groups. This phenomenon is called the Zellballen growth pattern (7). The presence of numerous collagen fibers and connective tissue around neoplastic cells usually prevents the formation of distant metastases, as occurred in our case.

On the basis of tumor morphology (strong cellular polymorphism, confluent areas of necrosis, high anisocytosis and anisokaryosis) and positive immunoreactivity of the neoplastic cells with CGA, NSE, and S100 proteins, malignant pheochromocytoma was diagnosed, despite the low mitotic activity and the absence of metastases. In conclusion, due to the specific biology of the tumor, future studies should be focused on finding specific and reliable methods to differentiate benign and malignant pheochromocytoma.

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