

## Immunohistochemical Detection of Proteins Associated with Multidrug Resistance to Anti-Cancer Drugs in Canine and Feline Primary Pulmonary Carcinoma

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**ABSTRACT.** Fifty-two canine and eighteen feline primary pulmonary carcinomas were evaluated immunohistochemically for the expression of proteins associated with multidrug resistance to anti-cancer drugs. P-glycoprotein (PGP), multidrug resistance-related protein (MRP) and lung resistance-related protein (LRP) expression were frequently observed in neoplastic cells of all carcinoma types, and metallothionein (MT) expression was observed in about half of each carcinoma type. Furthermore, overlapping expression was detected in all positive cases. These results indicate that most canine and feline primary pulmonary carcinomas may have strong multidrug resistance, which is related to the PGP, MRP, LRP or MT expression. It might be difficult to treat canine and feline primary pulmonary carcinomas using anti-cancer drugs because of multidrug resistance.

**KEY WORDS:** immunohistochemistry, lung resistance-related protein, metallothionein, multidrug resistance-related protein, P-glycoprotein.

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Human primary pulmonary carcinoma is divided into 2 groups; small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). Surgical resection is not suitable for SCLC because of metastasis at the time of diagnosis in many cases; therefore, chemotherapy is recommended generally. For NSCLC, however, including adenocarcinoma, adenosquamous carcinoma and squamous cell carcinoma, chemotherapy is less effective; therefore, surgical resection is recommended generally [12]. Primary pulmonary tumors in domestic animals are uncommon to extremely rare, with a prevalence of 0.1–0.9% in dogs, a little higher than in cats [23]. In the treatment of canine and feline primary pulmonary carcinoma, surgical resection is recommended [18], but to reduce the risk of local recurrence and metastasis, adjuvant chemotherapy is required similarly to the treatment for human NSCLC.

P-glycoprotein (PGP), a 150–170 kDa plasma membrane protein, belongs to the ATP binding cassette (ABC) superfamily of transporter proteins, and can extrude a range of hydrophobic anti-cancer drugs from the cell [20]. Overexpression of PGP is associated with resistance to different types of anti-cancer drugs (e.g. daunorubicin, doxorubicin, mitoxantrone, etoposide, teniposide, vinblastine, vincristine, mitomycin C, paclitaxel, actinomycin D and topotecan) [6]. Multidrug resistance-related protein (MRP), which belongs to the ABC superfamily as PGP, is a 190 kDa membrane-bound glycoprotein. MRP probably works by direct extrusion of cytotoxic drugs from the cell and/or by mediating sequestration of the drugs into intracellular compartments, both leading to a reduction in effective intracellular drug concentrations [16]. Overexpression of MRP is associated with resistance to doxorubicin, vincristine, actinomycin

D, etoposide and colchicine [7, 22]. Lung resistance-related protein (LRP) is a 110 kDa protein found in multidrug-resistant cell lines not expressing PGP. LRP is the main component of vault protein, and plays a role in drug resistance by regulating the nucleocytoplasmic transport of drugs [8]. The expression of LRP is involved in resistance to doxorubicin, vincristine, etoposide, paclitaxel, and gramicidin D [10]. In one previous report, there was no relationship between the mRNA expression level of LRP and the phenotype of adriamycin resistance in feline lymphoma cell line [5]. Metallothionein (MT) is a low molecular weight protein (10 kDa) with a high cysteine content (30%), exhibiting selective binding affinity for Zn, Cu and other group II heavy metals. MT is thought to play essential roles in Zn and Cu metabolism, heavy metal transport (particularly in copper-loaded animals), and to protect cells from oxidative stress [2]. According to previous reports, in dogs, MT immunoreactivity was observed in the central nervous system, liver, kidney etc., and MT cDNA obtained from the liver of a cadmium-treated beagle was cloned and sequenced [11, 21]. MT expression increases following chemotherapy and may confer resistance in lung cancer, especially NSCLC [14]. Tumor cell lines with acquired resistance to cisplatin overexpressed MT [9]. MT expression has been associated with poor prognosis in a variety of tumors [4].

In dogs and cats, there are reports of the immunohistochemical study of the expression of proteins associated with multidrug resistance in mast cell tumor, mammary gland tumor and melanocytic tumor, but there are no reports about canine and feline primary pulmonary carcinoma [2, 15]. The aim of this study was to investigate the expression of PGP, MRP, LRP and MT using immunohistochemical methods in canine and feline primary pulmonary carcinoma, and to estimate the effectiveness to chemotherapy.

Fifty-two canine and eighteen feline primary pulmonary

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carcinoma tissue samples were obtained from private animal hospitals. All samples were fixed in 10% or 10% neutral-buffered formalin, embedded in paraffin wax and sectioned at 3  $\mu\text{m}$ . Sections were stained with hematoxylin and eosin (HE), and classified histopathologically based on WHO classification [3]. There was no clinical information of patients after surgical resection. When some samples of the same tumor were available, only one block from each tumor was selected for immunohistochemical staining.

For immunohistochemical staining, all paraffin sections cut at 3  $\mu\text{m}$  were mounted on MAS-coating slides (S9215; Matsunami Glass, Ind., Ltd., Osaka, Japan). After deparaffinization and rehydration, all sections were pretreated by autoclaving for 15 min at 121°C in 0.01 M citrate buffer solution (pH 6.0), and endogenous peroxidase activity was quenched by immersion in 0.3% hydrogen peroxide diluted with methanol for 10 min. As primary antibodies, anti-PGP monoclonal antibody (C494, 1:200; DAKO Corporation, CA, U.S.A.), anti-MRP monoclonal antibody (m6, 1:50; Nichirei, Tokyo, Japan), anti-LRP monoclonal antibody (1014, 1:50; Santa Cruz Biotechnology, Inc., CA, U.S.A.), and anti-MT monoclonal antibody (E9, 1:25; DAKO) were used. The slides were incubated with anti-MT monoclonal antibody by microwaving for 30 min, and with anti-PGP, MRP and LRP monoclonal antibodies at 4°C overnight. The slides were incubated with EnVision polymer reagent (DAKO) by microwaving for 6 min with the anti-MT monoclonal antibody, and were incubated for 30 min at room temperature with anti-PGP, MRP and LRP monoclonal antibodies. All slides were incubated for 10 min in a solution of 0.075% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-HCl (pH 7.5) with 0.0225% hydrogen peroxide and counterstained with Mayer's hematoxylin. Normal proximal tubular epithelial cells of canine or feline kidney for anti-PGP, MRP and MT and normal bronchial epithelial cells of canine or feline lung for anti-LRP were used as positive controls. Tumor was considered to positive for the antibodies when more than 10% of the neoplastic population was positive, as reported previously [15].

The 52 canine primary pulmonary carcinomas were classified into 41 adenocarcinomas, 7 adenosquamous carcinomas, 2 squamous cell carcinomas and 2 combined carcinomas. The 18 feline primary pulmonary carcinomas were classified into 13 adenocarcinomas and 5 adenosquamous carcinomas. PGP expression was found in the cell membrane of adenocarcinoma and combined carcinoma, and in the cytoplasm of adenosquamous carcinoma and squamous cell carcinoma (Fig. 1). MRP and LRP expressions were found in the cytoplasm, and LRP expression was only located in tumor cells of the luminal structure in canine combined carcinoma (Figs. 2 and 3). MT expression was found in the cytoplasm only or both the cytoplasm and nucleus (Fig. 4). In canine primary pulmonary carcinomas, the ratio of the expressions of PGP, MRP, LRP and MT was 48/52 (92.3%), 50/52 (96.2%), 44/52 (84.6%), and 25/52 (48.1%), respectively (Table 1). In feline primary pulmonary carcinomas, the ratio of the expressions of PGP, MRP,

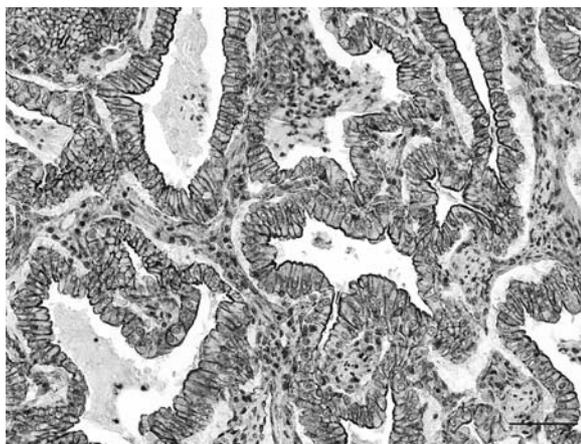


Fig. 1. Immunohistochemistry of neoplastic cells from feline adenocarcinoma using anti-PGP antibody. Neoplastic cells show a positive reaction in the cell membrane. Mayer's hematoxylin counterstaining. Bar=50  $\mu\text{m}$ .

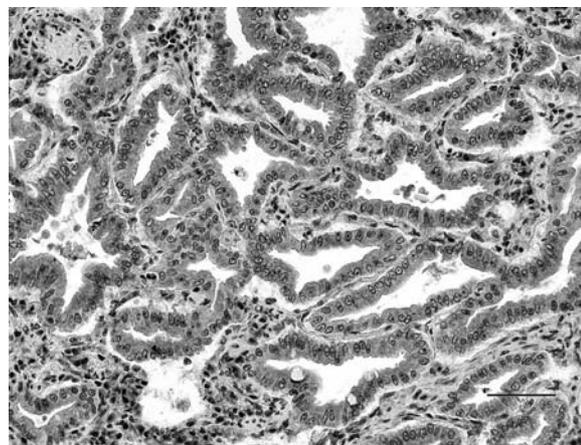


Fig. 2. Immunohistochemistry of neoplastic cells from feline adenocarcinoma using anti-MRP antibody. Neoplastic cells showing intracytoplasmic immunoreactivity. Mayer's hematoxylin counterstaining. Bar=50  $\mu\text{m}$ .

LRP and MT was 18/18 (100%), 18/18 (100%), 18/18 (100%), and 9/18 (50.0%), respectively (Table 2). In addition, more than two proteins were coexpressed in all positive cases. In both canine and feline primary pulmonary carcinoma, the expressions of PGP, MRP, LRP and MT were almost the same for each histopathological characteristic; thus, there was no correlation between their expression and the histopathological characteristics.

In human NSCLC, cisplatin-based chemotherapy (e.g. cisplatin and vinorelbine) is used for adjuvant chemotherapy as a standard protocol [13]. In contrast, there is no effective protocol of chemotherapy for primary pulmonary carcinoma in veterinary medicine [18]. Platinum-containing drugs, such as cisplatin and carboplatin, have been used to treat canine primary pulmonary carcinoma, but positive results were not achieved [19]. There is only one report of a

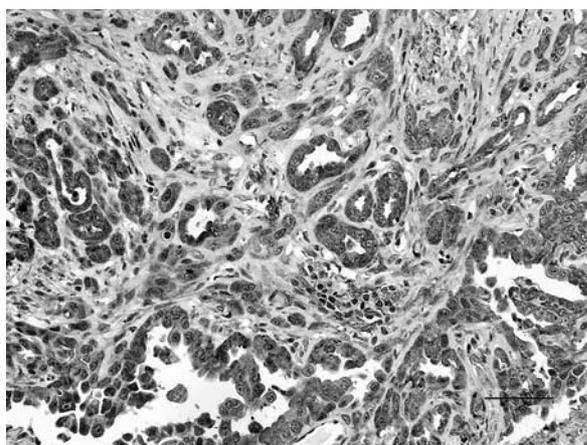


Fig. 3. Immunohistochemistry of neoplastic cells from canine adenocarcinoma using anti-LRP antibody. Neoplastic cells showing intracytoplasmic immunoreactivity. Mayer's hematoxylin counterstaining. Bar=50  $\mu$ m.

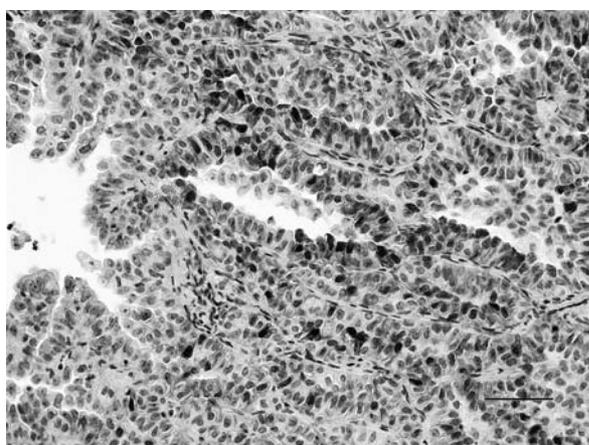


Fig. 4. Immunohistochemistry of neoplastic cells from canine adenocarcinoma using anti-MT antibody. Neoplastic cells show a positive reaction in both cell membrane and cytoplasm. Mayer's hematoxylin counterstaining. Bar=50  $\mu$ m.

cat with well-differentiated pulmonary adenocarcinoma surviving for more than 1040 days (>115 days; overall median survival time in cat with pulmonary carcinoma) with no evidence of metastatic disease by pneumonectomy and adjuvant mitoxantrone chemotherapy [1]. Considering these previous reports, chemotherapy for canine and feline primary pulmonary carcinoma may be less effective. In human pulmonary carcinoma, there is a report on immunohistochemistry investigating the correlation between the expression of proteins associated with multidrug resistance and the response to chemotherapy, which indicated that coexpressed proteins associated with multidrug resistance seemed to have a negative impact on the response to chemotherapy [17]. As a result of this study, in both canine and feline primary pulmonary carcinomas, PGP, MRP, LRP were expressed in most cases and MT was expressed in about 50% of cases, and more than two different proteins were coexpressed in all positive cases. Therefore, this result may raise the possibility of strong multidrug resistance in canine and feline primary pulmonary carcinomas. As it was uncertain whether patients received chemotherapy before surgical resection in this study, we could not detect whether multidrug resistance is instinctive or acquired.

In conclusion, there may be strong multidrug resistance without relation to histopathological characteristics in canine and feline primary pulmonary carcinomas, which may lead to inefficient chemotherapy. Further investigation of other multidrug resistance markers (e.g. glutathione S-transferase, topoisomerase II) in canine and feline primary pulmonary carcinomas and analysis of the correlation between the expression of proteins associated with multidrug resistance and the response to chemotherapy as clinical treatment is required.

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Table 1. Expression of proteins associated with multidrug resistance to anti-cancer drugs in canine primary pulmonary carcinomas

Protein	Overall [n=52] (%)	Adenocarcinoma [n=42] (%)	Adenosquamous carcinoma [n=7] (%)	Squamous cell carcinoma [n=2] (%)	Combined carcinoma [n=2] (%)
PGP	48 (92.3)	38 (92.3)	6 (85.7)	2 (100)	2 (100)
MRP	50 (96.2)	39 (95.1)	7 (100)	2 (100)	2 (100)
LRP	44 (84.6)	34 (82.9)	7 (100)	2 (100)	1 (50.0)
MT	25 (48.1)	18 (43.9)	5 (71.4)	1 (50.0)	1 (50.0)

Table 2. Expression of proteins associated with multidrug resistance to anti-cancer drugs in feline primary pulmonary carcinomas

Protein	Overall [n=18] (%)	Adenocarcinoma [n=13] (%)	Adenosquamous carcinoma [n=5] (%)
PGP	18 (100)	13 (100)	5 (100)
MRP	18 (100)	13 (100)	5 (100)
LRP	18 (100)	13 (100)	5 (100)
MT	9 (50.0)	6 (46.2)	3 (60.0)

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