FULL PAPER

Pathology

Immunohistochemical features of canine ovarian papillary adenocarcinoma and utility of cell block technique for detecting neoplastic cells in body cavity effusions

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ABSTRACT. Dogs with ovarian papillary adenocarcinoma occasionally present with ascites and/ or pleural effusion. These aspirated fluids often contain a large number of cells, and distinction between neoplastic cells and activated mesothelial cells can be difficult. In this study, 7 cases of canine ovarian papillary adenocarcinoma, including 3 with ascites and pleural effusion, were immunohistochemically examined. Ovarian tumor cells were positive for cytokeratin CAM5.2 (CAM5.2), Wilms' tumor 1 (WT-1) and progesterone receptor (PR) in all 7 cases. A metastatic lesion of the mediastinum in one case was also positive for CAM5.2, WT-1 and PR. Immunohistochemistry on cell blocks obtained from ascites and/or pleural effusion of 2 cases revealed the presence of PR-positive epithelial cells. Whereas, activated mesothelial cells in ascites or pleural effusion collected from dogs without neoplastic lesions were negative for PR. In addition, surface epithelium and subsurface epithelial structures (SES) of normal canine ovaries, that are considered to be the cell of origin for ovarian papillary adenocarcinoma, were also positive for CAM5.2, WT-1 and PR. These results indicate that, together with CAM5.2, WT-1 and PR is a useful diagnostic marker for canine ovarian papillary adenocarcinoma. Expression of PR may be associated with progesterone-dependent nature of canine ovarian papillary adenocarcinoma.

KEY WORDS: cell block, dog, ovarian papillary adenocarcinoma, progesterone receptor, Wilms' tumor 1

Canine ovarian papillary adenocarcinoma is considered to arise from the surface epithelium and subsurface epithelial structures (SES) which are modified mesothelium [1]. Histopathologically, tumor cells are arranged in papillary and glandular pattern, which is comparable to human serous carcinoma. Human serous carcinoma is characterized by immunopositivity for CAM5.2, cytokeratin 7, WT-1, estrogen receptor (ER) and Paired box gene 8 (PAX8) [17, 25]. Progesterone receptor (PR) is expressed in some cases. However, these markers have not been examined in canine ovarian papillary adenocarcinoma.

Dogs with ovarian papillary adenocarcinoma occasionally present with ascites and/or pleural effusions [4, 12–14, 21]. Aspirated fluid contains a large number of cells, that may consist of neoplastic epithelial cells, activated mesothelial cells and inflammatory cells. In addition, as is often the case with other malignant epithelial tumors of the visceral organs, morphological distinction between epithelial tumor cells and activated mesothelial cells can be difficult on cytological preparations. When human patients with carcinoma develop ascites and pleural effusions, the cell block technique is generally used to confirm tumor cells in aspirated fluids.

The aim of this study is to characterize the immunohistochemical features of canine ovarian papillary adenocarcinoma and compare with that of normal canine ovary and oviduct tissues. In addition, immunohistochemistry was performed on cell blocks obtained from ascites and pleural effusion of dogs with ovarian papillary adenocarcinoma and dogs without neoplastic diseases. Diagnostic markers and cell of origin for canine ovarian papillary adenocarcinoma are discussed together with that of human ovarian serous carcinoma.

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MATERIALS AND METHODS

Histology and cytology samples

Ovaries from 7 dogs with ovarian papillary adenocarcinomas (cases 1–7) were examined (Table 1). In one case (case 1), a mediastinal mass was also resected and examined together. Three cases (cases 1–3) presented with ascites and pleural effusions before surgery. Cytology and cell block specimens were prepared from aspirated fluids in 3 cases (cases 1–3) and 2 cases (cases 2 and 3), respectively. For control tissues (Table 2), cell block specimens were prepared from ascites or pleural effusions from 4 dogs without neoplastic lesions (cases 8–11). In 3 of the 7 tumor samples (cases 2, 4 and 6), the sections contained adjacent normal ovary and oviduct tissues. The ovaries and oviducts from these 3 cases and 3 dogs without neoplastic lesions (cases 12–14) were used for normal tissues (Table 2). All the samples were submitted to Shikoku Cytopathologic Laboratory or Veterinary Pathology Diagnostic Center between 2018 and 2021 for histopathological examination. The sizes of each formalin-fixed tumor were recorded by measuring the longest diameter.

Histopathology and immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were cut at a thickness of 4 μm for histopathological and immunohistochemical examinations. For histopathological examination, sections were stained with hematoxylin and eosin (HE). Diagnosis of ovarian papillary adenocarcinoma was based on characteristic morphologic features [1]. Mitotic index was defined as the number of mitoses per ten high-power fields (HPF; ×400, 0.237 mm^2) under a light microscope.

The primary antibodies used for immunohistochemistry are listed in Table 3. Immunohistochemical processing was performed using standard techniques on an automated immunohistochemistry stainer (Leica Bond-III, Leica Biosystems, Melbourne, Australia) with a polymer with diaminobenzidine (DAB) chromogen and hematoxylin (Bond Polymer Refine Detection kit, Leica Biosystems, Newcastle Upon Tyne, UK). For antigen retrieval, all sections were treated for 20 min using ER2 (Bond Epitope Retrieval Solution 2, Leica Biosystems). The canine uterus with no gross lesion was used as a control for PR and ER.

Evaluations of immunoreactivity

Cytoplasmic staining was judged as positive for CAM5.2; nuclear staining was judged as positive for WT-1, PR, ER and PAX8 under low-power magnification (×100). The percentage of positive cells on each specimen was scored as follows: +, <10% positive cells; ++, 10–70% positive cells; +++, >70% positive cells; and -, negative. In addition, the immunohistochemical positive rates of ovarian tumors (n=7), normal tissues (n=6), and cell blocks of body cavity fluid samples (n=4) were calculated.

Cytology

After centrifugation of the body cavity fluids, supernatant was discarded. A drop of the sediment was placed on a glass slide and a routine pull smear was made. Smears were dried and then stained with Wright-Giemsa stain.

Table 1. Clinical information of 7 dogs with ovarian papillary carcinoma

Case	Breed	Age (years)	Lesion site	Tumor size (cm)	Corpus luteum	Complications
1	Bernese Mountain Dog	11	Unilateral ovary	4.2	Absent	Histiocytic sarcoma, granulosa cell tumor, ovarian cystic carcinoma
2	Shih Tzu	5	Bilateral ovaries	2.2 and 2.0	Present	None
3	Papillon	13	Unilateral ovary	2.0	Present	None
4	Yorkshire Terrier	11	Unilateral ovary	4.0	Present	Benign mammary tumor
5	Shiba	11	Unilateral ovary	3.5	Absent	Pyometra
6	Miniature Dachshund	13	Bilateral ovaries	4.3 and 3.3	Present	Malignt and benign mammary tumors
7	Mix	14	Unilateral ovary	8.0	Absent	Pyometra, Malignant mammary tumor

Table 2. Clinical information of the control dogs

Case	Breed	Age (years)	Cell/tissue source	Present illness
8	Mix	11	Ascites	Heart failure
9	Pomeranian	9	Ascites	Pyometra
10	Pomeranian	11	Pleural effusion	Unknown
11	Golden Retriever	11	Pleural effusion	Unknown
12	Toy Poodle	9	Normal ovary and oviduct	Adenomyosis of uterus
13	Mix	10	Normal ovary and oviduct	Benign mammary tumor
14	Chihuahua	12	Normal ovary and oviduct	Uterine adenomyosis and endometrial hyperplasia

Cell blocks were prepared from effusion samples of cases 8–11.

Cell block technique

The 20 ml fluids were centrifuged at 1,000 rpm for 15 min and the supernatant was discarded. The sediments were fixed in 10% formalin; fixation period was within 3 days for cases 3 and 8–11, and 16 days for case 2. Subsequently, the sediments were embedded in paraffin blocks. The tissues were cut and stained by the same methods used for histopathology and immunohistochemistry.

RESULTS

Clinical and gross findings

Clinical information of the 7 dogs diagnosed with ovarian papillary adenocarcinoma (cases 1–7) is summarized in Table 1. The median age of the 7 dogs was 11 years (range: 5–14 years). The median tumor diameter was 3.5 cm (range: 2–8 cm). In case 1, 3 and 4, the tumors appeared multinodular enlargements. In other cases, the tumors demonstrated papillary growth and cauliflower-like appearances. These tumors sometimes included small and large cysts (cases 1,5 and 6). Cases 1–3 presented with ascites and pleural effusions at the time of surgery. These body cavity fluids resolved soon after ovariectomy. In case 2, ascites and pleural effusion reaccumulated 7 months after surgery. In case 3, the pleural effusion reaccumulated 4 months after surgery. Ascites and pleural effusions were not present at surgery in cases 4–7.

Histopathology

Histologically, tumor tissue showed glandular and/or papillary pattern. The glands were slit-like or irregular-shaped. The papillae were irregularly branching. There were frequent papillary structures with narrow stromal core and micropapillary growth pattern in cases 1, 2, 3, 6 and 7 (Table 4 and Fig. 1). Tumors in case 4 and 5 were mainly composed of large stromal core covered by tumor cells (Table 4 and Fig. 2). The tumor cells had a small amount of cytoplasm, round to oval nuclei with mild or moderate atypia. Mitotic index was low in all cases (range: 1–4/10HPFs). In case 2, the tumor cells infiltrated the lymphatic vessels and the oviduct.

In the mediastinal mass of case 1, tumor tissue infiltrated the lymph node. The tumor cells were morphologically similar to the tumor cells in the ovary, and were arranged in glandular or micropapillary pattern.

Table 3. List of primary antibody used for immunohistochemistry

Antibody	Host	Source	Clone	Dilution
CAM5.2	Mouse	Becton Dickinson, Franklin Lakes, NJ, USA	CAM5.2	RTU
WT-1	Mouse	Leica Biosystems, Newcastle Upon Tyne, UK	WT49	1:80
PR	Mouse	Roche, Basel, Switzerland	1E2	RTU
ER	Mouse	Leica Biosystems	6F11	1:120
PAX8	Mouse	Roche	MRQ-50	RTU

CAM5.2, cytokeratin CAM5.2; WT-1, Wilms' tumor 1; PR, progesterone receptor; ER, estrogen receptor; PAX8, Paired box gene 8; RTU, ready to use.

Table 4. Immunohistochemical and histopathological results of ovarian papillary carcinoma cases

Case	Neoplastic cells from	Immunoreactivity for					Histopathology	
		CAM5.2	WT-1	PR	ER	PAX8	Mitotic count	Histological pattern
1	Ovary	+++	+++	+++	-	-	1	N
	Mediastinum	+++	+++	+++	-	-	0	M
2	Ovary	+++	+++	++	+	+	4	N
	Pleural effusion	+++	-	++	-	-	0	M
	Ascites	+++	-	++	-	++	0	M
3	Ovary	+++	+++	++	++	-	1	N
	Pleural effusion (recurrence)	+++	+++	++	-	-	7	M
4	Ovary	+++	+++	+++	+	-	1	L
5	Ovary	+++	+++	++	-	-	0	L
6	Ovary	+++	+++	++	-	-	2	N
7	Ovary	++	++	+++	-	-	4	N

CAM5.2, cytokeratin CAM5.2; WT-1, Wilms' tumor 1; PR, progesterone receptor; ER, estrogen receptor; PAX8, Paired box gene 8; reccurence,this pleural effusion recurrenced 4 months after surgery; N, papillary structures with narrow stromal core and micropapillary growth; L, papillary structures with large stromal core; M, micropapillary or ball-shaped.; +, <10% positive cells; ++, 10–70% positive cells; +++, >70% positive cells; -, negative.

Cytology and cell blocks of the body cavity effusions

On cytology specimens of body cavity effusions from cases 1–3, a large number of papillary, glandular and spherical clusters of variable sizes were observed (Fig. 3). The clusters were several cells thick with some nuclear overlapping. The cytological features of individual cells were increased nuclear to cytoplasmic ratio, round to oval nuclei in uniform size with occasionally prominent nucleoli. The nuclei arranged radially. Few mitotic figures were observed. In effusion cytology, reactive mesothelial cells also form small or large spherical clusters. They have basophilic cytoplasm and central round nuclei. However, these reactive mesothelial cells with high nuclear to cytoplasmic ratio may be sometimes confused with malignant cells.

The effusion cell blocks of cases 2 and 3 contained many epithelial-like cells with mild to moderate atypia forming round-shaped and micropapillary clusters (Fig. 4). Most of the nuclei were unevenly distributed.

Immunohistochemistry

Detailed immunohistochemical results of ovarian papillary adenocarcinoma are summarized in Table 4. The tumor cells in all cases were positive for CAM5.2, WT-1 and PR (Fig. 5). In case 1, the tumor cells in the mediastinum were positive for CAM5.2, WT-1 and PR. Immunoreactivity for ER was positive in cases 2, 3 and 4; PAX8 was positive only in case 2.

Epithelial-like cells in cell block of body cavity effusion were positive for CAM5.2 (Fig. 5). On cell block of case 2, these cells were negative for WT-1 and some were positive for PR. In pleural effusion of case 3, the majority of epithelial-like cells were positive for WT-1 and some were positive for PR. On cell block of body cavity effusions from dogs without ovarian tumors, mesothelial cells were positive for CAM5.2 and WT-1, and negative for PR (Table 5 and Fig. 6).

The results of immunohistochemical examinations on normal ovaries, oviducts and mesothelium (cases 2, 4, 6, 12, 13 and 14) are summarized in Table 6. CAM5.2 expression was observed in ovarian surface epithelium, SES, oviduct epithelium and mesothelium (100%). Immunoreactivity for WT-1 was observed in SES and surface epithelium (100%); oviduct and mesothelium (83.3%); and granulosa cell and corpus luteum (50%). Immunoreactivity for PR was observed in ovarian surface epithelium and SES (100%); oviductal epithelium (66.7%); and theca cell (50%). The intensity of immunoreactivity for PR was strong in SES and weak in surface epithelium in the same case. Immunohistochemical positive rate of ER was low, and the intensity was weak to moderate in the ovaries and oviducts. Immunoreactivity for PAX8 was observed in oviductal epithelium (100%); and SES (66.7%).

DISCUSSION

The present study revealed that the tumor cells in ovarian papillary adenocarcinoma were positive for CAM5.2, WT-1 and PR, suggesting the diagnostic utility of these molecules. The surface epithelium and SES, which were supposed to be the origin of ovarian papillary adenocarcinoma, were also positive for these molecules. Canine papillary adenocarcinoma is histologically similar to human serous carcinoma. Human serous carcinoma is commonly immunopositive for CAM5.2, WT-1, PAX8 and ER [17, 25]. Also, about 60% of low-grade serous carcinoma cases and 30% of high-grade serous carcinoma cases are positive for PR [23]. In canine ovarian papillary adenocarcinoma cases, PAX8 and ER positivity were low. The present study shows that canine ovarian papillary adenocarcinoma and human serous carcinoma share some immunohistochemical characteristics, such as CAM5.2 and WT-1 positivities, although immunoreactivity to PAX8 and ER were different.

Histopathologically and cytologically, it was difficult to differentiate adenocarcinoma cells from reactive or neoplastic mesothelial cells in the body cavity effusion. Therefore, cell blocks were used to immunohistochemically characterize the cells collected from body cavity effusions. In human medicine, the cell block technique is a generalized method utilized for diagnostic evaluation of body cavity effusions. The advantage of cell block specimens is that it allows to prepare multiple specimens and perform immunohistochemistry using different antibodies on consecutive sections. Expression of CAM5.2 and WT-1 were detected in both adenocarcinoma cells and mesothelial cells. However, expression of PR was limited to adenocarcinoma cells. The results suggest that combination of CAM5.2, WT-1, and PR immunohistochemistry on cell blocks is useful for differentiating mesothelial cells from ovarian papillary adenocarcinoma cells in body cavity fluids of dogs.

Although accurate evaluation of immunohistochemical staining intensity was difficult because of inconsistent fixation conditions, the staining intensity of exudative cells tended to be weak in comparison to primary tumors and original cells. Moreover, the cells collected from body cavity effusion in case 2 were negative for WT-1 but positive for PR. This fluid sediments were fixed in formalin for 16 days and then embedded in paraffin. Therefore, it is possible that prolonged fixation may have caused reduction of WT-1-positive cells. Previous studies have shown that prolonged formalin fixation results in decreased antigenicity [3, 29]. Delayed fixation is a problem for increased proteolytic and nucleic acid degeneration in clinical tissue samples [11]. We compared WT-1 immunohistochemistry in 2 normal ovaries and oviducts in various durations of formalin fixation in order to examine the effect of prolonged fixation. A decrease in number of WT-1-positive epithelial cells was observed in 16-days-long to 5-months-long fixation period (Data not shown). Prolonged fixation might lead to a reduction in WT-1 immunoreactivity.

In the present study, 3 cases of ovarian papillary adenocarcinoma presented with ascites and pleural effusion, which resolved after ovariectomy. Similar conditions were also reported in previous canine cases of ovarian papillary adenocarcinoma [12–14, 21]. The phenomena are thought to be associated with Meigs or pseudo-Meigs syndrome in veterinary medicine. In 1937, Meigs and Cass [20] reported a series of seven cases of ovarian fibroma associated with ascites and pleural effusion. It was later termed Meigs syndrome by Rhoades and Terrell [22]. The following criteria are to be met for the diagnosis of Meigs syndrome: 1) presence of benign ovarian tumor, 2) ascites, 3) pleural effusion, 4) resolution of ascites and pleural effusion after removal of the tumor [18]. Besides, pseudo-Meigs syndrome is a similar condition associated with other types of tumors [19]. However, the mechanisms
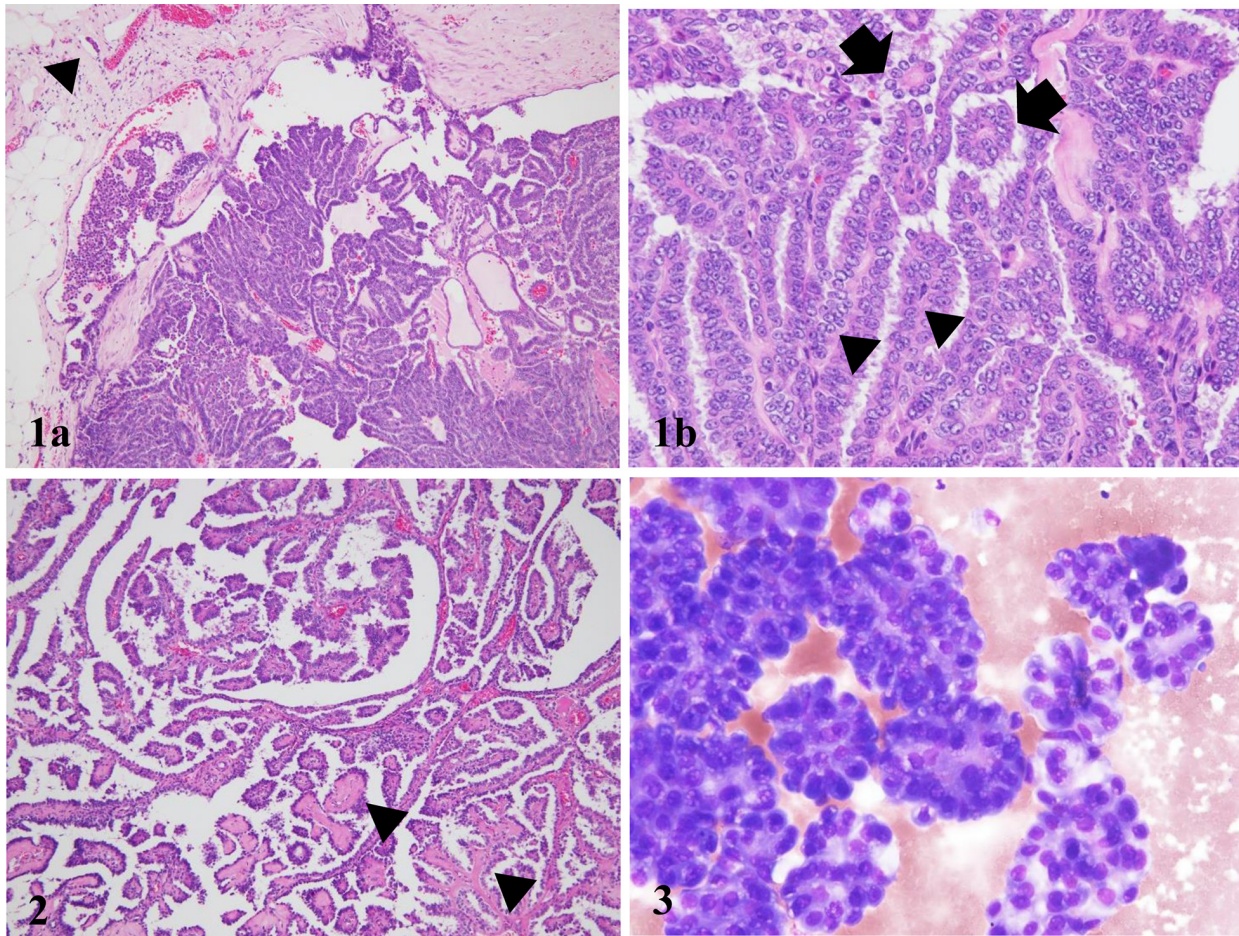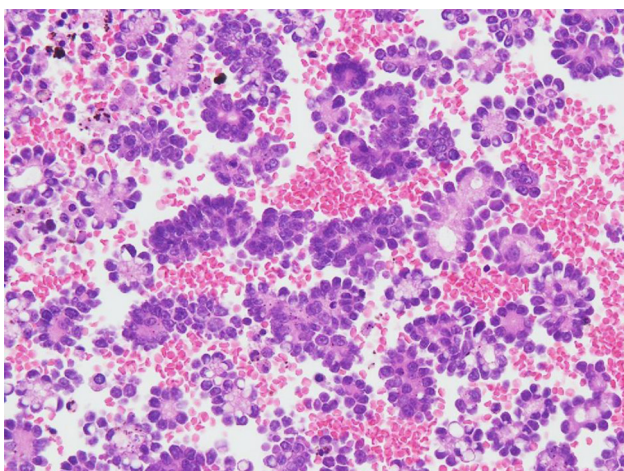
Fig. 1. Histological features of papillary adenocarcinoma (case 2). **a**) Papillary structures with narrow stromal core, micropapillary growth pattern and vascular infiltration (arrowhead). Hematoxylin eosin (HE). ×100. **b**) Tumor cells have small amount of cytoplasm and round to oval nuclei. Papillary structures with narrow stromal core (arrowheads) and micropapillary growth pattern (arrows). HE. ×400.

Fig. 2. Histological features of papillary adenocarcinoma without body cavity effusion (case 4). Papillary structures with large stromal core (arrowheads). Hematoxylin eosin. ×100.

Fig. 3. Cytological features of the epithelial-like cells in pleural effusion (case 1). Round-shaped clusters and large cohesive clusters with papillary configuration are observed. These clusters compose of atypical cells with high nuclear to cytoplasmic ratio. Wright-Giemsa. ×400.

Table 5. Immunocytochemical results of mesothelial cells in body cavity effusion

Case	Cell source	Immunoreactivity for		
		CAM5.2	WT-1	PR
8	Ascites	+++	+	-
9	Ascites	+++	+	-
10	Pleural effusion	+++	++	-
11	Pleural effusion	+++	++	-

CAM5.2, cytokeratin CAM5.2; WT-1, Wilms' tumor 1; PR, progesterone receptor; +, <10% positive cells; ++, 10–70% positive cells; +++, >70% positive cells; -, negative.
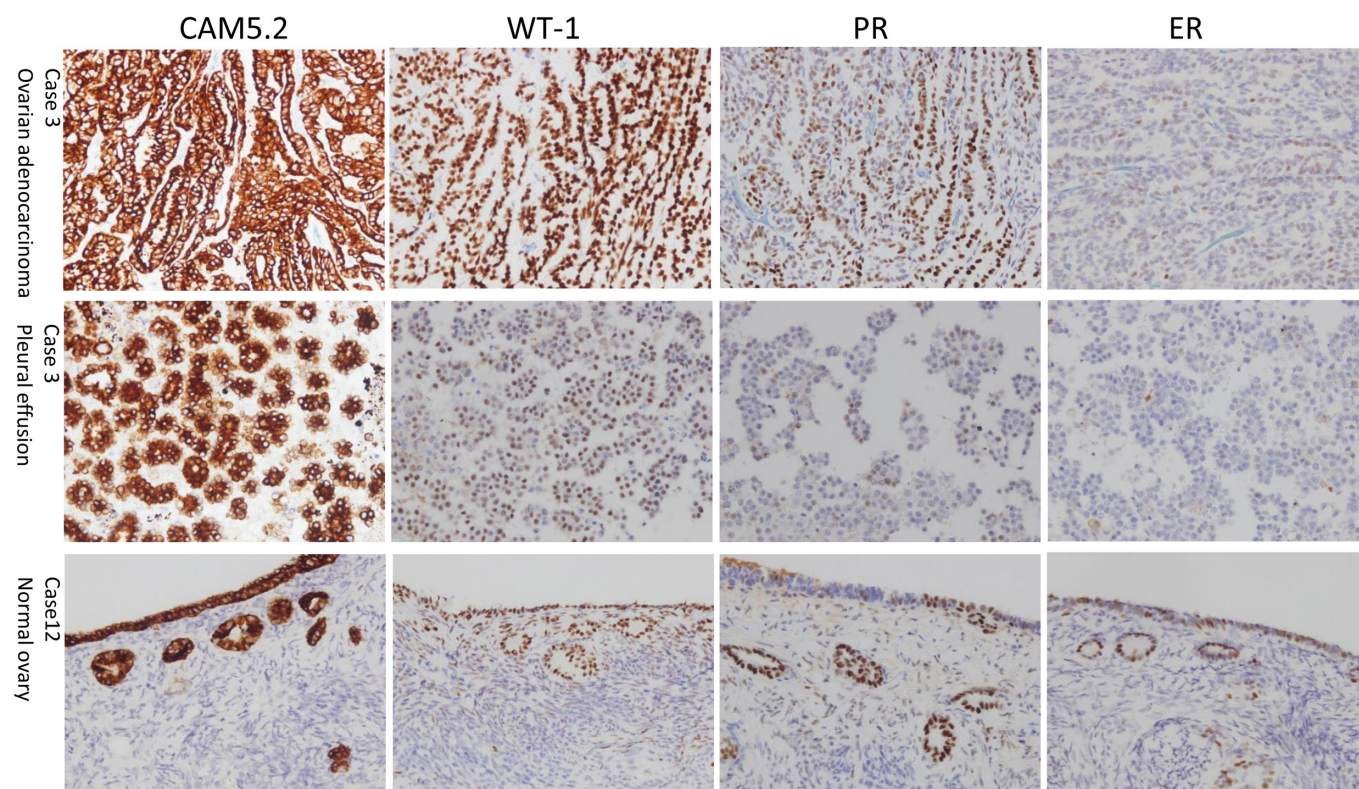
Fig. 4. Histological features of the epithelial-like cells on cell block of pleural effusion (case 2). A large number of round-shaped and micropapillary clusters are observed. These clusters consist of epithelial-like cells with mild or moderate atypia. Most of the nuclei are unevenly distributed. Hematoxylin eosin. ×400.
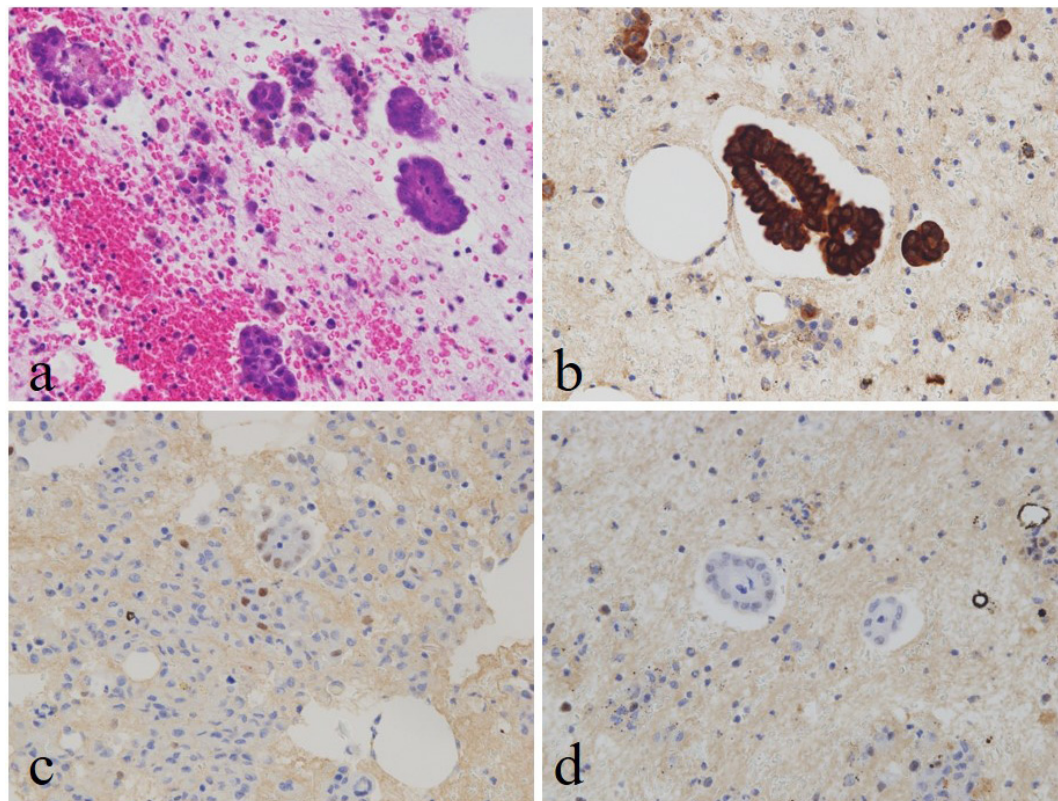
Fig. 5. Representative images of immunohistochemical staining for cytokeratin CAM5.2 (CAM5.2), Wilms' tumor 1 (WT-1), progesterone receptor (PR) and estrogen receptor (ER). ×400. The majority of neoplastic cells are positive for CAM5.2, WT-1 and PR. Some epithelial-like cells in effusion are positive for PR. Few neoplastic cells are positive for ER, and epithelial-like cells in effusion are negative for ER. Ovarian surface epithelium and subsurface epithelial structures (SES) are positive for CAM5.2, WT-1, PR and ER. The staining intensity for PR is strong in SES and weak in surface epithelium.

of how the ascites and pleural effusion develop in these conditions are poorly understood. From the immunohistochemical examination of this study, the epithelial-like cells in body cavity effusions and mediastinal mass appeared to be the malignant cells of ovarian papillary adenocarcinomas. Therefore, it is considered that the dogs suffering from ovarian papillary adenocarcinoma with malignant effusions have specific clinical symptoms different from Meigs or pseudo-Meigs syndrome.

Canine ovarian papillary adenocarcinomas were consistently positive for PR. ER and PR are members of the nuclear receptor superfamily of transcription factors that mediate the physiological effects of steroid hormones [8]. Estrogen and progesterone are essential for the development and cyclical regulation of hormone-responsive tissues including the breast and reproductive tract. They play roles in the development and control of animal and human tumors arising in their target organs, for example, the mammary glands of dogs and cats [10], the breast and uterus in human [7]. The role of progesterone in ovarian cancer is not well understood; both proliferative and inhibitory actions of progesterone have been reported in human [6]. A higher PR status correlated with increased survival in the cases of human epithelial ovarian carcinoma [2, 23, 24, 28] Several independent *in vitro* studies demonstrated anti-proliferative actions of progesterone at higher concentrations (≥1 µM) in ovarian cancer cells [5, 15, 26], while fewer studies reported progesterone as proliferative in these cells at lower concentrations [9, 27]. Additionally the recent study in mice model of high-grade serous carcinoma suggests that progesterone helps to drive the development of metastatic ovarian cancer, and blocking progesterone signaling may be a useful strategy for preventing this [16].

PR expression in neoplastic cells and resolution of body cavity effusion after ovariectomy suggest that progesterone secreted from the ovaries may contribute to proliferation of ovarian papillary adenocarcinoma in dogs. Progesterone is a steroid hormone that is produced primarily by the corpus luteum in the ovaries. It is also produced, to a lesser extent, in the adrenal glands and the placenta. Therefore, ovariectomy may be a beneficial treatment for dogs with ovarian papillary carcinoma. Expression of hormonal receptors predicts response to anti-progesterone therapy. The treatment with PR antagonist such as aglepristone (RU534) used as an abortifacient in pregnant animals, may be effective. Further investigations are required to clarify the effect of progesterone and the possible role of PR in the development and progression of canine ovarian papillary adenocarcinoma.

Fig. 6. Mesothelial cells in pleural effusion in case 11. **a**) Histological features of mesothelial cells. A few and small round-shaped clusters are observed. Each mesothelial cell is similar to epithelial cell. HE. ×400. Representative images for canine mesothelial cells of immunohistochemical staining for cytokeratin CAM5.2 (CAM5.2), Wilms' tumor 1 (WT-1) and progesterone receptor (PR). **b**) Mesothelial cells are positive for CAM5.2. ×400. **c**) Mesothelial cells are positive for WT-1. ×400. **d**) Mesothelial cells are negative for PR. The staining intensity of mesothelial cells is equivalent to that of background. ×400.

Table 6. Immunohistochemical results in ovarian papillary adenocarcinoma and normal tissues

Cell type	No. of cases	% (No. of cases) Immunoreactivity for				
		CAM5.2	WT-1	PR	ER	PAX8
Ovarian papillary adenocarcinoma	7	100 (7)	100 (7)	100 (7)	42.9 (3)	14.3 (1)
Surface epithelium	6	100 (6)	100 (6)	100 (6)	16.7 (1)	0 (0)
SES	6	100 (6)	100 (6)	100 (6)	16.7 (1)	66.7 (4)
Oviductal epithelium	6	100 (6)	83.3 (5)	66.7 (4)	16.7 (1)	100 (6)
Mesothelium	6	100 (6)	83.3 (5)	0 (0)	0 (0)	0 (0)
Mesothelium in body cavity effusion	4	100 (4)	100 (4)	0 (0)	ND	ND
Granulosa cell	6	0 (0)	50 (3)	0 (0)	0 (0)	0 (0)
Theca cell	6	0 (0)	0 (0)	50 (3)	0 (0)	0 (0)
Corpus luteum	6	0 (0)	50 (3)	0 (0)	16.7 (1)	0 (0)

CAM5.2, cytokeratin CAM5.2; WT-1, Wilms' tumor 1; PR, progesterone receptor; ER, estrogen receptor; PAX8, Paired box gene 8; SES, subsurface epithelial structure; ND, not done.

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