

Expression of Ccdc85C, a causative protein for murine hydrocephalus, in the mammary gland tumors of dogs

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Summary. Coiled-coil domain containing 85c (*Ccdc85c*) is a causative gene for hemorrhagic hydrocephalus mouse which shows hydrocephalus with frequent brain hemorrhage and formation of subcortical band heterotopia. A previous study revealed that Ccdc85C protein is expressed in the systemic simple epithelial cells with proliferative activity in rats and suggested that Ccdc85C expression may be related to the cell proliferation of simple epithelial cells. To reveal the roles of Ccdc85C in the proliferative lesion, we examined the expression patterns of Ccdc85C in the mammary gland tumor of dogs, a common representative tumor derived from simple epithelial cells. In canine mammary gland tumors, Ccdc85C was expressed at the apical junctions of the luminal epithelial cells. Ccdc85C was also distributed throughout the entire cytoplasm of the myoepithelial cells. Ccdc85C expression was observed at the epithelial cells with luminal structures, but was not observed at the epithelial cells forming sheet growth pattern without luminal structure. In carcinomas, Ccdc85C expression in mammary tumor tissue tended to be weaker than that in surrounding normal mammary gland tissue. Ccdc85C is known to cause neurological diseases such as hydrocephalus, and subcortical heterotopia, and the present study is the first to demonstrate Ccdc85C expression in canine mammary tumors and a relationship between Ccdc85C expression and tumor malignancy.

Key words: Apical junction, Ccdc85C, Tumor, Malignancy, Dog

Introduction

Hemorrhagic hydrocephalus (*hhy*) mouse is a spontaneous mutant with hemorrhagic hydrocephalus and formation of subcortical band heterotopia (Kuwamura et al., 2004). A previous study demonstrated that hydrocephalus is caused by an ependymal agenesis and the subcortical heterotopia is due to an abnormal migration of neurons in *hhy* mice (Mori et al., 2012). Coiled-coil domain-containing 85C (*Ccdc85c*) on mouse chromosome 12 is a causative gene for *hhy* mice and encodes 420 amino acid Ccdc85C protein. In *hhy* mice, exon 2-containing region of *Ccdc85c* was replaced by a 1.5-kb retrotransposon-like sequence, which resulted in frame shifts of Ccdc85c starting at the exon 1-3 junction (Mori et al., 2012).

Our previous study demonstrated that Ccdc85C protein is expressed in fetal and neonatal systemic simple epithelial cells (Tanaka et al., 2015). We also clarified that Ccdc85C expression becomes weak with the advancing development of most organs, but Ccdc85C keeps strong expression at the intestine from fetus to adult. Taken together, we suggested that Ccdc85C plays an important role in the proliferative property of simple epithelial cells. A recent study also revealed a relationship between CCDC85C and Yes-associated protein 1 (YAP1) in humans (Wang et al., 2014). YAP1 is a key effector of the Hippo pathway that controls organ size and is known to play important roles in tumorigenesis (Barron et al., 2014). In light of the

results of these studies, we hypothesized that Ccdc85C may have some roles in neoplastic tissue in addition to normal proliferating cells.

In this study, we examined the Ccdc85C protein expression patterns in tumors to clarify the function of Ccdc85C in the tumor. Because Ccdc85C is expressed in the simple epithelial cells of normal tissue, we examined mammary gland tumors of dogs as a common, representative epithelial tumor. We clarified that Ccdc85C was expressed in the luminal epithelial cells and myoepithelial cells in canine mammary tumors and Ccdc85C expression was related to the malignancy of the mammary tumors.

Materials and methods

Tumor samples

Spontaneous mammary gland tumors in dogs were collected from private veterinary hospitals and the Veterinary Medical Center at Osaka Prefecture University. Prophylactically removed surrounding normal mammary gland tissues were also sampled. Half of each sample was frozen in TISSU MOUNT® (Chiba medical, Japan) at -80°C, and the remaining half of each sample was fixed in 10% neutral buffered formalin overnight. The formalin fixed tissues were processed routinely, embedded in paraffin wax.

Histopathological diagnoses

Hematoxylin and eosin (HE)-stained sections were made from formalin-fixed paraffin-embedded samples of canine tumors. The mammary gland tumors were then diagnosed histopathologically by board-certified pathologists. The canine mammary gland tumors were diagnosed according to the WHO classification of dog and cat mammary tumors (Misdorp et al., 1999). Information about the cases and the diagnoses is provided in Table 1.

Immunohistochemistry

Immunofluorescence was performed as previously described (Tanaka et al., 2015). Sections were incubated with rabbit polyclonal antibody against rat Ccdc85C made by ourselves (1:10,000; Tanaka et al., 2015), mouse monoclonal antibodies against E-cadherin (1:500; BD Biosciences, USA) or alpha-smooth muscle actin (α -SMA; 1:1000, Dako, Denmark) overnight at 4°C. Signals were scanned with a virtual slide system (VS1-20-S5, Olympus, Japan) and a confocal imaging system (C1Si; Nikon, Japan).

Comparison of the expression intensity of Ccdc85C between normal and mammary gland tumor tissues

We examined the Ccdc85C expression in the luminal epithelial cells in each canine mammary gland tumors by

evaluating the double immunofluorescence for Ccdc85C and E-cadherin. The tumors were then classified into the following three groups: *High*, the neoplastic cells intensely expressed Ccdc85C at a higher level compared to normal mammary gland tissues; *Equal*, the Ccdc85C expression in neoplastic cells had almost the same intensity as that in normal mammary gland tissues; *Low*, the neoplastic cells expressed less Ccdc85C than normal mammary gland tissues. The number of cases in each classified group was counted.

Results

Ccdc85C protein is expressed in mammary gland tumors of dogs

Eight complex carcinomas, one simple carcinoma, six complex adenomas and three simple adenomas were diagnosed in dogs (Table 1). The double immunofluorescence results for Ccdc85C and E-cadherin demonstrated that Ccdc85C was expressed at apical junctions in the neoplastic luminal epithelial cells (Fig. 1A-C). Ccdc85C expression was also detected in the luminal epithelial cells in the surrounding normal mammary glands (Fig. 1D-F).

In addition to luminal epithelial cells, some of the α -SMA-positive myoepithelial cells expressed Ccdc85C protein (Fig. 2). Ccdc85C expressing myoepithelial cells were observed in both normal and neoplastic mammary glands (Fig. 2A-F, arrowheads). In myoepithelial cells, Ccdc85C protein was diffusely distributed throughout the entire cytoplasm (Fig. 2A-F, arrowheads). Intense Ccdc85C expression was observed in the normal and neoplastic myoepithelial cells surrounding the luminal epithelial cells (Fig. 2A-C). In contrast, weak expression was seen at the neoplastic myoepithelial foci in the

Table 1. Information and diagnoses of canine mammary gland tumor cases.

Dog No.	Breed	Age	Diagnosis
1	Border collie	11y2m	complex carcinoma
2	Siberian husky	12y7m	complex carcinoma
3	Beagle	12y	complex carcinoma
4	Papillon	9y9m	complex adenoma
5	Miniature dachshund	11y7m	complex carcinoma
6	Toy poodle	7y9m	complex adenoma
7	Shiba Inu	15y11m	simple carcinoma
8	Chihuahua	12y4m	simple adenoma
9	Chihuahua	10y2m	complex carcinoma
10	Mix	7y	complex carcinoma
11	Miniature dachshund	9y11m	complex adenoma
12	Miniature dachshund	7y	complex adenoma
13	Chihuahua	12y8m	simple adenoma
14	Miniature dachshund	11y10m	simple adenoma
15	Shih Tzu	16y8m	complex carcinoma
16	Boston terrier	9y10m	complex adenoma
17	Miniature dachshund	9y10m	complex adenoma
18	Cavalier	10y11m	complex carcinoma

Ccdc85C expression in canine mammary tumors

canine complex tumors (Fig. 2D-F).

Ccdc85C expression patterns in the neoplastic cells

In the mammary gland tumors, neoplastic luminal epithelial cells showed papillary, tubular, and sheet growth patterns. Ccdc85C-expressing luminal epithelial cells formed a lumen with papillary and tubular growth patterns (Fig. 3A-D). A part of the luminal epithelial cells forming a lumen did not express Ccdc85C (Fig. 3A-C,E). Ccdc85C expression was not observed in the luminal epithelial cells which lacked a luminal structure and showed the sheet growth pattern (Fig. 3A-C,F).

Relationship between Ccdc85C expression and mammary gland tumor malignancy

To clarify the relationship between Ccdc85C expression and mammary gland tumor malignancy, we

compared the expression intensity of Ccdc85C between mammary gland tumors and normal mammary glands. The results of the comparison of Ccdc85C expression intensity in each tumor are shown in Table 2. In the carcinomas, the number of cases classified as *High* was almost the same as that classified as *Low* (Fig. 4A). In contrast, among the adenomas, most cases (seven of nine) were judged as *High* and only one case was classified as *Low* (Fig. 4B).

Discussion

Ccdc85C in dogs

Immunofluorescence using the anti-rat Ccdc85C antibody produced by ourselves showed signals in mammary glands and mammary gland tumors of dogs. The signals were observed at apical junctions of the luminal epithelial cells. Based on these results, we

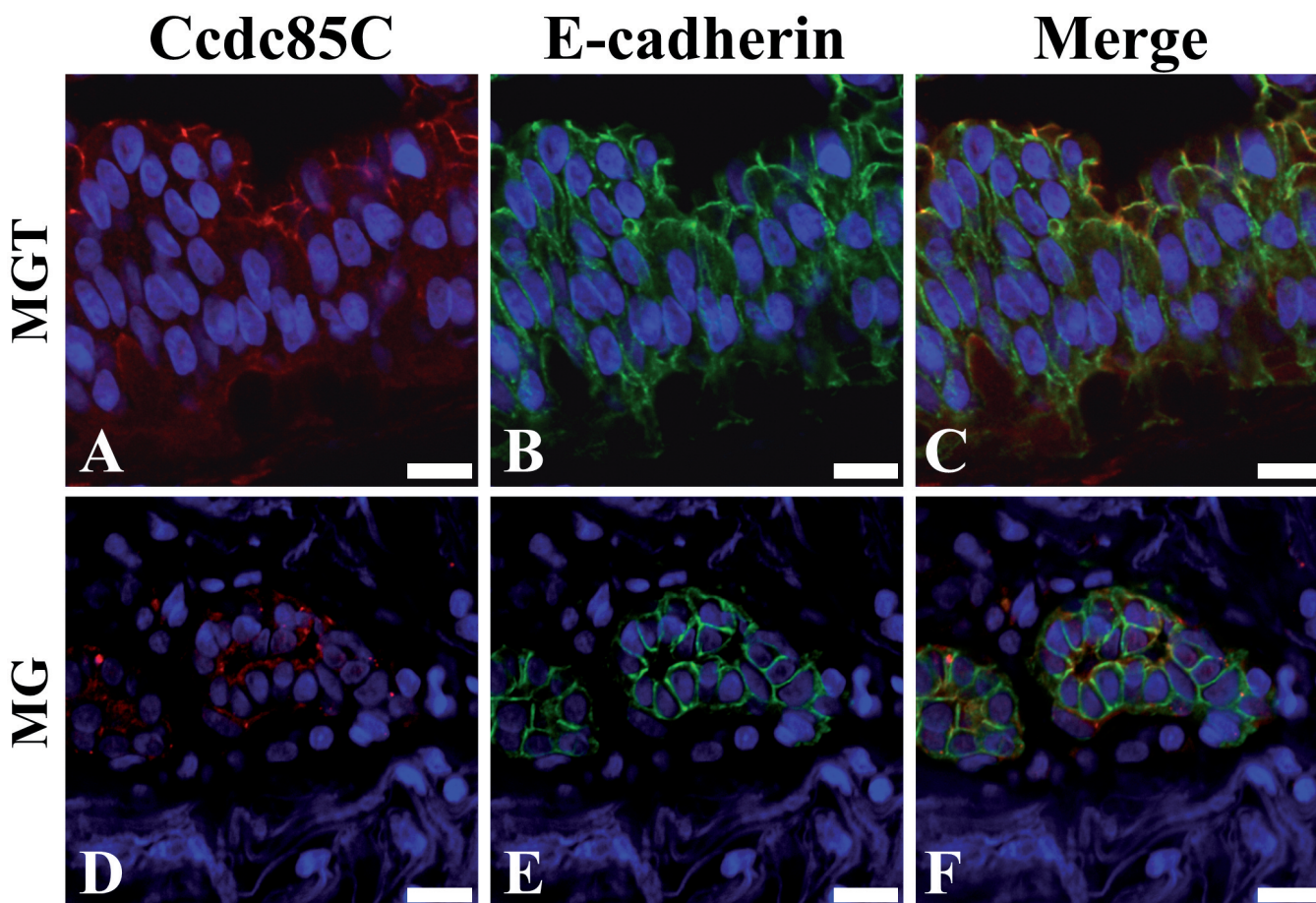


Fig. 1. Ccdc85C expression in the neoplastic luminal epithelial cells of canine mammary gland. Double immunohistochemistry for Ccdc85C and E-cadherin to canine mammary gland tumors (A-C, dog No. 8, simple adenoma) and surrounding normal mammary gland of same dog (D-F, dog No. 8, simple adenoma). Ccdc85C is expressed at the apical junctions of the luminal epithelial cells in both normal mammary gland and mammary gland tumor of dogs. MG, normal mammary gland; MGT, mammary gland tumor. Bars: 20 μ m.

concluded that the Ccdc85C antibody cross-reacts to Ccdc85C in dogs. A study of Ccdc85C in dogs has not been reported prior to the present study, and the antibody that we developed would be useful for further examination of Ccdc85C in dogs.

The present findings revealed that in the luminal epithelial cells of canine normal and neoplastic mammary glands, the expression patterns were similar to those in normal rat epithelial cells. An alignment search for the rat Ccdc85C protein using the Basic Local Alignment Search Tool (BLAST) on the website of the U.S. National Center for Biotechnology Information (NCBI) revealed that the Ccdc85C proteins of dogs have relatively high homology to rat Ccdc85C (identities in protein: 90%). These results suggest that the Ccdc85C in the luminal epithelial cells of mammary glands of dogs has biological characteristics that are similar to those of rats.

Ccdc85C expression in myoepithelial cells

Ccdc85C was expressed in the myoepithelial cells of dogs, and the expression pattern of Ccdc85C in the myoepithelial cells was different from those of luminal epithelial cells; i.e., the Ccdc85C was diffusely distributed in the myoepithelial cytoplasm. Myoepithelial cells surround the luminal epithelial cells and contribute to the ejection of milk (Emerman and Vogl, 1986). Myoepithelial cells have epithelial characteristics such as the expression of epithelial keratins and the presence of cell-cell junctions. Myoepithelial cells also have characteristics similar to those of smooth muscle cells such as the expression of α -SMA (Adriance et al., 2005). Thus, the unique expression pattern of Ccdc85C in the myoepithelial cells may be related to the function of myoepithelial cells.

In this study, Ccdc85C expression in the

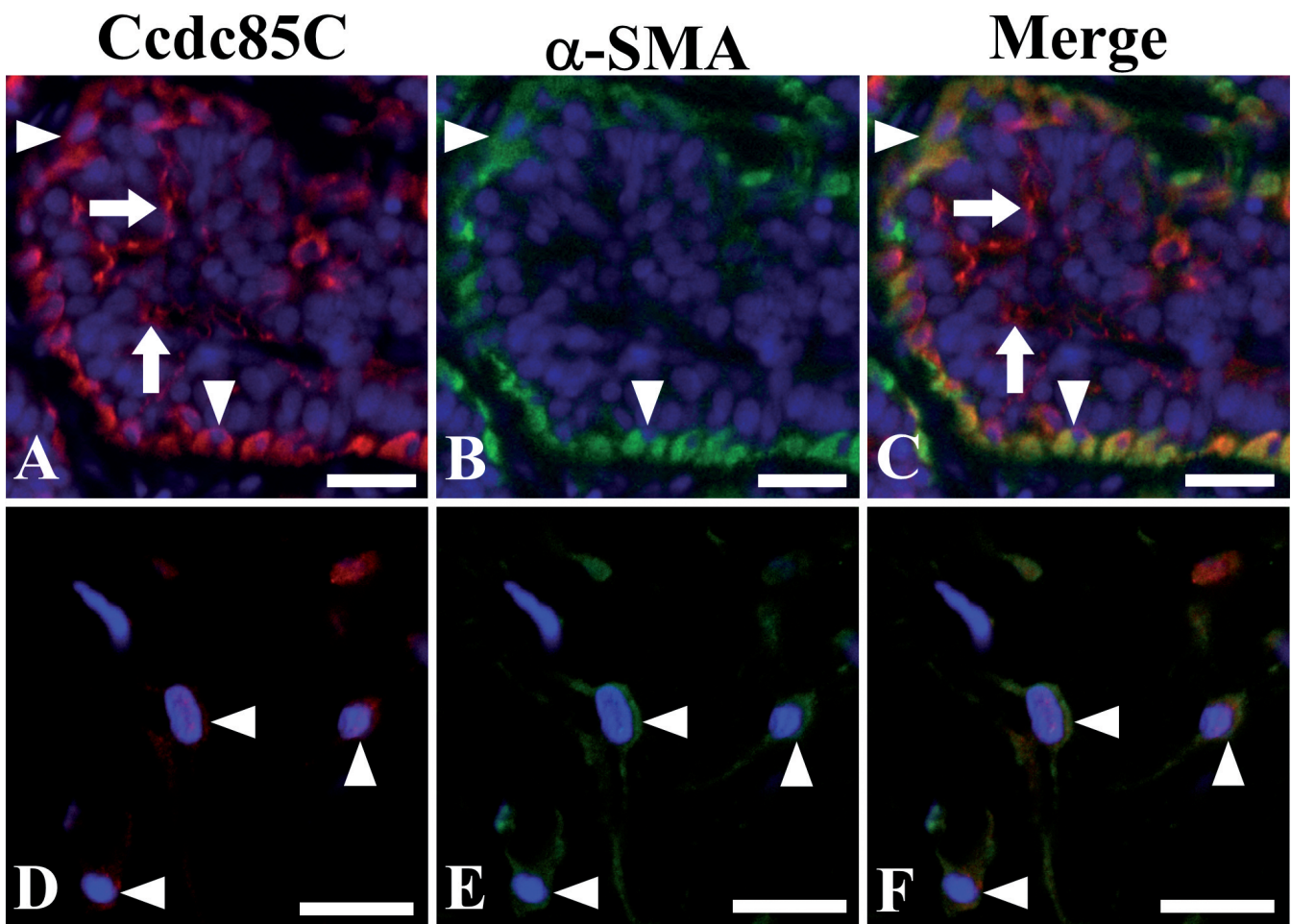


Fig. 2. Ccdc85C expression in the myoepithelial cells in dogs. Double immunohistochemistry for Ccdc85C and α -SMA to mammary gland tumors of dog (A-C, dog No. 13, simple adenoma; D-F, dog No. 15, complex carcinoma). α -SMA positive canine myoepithelial cells diffusely express Ccdc85C in their cytoplasm (A-F, arrowheads). Canine myoepithelial cells in myoepithelial foci in the complex tumors weakly express Ccdc85C (D-F, arrowheads). Arrows indicate Ccdc85C expression in the luminal epithelial cells. Bars: 20 μ m.

myoepithelial cells was not observed in all myoepithelial cells. Moreover, myoepithelial cells intensely expressing Ccdc85C were adjacent to luminal epithelial cells and Ccdc85C was weakly detected at the myoepithelial foci in the canine complex tumors. These results indicate that

the expression of Ccdc85C was not constitutive in the myoepithelial cells and may be associated with an interaction between myoepithelial cells and luminal epithelial cells.

In addition, Ccdc85C-expressing myoepithelial cells

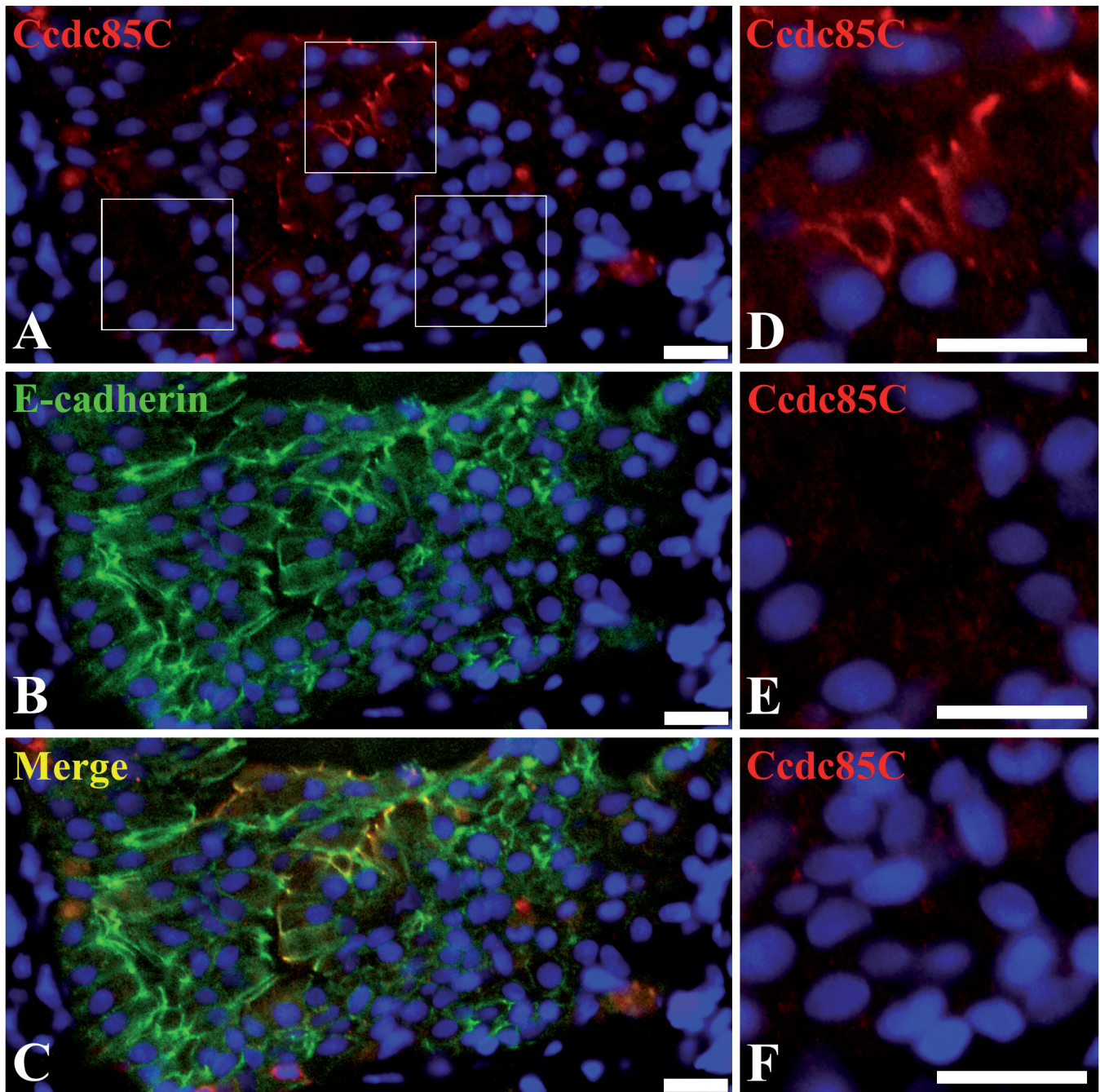


Fig. 3. Loss of Ccdc85C expression in the neoplastic luminal epithelial cells with sheet growth pattern. Double immunohistochemistry for Ccdc85C and E-cadherin to mammary gland tumor of dog (A-F, dog No. 15, complex carcinoma). Expression of Ccdc85C is observed in the luminal epithelial cells forming lumen (D), however, expression of Ccdc85C is not always detected in the lumen forming luminal epithelial cells (E). In contrast, Ccdc85C is not expressed in the luminal epithelial cells with sheet growth pattern. D, E and F are higher magnification of squares in A. Bars: 20 μ m.

were observed in dogs but not in rats (Tanaka et al., 2015). The characteristics of myoepithelial cells differ among species. In this study, the tumors which showed neoplastic proliferation of luminal epithelial cells but not myoepithelial cells were diagnosed as simple adenoma or simple carcinoma. In contrast, the tumors with neoplastic proliferation of both luminal epithelial cells and myoepithelial cells were diagnosed as complex adenoma or carcinoma. It is known that the neoplastic proliferation of myoepithelial cells is common in canine mammary tumors (Nerurkar et al., 1989). In this study, 78% of canine cases were diagnosed as complex tumors. The present findings may reflect species difference in myoepithelial characteristics in the mammary tumors.

Ccdc85C expression in the mammary gland tumors

Ccdc85C expression was observed in the neoplastic luminal epithelial cells that formed lumens as well as in the normal mammary glands. However, Ccdc85C expression was absent in the luminal epithelial cells without glandular structures. The Ccdc85C protein is expressed in the simple epithelial cells but not stratified epithelial cells (Tanaka et al., 2015). Taking all of these findings together, we propose that the Ccdc85C loss at the neoplastic luminal epithelial cells with sheet growth reflects the loss of polarity of epithelial cells found in the normal simple epithelial cells. It is not yet clear whether the absence of Ccdc85C expression is a primary or secondary change in the structural atypia of luminal epithelial cells. Further studies are necessary to reveal the relationship between Ccdc85C and tumorigenesis.

Table 2. Comparison of Ccdc85C expression intensity in canine tumors.

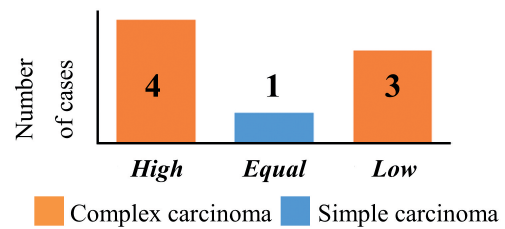
Dog No.	Diagnosis	Ccdc85C expression intensity	Group
1	complex carcinoma	MG < MGT	High
2	complex carcinoma	MG < MGT	High
3	complex carcinoma	MG > MGT	Low
4	complex adenoma	MG < MGT	High
5	complex carcinoma	MG < MGT	High
6	complex adenoma	MG < MGT	High
7	simple carcinoma	MG = MGT	Equal
8	simple adenoma	MG < MGT	High
9	complex carcinoma	No data *	
10	complex carcinoma	MG < MGT	High
11	complex adenoma	MG < MGT	High
12	complex adenoma	MG < MGT	High
13	simple adenoma	MG < MGT	High
14	simple adenoma	MG = MGT	Equal
15	complex carcinoma	MG > MGT	Low
16	complex adenoma	MG > MGT	Low
17	complex adenoma	MG < MGT	High
18	complex carcinoma	MG > MGT	Low

MG, normal mammary gland; MGT, mammary gland tumor. High: neoplastic cells expressed Ccdc85C more intensely than normal mammary gland. Equal: Ccdc85C expression at neoplastic cells was almost same as that at normal mammary gland. Low: neoplastic cells expressed Ccdc85C less intensely than normal mammary gland. * We could not find surrounding normal mammary gland tissue.

Ccdc85C expression and tumor malignancy

Among the nine canine mammary gland adenomas, 11% of the cases showed weak Ccdc85C expression compared to the surrounding normal mammary gland. On the other hand, in the canine mammary gland carcinomas, 37.5% of eight cases showed weaker Ccdc85C expression compared to the surrounding normal mammary gland. These results suggest that Ccdc85C expression in carcinomas tends to be weaker than that in adenomas. The present results demonstrated that Ccdc85C expression was absent in the neoplastic cells without glandular structures. Among the criteria used to determine the histologic malignant grade of canine mammary neoplasms, tubule formation is described as important (Goldschmidt et al., 2011). Carcinomas forming less tubules are considered likely to be more malignant. Taking the past and present findings into account, we speculate that the structural atypia may be associated with the weaker expression of Ccdc85C in carcinomas. However, the neoplastic luminal epithelial cells with tubular growth pattern observed in the present study did not necessarily express Ccdc85C strongly, and thus other factors affecting Ccdc85C expression in neoplasms should be investigated.

A, canine adenocarcinomas



B, canine adenomas

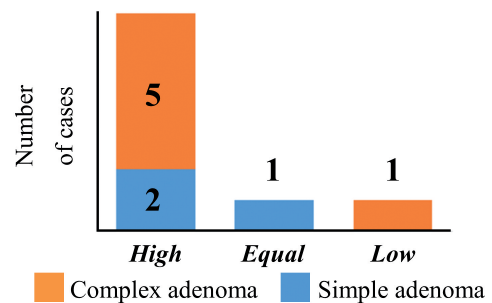


Fig. 4. Relationship between Ccdc85C expression intensity and tumor malignancy. In carcinomas, almost the same number of cases are classified as *High* and *Low* (A). In contrast, most cases are classified into *High* and only one case is judged as *Low* in adenoma (B). *High*: the intensely expressed Ccdc85C at a higher level compared to normal mammary gland tissue. *Equal*: the Ccdc85C expression in neoplastic cells had almost the same intensity as that in the normal mammary gland. *Low*: the neoplastic cells expressed less Ccdc85C than the normal mammary gland.

Ccdc85C expression in canine mammary tumors

Ccdc85C, neurological diseases and malignant transformation of tumors

Our previous studies showed that *Ccdc85C* is expressed at various simple epithelia including neuroepithelium of mice and rats (Mori et al., 2012; Tanaka et al., 2015). Lack of *Ccdc85C* causes hydrocephalus and subcortical heterotopia in mice (Mori et al., 2012). Our previous study also demonstrated that *Ccdc85C* is strongly expressed at the apical junction of simple epithelia with high proliferative activity and suggested that *Ccdc85C* might correlate with cell polarity (Tanaka et al., 2015). In this study, neoplastic luminal epithelial cells, which showed sheet growth pattern, did not express *Ccdc85C*. This result also suggests that *Ccdc85C* expression is related to cell polarity of simple epithelia. Therefore we speculated that *Ccdc85C* expression interacts with cell polarity of simple epithelia, and some changes in the interaction might cause neurological diseases and malignant transformation of tumors.

In conclusion, the results of the study described herein demonstrated that *Ccdc85C* protein was expressed in the neoplastic luminal epithelial cells of dogs. The findings also demonstrated different expression patterns of *Ccdc85C* in canine myoepithelial cells, and a relationship between *Ccdc85C* expression and tumor malignancy. *Ccdc85C* is known to cause neurological diseases such as hydrocephalus, subcortical heterotopia and retinal dysplasia, and the present study is the first to demonstrate that *Ccdc85C* plays a role in mammary tumors.

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