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Running title: Tight junction protein in sweat gland tumors

Abstract

Primary cutaneous mucinous carcinoma (PCMC) is a rare sweat gland tumor characterized by the presence of abundant mucin around the tumor islands, but the molecular mechanisms for this structure are not well elucidated. Because that mucin is epithelial in nature, it is likely to be produced by epithelial tumor cells, not by surrounding stromal cells. We hypothesized that the abundant mucin is a result of reversed cellular polarity of the tumor. To test this hypothesis, we conducted an immunohistological study to investigate expression of tight junction (TJ) proteins occludin and ZO-1 in PCMC, as well as in normal sweat glands and other sweat gland tumors. Dot-like or linear expression of TJ proteins was observed at ductal structures of sweat glands, and ductal or cystic structures of related tumors. In PCMC, however, TJ protein expression was clearly visible at the edges of tumor cell islands. This study provides evidence to show that the characteristic histological structure of PCMC is caused by inverse polarization of the tumor cells, and that TJ proteins are useful markers of ductal differentiation in sweat gland tumors.

Key words: cell polarity, immunohistochemistry, mucinous adenocarcinoma, sweat gland neoplasms, tight junctions

Introduction

Primary cutaneous mucinous carcinoma (PCMC) is a rare neoplasm with a highly characteristic histologic appearance: Numerous nests of the tumor cells with glandular differentiation are surrounded by an abundance of mucin as if floating in lakes.[1] Histochemical studies have shown mucin to be sialomucin, an epithelial mucin produced by neoplastic cells, rather than sulfated acid mucopolysaccharides produced by connective tissues.[2] The thin fibrous stroma around the mucin lakes contains only sparsely distributed fibroblasts. Therefore, the tumor cells appear to secrete mucin externally as opposed to secreting mucin to the luminal side. Adsay *et al.* analyzed mucinous carcinoma of the breast and pancreas and found CEA and MUC1-expression and lack of basal lamina formation in the stroma-facing surfaces of tumors, supporting "inverse polarization" theory.[3] We reasoned that this inversed polarity hypothesis can be more directly verified by examining tight junctions (TJs), which are located at the most-apical part of the lateral membrane and involved in polarized secretion.[4] In this study, we first tested whether immunohistochemical staining of TJ-related proteins, ZO-1 and occludin, can visualize the apical side of the sweat glands, and then examined PCMC as well as some other sweat gland tumors.

Methods

Cases

With the approval from the Medical Ethics Committee of the Asahikawa Medical University (#1572), we retrieved eight cases of PCMC, four cases of apocrine cyst adenoma, four cases of nodular hidradenoma, three cases of eccrine poroma, four cases of porocarcinoma, and three cases of extramammary Paget disease from the archives of the Department of Dermatology, Asahikawa Medical University. For comparative purposes, surgical specimens containing normal sweat glands were also included.

Histological and immunohistochemical staining

Skin tumors were fixed in 10% buffered formalin and embedded in paraffin. The deparaffinized and rehydrated tissue sections mounted on glass slides were either stained with hematoxylin and eosin (HE) for histopathological analyses, or used for colloidal ion staining following a standard protocol, or immunohistochemical staining as follows. The sections were pre-incubated with 3% hydrogen peroxide in methanol for 10 min, and then with 10% normal goat serum for 15 min at room temperature. The sections were incubated with one of the following primary antibodies for 1 h at 37℃: rabbit polyclonal anti-occludin (1:100 dilution; Zymed Laboratories, San Francisco, CA,

USA), rabbit polyclonal anti-ZO-1 (1:100 dilution; Zymed Laboratories), mouse monoclonal anti-human Ki-67 (1:100 dilution; Dako, Carpinteria, CA, USA), and mouse monoclonal epithelial membrane antigen (EMA) antibodies (Dako). For occludin and ZO-1 staining, digestion with pronase E (0.2 mg/ml, 5 min at 37℃, Sigma-Aldrich, St. Louis, MO, USA) and pretreatment with 0.3% TritonX-100 in PBS (5 min at room temperature) were added before the incubation with the blocking serum. We used normal immunoglobulin fraction of mouse or rabbit serum as a negative control. The sections were then stained with a standard streptavidin-biotin method (Histofine, Nichirei Biosciences, Tokyo, Japan) using diaminobenzidine as a substrate for peroxidase (Dako). Nuclei were counterstained with hematoxylin.

Electron microscopy

Formalin-fixed paraffin-embedded tissues of two cases of PCMC were deparaffinized in xylene and re-embedded in epoxy resin for transmission electron microscopy and examined using a standard protocol.[5]

Results

TJ protein expression in sweat gland and sweat gland tumors

We first tested whether TJ protein expression can be detected immunohistochemically in formalin-fixed skin tissue samples. We found that both ZO-1 and occludin antibodies stained the luminal side of eccrine and apocrine sweat glands in a dot-like or linear fashion (Fig. 1). We next examined TJ protein expression in some benign sweat gland tumors (Fig. 2). The expression of ZO-1 and occludin was observed at luminal membranes of various sized cystic structures in apocrine cyst adenoma, nodular hidradenoma and eccrine poroma. Luminal staining of TJ protein was also detected in malignant tumors, porocarcinoma and extramammary Paget disease (Fig. 3). In fact, glandular differentiation was more clearly visible with this immunostaining than with HE staining.

TJ protein expression in PCMC

We then studied TJ protein expression in PCMC (Fig. 4, 5, Table 1). In five cases of low-grade PCMC (cases 1 to 5, grade 1 to 2 of Kazakov's grading system),[1] both ZO-1 and occludin expression was detected at the edge of the tumor cell nest surrounded by mucin-rich stroma, as well as at the edge of luminal structures inside the nests. In an *in situ* component found in a case of low-grade PCMC (case 5), where tumor nests were surrounded by myoepithelial cells, but not by mucinous stroma, TJ protein staining was

found only at the edge of lumen inside of the nest. In two cases of high-grade PCMC (cases 7 and 8, Kazakov's grade 3), TJ protein expression was weakly and diffusely seen all over the tumor cells. We also performed immunochemical staining of Ki-67 and EMA in PCMC. In the low-grade cases, Ki-67 labeling index was lower than in the high-grade case (Table 1). EMA staining showed similar staining patterns to those of TJ protein (Table 1 and Fig. 6).

Ultrastructure of PCMC

We examined two cases of PCMC using electron microscopy to see whether TJ structures can be detected ultrastructurally. No typical TJ structures ("kissing point") between the plasma membranes of two adjacent cells could be found between the cells facing either the lumen or the mucinous stroma possibly because of poor tissue preservation (data not shown). Lamina densa was not detected around the tumor island (data not shown).

Discussion

In the present study, we demonstrated that immunohistochemical staining of TJ proteins can delineate cellular polarity and glandular differentiation in normal sweat

glands and sweat gland neoplasm. This staining validated “inverse polarization” of PCMC.

TJ protein expression has been detected in normal human sweat duct using immunofluorescent staining in experimental models,[6] but not immunohistochemically in formalin fixed tissues processed for routine pathological diagnostics. TJ protein expression has been examined in adenocarcinomas in other organs, such as breast, bile duct, esophagus, and colon,[7,8] but not in sweat gland tumors. Reversed expression of TJ protein has not been reported in mucinous carcinoma of other tissues, either. In nodular hidradenoma, various-sized cleavage-like, cystic structures have been regarded as resulting from tissue degeneration by some authors,[9] but expression of TJ protein at the luminal side observed in our study indicated that they are in fact dilated ducts or glands. TJ protein expression in a malignant tumor was also observed at luminal edges, suggesting that it can serve as a glandular differentiation marker in sweat gland tumors.

A PCMC *in situ* component did not show TJ protein expression at the edge of tumor nests, but only at the luminal edges. TJ protein staining around the outer edge of the tumor nests was only observed in the area surrounded by mucin, indicating association between characteristic histological features of PCMC and reversed TJ protein

expression. EMA and MUC2 showed a similar staining pattern at the outer edge of the tumor nests in PCMC.[1,9] Adsay et al. have reported similar results with antibodies against several glycoproteins in mucinous carcinoma of breast and prostate glands.[3] These data indicate close association between specific localization of mucin and cellular polarity demonstrated by TJ protein staining. Polarized expression of TJ protein and EMA was lost in a high-grade case of PCMC, indicating that dedifferentiated tumor cells lose cellular polarity. Since clear TJ structures were not detected by ultrastructural examination in the present study, TJ protein immunostaining is an easier and more reliable method to examine tissue polarity in clinical tissue samples.

In conclusion, the present study clearly demonstrated “inverse polarization” of the cells in PCMC. TJ protein immunostaining can be a useful means of characterizing histogenesis of glandular tumors.

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CONFLICT OF INTEREST: None declared

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Figure Legends

Figure 1. Immunohistochemical staining of normal sweat glands. (a) ZO-1 staining of eccrine gland shows dot-like (red arrowheads) and linear patterns (red arrows) at the luminal side of the gland. (b) ZO-1 staining of an apocrine sweat gland at the luminal side. (c) Occludin staining of an eccrine sweat duct shows dot-like and linear patterns at the luminal edge.

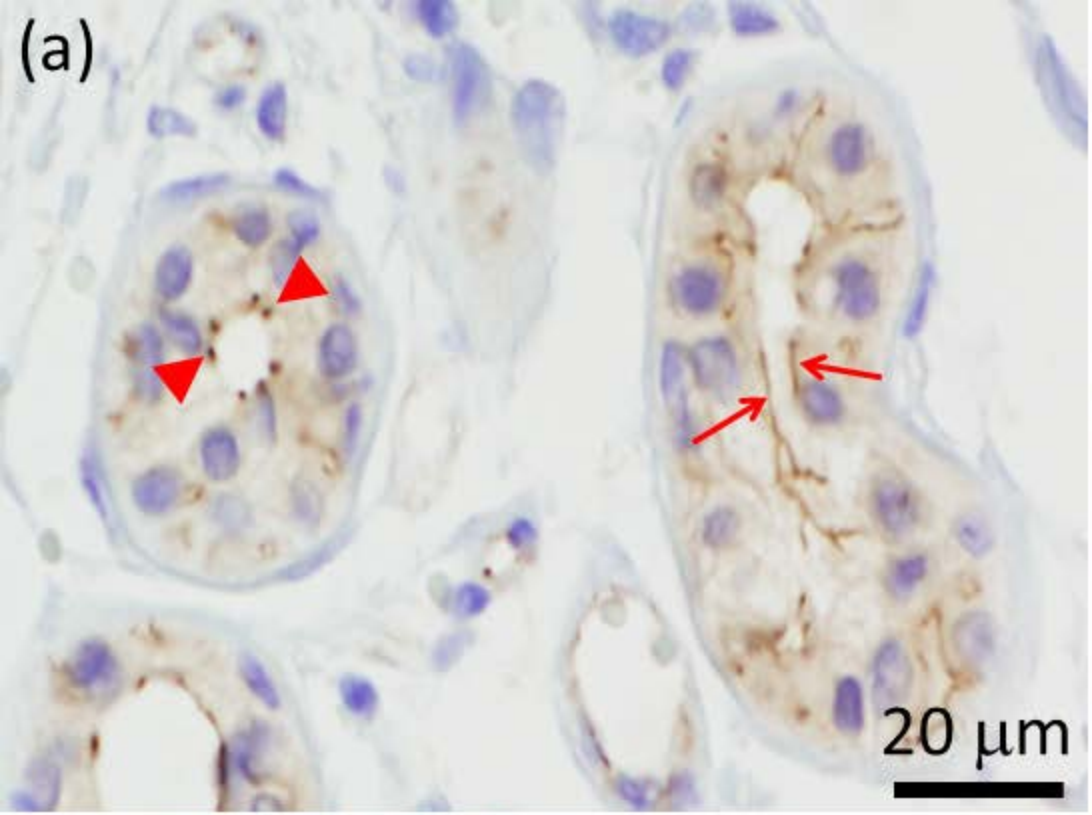
Figure 2. Hematoxylin and eosin (HE) stain (a – c) and immunohistochemical staining (d – i) of benign sweat gland tumors. (a, d, g) apocrine cyst adenoma, (b, e, h) nodular hidradenoma and (c, f, i) eccrine poroma. ZO-1 (d, e, f) and occludin (g, h, i) immunostaining. Tight junction protein expression is noted at the luminal side of various sized cystic structures (*).

Figure 3. HE stain and immunohistochemical staining of malignant sweat gland tumors. HE stain of eccrine porocarcinoma (a) and extramammary Paget's disease (b). ZO-1 staining of eccrine porocarcinoma (c) and extramammary Paget's disease (d). ZO-1 expression is observed at the luminal side of various sized cystic spaces (*).

Figure 4. HE stain and immunohistochemical staining of primary cutaneous mucinous carcinoma (PCMC). HE stain of low-grade PCMC (a, grade 1-2), an *in situ* component (b), and a high-grade PCMC (c, grade 3). (a) Nests of tumor cells with small round nuclei are surrounded by an abundance of extracellular mucin. (b) The tumor nests containing cystic structures (*) are surrounded by myoepithelial cells (green arrows) at an *in situ* component. (c) High-grade PCMC has irregular shaped tumor cells with various sized nuclei. (d) ZO-1 stain is seen not only at the luminal edge within the nests (a red arrow), but also at the outer edge of the tumor nests (arrowheads). (e) Various sized lumen (*) in tumor islands are lined with occludin stain. The outer edge of the nests are lined with occludin negative myoepithelial cells (green arrow). (f) Occludin expression is weak and diffuse in high-grade PCMC.

Figure 5. ZO-1 immunostaining (a) and colloidal iron staining (b) in serial sections of low-grade PCMC. ZO-1 expression is seen at the edge of the tumor cell islands surrounded by characteristic mucin-rich stroma as well as at the edge of luminal structures inside in PCMC.

Figure 6. Epithelial membrane antigen (EMA) immunostaining in low-grade (a) and high-grade PCMC (b). EMA is positive at the luminal edge (arrows) and tumor island edge (arrowheads) in low-grade PCMC, but diffusely positive in high-grade PCMC.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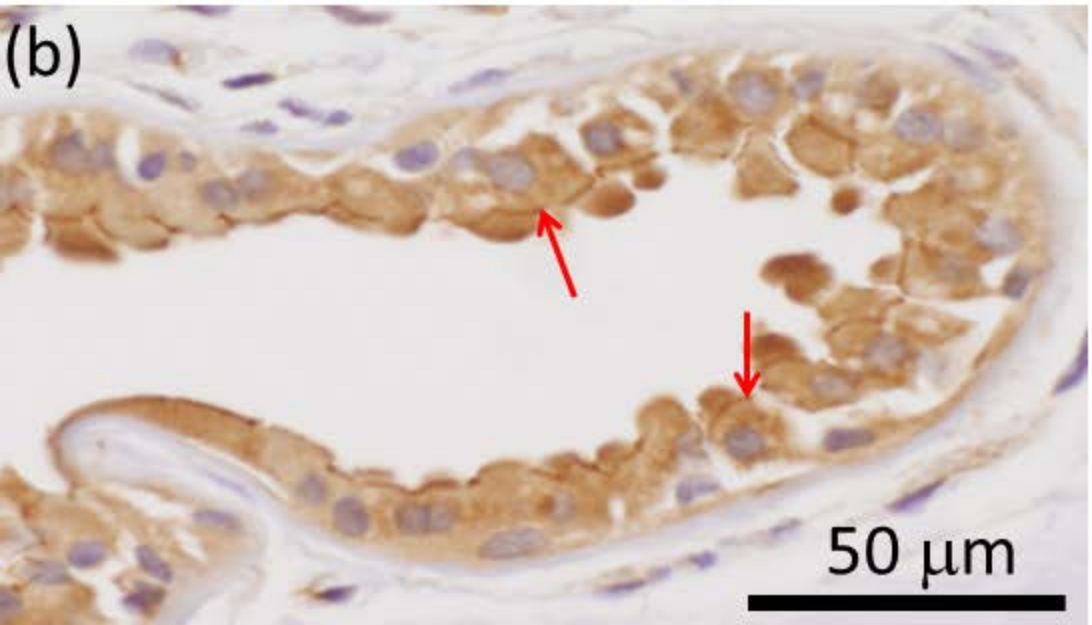
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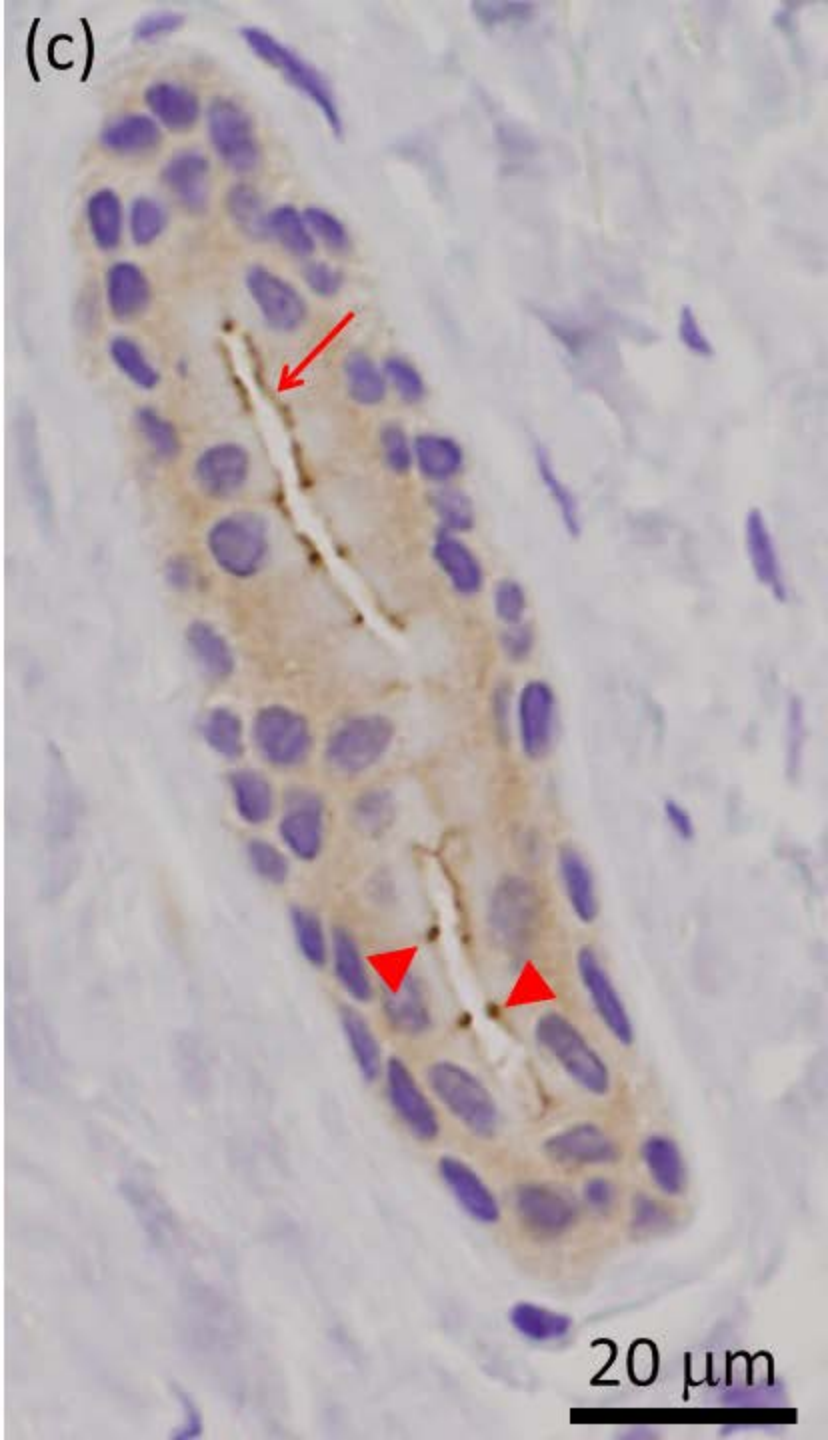
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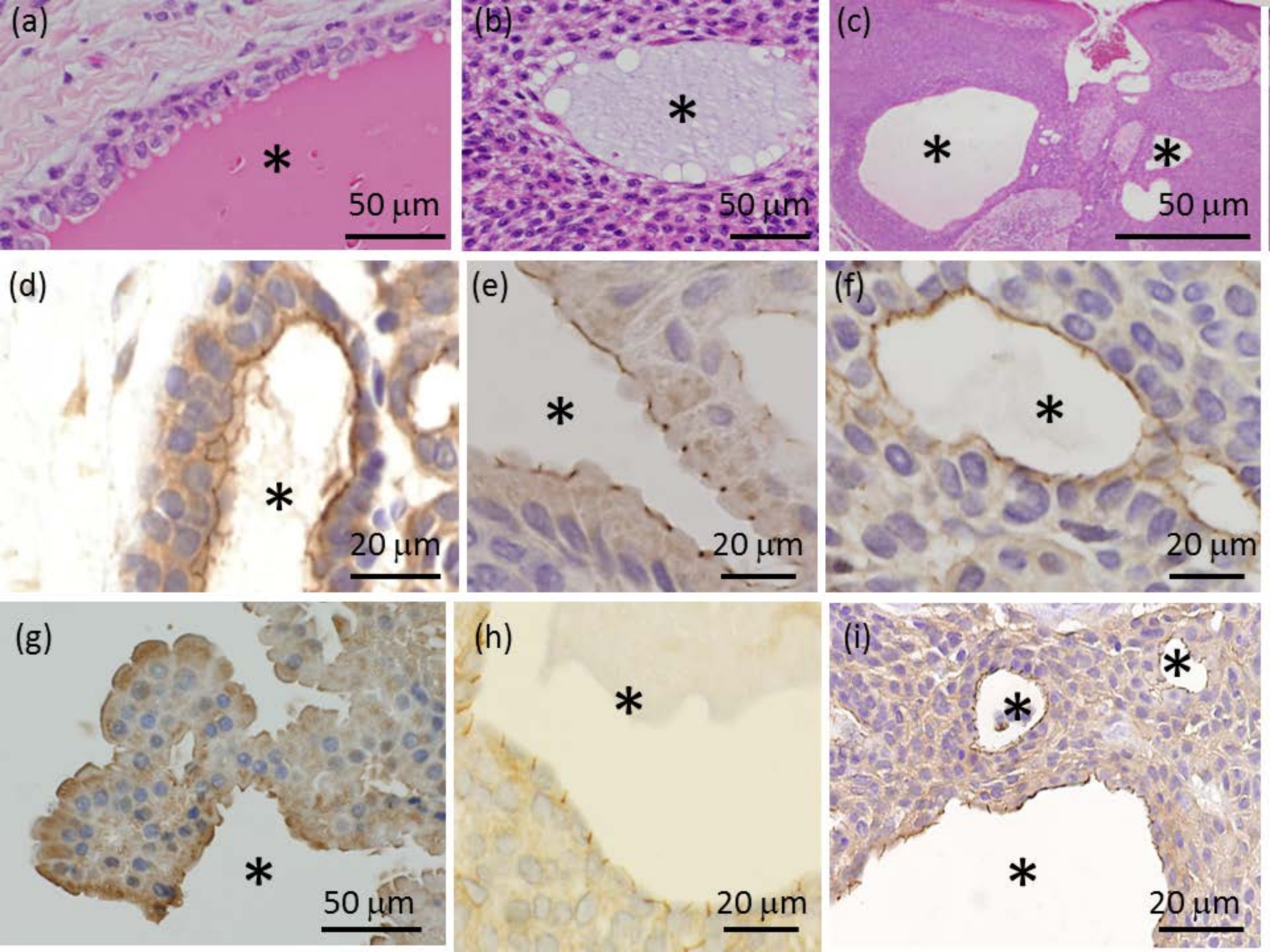
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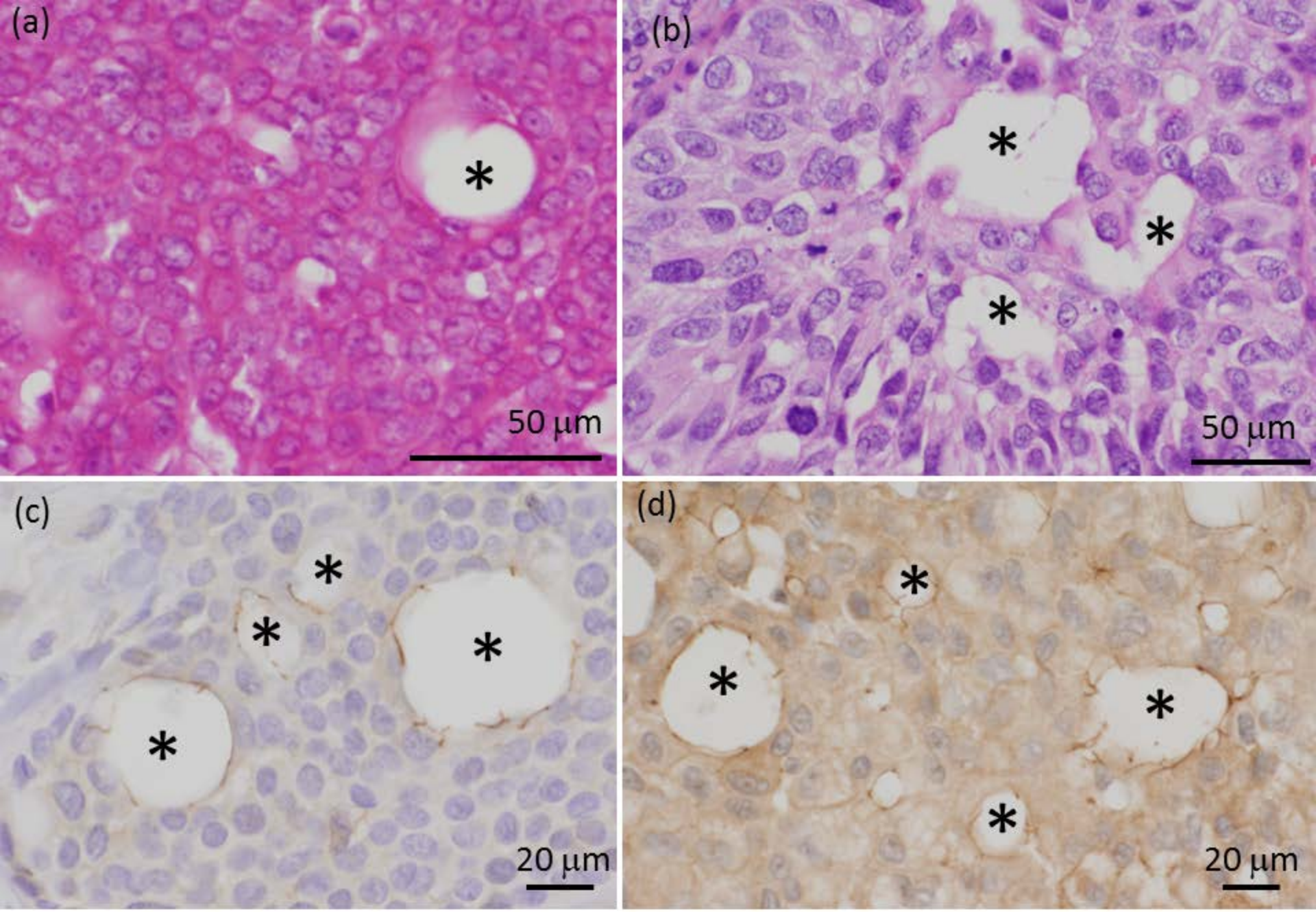
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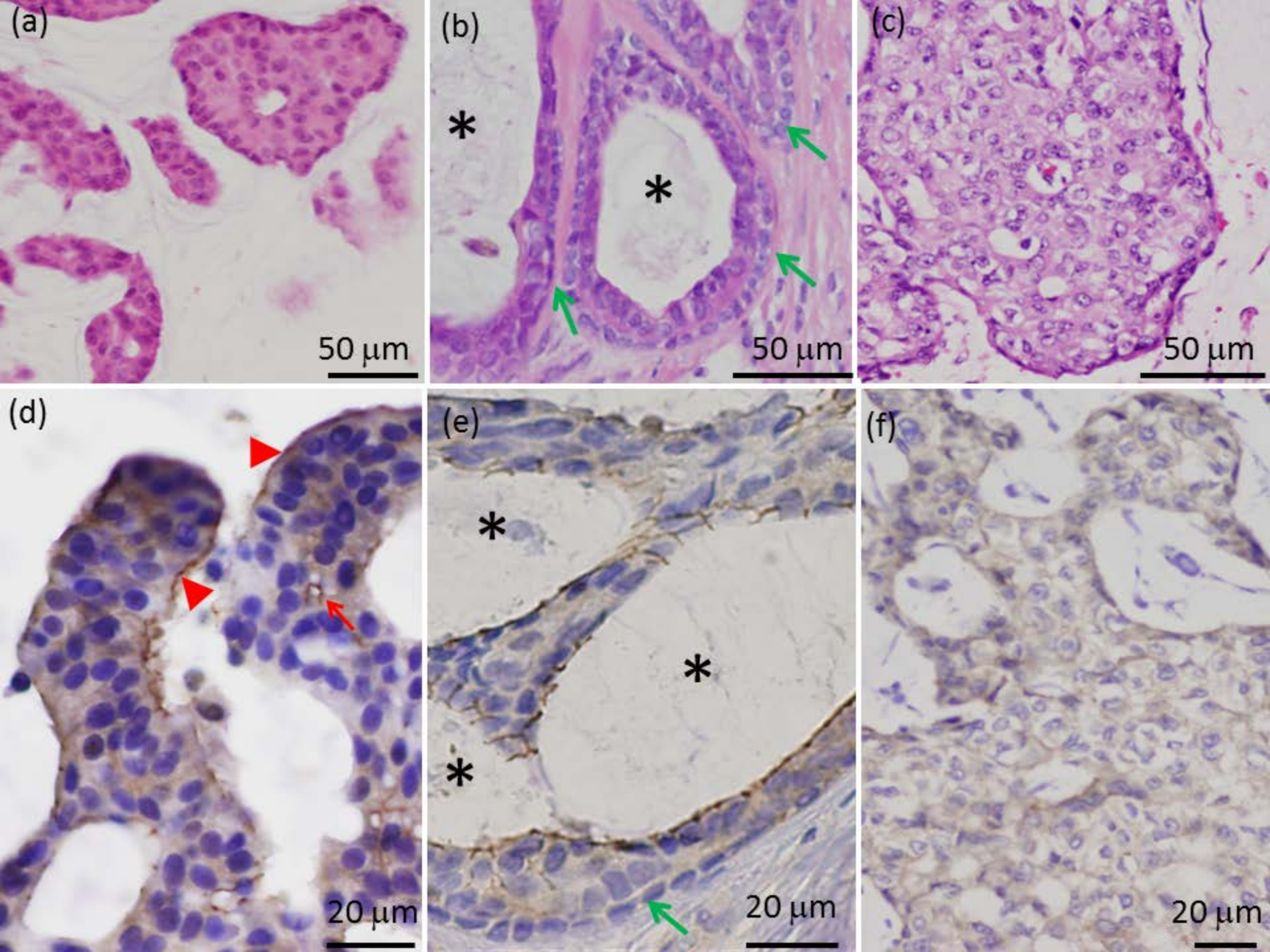
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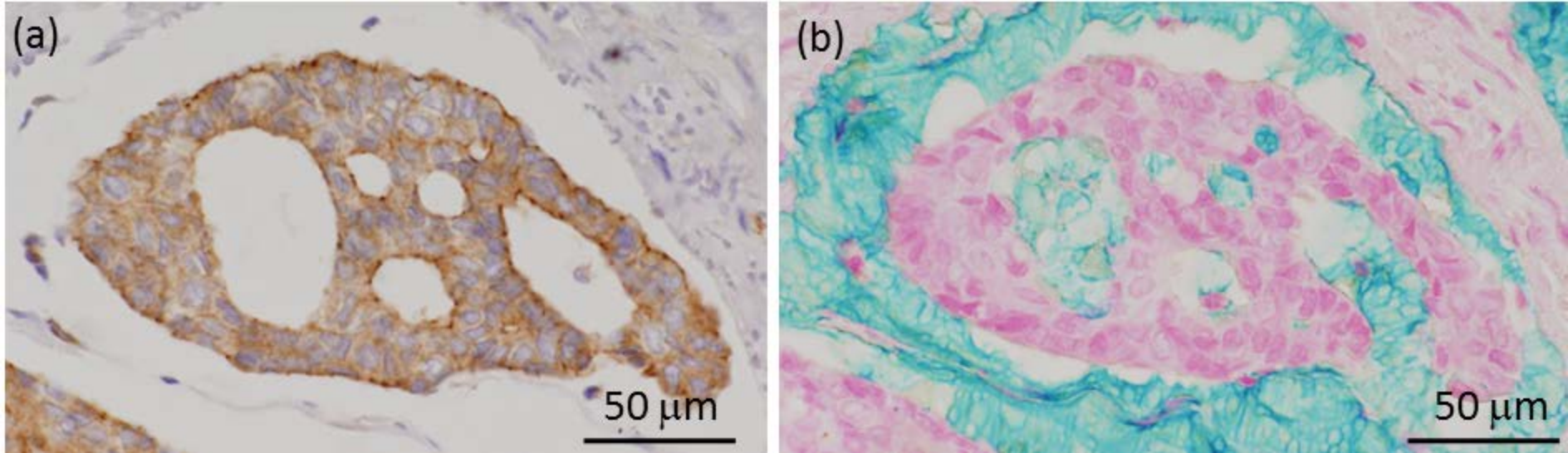
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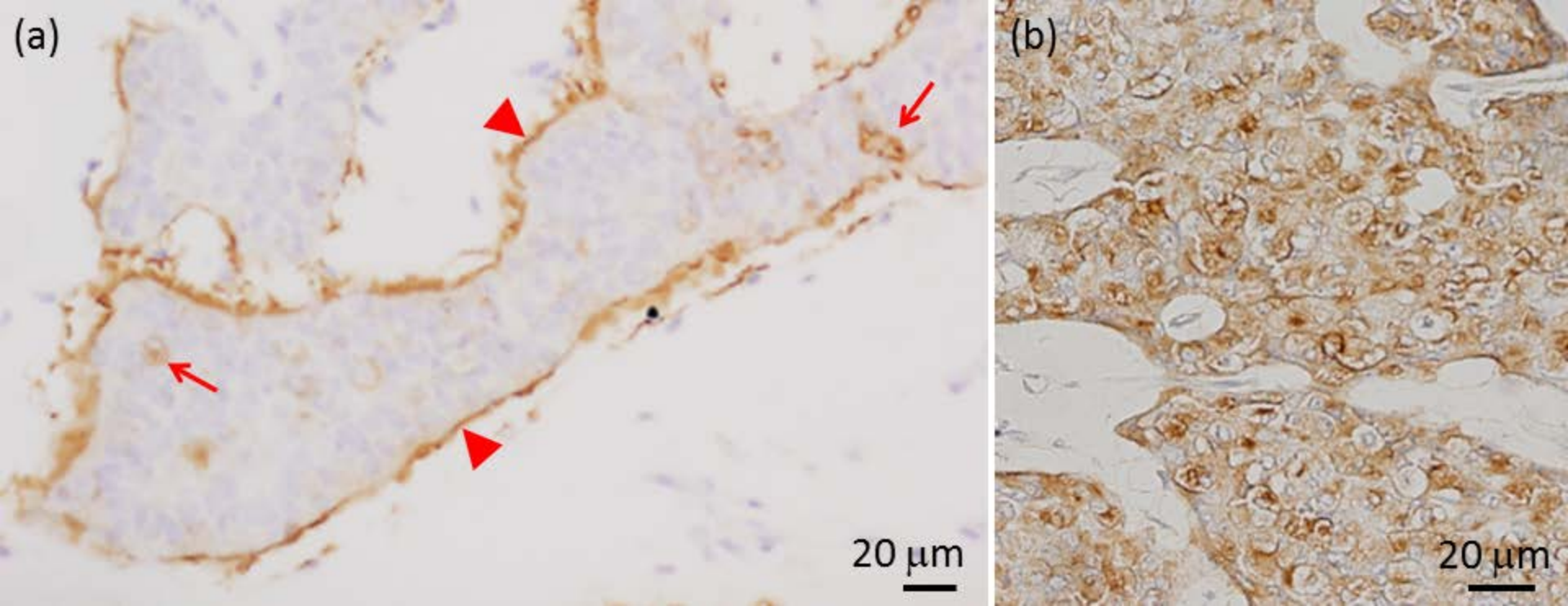
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