CASE REPORT

GALT carcinoma: three case reports with glycoprotein 2 immunohistochemistry and electron microscopic observations.

Running title: *Three cases of GALT carcinoma*

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Abstract

Gut-associated lymphoid tissue (GALT) carcinoma is a rare colorectal tumor that arises in the epithelium covering GALT. GALT carcinoma is a differentiated tubular adenocarcinoma with dense lymphoid tissue with a characteristically well-demarcated margin. To date, twenty-six cases of GALT carcinoma, including the three cases discussed here, have been reported. Most of them (24/26) were discovered at early stages and none of the cases have documented any metastases. This suggests that GALT carcinoma may have a favorable prognosis. It is hypothesized that GALT carcinoma originates from M cells in specialized epithelia covering GALT. However, this hypothesis has yet to be confirmed. In this study, we examined three cases of GALT carcinoma by immunohistochemistry detection of glycoprotein 2, a specific marker for M cells, and electron microscopy. Our findings showed that the tumor cells of GALT carcinoma in all three cases were negative for M cells. We thus concluded that GALT carcinoma may be a tubular adenocarcinoma arising in the GALT by chance. This unique carcinoma is a diferentiated adenocarcinoma that slowly grows with development of GALT. We propose that GALT carcinoma should be classified separately because of its histologic setting and good prognosis.

Keywords: gut-associated lymphoid tissue, M cell, colon, adenocarcinoma, glycoprotein 2

Introduction

Gut-associated lymphoid tissue (GALT) carcinoma is a rare colorectal adenocarcinoma comprising of differentiated tubular adenocarcinoma and dense lymphoid tissue with a characteristically well-demarcated margin. Most GALT carcinomas present at early stages, and all patients have a favorable clinical outcome. [1,2] The characteristic appearance of these tumors has led to the hypothesis that they originate from M cells in the follicle-associated epithelium (FAE) covering GALT. [3] Recent research has shown that M cells play a pivotal role in intestinal antigen presentation. [4–6] However, the origin of GALT carcinoma from M cells remains indeterminate. Here we report three cases of GALT carcinoma. Our immunohistochemical and ultrastructural findings strongly suggest that GALT carcinoma does not show features of M cells.

Materials and methods

Case selection

After approval for the study was obtained from the Institutional Review Board, we retrospectively searched pathology database reports of Sasebo City General Hospital and Saga National Hospital from 2006 to 2016 and found three cases of GALT carcinoma. These patients had undergone endoscopic mucosal resection, endoscopic submucosal dissection, and laparoscopic high anterior resection, respectively. Normal colon tissue

was obtained from the surgical margins in cases of standard colorectal adenocarcinoma as a positive control for glycoprotein 2 immunostaining. Hematoxyline and eosin-stained histologic slides prepared from the specimens were reviewed for pathologic analysis. Clinical data including patients' treatments and outcomes were retrieved from their medical records.

Immunohistochemistry

Glycoprotein 2 is a specific marker for M cells and is expressed exclusively at the apical surface and in the cytoplasm of M cells among the intestinal epithelium, including the FAE.[5,6] We therefore detected glycoprotein 2 by immunohistochemistry in the current case series.

Paraffin sections of the samples were mounted on glass microscope slides, deparaffinized, and immersed in absolute methanol containing 0.15% H_2O_2 for 15 min at room temperature to block endogenous peroxidase activity. After washing, the sections were immersed in phosphate-buffered saline then treated with mouse monoclonal antibodies against human anti-glycoprotein 2 antibody (1:400; MBL, Nagoya, Japan). The antibody was applied to the sections overnight at 4ºC, followed by a Histofine® Simple Stain *MAX PO* (Nichirei, Tokyo, Japan) for 30 min at room

temperature. All specimens were color developed using a *3,3′*-diaminobenzidine *tetrahydrochloride* chromogen *kit* (*Dako, CA, USA*), and counterstained with Mayer's hematoxylin.

Transmission Electron Microscopy

Transmission electron microscopy was performed retrospectively on specific areas of formalin-fixed, paraffin-embedded blocks from the three cases. For the electron microscopic examination, tissue fragments of 1 mm^3 were cut with a sharp blade from distinct, well-chosen areas of the paraffin block. The tissues were deparaffinized in 100 % xylene for 30 min × 2 prior to rehydration using a descending alcohol series. After the tissue specimen was fixed with 1 % osmium tetroxide for 2 h, it was dehydrated using an ascending alcohol series. Epoxy resin infiltration was performed and the resin was polymerized to make electron microscopy blocks. The blocks were thin-sectioned and examined with a JEOL JEM-1200EX transmission electron microscope.

***In situ* hybridization**

In situ hybridization was performed using Bond™ Ready-to-Use ISH EBER probe (Leica Biosystems Inc., Newcastle, United Kingdom) to detect *Epstein–Barr virus-encoded RNA,* in accordance with the manufacturer's instructions. Positive staining was identified as brown dots in the nucleus.

Results

Case 1

Clinical history

A 70-year-old woman with diabetes mellitus, hypertension, and hyperlipidemia visited our hospital for a follow-up colonoscopy. Two years previously, a flat lesion in the cecum had been endoscopically removed. Pathological examination revealed a well-differentiated tubular adenocarcinoma with submucosal invasion (60 μm). The margins of the specimen were tumor-free, with no intravascular invasion. During follow-up colonoscopy, a 12–13-mm submucosal tumor-like lesion was observed, which protruded into the luminal side of the sigmoid colon (Fig. 1a). A reddish mucosa on the surface of the tumor was observed. An epithelial tumor was suspected and endoscopic mucosal resection was performed. The pathological tumor stage was T1N0M0. The patient had an uneventful recovery and no recurrence or metastasis had occurred after two years of

follow-up.

Pathologic findings

Examination with a magnifying glass indicated that the tumor was conspicuously demarcated and accompanied by dense lymphoid tissue in the submucosa (Fig. 1b). Most of the tumor surface comprised of normal mucosa, but the mucosa was partially ruptured, exposing some tumor glands to the surface as a result (Fig. 1b). A number of secondary follicles with germinal centers had developed in the lymphoid tissue, which enclosed tumor nests entirely. Columnar epithelium with significant atypia and eosinophilic cytoplasm formed fusiform glands within the lymphoid tissue (Fig. 1c). Eosinophilic secretion and calcification could be seen in the glands and some glands were cystically dilated (Fig. 1d). Notably, a few glands within the tumor showed slight nuclear atypia (Fig. 1e). The tumor appeared to be surrounded by muscularis mucosa, but immunostaining with desmin showed absence of smooth muscle bands surrounding the tumor (data not shown).

Sections were immunostained for glycoprotein 2 to determine if the GALT carcinomas originated from M cells. In GALT carcinoma of case 1, no tumor cells stained positive for glycoprotein 2 (Fig. 1f). Normal GALT follicles from a control colectomy for

colorectal adenocarcinoma were used as a positive control for assessing the results of glycoprotein 2 immunohistochemistry. In the positive control, some cells in the cytoplasm and plasma membrane of the epithelia covering GALT were positive for glycoprotein 2 (Supplemental figure).

Case 2

Clinical historyA 72-year-old man with a history of distal gastrectomy for gastric cancer was referred to our hospital because of a positive fecal occult blood test. At colonoscopy, an elevated 4-mm diameter lesion in the rectum was identified. Adenocarcinoma was suspected and endoscopic submucosal dissection was performed. The pathological specimen disclosed a possible positive margin. A low anterior resection was subsequently performed. No remnant tumor or lymph node metastasis was detected in the surgical specimen, and the tumor stage was determined to be T1N0M0. There was no recurrence at 7 years of follow-up.

Pathologic findings

On examination with a magnifying glass, the tumor formed a nodular lesion mainly in the submucosa and the tumor margin was clearly demarcated (Fig. 2a). The

surface of the tumor was partially eroded and adenocarcinoma, as well as normal mucosa, could be seen in the epithelia covering the tumor (Fig. 2b). Atypical fusiform glands proliferated in the dense lymphoid tissue (Fig. 2c). Tumor cells showing little atypia were also seen, similar to case 1. The tumor was negative for glycoprotein 2 in immunohistochemistry. (Fig. 2d).

Case 3

Clinical historyA 58-year-old man was referred to our hospital for bloody stools. At colonoscopy a rectal cancer invading the submucosa was suspected. A laparoscopic high anterior resection was performed. The pathological tumor stage was T1N0M0. There was no recurrence after a year of follow-up.

Pathologic findings

Examination with a magnifying glass showed a pedunculated polyp (Fig. 3a). The tumor exhibited a nodular lesion in the submucosa, which was surrounded by well-circumscribed, dense lymphoid tissue. The columnar cells with irregular glands showed eosinophilic material (Fig. 3b). The tumor was negative for glycoprotein 2 (Fig. 3c).

Electron microscopy

We examined these three cases ultrastructurally to clarify whether the GALT carcinoma had features of M cells. It has been described in the literature that M cells have distinct microfolds, which are more sporadic and shorter than the microvilli of other enterocytes. [7] In addition, the plasma membrane of M cells are characteristically invaginating the cell and contains lymphocytes and/or dendritic cells within it. [8]

We prepared the specimens from formalin-fixed, paraffin-embedded tissues. Unfortunately, the quality of the electron micrographs were suboptimal. Cellular architecture of adequate quality could not be obtained. Regardless, the samples were analysed to the best of our ability.

The sample from case 1 revealed that adenocarcinoma cells lined forming glands and numerous lymphocytes surrounded the tumor nests (Fig. 4a). The tumor cells had well-developed microvilli and retained polarity toward the base of the epithelium. Some tumor cells were filled with abundant mucus droplets in the cytoplasm (Fig. 4b). In case 2, although the sample was of poor quality due to the old sample being paraffin-embedded for an extended duration, dense-packed microvilli at the apical sites of tumor cells were discernible (Fig. 4c). The specimen from case 3 also showed well-developed

microvilli retaining polarity toward the base of the epithelium (Fig. 4d). Tumor cells engulfing lymphocytes or dendritic cells were hardly discernible in any specimen.

in situ hybridization for Epstein-Barr virus

We carried out *in situ* hybridization to detect Epstein–Barr virus RNA in the tumor glands and lymphocytes in all three GALT carcinoma with negative results in all tested cases.

Discussion

Since the first case of GALT carcinoma described by Rubio [9] in 1984, twenty-six cases of GALT carcinoma including three of our cases have been reported to date. [1,2] Most of these cases (24/26) have been confined to the submucosa, and notably, metastases has not occurred in any of the patients. [1,2] This suggests that this tumor type has a good prognosis compared with standard colorectal adenocarcinomas.

GALT forms dome-shaped nodules in the normal intestinal mucosa and is covered with FAE. [7] FAE has a low absorption capacity, few goblet cells, and some intraepithelial lymphocytes. M cells reside sporadically and almost exclusively within the FAE. [5] Under electron microscopy analysis, M cells engulf lymphocytes and/or dendritic

cells within it via their invaginated plasma membranes.[8] M cells take up and transport intraluminal antigens to the adjacent GALT system through lymphocytes and/or dendritic cells. M cells thus play an important role in intestinal immunosurveillance.[5,6] Recent studies have shown that M cells are derived from intestinal epithelial stem cells as well as other enterocytes, but are specifically influenced by GALT.[5]

GALT carcinoma has been hypothesized to originate from M cells because it contains GALT-like lymphoid tissue, lacks goblet cells, and is morphologically similar to experimental animal tumors.[2,3,10,11] This has remained a hypothesis because of the lack of direct immunohistochemical evidence distinguishing M cells with specific markers. Today, glycoprotein 2 is widely recognized as a specific M cell antigen.[5–7] Glycoprotein 2 is a receptor that can bind to infectious Gram-negative bacilli such as *Escherichia coli* or *Salmonella enterica*.[4] M cells are the only cells in the intestinal epithelium that highly expresses glycoprotein 2.[6,7] We found no glycoprotein 2-positive cells in any of the three GALT carcinoma tumor samples. The detection of glycoprotein 2-positive cells in the control specimen confirmed that the glycoprotein 2 immunostain was effective.

Additionally, we examined three cases of GALT carcinoma with electron microscopy. In the present study, we observed the samples from formalin-fixed, paraffin-embedded tissue because all specimens of GALT carcinoma of the three cases had been

used for paraffin blocks. As such, the overall quality of the micrographs was decreased and cellular architecture was difficult to appreciate. However, we were able to analyze the samples to a certain extent. A characteristic feature of M cells is the presence of lymphocytes or dendritic cells within it. [7,8] We confirmed that tumor cells of GALT carcinoma hardly contained lymphocytes or dendritic cells but showed well-developed microvilli. These findings suggest that GALT carcinoma may in fact be an ordinary tubular adenocarcinoma.

Some hypotheses regarding the pathogenesis of GALT carcinomas and/or submucosal tumor-like carcinomas have been proposed: 1) the carcinomas develop from glandular epithelia embedded during mucosal repair, 2) tumors that arise in the deepest layer of the mucosa or 3) from ectopic epithelium, such as a remnant carcinoma or 4) a carcinoma with a strong tendency to invade the submucosa. [12,13] Findings from the current study demonstrated sporadic tumor glands among the dense lymphoid tissue, suggesting that the carcinoma may have proliferated slowly along with lymphoid tissue development. In a fast-growing carcinoma, tumor cells are likely to overtake the lymphoid tissue. This suggests that our observations of an even distribution of glands and lymphoid tissue could develop in a slow growing tumor type. Furthermore, GALT carcinomas showed unusual demarcation as observed in case 1 in this study, where lymphocytic stroma appeared to

be set before invasion by the carcinoma. This indicates that the lymphoid tissue originated from the GALT, and that the carcinoma grew while maintaining the architecture of GALT. Since GALT in the normal intestine often penetrates the muscularis mucosa, adenocarcinomas arising in GALT could easily grow in the submucosa rather than the mucosa. GALT carcinoma could therefore be a tubular adenocarcinoma arising in GALT by chance, and subsequently within and in parallel with the GALT.

Interestingly, two of three cases showed some glands within the tumors with very little nuclear atypia. These may have been normal glands entrapped during adenocarcinoma invasion of the submucosa, or they may have been entrapped glands from which the adenocarcinoma subsequently arose.

GALT carcinoma may be included in the same category of lymphoepithelioma-like carcinoma or medullary carcinoma because of the morphological similarity. However, both lymphoepithelioma-like carcinoma and medullary carcinoma are described as high-grade undifferentiated adenocarcinomas with infiltrative features. [14,15] In contrast, GALT carcinoma is a differentiated tubular carcinoma with a clearly demarcated margin. In addition, lymphoid tissue in GALT carcinoma includes many developed follicles with germinal centers considered to be preexisting lymphoid tissue. This finding is also distinct from high microsatellite instability colorectal carcinoma which often accompanied with

lymphoid infiltration. With regard to the microsatellite status of GALT carcinoma, most cases have been reported to be stable. [1] Given that carcinomas that are accompanied with dense lymphoid tissue generally have a relatively good prognosis, GALT carcinomas are also likely to have a much better prognosis than lymphoepithelioma-like carcinomas and medullary carcinomas.

Although we found no evidence to suggest that GALT carcinoma originated from M cells, it is possible that it may arise from the FAE covering the GALT. The FAE has been reported to be directly stimulated by subepithelial mesenchymal cells through receptor activator of nuclear factor-κB ligand (RANKL), and expresses high levels of the epithelial chemokine CCL20.[16] Given that both the nuclear factor-κB signaling pathway and CCL20 have been reported to be upregulated in colorectal adenocarcinoma,[17–19] it is possible that GALT may promote the occurrence of adenocarcinoma in the FAE.

Rubio et al. offered a hypothesis that GALT carcinoma may develop through "the third pathway" other than the conventional adenoma-carcinoma or serrated adenoma-carcinoma pathway since embedded epithelium are often found in GALT of colectomy specimens of inflammatory bowel disease. [2] In addition, GALT carcinoma occurred with high frequency in dimethylhydrazine-treated Spraque-Dawley rats. [11] Some inflammatory conditions may induce proliferation of GALT, injury and repair of mucosa,

and eventual development of GALT carcinomas.

Conclusion

Our study of three cases supports the hypothesis that GALT carcinoma may not originate from M cells but be a differentiated adenocarcinomas that rarely occurs on GALT. GALT carcinoma has characteristic features and a favorable prognosis, warranting further investigations.

Compliance with ethical standards: Upon receipt of the sample, we set up a website including the relevant information allowing patients to opt out of participation in the study. The ethical committee of Nagasaki University Graduate School of Biomedical Sciences approved the protocol in accordance with the Declaration of Helsinki principles. Written informed consent was not required.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Author contribution

HH did the immunohistochemical and ultrastructural evaluation and wrote the manuscript. All other authors have contributed to data collection and interpretation, and critically reviewed the manuscript. All authors approved the final version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Figure legends

Fig. 1 Colonoscopy findings, histological and immunohistochemical features in case 1. (a) Colonoscopy photograph showing a 12–13-mm diameter polypoid lesion with a partially red mucosa. (b) Examination with a magnifying glass shows a well-demarcated tumor with dense lymphoid tissue localized in the submucosa. Most of the lesion surface comprises of normal epithelium. (c) Fusiform glands are identified sporadically within the lymphoid structure with germinal centers. (d) A cystically dilated gland with eosinophilic material and calcification is shown. (e) Some tumor glands show little nuclear atypia. (f) No tumor cells were positive for glycoprotein 2 in immunohistochemistry.

Fig. 2 Histological and immunohistochemical features in case 2. (a) A well-demarcated tumor shows dense lymphoid tissue extending into the submucosa. The surface was focally eroded. (b) Beneath the tumor surface, adenocarcinomas can be observed in the mucosa. (c) Cribriform glands with nuclear atypia are shown. (d) A few tumor glands show little nuclear atypia. (e) No tumor cells were positive for glycoprotein 2 in immunohistochemistry.

Fig. 3 Histological and immunohistochemical features in case 3. (a) A pedunculated polypoid lesion is shown. A tubular adenocarcinoma shows dense lymphoid tissue proliferating in the head of the polyp. (b) Irregularly shaped atypical glands containing eosinophilic material are shown. (c) No tumor cells were positive for glycoprotein 2 in immunohistochemistry.

Fig. 4 Electron micrographs of GALT carcinoma. (a) The specimen from case 1 revealed adenocarcinoma cells with well-developed microvilli and many lymphocytes surrounding the tumor glands. Intraepithelial lymphocytes are scarcely seen. (b) Some tumor cells filled with mucin droplets are shown. (c) The specimens from case 2 show dense microvilli despite poor sample quality. (d) Tumor cells from case 3 retain nuclear polarity and well-developed microvilli are also observed.

Supplemental figure legends

Supplemental figure In the control samples representing normal mucosa, some cells in the epithelium covering GALT are glycoprotein 2 positive in immunohistochemistry.

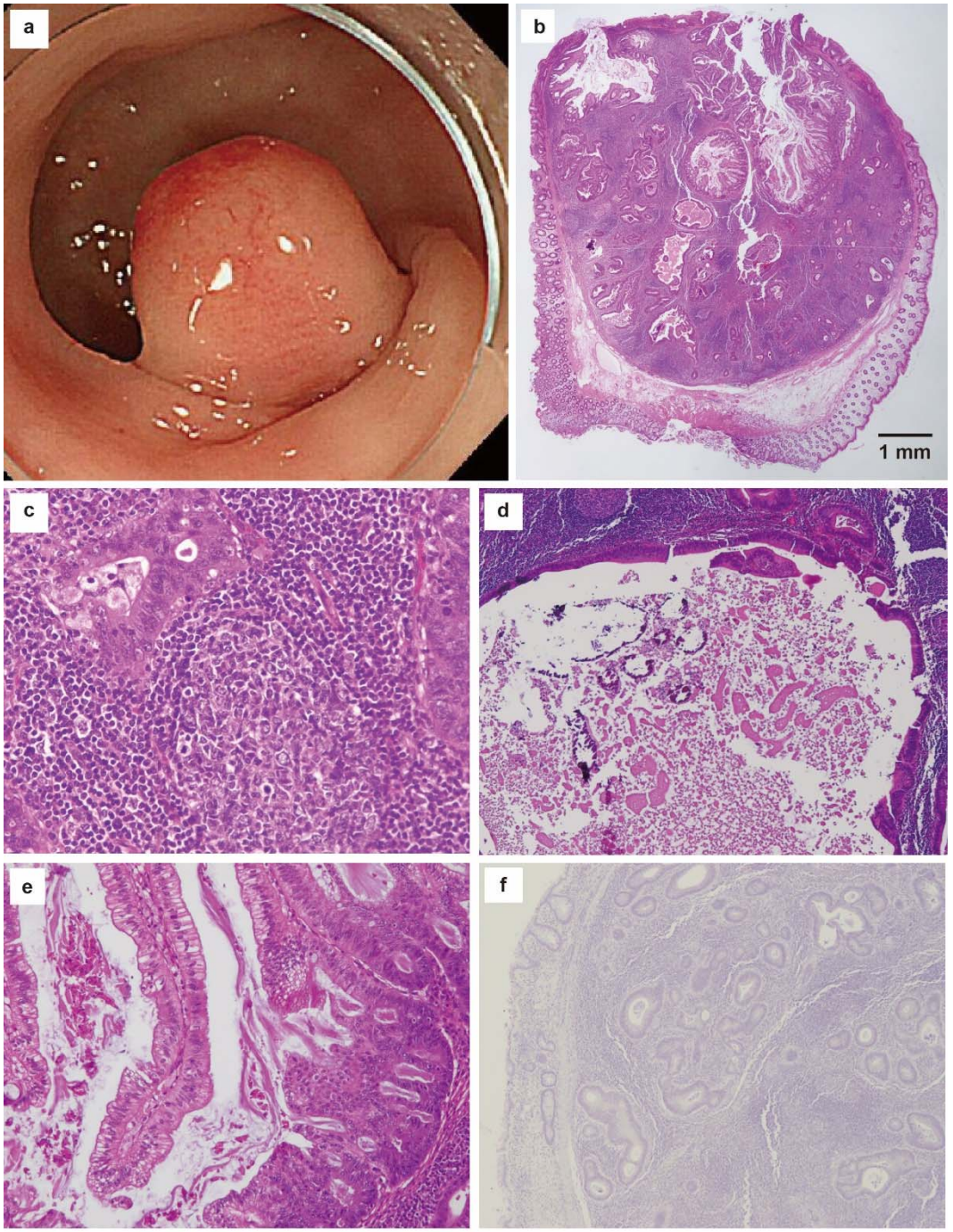


Fig. 1

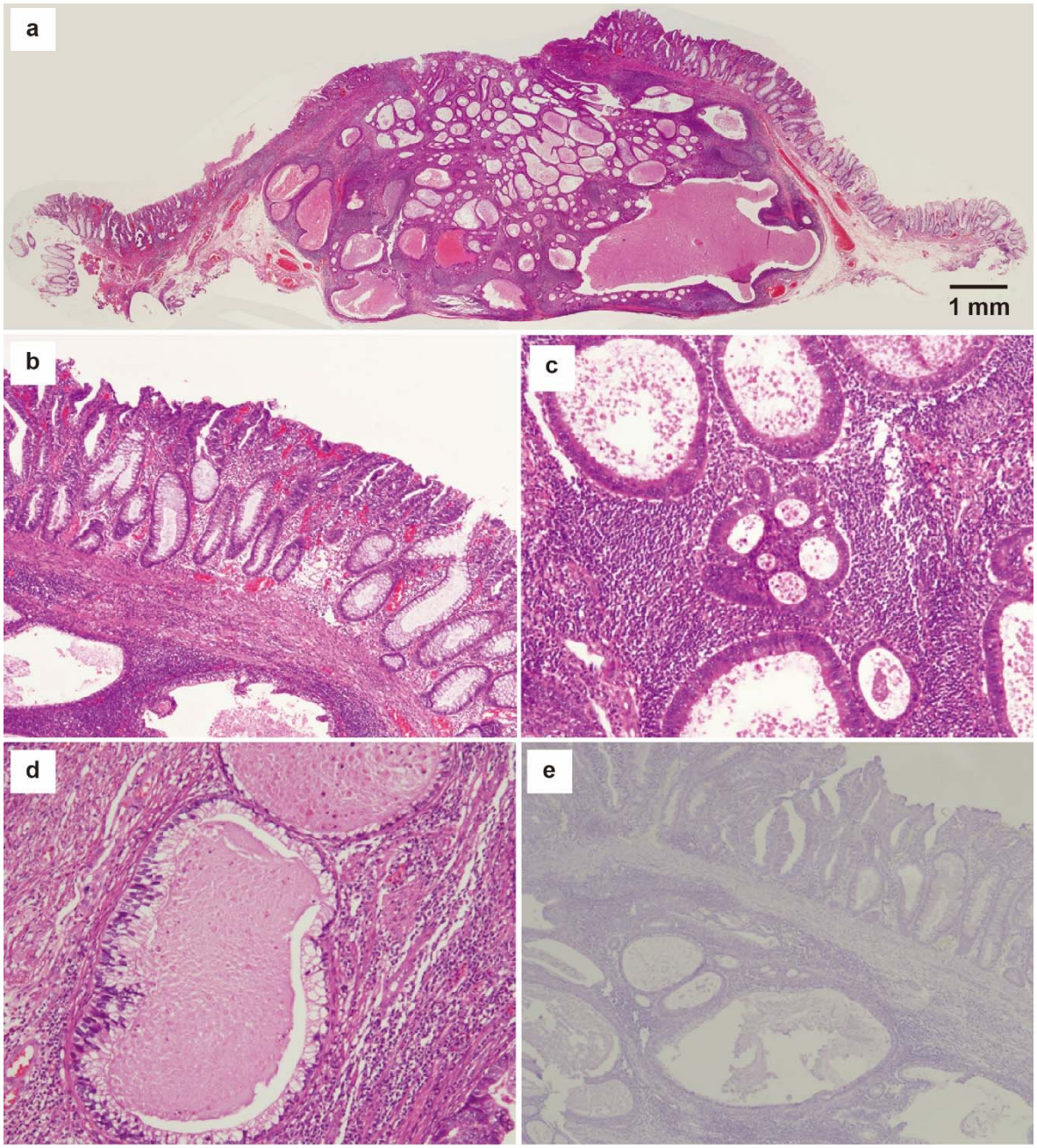


Fig. 2

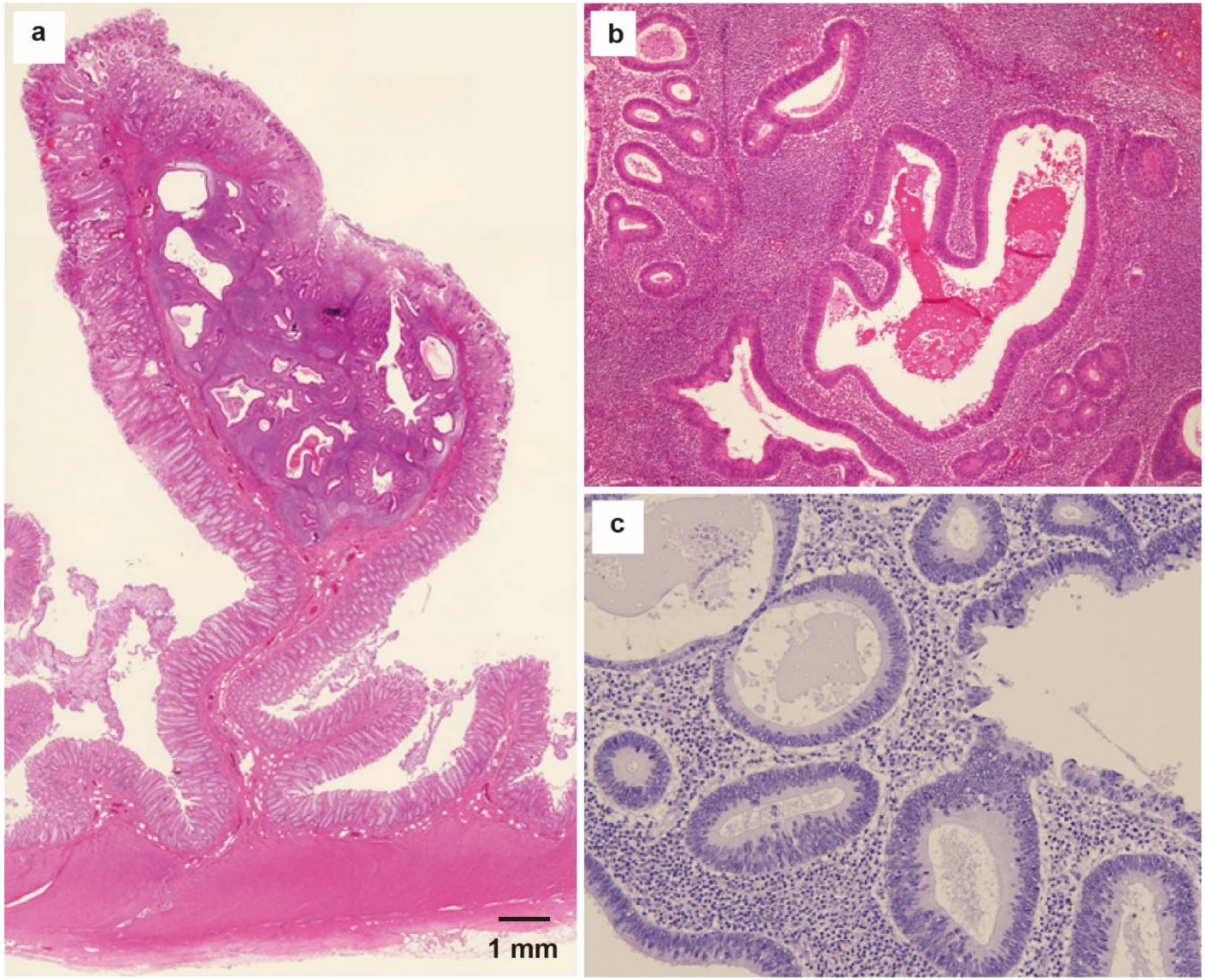


Fig. 3

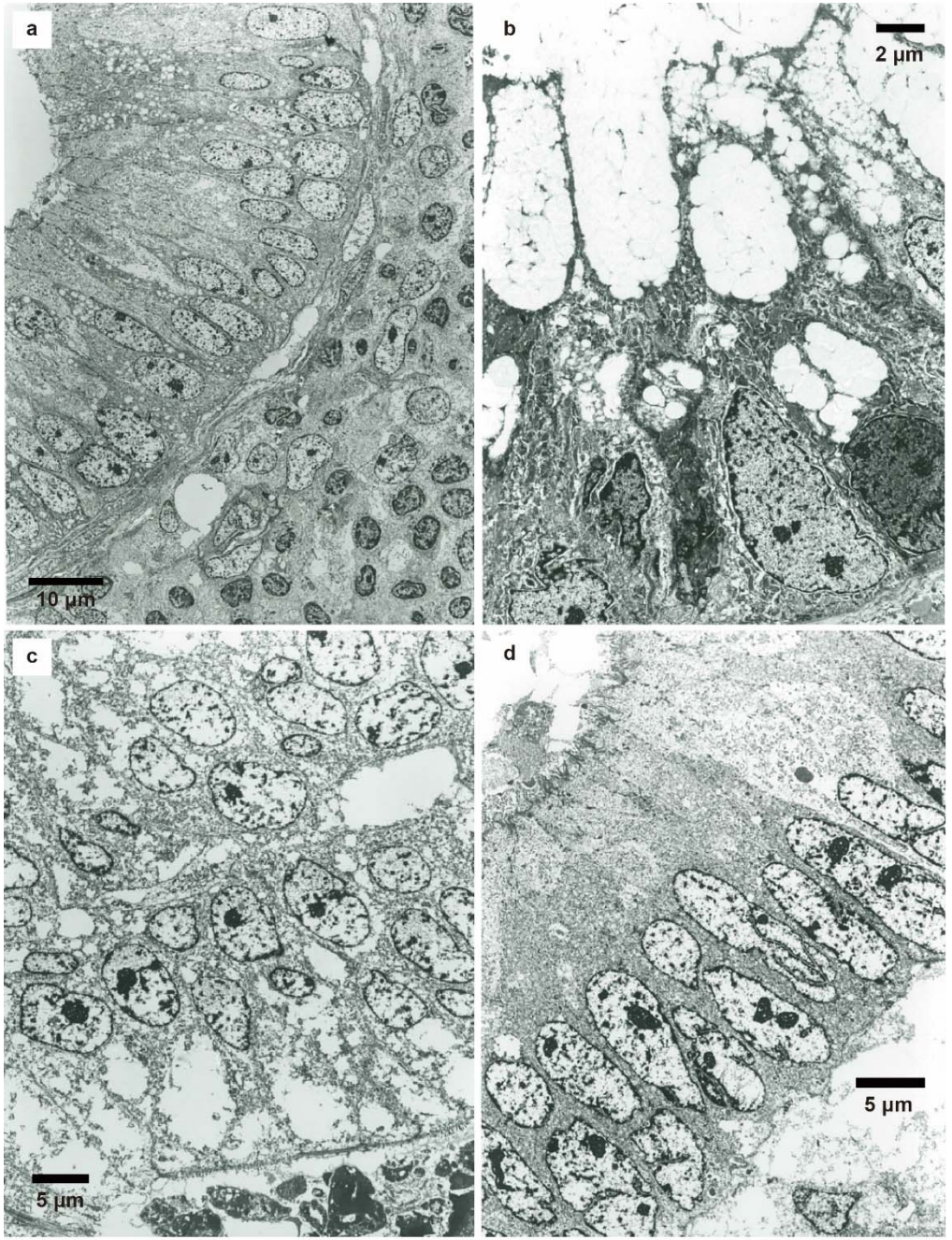
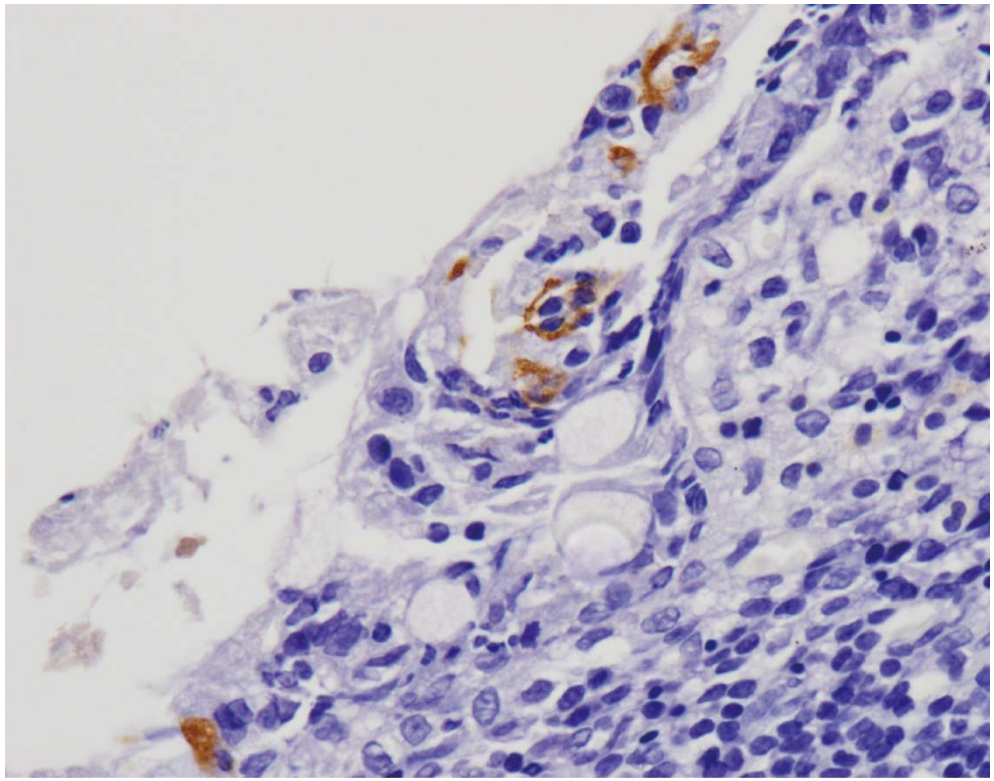


Fig. 4



Supplemental figure