distributions of 37 GISTs were 2 very low, 26 low, 7 intermediate, and 2 high risk.

Recurrence and overall survival. At a median follow-up of 74 months (range, 1–181), 5-year disease-free survival for 37 patients with GISTs was 100%. No port-site recurrences developed in any of the GIST patients. Pathologic margins were microscopically negative for all patients; however, 1 patient with an endoluminal GIST of intermediate risk developed a local recurrence 49 months after intragastric stapled wedge resection. Open proximal gastrectomy followed by reconstruction with jejunal pouch interposition was performed with no recurrence for the 94-month follow-up.

DISCUSSION

Gastric GISTs, a relatively rare entity of nonepithelial, mesenchymal neoplasm, account for less than 3% of all gastrointestinal neoplasms.[15] Local gastric resection with gross negative surgical margin is accepted worldwide for the treatment of GISTs.[1-6] Multiple studies have demonstrated the feasibility of laparoscopic resections for gastric GISTs. Sexton et al[16] demonstrated successful laparoscopic resections in 98% of 61 patients, with a morbidity rate of 16% and mortality rate of 2%. Operative indications and treatment guidelines for GIST by a laparoscopic approach are not clear.

The GIST Consensus Conference (2004) recommended limiting laparoscopic resection to tumors smaller than 2 cm due to the increased risk of tumor rupture and spillage.[17] Novitsky et al,[5] however, reported, a 92% disease-free long-term survival despite a mean tumor size of 4.4 cm (range, 1–8.5) for 50 gastric GIST patients of laparoscopic resections. Similar results were reported by Otani et al,[4] with an excellent survival after laparoscopic wedge resection for 2–5 cm gastric GISTs.

In the present study, we proposed a standardized system of operation selection based on tumor size, location, and growth morphology for suspected gastric GISTs. Our tailored laparoscopic gastric resections proved safe and feasible, resulting in a low conversion rate (2%), low morbidity rate (2%), and a favorable oncologic outcome. As a note of caution in the present study, intraoperative gastric "perforation" occurred in 2 patients with intragastric resections for endoluminal GISTs located near the EGJ; however, a good field of intragastric view can be obtained, because the full-thickness area of resected gastric wall was very small, and the perforation was closed with intragastric suture (no leakage was observed due to the perforation). We also experienced 1 local recurrence after intragastric stapled resection of a 3-cm GIST, with 5 mitoses/50 HPFs, and a surgical margin of 3 mm. This might be attributed to a small capsular injury at the bottom of the tumor caused by transgastric resection with an endoscopic linear stapler. Careful indication for intragastric stapled wedge resection is necessary, from the experience of this case, and it is particularly important to eliminate intraluminal or mixed growth types by operative findings. To prevent tumor rupture during partial gastrectomy, it is important to achieve a gross negative margin on the tumor. For the exoluminal type, traction of the normal gastric wall at the bottom of the tumor is performed using a mini loop retractor. For the endoluminal type, full-thickness resection should be considered if it is difficult to insert an endoscopic linear stapler due to the risk of tumor rupture.

Tumors located near the EGJ or pylorus are challenging and difficult to treat laparoscopically because of the risk of gastric inlet or outlet narrowing.[18-20] For small lesions, we recommend manual resection using an ultrasonic coagulating shears, with the resection margin paralleling the round edge of the tumor to limit the amount of healthy tissue loss and to avoid luminal narrowing. Transgastric or manual resections can be considered to provide a satisfactory postoperative functional reservoir of the stomach. An open operative approach is recommended for mixed type GIST near the EGJ, because laparoscopic surgery is technically more complicated. The condition may be different with laparoscopy-assisted proximal gastrectomy if operational experience is extensive with endoscopic surgery.

We report our first initial clinical experiences of SILAS for gastric sbmucosal tumors. Peri-operative results obtained in our cases are comparable to previously reported laparoscopic series.[7] SILAS have the potential to provide patients with improved cosmesis and a low level of postoperative pain, satisfying their growing demand for less invasive surgical procedures.[21,22]

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ORIGINAL PAPER

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Pancreatic-type mixed acinar-endocrine carcinoma with alpha–fetoprotein production arising from the stomach: a report of an extremely rare case

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Abstract An extremely rare case of mixed acinar-endocrine carcinoma (MAEC) arising from the stomach in a 56-year-old Japanese woman is herein presented. An endoscopic examination and computed tomography showed a protruding gastric tumor and a large extragastric mass, respectively. Macroscopic observation on the surgical specimen revealed the extragastric cystic mass was continued to the intragastric tumor. Histologically, the intragastric tumor consisted of large or small solid nests with acinar appearance. The cancer cells had an ovoid nuclei and polygonal cytoplasm, which was frequently amphophilic. Immunohistochemical examination showed that the cancer cells were positive for chromogranin-A, synaptophysin, alpha-amylase, lipase, and alpha-fetoprotein (AFP) but were negative for CD56, insulin, and other hormones. Ultrastructurally, the cancer cells contained 500-nm electron-lucent zymogen granules and 230-nm electron-dense neuroendocrine granules. This tumor was finally diagnosed to be MAEC with AFP production of the stomach. Although no ectopic pancreas was found in the stomach, this tumor may originate from ectopic pancreas. As another theory, it is possible for this tumor to originate from the pluripotent stem cells in the stomach. A gastric MAEC with AFP production has not been reported previously.

Key words Acinar-endocrine carcinoma · Alpha-fetoprotein · Stomach · Immunohistochemistry · Ultrastructure

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Introduction

Pancreatic acinar cell carcinoma (ACC) is a rare neoplasm, accounting for only 1%–2% of all exocrine tumors of the pancreas.[1] Pancreatic ACC usually appears as an exophytic, oval or round, well-circumscribed and hypovascular mass on computed tomography (CT) and magnetic resonance imaging (MRI).[2] Pancreatic-type ACC can occur in other organs, including the stomach.[3–9] Adenocarcinomas originating from an ectopic pancreas have been reported in fewer than 30 cases in the literature,[8–11] and a few reports have shown gastric ACC and neuroendocrine carcinoma arising from an ectopic pancreas.[3,12,13] So far, there have been only rare case reports of pancreatic-type carcinomas arising from the stomach.

This report presents one extremely rare case with a mixed acinar-endocrine carcinoma (MAEC) with alpha-fetoprotein (AFP) production of the stomach. Only four cases of MAEC or composite glandular and endocrine carcinoma with acinar differentiation (CGEA) have been reported.[14,15] This report is the first case of gastric pure MAEC with AFP production studied by electron microscopy and immunohistochemistry, especially by double-immunostaining for exocrine and endocrine markers.

Materials and methods

A case of MAEC was surgically resected under the diagnosis of advanced gastric cancer and processed for routine surgical pathology. Detailed clinical information was obtained from the medical record. All the specimens of the resected stomach were routinely investigated by hematoxylin and eosin (H&E) stain, and periodic acid–Schiff (PAS) and Alcian blue staining was also performed. An immunohistochemical analysis for the tumor was performed using EnVision + (DakoCytomation, Carpinteria, CA, USA) on formalin-fixed, paraffin-embedded 4-μm sections. The antibodies used in this study are summarized in Table 1. Fluorescence double-immunostaining for alpha-amylase

Table 1. Antibodies in this immunohistochemical study and the results

Antibody to:	Clone	P/M	Source	Antigen retrieval	Results
pan-CK	AE-1/3	M	Signet Laboratories (Dedham, MA, USA)	Autoclave*	++
HMWK	34betaE12	M	DakoCytomation (Carpinteria, CA, USA)	Autoclave*	–
CK8,18	CAM5.2	M	Becton Dickinson (San Jose, CA, USA)	Autoclave*	+
CK7	OV-TL12/30	M	DakoCytomation (Carpinteria, CA, USA)	Autoclave*	++
CK19	Ks19.1	M	Progen Biotechnik (Geiderberg, Germany)	Autoclave*	++
CK20	IT-Ks20.8	M	Progen Biotechnik (Geiderberg, Germany)	Autoclave*	–
CD56	Lu243	M	Nihon-Kayaku (Tokyo, Japan)	Autoclave*	–
Chromogranin-A		P	DakoCytomation (Carpinteria, CA, USA)		+
Synaptophysin		P	Signet Laboratories, Inc (Dedham, MA, USA)	Autoclave*	+
Alpha-amylase		P	Biomeda (Foster City, CA, USA)		+
Lipase		P	Abcam (Cambridge, UK)		+
Trypsin		P	Abcam (Cambridge, UK)		f+
Alpha-1AT		P	DakoCytomation (Carpinteria, CA, USA)		f+
AFP		P	DakoCytomation (Carpinteria, CA, USA)	Autoclave*	p+
CA19-9	116NS199	M	DakoCytomation (Carpinteria, CA, USA)		p+
CA125	OC125	M	DakoCytomation (Carpinteria, CA, USA)	Autoclave*	–
CEA	CEM10	M	DakoCytomation (Carpinteria, CA, USA)		–
CD117 (c-kit)		P	DakoCytomation (Carpinteria, CA, USA)	Autoclave*	–
CD34	My10	M	Becton Dickinson (San Jose, CA, USA)	Autoclave*	–
Caldesmon	h-CD	M	DakoCytomation (Carpinteria, CA, USA)	Autoclave*	–
Insulin		P	DakoCytomation (Carpinteria, CA, USA)		–
Glucagon		P	IBL(Gunma, Japan)	Autoclave*	–
PP		P	DakoCytomation (Carpinteria, CA, USA)		–
Somatostatin		P	DakoCytomation (Carpinteria, CA, USA)		–
Serotonin	5HT-H209	M	DakoCytomation (Carpinteria, CA, USA)		–
MUC1	NCL-Mab695MUC1	M	Novocastra Laboratories (Newcastle upon Tyne, UK)	Autoclave*	–
MUC2	NCL-CLP58MUC2	M	Novocastra Laboratories (Newcastle upon Tyne, UK)	Autoclave*	–
MUC4	1G8	M	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	Autoclave*	–
MUC5AC	CLH2MUC5AC	M	Novocastra Laboratories (Newcastle upon Tyne, UK)	Autoclave*	–
MUC6	CLH5MUC6	M	Novocastra Laboratories (Newcastle upon Tyne, UK)	Autoclave*	–
Ki-67 L.I.	MIB-1	M	DakoCytomation (Carpinteria, CA, USA)	Autoclave*	34.5%

CK, cytokeratin; HMWK, high molecular weight keratin; alpha-1AT, alpha-1 antitrypsin; AFP, alpha-fetoprotein; CEA, carcinoembryonic antigen; PP, pancreatic polypeptide; Ki-67 L.I., Ki-67 labeling index; P, polyclonal; M, monoclonal; autoclave*, autoclave in 0.1 mol/l citrate buffer, pH 6.0; –, negative; f+, focally positive; p+, partially positive; +, positive; ++, strongly and diffusely positive

and chromogranin-A was performed with the indirect immunofluorescence method using fluorescein isothiocyanate (FITC)-conjugated swine antirabbit immunoglobulins (code no. F2 205; DakoCytomation) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine antirabbit immunoglobulin (code no. R0156; DakoCytomation). For transmission electron microscopic analysis, small blocks, ~1 mm^3, of the formalin-fixed specimens were refixed in 2% glutaraldehyde in phosphate-buffered saline (PBS) at 4°C overnight and then for 2 h at 4°C with 1% osmium tetraoxide in PBS. After dehydration, the sections were transferred to propylene oxide and embedded in epoxy resin Quetol 812 (Nisshin EM, Tokyo, Japan). Ultrathin sections were cut with an Ultracut UCT (Leica Microsystems, Vienna, Austria), doubly stained with uranyl acetate and lead citrate, and observed with a JEM-1230 electron microscope (JEOL, Tokyo, Japan).

Results

Clinical findings

A 56-year-old Japanese woman was shown to have a gastric tumor by endoscopic examination; endoscopic biopsy revealed it to be "poorly differentiated adenocarcinoma," and a CT scan revealed a large extragastric mass. She was admitted to our hospital for surgery. She had not experienced nausea, vomiting, or dysphagia, except for a slight fever. Routine blood and liver function tests showed elevated levels of CA125 (229 U/ml: normal range, 0–35 U/ml), amylase (573 U/l: pancreatic amylase, 93 U/dl, normal range, 45–116 U/dl), and AFP (318 ng/ml: normal range, 0–10.0 ng/ml). Her medical history included hypertension, a total hysterectomy for reasons of endometriosis, and an appendectomy. Upper endoscopy revealed a 6.0-cm protruding tumor in the gastric body. A biopsy specimen was pathologically suspected to be "well-differentiated neuroendocrine carcinoma." A CT scan revealed a large extragastric mass, suggestive of a gastrointestinal stromal tumor (GIST) between the stomach and pancreas, which showed irregularly low intensity in the internal area of this mass (Fig. 1). The clinical diagnosis was an advanced gastric cancer associated with GIST and the clinical stage was estimated to be cT3N2M0. A CT scan did not find any metastatic foci in the lung and liver and showed no findings of a head and neck primary lesion. A total gastrectomy, splenectomy, right-half colonectomy, and lymph node dissection were performed. At surgery, bloody ascites were observed from the ruptured extragastric mass, which was

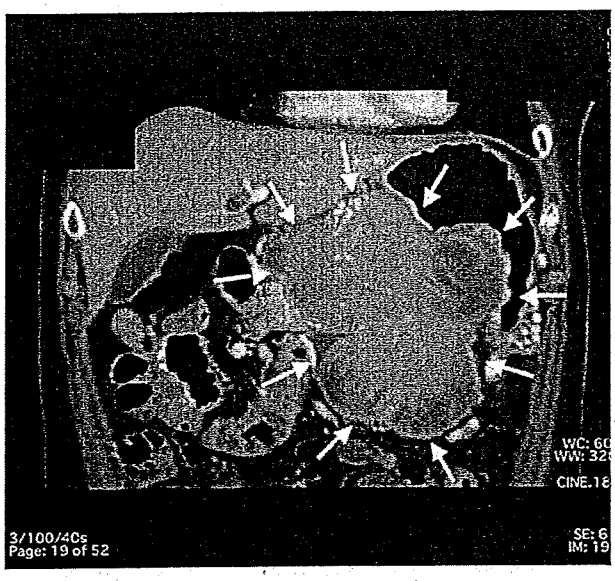

Fig. 1. Computed tomography showed a large extragastric mass (*arrows*), which seemed to be adherent to the pancreas, but the tumor originated from the stomach

not connected with the pancreas. After 1 month, chemotherapy (TS-1 and CDDP) was initiated. After 2 months, she experienced severe vomiting and was admitted to the emergency room. At that time, a CT scan showed marked peritoneal dissemination and multiple liver metastases. After 1 month, axillary skin metastases emerged, and thereafter the patient died of the disease. Autopsy was not permitted.

Pathological findings

The gastric tumor and the extragastric mass were in the same region of the stomach. Macroscopically, a direct connection between the gastric tumor and the extragastric mass was confirmed (Fig. 2). The extragastric mass was cystic and contained bloody clots. The cut surface of both tumors appeared smooth and grayish-white to tan in color.

By microscopic examination, the gastric and extragastric tumors showed similar histology and therefore the extragastric tumor was not a GIST (Fig. 3a). The predominant circumscribed cellular islands composed of large and small solid nests of polygonal cells were surrounded by numerous small blood vessels, and an acinar pattern was frequently seen (Fig. 3b,c). The tumor exhibited expansive growth. The tumor cells had moderate amounts of cytoplasm and round to oval nuclei with minimal to moderate atypia and one or two large nucleoli. The cytoplasm varied from abundantly amphophilic to moderately abundant, granular, and eosinophilic (Fig. 3d,f). Tumor cells with pyknotic nuclei were present at the periphery of the solid nests. The present case showed no differentiated component characteristic of a tubular adenocarcinoma. The mitotic rate was 1 per 10 high-power fields. There was no necrosis and vascular or perineural invasion, but there was mild lymphatic involvement. Although all the resected specimens were examined, neither an ectopic pancreas nor a pancreatic acinar metaplasia was found in any of the gastrectomy specimens. The nontumorous gastric mucosa showed chronic gastritis with intestinal metaplasia but revealed no findings of *Helicobacter pylori* infection.

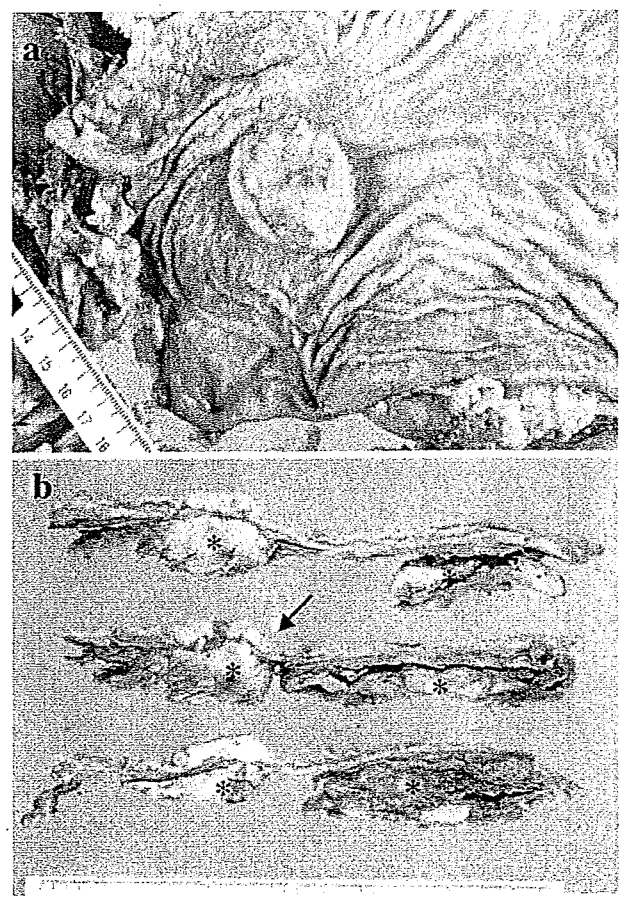

Fig. 2. Macroscopic findings **a** The gastric lesion showed a Bormann type 1-like protruded tumor. **b** The intragastric tumor was continuous with the extragastric mass (*asterisks* and *arrows*, respectively). Both tumors showed a grayish-white to tan appearance and a relatively pushing growth pattern

The histological appearance of the lymph node metastasis was identical to that of the primary tumor. No direct invasion or metastasis to the colon and spleen was seen. Therefore, pathological tumor stage was pT3N1M0 (stage IIIA).

Histochemical and immunohistochemical analysis

The cancer cells showing solid nests were negative for PAS and Alcian blue stains, whereas they were focally positive for PAS with a granular pattern (Fig. 4a). Results of immunohistochemical analysis are shown in Table 1. Strong immunoreactivity for pan-cytokeratins (CKs) CK7, CK8, 18, and CK19 was observed, but no immunoreactivity for CK20 and high molecular weight keratin was seen (Fig. 4b).

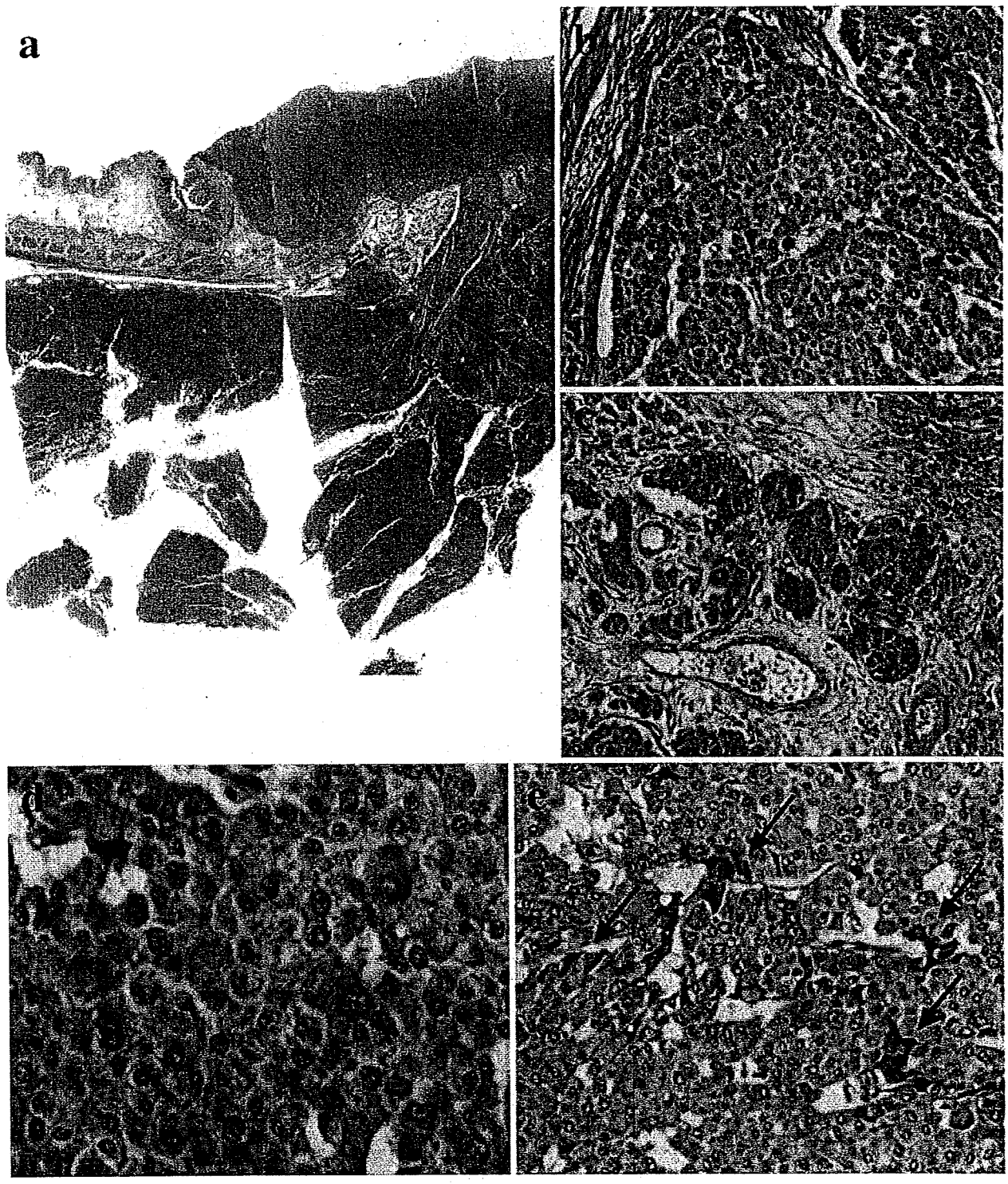

Fig. 3. Microscopic findings. **a** The intragastric tumor showed an expansive growth pattern, which was continuous with the extragastric tumor (*asterisk*). **b** The tumor showed a solid nest pattern with expansive and medullary growth. A small stroma composed of blood vessels and connective tissues was observed. **c** The tumor cells partially showed an acinar pattern, which was similar to pancreatic acinar cell carcinoma. **d** The tumor cells revealed ovoid and abundant amphophilic cytoplasm and round nuclei with one large nucleolus. **e** The cytoplasm of the tumor cells showed a partly eosinophilic appearance. Such tumor cells, which were present in the nests, had pyknotic nuclei (*arrows*). **a** ×5; **b, d, e** ×200; **c** ×100

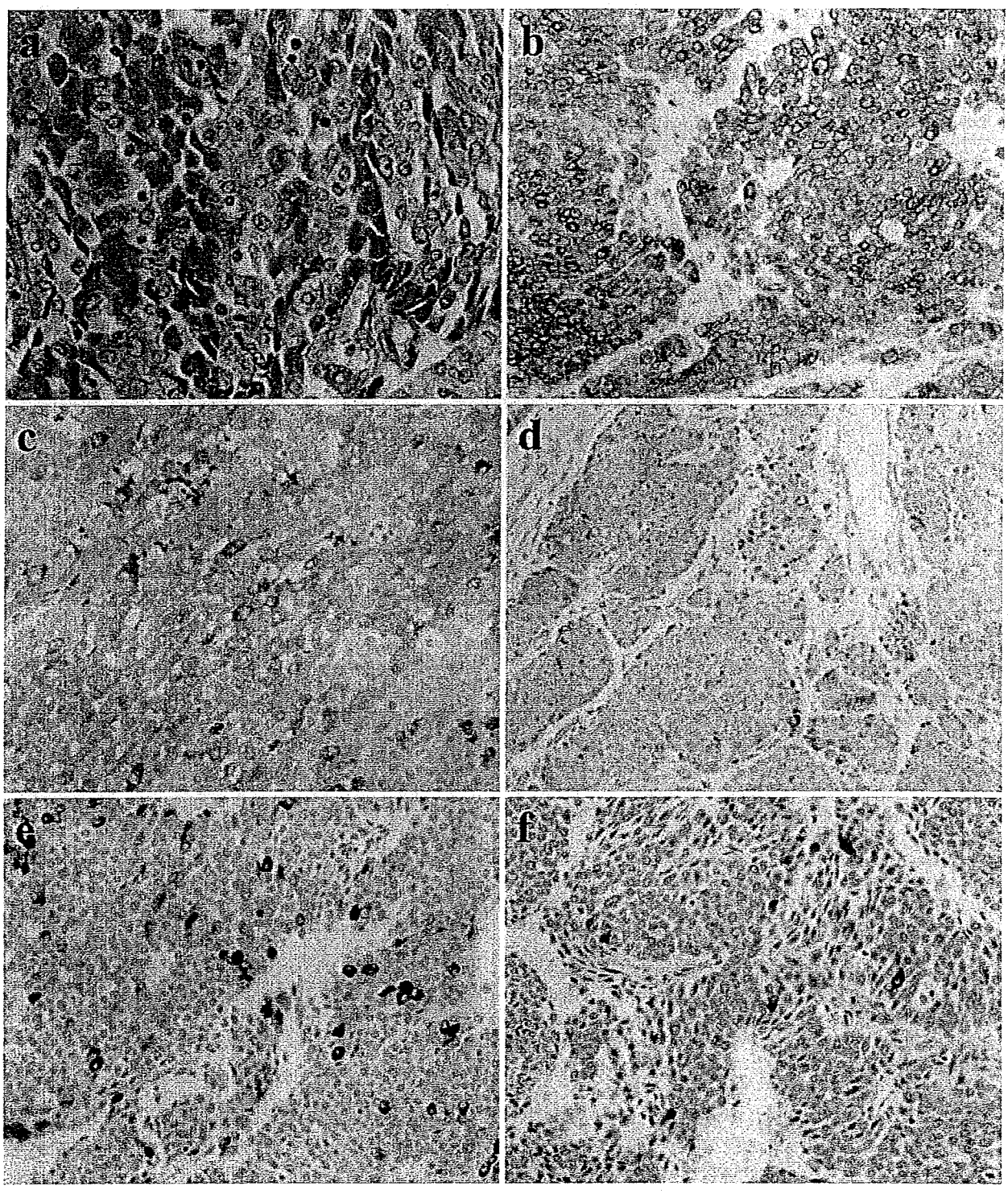

Fig. 4. Histochemical and immunohistochemical findings. **a** Periodic acid–Schiff (PAS) stains revealed fine granules in the cytoplasm that were resistant to diastase digestion. **b** The tumor cells showed strong and diffuse immunoreactivity for cytokeratin 7. **c** Tumor cells focally showed strong immunoreactivity for CA19-9. **d** Tumor cells showed diffuse immunoreactivity for synaptophysin. **e** Tumor cells partially showed strong immunoreactivity with a granular pattern for lipase. **e** Tumor cells showed strong and scattered immunoreactivity for alpha-fetoprotein. a–e ×200

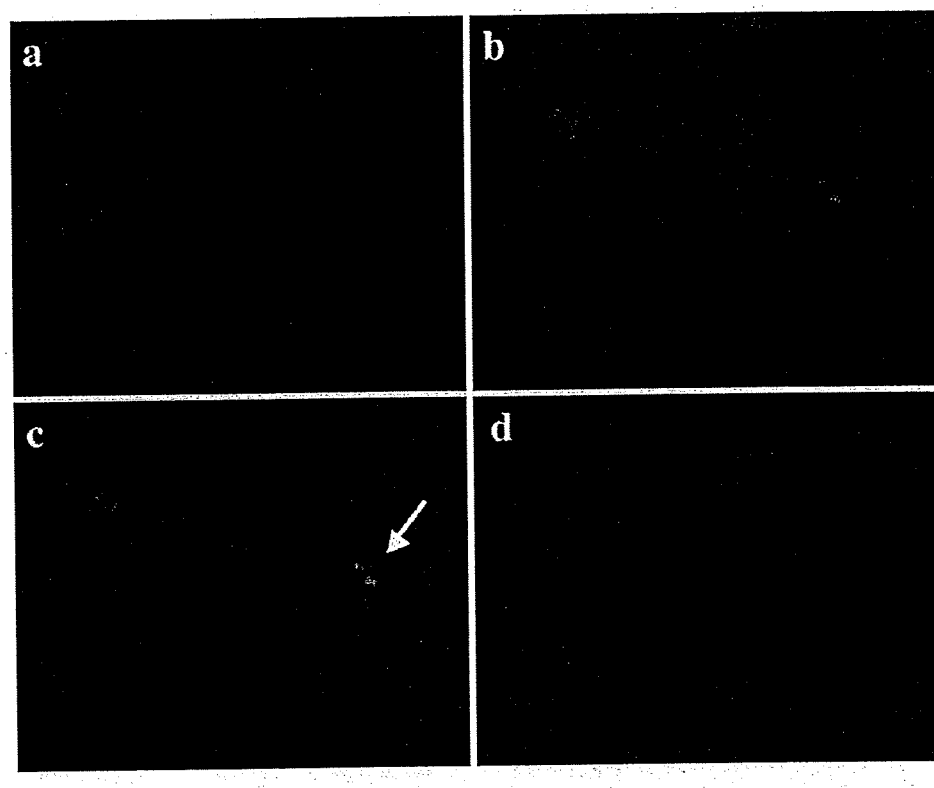

Fig. 5. Results of fluorescence double-immunostaining of alpha-amylase and chromogranin-A. **a** Some tumor cells showed red fluorescent signals for alpha-amylase. **b** Some tumor cells showed green fluorescent signals for chromogranin-A. **c** The tumor cells showed immunoreactivity for alpha-amylase (*red* signals) and chromogranin-A (*green* signals), respectively, whereas a few cells showed fluorescent labeling in the same cells (*yellow* signals: *arrow*). **d** The negative control, in which the primary antibodies were replaced with normal rabbit serum, showed no signals for alpha-amylase or chromogranin-A. **a–d** ×200

Immunoreactivity for endocrine markers (chromogranin-A and synaptophysin) was noted in the solid and nested cells (Fig. 4c), but no immunoreactivity for CD56 was seen. Partial immunoreactivity for pancreatic exocrine markers (alpha-amylase, lipase, trypsin, and alpha-1-antitrypsin) was observed in the cytoplasm of cancer cells with a coarse granular pattern (Fig. 4d). Patchy immunoreactivity for AFP was observed in solid nests (Fig. 4e). Although the cancer cells were focally positive for CA19-9 (Fig. 4f), no immunoreactivity for CA125 and CEA was seen. There was no immunoreactivity for CD117 (c-kit), CD34, caldesmon, insulin, glucagons, pancreatic polypeptide, somatostatin, serotonin, MUC1, MUC2, MUC4, MUC5AC, or MUC6.

Fluorescence double-immunostaining of alpha-amylase and chromogranin-A indicated that a few tumor cells coexpressed both exocrine and endocrine markers, but most tumor cells were positive for either exocrine or endocrine markers (Fig. 5).

Ultrastructural examination

Ultrastructurally, the cancer cells contained both 500-nm electron-lucent granules and 230-nm electron-dense granules in the same cells (Fig. 6). The latter had markedly electron-dense cores. The cancer cells showed polygonal cytoplasm, which contained small amounts of rough endoplasmic reticulum and ribosomes. Intercellular desmosome-like junctions were infrequently seen between the cancer cells.

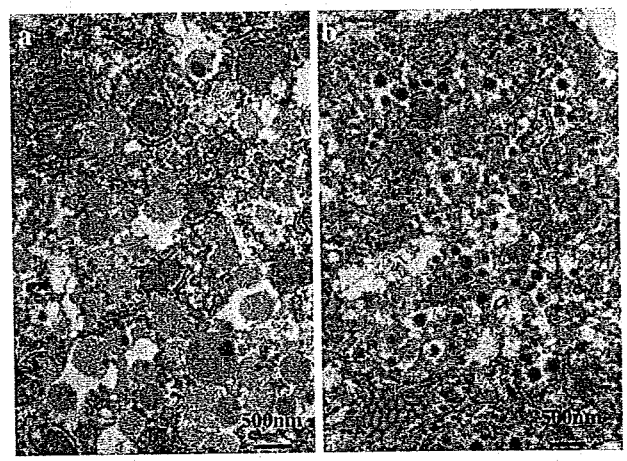

Fig. 6. Transmission electron microscopic findings. The tumor cells contained electron-lucent granules measuring 500 nm in diameter (**a**), whereas the tumor cells contained electron-dense granules measuring 230 nm in diameter (**b**). The former were exocrine (zymogen) granules and the latter were neuroendocrine granules, which had electron-dense cores

Discussion

The present gastric tumor was histologically very similar to an MAEC of the pancreas.[16–17] It showed solid nests, which frequently included an acinar pattern, and abundant amphophilic or eosinophilic cytoplasm with a granular appearance.

Immunohistochemically, the tumor not only expressed the pancreatic enzymes, lipase, trypsin, alpha-1-antitrypsin, and the exocrine marker (alpha-amylase) but also expressed endocrine markers (chromogranin-A and synaptophysin). However, the tumor cells showed no immunolocalization of gastrointestinal and pancreatic hormones in the extent of the present examination. Fluorescence double-immunostaining for alpha-amylase and chromogranin-A and ultrastructural observation elucidated both exocrine and endocrine differentiations of the tumor cells. Because the ultrastructural examination showed neuroendocrine granules in the tumor cells, it is possible that this tumor produces some hormones and prohormones. However, we could not confirm what kinds of hormones or prohormones in the extent of our immunohistochemical panel. As endocrine markers are focally expressed in 42% of ACCs,[18–21] some of the previously reported pancreatic ACCs might be reclassified as MAECs.

Only four cases of gastric MAEC have been reported so far. The first case of MAEC was reported as a "gastric carcinoma resembling pancreatic mixed acinar-endocrine carcinoma" by Fukunaga in 2002,[14] and then three other cases were reported as "composite glandular and endocrine tumors of the stomach with pancreatic acinar differentiation" by Jain et al. in 2005.[15] The previous cases had glandular components but were not examined for their AFP immunoreactivity and ultrastructure. The current case showed solid nest components with AFP production without any glandular components. Gastric carcinomas with hepatoid features frequently show AFP production, which are termed "AFP-producing carcinoma" or "hepatoid adenocarcinoma".[22] Because such gastric carcinomas are high-grade carcinomas, gastric carcinoma with AFP production has a poor prognosis. Ooi et al. reported a medullary tumor with gastrointestinal tract-specific AFP,[23] and their cases might be MAECs with AFP production. As our present patient also died of the disease, the AFP-producing ability in gastric cancers plays an important role in the prognosis.

The origin of gastric ACC was considered to be an ectopic pancreas.[3,12] On the other hand, Jain et al. suggested that CGEA might develop from a pluripotent stem cell with the potential for divergent differentiation.[15] An ectopic pancreas is common in the stomach, and a few examples of carcinomas arising in ectopic pancreas have been described.[3,10–13] However, extensive study in all these cases failed to reveal any evidence of nonneoplastic pancreatic tissue. One could argue that the tumor may have overgrown the pancreatic rests. The incidence of ectopic pancreas in the stomach in autopsies ranges from 0.5% to 13.7%, being more common at the age of 35–50 years, with male predominance.[24] The most common accepted theory suggests that, during the development of the normal pancreas from several evaginations originating from the wall of the primitive duodenum, one or more evaginations may remain in the bowel wall. The migration of this embryonic remnant along with the development of the gastrointestinal tract gives rise to the ectopic pancreatic tissue.[25] Another theory suggests that during embryogenesis pancreatic metaplasia of the endodermal tissues localized in the gastric submucosa may occur. The latter is associated with the foregoing pluripotent stem cell theory. However, we could not find pancreatic metaplasia in this case, and it remains unclear whether the origination of the current tumor was related to pluripotent stem cells in the stomach.

Metastasis or direct invasion from a pancreatic primary lesion seems unlikely, as imaging and surgical findings indicated was no space-occupying lesion to exist in the pancreas or no connection with the tumor and the pancreas. In the present case, no primary lesion existed in the head and neck region. Therefore, this case was not metastasis of an acinic cell carcinoma of the head and neck.

The possibility of pancreatic-type differentiation seems most likely. The presence of exocrine enzymes in both the endocrine and glandular cells suggests that the lesion may have originated from a common pluripotent stem cell. Pancreatic metaplasia in the stomach subsequent to chronic inflammation of the stomach is well recognized. Pancreatic metaplasia containing lipase-producing cells has been described with chronic atrophic gastritis.[26,27] Lipase has been demonstrated in pancreatic metaplasia, although it is still controversial whether pancreatic-type acinar cell are normal or metaplastic.[28] Although no pancreatic metaplasia or ectopic pancreas was observed in any sections of the resected stomach, the present tumor has originated from ectopic pancreatic tissue in the stomach. There are about 30 reported cases of adenocarcinomas and neuroendocrine carcinomas that developed from ectopic pancreas;[3,10–13] in such cases, heterotopia had been confirmed at the gastric mucosa near the tumor, whereas some reports failed to find an ectopic pancreas near the tumor. During tumor progression, the ectopic pancreatic tissue might be destroyed and vanish.

In summary, this was the first reported case of a gastric MAEC with AFP production that included immunohistochemical and ultrastructural examination. Although the preoperative biopsy specimen indicated well-differentiated neuroendocrine carcinoma, this tumor was finally diagnosed to be MAEC with AFP production of the stomach. The gastric MAEC may originate from ectopic pancreatic tissue or pluripotent stem cells. This tumor expressed both exocrine and endocrine markers with AFP immunoreactivity. Although pancreatic ACC has a relatively good prognosis, the present case had a poor prognosis.

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Original article

Expression of tight-junction-associated proteins in human gastric cancer: downregulation of claudin-4 correlates with tumor aggressiveness and survival

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Abstract

Background. **Claudin, occludin, and zonula occludens (ZO)-1 are known as tight-junction-associated proteins. The aim of this study was to examine the expression of these proteins in gastric carcinoma.**

Methods. **Gastric cancer tissues ($n = 124$) were obtained from 124 patients who underwent gastrectomy at our hospital between January 2000 and December 2004. The expression of the above tight-junction-associated proteins in carcinoma, normal mucosa, and metaplastic epithelium was examined using immunohistochemistry. In addition, the expression of claudin-4 mRNA was examined in fresh frozen tissue obtained from 34 patients.**

Results. **Significant correlations were seen between the expression of claudin-4, occludin, and ZO-1. In regard to claudin-4, significant correlations were seen between the expression of claudin-4 evaluated by immunohistochemistry and the expression of claudin-4 mRNA. Claudin-4 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma, advanced T stage, lymph node metastasis, and peritoneal metastasis. Occludin and ZO-1 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma. Overall survival was significantly shorter in patients with low claudin-4 expression. Cox multivariate analysis revealed that low claudin-4 expression was independently associated with significantly decreased overall survival.**

Conclusion. **Tight-junction-associated proteins, particularly claudin-4, may play important roles in determining invasiveness, metastatic potential, and survival in gastric cancer.**

Key words Occludin · Zonula occludens-1 protein · Metastasis

Introduction

Tight junctions, adherens junctions, gap junctions, and desmosomes are the known cell membrane structures that participate in cell-to-cell adhesion. Tight junctions are present in epithelial and endothelial cell membranes, forming a component of intercellular junctional complexes and playing important roles in barrier function, cell polarity, and cell signaling pathways [1].

Claudins are major tight-junction constituents and display four transmembrane domains. This multiple gene family is expressed in a tissue-specific pattern. To date, 24 members of the claudin family have been identified [1].

Occludin is another constituent of tight junctions [1, 2], and again has four transmembrane domains. To date, no occludin isotypes have been identified in any species [1]. Occludin-deficient epithelial cells demonstrate a well-developed network of tight junction strands [3], suggesting that occludin is an accessory protein in terms of tight-junction strand formation [1].

Zonula occludens (ZO)-1, -2 and -3 are membrane-associated proteins that connect tight junctions to the cytoskeleton [2]. ZO-1 establishes a link between occludin and the actin cytoskeleton [3], and is part of a signaling pathway linking tight junctions to the regulation of gene expression [4].

In normal gastric mucosa, positive immunostaining has been detected for claudin-18, but not for claudin-4 [5]. We have previously revealed, using an oligonucleotide microarray, that claudin-4 is upregulated in gastric cancer [6], and another study using an oligonucleotide microarray has reported that claudin-4 is upregulated in intestinal-type gastric cancer [7]. Moreover, claudin-4 is reportedly highly expressed in gastric intestinal-type adenocarcinoma [5, 8, 9]. To date, several studies have been reported regarding the biological functions of claudin-4 in gastric cancer. For example, it has been reported that *Helicobacter pylori* was able to increase paracellular permeability by disrupting occludin, claudin-4, and claudin-5 [10], and it has been shown that Cdx2 plays an important role in the regulation of intestinal claudin expression, not only

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in gastric mucosa with intestinal metaplasia but also in gastric carcinoma [11].

Considering the circumstances mentioned above, claudin-4 may play an important role in gastric carcinogenesis. However, the exact function of claudin-4 in gastric cancer is still unclear. As previously reported, it is probably true that claudin-4 expression is decreased in diffuse-type adenocarcinoma [5, 7, 8, 12]. In terms of the aggressiveness of gastric carcinoma, a few studies have reported about it. For example, a trend was observed between the overexpression of claudin-4 and lymph node metastasis [13], and a trend was observed between reduced claudin-4 expression and advanced T stage [12]. For patient survival, Lee et al. [12] reported that reduced expression of claudin-4 showed an associative tendency with a high cumulative recurrence rate in patients with gastric carcinoma, whereas Resnick et al. [8] reported a significant association between claudin-4 expression and poor survival.

To sum up, the correlation between claudin-4 expression and the aggressiveness of gastric cancer, shown by such features as invasion, metastasis, and survival, is still controversial.

Therefore, the aim of this study was to examine the expression of claudin-4 in gastric carcinoma, normal mucosa, and metaplastic epithelium, and to correlate the expression of this protein with features of the aggressiveness of gastric cancer such as invasion, metastasis and survival. In addition to the expression of claudin-4, the expression of occludin and ZO-1 is reportedly reduced in poorly differentiated gastric adenocarcinoma [14], and there is very little combined analysis of claudin-4, occludin, and ZO-1 expression. We therefore also examined the expression of occludin and ZO-1, as known components of tight junctions, in gastric carcinoma to provide some insights into gastric carcinogenesis.

Patients, materials and methods

Tissue samples

A total of 124 gastric cancer tissues were obtained from 124 patients who underwent gastrectomy at Fukushima Medical University between January 2000 and December 2004.

Written informed consent was obtained from all patients and ethical approval of this study was obtained from the ethics committee of Fukushima Medical University. Patient characteristics are shown in Table 1. The mean age of the patients (89 men and 35 women) was 66.8 years (range, 18–86 years). Follow-up time ranged from 31 to 1931 days (median value was 666.5 days). There were 57 cases of total gastrectomy, 54 cases of distal gastrectomy, 5 cases of proximal gastrectomy, and 7 cases of pylorus-preserving gastrectomy. One hundred and nine patients received curative operations and 15 received noncurative operations.

According to the *Japanese classification of gastric carcinoma* (2nd English edition; Table 1) [15], there were 52 cases of stage IA, 27 cases of stage IB, 11 cases of stage II, 9 cases of stage IIIA, 4 cases of stage IIIB, and 21 cases of stage IV. Histological type was classified as: (a) differentiated-type adenocarcinoma, including papillary adenocarcinoma and tubular adenocarcinoma; and (b) undifferentiated-type adenocarcinoma, including poorly differentiated adenocarcinoma, mucinous adenocarcinoma, and signet ring cell carcinoma. According to this classification, there were 70 cases of differentiated-type adenocarcinoma and 54 cases of undifferentiated adenocarcinoma. Five-year survival stratified according to pT and pN is shown in Table 2.

Immunohistochemistry

Resected specimens were fixed with 10% buffered formalin and embedded in paraffin. Tissue blocks were then sliced into 4-μm sections and mounted on glass slides. The tissue sections were dewaxed and rehydrated through graduated changes of xylene and graded alcohol, then washed with phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by incubating the sections with 0.3% hydrogen peroxide for 10 min, and then the sections were washed with PBS. The sections were pretreated in 10-mM citrate buffer (pH 6), using a microwave-based antigen retrieval method for 15 min. After being washed with PBS, to block nonspecific background reactions, the sections were exposed to normal bovine serum for 10 min, and then incubated with monoclonal antihuman claudin-4, occludin, ZO-1 antibody (Zymed Laboratories, South San Francisco, CA, USA) overnight at a dilution of 1: 100. After being washed with PBS, the sections were incubated with biotinylated secondary antibody for 10 min. After a further wash with PBS, the sections were incubated with peroxidase-conjugated streptavidin for 5 min. The peroxidase reaction was carried out in a solution of hydrogen peroxide as a substrate and 3, 3′ diaminobenzidine tetrahydrochloride as a chromogen. Positive controls were colonic mucosa and negative controls were the same specimens in which the primary antibody was replaced with nonreactive antibodies. We used a Histofine SAB-PO kit (Nichirei, Tokyo, Japan), which contains blocking antibody, biotinylated secondary antibody, and peroxidase-conjugated streptavidin.

Table 1. Patient characteristics

Sex		Lymph node metastasis	
Male	89	pN0	80
Female	35	pN1	26
Age (years)	18–86 (Mean, 66.8)	pN2	12
Type of surgery		pN3	6
TG	57	Histological type	
DG	54	pap	5
PG	5	tub1	40
PPG	7	tub2	25
Other	1	por1	10
Peritoneal metastasis		por2	18
P0	112	sig	18
P1	12	muc	7
Hepatic metastasis		Other	1
H0	118	Stage	
H1	6	IA	52
Depth of invasion		IB	27
pT1	59	II	11
pT2	35	IIIA	9
pT3	23	IIIB	4
pT4	7	IV	21
		Curability	
		A	85
		B	24
		C	15

A total of 124 patients who underwent gastrectomy at Fukushima Medical University from 2000 to 2004 were included. Patient characteristics were classified according to the *Japanese classification of gastric carcinoma* (2nd English edition) [15].
pap, papillary adenocarcinoma; tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; por1, poorly differentiated adenocarcinoma (solid type); por2, poorly differentiated adenocarcinoma (non-solid type); sig, signet ring cell carcinoma; muc, mucinous adenocarcinoma; TG, total gastrectomy; DG, distal gastrectomy; PG, proximal gastrectomy; PPG, pylorus-preserving gastrectomy

Table 2. Relationship of pT, pN and patient survival

	5-Year survival (%)
pN1	78.4
pN2	85.3
pN3	27.2
pT1	78.5
pT2	68.9
pT3	41.6
pT4	—

RNA extraction and cDNA synthesis

RNA was extracted from fresh frozen cancer tissue obtained from 34 patients, using an RNeasy Mini Kit and QIA shredder (Qiagen, Hilden, Germany). We extracted the samples from the invasive front of cancer without necrosis. Total RNA (1 μg) was reverse-transcribed using random hexamer, SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 10 M dNTP (Sigma-Aldrich, St. Louis, MO, USA). A reverse transcriptase-polymerase chain reaction (RT-PCR) assay was performed using the Gene Amp PCR System (Applied Biosystems, Foster City, CA, USA). Integrity of the isolated RNA was established by RT-PCR analysis of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Real-time quantitative polymerase chain reaction (RQ-PCR)

An RQ-PCR assay was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). PCR reactions proceeded in a mixture (50 μl) containing 2.5 μl of TaqMan gene expression assays (Applied Biosystems) including claudin-4-specific oligonucleotide primers (assay ID Hs00533616 s1), 25 μl of Taq Man Universal PCR Master Mix (Applied Biosystems), and 2 μl of cDNA sample. Amplification was performed for 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and then claudin-4 mRNA expression level was normalized against quantified GAPDH mRNA expression. Non-template control was used as a negative control. We confirmed that there was no decomposition of RNA using electrophoresis (data not shown).

Interpretation of immunohistochemistry and RQ-PCR

In immunohistochemistry, staining was scored by a coauthor without knowledge of clinical factors. Carcinoma, adjacent normal fundic mucosa without gastritis or metaplasia, and metaplastic epithelium in the same slides were reviewed when present. Whole mucosa was evaluated, not only surface epithelia. In accordance with previously published criteria, the incidence of positively stained cells was graded as follows: 0, less than 10%; 1, 10%-50%; 2, 50%-90%; 3, more than 90% [12, 14], and for the purpose of data analysis, the incidence of positively stained cells was graded into two groups: low expression, fewer than 50% of cells stained (incidence score 0 or 1); and high expression, more than 50% of cells stained (incidence score 2 or 3) [12]. Representative staining patterns of tight-junction-associated-proteins in normal gastric mucosa, metaplastic epithelium, and carcinoma are shown in Fig. 1. In immunohistochemistry, as the staining patterns of normal mucosa and metaplastic epithelia are similar to that of carcinoma, we scored the expression of the tight-junction-associated proteins in normal mucosa and metaplastic epithelia in the same way as the carcinoma.

We then examined correlations between the expression of tight-junction-associated proteins and clinicopathological factors such as depth of tumor invasion, lymph node metastasis, hepatic metastasis, peritoneal metastasis, and overall survival.

In RQ-PCR, we examined the expression of claudin-4 mRNA to confirm claudin-4 expression estimated by immunohistochemistry.

Statistical analysis

Statistical analyses were performed using Dr. SPSS II for Windows (SPSS, Chicago, IL, USA). The χ^2 test for independence was used to test the correlations of claudin-4, occludin, and ZO-1 expression with clinicopathological factors, and this test was also used for testing correlations between the expression of claudin-4 as evaluated by immunohistochemistry and the expression of claudin-4 mRNA.

Survival time was estimated using the Kaplan-Meier method, and the log-rank test was used for testing differences in survival time between groups. The multivariate Cox proportional hazard model was applied to detect independent predictors of survival. Values of $P < 0.05$ were considered statistically significant.

Results

All specimens had normal fundic mucosa, and 93 specimens had metastatic epithelia. In immunohistochemistry, as previously reported [5, 8, 12], the staining patterns for claudin-4 and ZO-1 in carcinoma, metaplastic epithelia, and normal mucosa were membranous, and nucleus and cytoplasm were faintly stained. For occludin, membranous staining was also observed in carcinoma, metaplastic epithelia, and normal mucosa (Fig. 1).

Claudin-4 was highly expressed in carcinoma and metaplastic epithelium. In contrast, the expression of claudin-4 was low in normal mucosa. Occludin and ZO-1 were highly expressed in normal mucosa as well as in carcinoma and metaplastic epithelium (Fig. 2).

Significant correlations were identified between the expression of claudin-4, occludin, and ZO-1 (Table 3).

For claudin-4, a significant correlation was seen between expression of claudin-4 as estimated by immunohistochemistry and the expression of claudin-4 mRNA ($P = 0.0030$; Fig. 3). Claudin-4 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P < 0.0001$), advanced T stage ($P = 0.0012$), lymph node metastasis ($P < 0.0001$), and peritoneal metastasis ($P < 0.0001$; Table 4).

Occludin expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P < 0.0001$), lymph node metastasis ($P = 0.0470$), and peritoneal metastasis ($P = 0.0232$; Table 4).

ZO-1 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P = 0.0030$) and advanced T stage ($P = 0.0049$; Table 4).

Relapse occurred in nine patients who received curative surgery, and recurrence patterns were peritoneal

Table 3. Correlation between expression of claudin-4, occludin and ZO-1

	Claudin-4				Claudin-4				Occludin		
	Low	High	*P*		Low	High	*P*		Low	High	*P*
Occludin			<0.0001	ZO-1			0.0046	ZO-1			0.0001
Low	28	9		Low	18	8		Low	18	8	
High	27	59		High	37	60		High	19	79	

A significant correlation was identified between the expression of claudin-4, occludin, and ZO-1. The χ^2 test for independence was used for statistical analysis

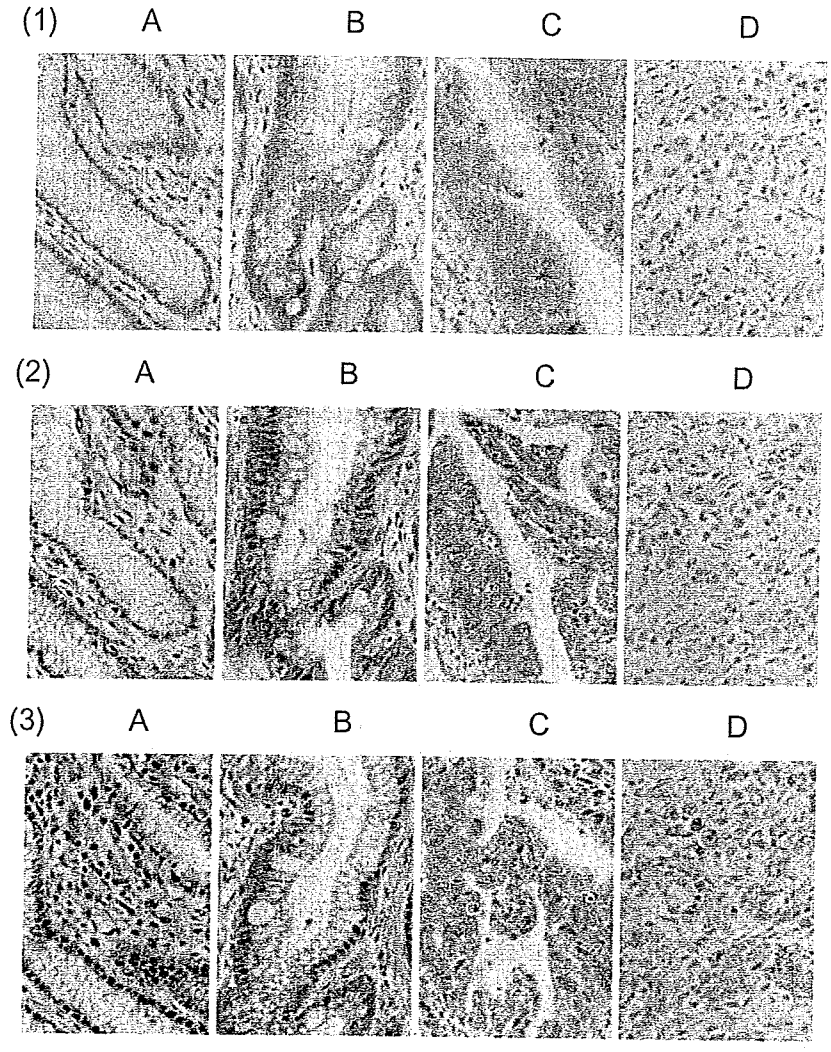

Fig. 1. Immunohistochemistry for claudin-4 (*1*), occludin (*2*), and zonula occludens-1 (ZO-1) (*3*). *A* Normal epithelium. *B* Metaplastic epithelium. *C* Well-differentiated adenocarcinoma (tub1). *D* Poorly differentiated adenocarcinoma (por2). × 200

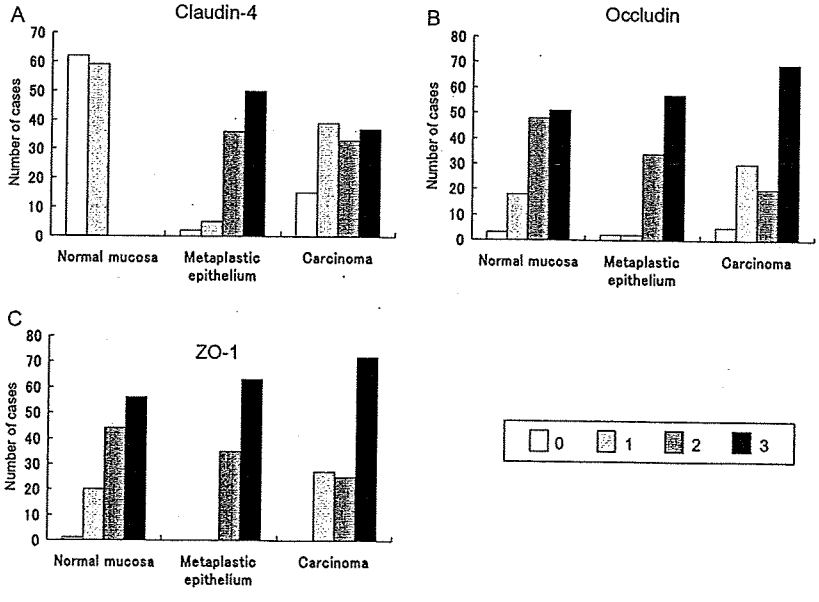

Fig. 2A–C. Expression of claudin-4, occludin, and ZO-1 in normal mucosa, metaplastic epithelium, and carcinoma. **A** Claudin-4 was highly expressed in carcinoma and metaplastic epithelium. In contrast, expression of claudin-4 was low in normal mucosa. **B, C** Occludin and ZO-1 were highly expressed in normal mucosa, as well as in carcinoma and metaplastic epithelium. *0*, less than 10%; *1*, 10%–50%; *2*, 50%–90% *3*, more than 90%

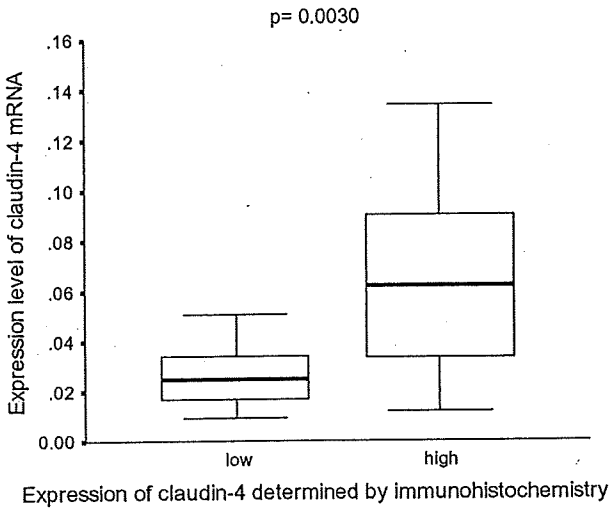

Fig. 3. Correlation between expression of claudin-4 as evaluated by immunohistochemistry and expression of claudin-4 mRNA. A significant correlation was seen between the expression of claudin-4 as determined by immunohistochemistry and the expression level of claudin-4 mRNA ($P = 0.0030$)

metastasis ($n = 5$), hepatic metastasis ($n = 3$), and lymph node metastasis ($n = 1$). Causes of death in all patients (including patients with noncurative operations) were peritoneal metastasis ($n = 9$), lymph node metastasis ($n = 4$), hepatic metastasis ($n = 4$), and other diseases ($n = 11$). Patients who underwent noncurative surgery received postoperative chemotherapy using S-1, cisplatin, paclitaxel, or irinotecan according to the choice of the physician.

Overall survival according to claudin-4 expression was significantly shorter in patients with low claudin-4 expression than in patients with high expression ($P = 0.0091$). No significant differences were seen according to the expression of occludin or ZO-1 ($P = 0.4031$ and $P = 0.3142$, respectively; Fig. 4).

Cox multivariate analysis for overall survival revealed that undifferentiated-type adenocarcinoma, lymph node metastasis, peritoneal metastasis, and low expression of claudin-4 were independently associated with significantly decreased survival ($P = 0.0089$, $P = 0.0377$, $P = 0.0290$ and $P = 0.0070$, respectively; Table 5).

Table 4. Correlation between expression of tight-junction-associated proteins and clinicopathological factors

	Claudin-4			Occludin			ZO-1		
	Low	High	*P*	Low	High	*P*	Low	High	*P*
Differentiated	4	65	<0.0001	9	61	<0.0001	8	62	0.0030
Undifferentiated	51	3		28	26		18	36	
T1	17	41	0.0012	13	46	0.0703	6	53	0.0049
T2/3/4	38	27		24	41		20	45	
N0	25	54	<0.0001	19	61	0.0470	13	67	0.0818
N1/2/3	30	14		18	26		13	31	
P0	43	68	<0.0001	30	82	0.0232	23	89	0.7181
P1	12	0		7	5		3	9	
H0	50	67	0.0511	36	82	0.4698	24	94	0.4456
H1	5	1		1	5		2	4	

Expression of tight-junction-associated proteins was classified as low expression (incidence of positively stained cells <50%) or high expression (incidence of positively stained cells >50%). The χ^2 test for independence was used for statistical analysis.
Claudin-4 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P < 0.0001$), advanced T stage ($P = 0.0012$), lymph node metastasis ($P < 0.0001$), and peritoneal metastasis ($P < 0.0001$). Occludin expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P < 0.0001$), lymph node metastasis ($P = 0.0470$), and peritoneal metastasis ($P = 0.0232$). ZO-1 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P = 0.0030$) and advanced T stage ($P = 0.0049$)

Table 5. Multivariate overall survival analysis (Cox proportional hazard model)

Variable	β	SE	*P*	HR	95% CI
Differentiated vs undifferentiated	2.0556	0.7861	0.0089	7.8112	1.6734–36.4611
T1 vs T2/3/4	−0.0814	0.5660	0.8856	0.9218	0.3039–2.7954
N0 vs N1/2/3	1.1265	0.5422	0.0377	3.0849	1.0660–8.9273
H0 vs H1	0.0410	0.6775	0.9517	1.0418	0.2761–3.9310
P0 vs P1	1.2811	0.5866	0.0290	3.6004	1.1402–11.3687
Claudin-4 expression; high vs low	2.0121	0.7461	0.0070	7.4787	1.7327–32.2791
Occludin expression; high vs low	0.0619	0.5745	0.9142	1.0639	0.3450–3.2802
ZO-1 expression; high vs low	0.1336	0.5228	0.7983	1.1429	0.4102–3.1842

Cox multivariate analysis revealed that undifferentiated adenocarcinoma, lymph node metastasis, peritoneal metastasis, and low expression of claudin-4 were independently associated with significantly decreased overall survival ($P = 0.0089$, $P = 0.0377$, $P = 0.0290$, and $P = 0.0070$, respectively)
SE, standard error; HR, hazard ratio; CI, confidence interval

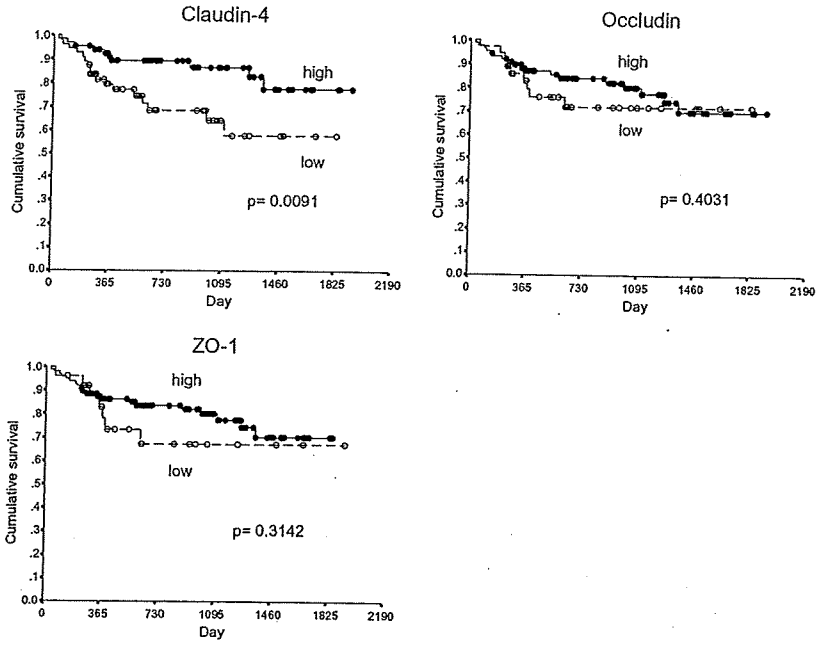

Fig. 4. Overall survival analysis according to tight-junction-associated proteins. Low claudin-4 expression was significantly associated with decreased overall survival ($P = 0.0091$). No significant correlation existed between the expression of occludin or ZO-1 and overall survival ($P = 0.4031$, $P = 0.3142$, respectively)

Discussion

Several studies have been reported about the relationship between tight-junction-associated proteins and gastric carcinogenesis. For example, it has been reported that *H. pylori* was able to increase paracellular permeability by disrupting occludin, claudin-4, and claudin-5 [10], and Cdx2 was shown to play an important role in the regulation of intestinal claudin expression not only in gastric mucosa with intestinal metaplasia but also in gastric carcinoma [11]. Moreover, ALL-1 fusion partner from chromosome 6 (AF-6), which is a Ras target, interacts with ZO-1 [16, 17]. As previously described, tight junctions play important roles in barrier function, cell polarity, and cell signaling pathways [1]. Therefore, disruption of the tight junction can cause the loss of cell polarity, resulting in an abnormal influx of growth factors, which could provide autocrine and paracrine stimulation to tumorigenic epithelial cells [5]. However, the exact roles of tight-junction-associated proteins in gastric cancer remain unclear [18].

In the present study, claudin-4 was highly expressed in differentiated adenocarcinoma and metaplastic epithelium, but was expressed very little in normal epithelium, as evaluated by immunohistochemistry. In gastric cancer, this result was confirmed by the result that a significant correlation was seen between the expression of claudin-4 estimated by immunohistochemistry and the expression of claudin-4 mRNA ($P = 0.0030$; Fig. 3). Claudin-4 is reportedly highly expressed in metaplastic epithelium and carcinoma, but little expressed in normal epithelium [5, 8, 9]. These results may be related to the fact that intestinal-type adenocarcinomas are derived from metaplastic epithelia [8].

Our study revealed that occludin and ZO-1 were expressed in almost all carcinoma, metaplastic epithelium, and normal mucosa specimens. Similar to our findings, ZO-1 was shown to be expressed in almost all carcinoma, metaplastic epithelium, and normal epithelium [8].

Claudin-4 is reportedly highly expressed in gastric intestinal-type adenocarcinoma [5, 7, 8, 12], and the expression of occludin and ZO-1 is reduced in poorly differentiated adenocarcinoma [14]. These results may indicate that tight junctions are important for the construction of gland structure [12].

As previously described, a few studies have reported on the correlation between claudin-4 expression and the aggressiveness of gastric cancer. Similar to our findings, a trend was observed between the reduced expression of claudin-4 and advanced T stage [12], but, contrasting with our results, a trend was observed between the overexpression of claudin-4 and lymph node metastasis [13]. However, there have been no reports about the relationship between occludin expression and the aggressiveness of gastric cancer. In regard to ZO-1, it has been reported that there was no apparent correlation between ZO-1 expression and advanced T stage or lymph node metastasis [12]. Our results suggest that decreased claudin-4 expression is related to undifferentiated-type adenocarcinoma, advanced T stage, lymph node metastasis, peritoneal metastasis, and poor survival. Regard-

ing occludin, we found that decreased expression of occludin was related to undifferentiated-type adenocarcinoma, lymph node metastasis, and peritoneal metastasis, and decreased occludin expression showed a tendency to be associated with advanced T stage and poor survival. Regarding ZO-1, decreased ZO-1 expression was related to undifferentiated-type adenocarcinoma and advanced T stage, and decreased ZO-1 expression showed a tendency to be associated with lymph node metastasis and poor survival. In addition, we showed significant correlations between the expression of claudin-4, occludin, and ZO-1. These results suggest that, of the tight-junction-associated proteins, not only claudin-4 but also occludin and ZO-1 may be related to the aggressiveness of gastric carcinoma.

Survival analysis showed that overall survival was significantly worse in patients with low expression of claudin-4. Cox multivariate analysis also revealed that low expression of claudin-4 was independently associated with significantly decreased overall survival. Contrasting with our results, a strong trend was detected between high claudin-4 expression and poor survival [8], but, similar to our findings, reduced expression of claudin-4 tended to be associated with a high cumulative recurrence rate [12], and the reduced expression of claudin-4 was found to be correlated with poor survival [5].

During the invasion process, loss of intercellular adhesion is one of the early critical steps toward metastasis [19]. If cellular polarity is maintained in cancer cells, cells adhere to each other at the adherens junction and form a basement membrane, then tight junctions at the apical borders are closed by tight junction proteins, and tubular gland structures are subsequently formed [12]. E-cadherin is known as the principal constituent of the adherens junction, and impairment of either the expression or the function of E-cadherin has been reported in cancer cell lines [20, 21]. Some human cancer cells may display impaired E-cadherin-mediated cell adhesiveness through the downregulation of α-catenin expression [22]. Adherens junctions are important for the adhesion of cell-to-cell junctions. In addition, the function of tight junctions may also be important for the construction of tubular gland structures and cell-to-cell adhesion [12]. As for the relationship between claudin-4 and E-cadherin, it has been reported that the reduced expression of claudin-4 and E-cadherin correlates with the disruption of glandular structure and loss of differentiation [12].

In tumor cells derived from rat mammary carcinoma, tight junctions were observed between weakly metastatic tumor cells and normal fibroblasts [23]. In pancreatic carcinoma, claudin-4 is overexpressed and this is associated with decreased invasiveness both in vitro and in vivo [24]. These findings, as well as the present results, suggest that reduced cell-to-cell adhesions formed by tight junctions lead to the dissociation of cancer cells from the original tumor, thus facilitating tumor invasiveness and metastatic potential [24].

In light of these observations, assuming that downregulated claudin-4 correlates with poor survival appears reasonable.

Gastric carcinogenesis may be related not only to claudin-4 but also to other tight-junction-associated proteins, because our study identified significant correlations between the expression of claudin-4, occludin, and ZO-1. The reason that only claudin-4 was associated with invasiveness, metastatic potential, and survival in our study may be related to the fact that claudin is major structural components of tight junction strands [2].

A limitation of this study is that we evaluated the expression of tight-junction-associated proteins using immunohistochemistry. Therefore, we confirmed the quality of immunohistochemistry by RQ-PCR, but we evaluated only claudin-4 because we had no data available about occludin and ZO-1.

Conclusions

We showed that the downregulation of tight-junction-associated proteins, especially of claudin-4, was associated with loss of differentiation in gastric carcinoma and tumor aggressiveness. Survival analysis revealed that the downregulation of claudin-4 was associated with poor survival by the multivariate Cox proportional hazard model as well as by the Kaplan-Meier method. In conclusion, the investigation of tight-junction-associated proteins, especially claudin-4 expression, in gastric carcinoma could be useful for predicting tumor aggressiveness, particularly for determining patient prognosis.

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Induction Chemotherapy with Docetaxel, 5-FU and CDDP (DFP) for Advanced Gastric Cancer

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Abstract. *Background: The aim of this study was to evaluate the feasibility and efficacy of modified triplet chemotherapy with docetaxel, 5-fluorouracil and cisplatin as induction chemotherapy for advanced gastric cancer (AGC). Patients and Methods: Treatment-naïve patients with AGC were eligible. The regimen consisted of 350 mg/m^2/day 5-FU by continuous infusion on days 1 to 5, 10 mg/m^2/day CDDP intravenously on days 1 to 5, and docetaxel at 60 mg/m^2/day intravenously on day 1. After 2 cycles (each cycle consisted of 4 weeks), surgical resection was attempted, 2-4 weeks after the completion of the regimen. Results: Eighteen patients were enrolled. Adverse events included grade 3 anorexia and nausea in 16.7% and 11.1% and grade 4 leukocytopenia and neutropenia in 5.6% and 27.8%, respectively. The overall response rate was 44.4%. Surgery was conducted in 15 patients. The 1- and 3-year survival rates were 75.6% and 51.1%, respectively. Conclusion: The modified triplet combination therapy is effective and well tolerated by patients with AGC.*

Although the incidence of gastric cancer is declining in Western countries, it is still the second most frequent cause of cancer-related death worldwide (1). Similar to other malignancies, the survival of patients with gastric cancer depends on the clinical stage of the disease. Surgery remains the treatment of choice for curing early-stage disease. On the other hand, the prognosis of patients with locally advanced or distant metastatic gastric cancer is still very poor even after surgery. Recent results of a randomized control trial showed that D2 lymphadenectomy plus extended para-aortic lymph node dissection provide no survival benefits compared to D2 alone (2), emphasizing the limited benefits of surgery for advanced gastric cancer (AGC). MacDonald *et al.* (3) have reported that postoperative adjuvant chemoradiotherapy significantly improved the relapse-free survival (RFS) and overall survival (OS) of patients with AGC compared with surgery alone. Furthermore, Cunningham *et al.* (4) reported in the results of the MAGIC trial that perioperative chemotherapy significantly improved both the RFS and OS of patients compared to surgery alone. These studies suggested that the selection of efficient perioperative chemotherapy for gastric cancer is important for the improvement of outcome of AGC.

Over the last decade, new active agents, including taxanes (paclitaxel (5, 6) and docetaxel (7, 8)), irinotecan (9), oxaliplatin (10), and S-1 (11, 12) have been developed and several randomized phase II/III studies have identified promising combination regimens for non-resectable cases of gastric cancer (13-18). Thus, in order to improve the rate of curative resection and to prolong the survival of patients after surgery, neoadjuvant chemotherapy (NAC) or induction chemotherapy should be investigated with chemotherapeutic regimens including novel active agents (19-22).

Docetaxel has shown promising activity when administered alone (response rate: 17-24%) (7, 8, 23, 24) or in combination with other agents (16, 25, 26). The phase III V325 study indicated that DCF (docetaxel, cisplatin and fluorouracil) was superior to CF (cisplatin and fluorouracil) in terms of response rate, time to progression, and OS (27). However, grades 3 to 4 treatment related adverse effects occurred in 82% and 57% of patients treated with DCF and CF, respectively (27). The original regimen of DCF in the V325 trial, was docetaxel at 75 mg/m^2 (1-hour intravenous infusion) plus CDDP at 75 mg/m^2 (1- to 3-hour intravenous infusion) on day 1, followed by 5-FU at 750 mg/m^2/day (continuous intravenous infusion) for 5 days every 3 weeks. To improve the feasibility of the triple-agent therapy, the dosage of docetaxel was reduced to 60 mg/m^2, which is the

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