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Insulin-Like Growth Factor II mRNA-Binding Protein 3 (IMP3) as a Useful Immunohistochemical Marker for the Diagnosis of Adenocarcinoma of Small Intestine

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The biological characteristics and roles of insulin-like growth factor II mRNA-binding protein 3 protein (IMP3) expression in small-intestinal adenocarcinoma were investigated. The value of IMP3 immunostaining in the diagnosis of small-intestinal epithelial lesions was also evaluated. Immunohistochemical expression of IMP3 in normal small-intestinal mucosa adjacent to adenoma and adenocarcinoma lesions, and inflamed duodenal and ileal mucosa was analyzed. Samples assessed were: duodenal ulcer (n=6), Crohn's disease (n=5), low-grade small-intestinal adenoma (n=10), high-grade small-intestinal adenoma (n=13), small-intestinal adenocarcinoma (n=23), lymph node metastases (LNM; n=7), and preoperative biopsies of small-intestinal adenocarcinoma (n=6). Immunohistochemical expression of Ki-67 and p53 was also analyzed in adenoma and adenocarcinoma samples. IMP3 was not expressed in normal epithelium, but weakly expressed in reparative epithelium. Meanwhile, increased IMP3 expression was associated with a higher degree of dysplasia in adenomas, higher T classification, LNM, Ki-67 positivity, histological differentiation, and lower 5-year disease-free survival, but not p53 expression in adenocarcinoma. IMP3 expression appears to be a late event in the small-intestinal carcinogenesis. Assessing the IMP3 staining pattern can be useful in the diagnosis of small-intestinal epithelial lesions when used in conjunction with other histological criteria.

Key words: adenocarcinoma, immunohistochemistry, IMP3, p53, small intestine

I. Introduction

Small-intestinal adenocarcinoma is uncommon despite approximately 90% of the surface area of the gastrointestinal tract being within the small intestine [18]. When adenocarcinoma does develop, the duodenum is most the frequently involved segment (55–82%), followed by the jejunum (11–25%) and ileum (7–17%) [1]. However, increase in the incidence of small intestinal adenocarcinoma has been recently reported, and this is largely attributable to an increase in duodenal adenocarcinomas [27].

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Even though small-intestinal adenocarcinomas are most often sporadic, some predisposing diseases have been identified, including Crohn's disease, familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, and celiac disease [1]. Additionally, the adenoma–carcinoma sequence model is believed to apply in both the small-intestinal and large-intestinal adenocarcinoma [31].

Endoscopic biopsy of the small intestine, —particularly of the duodenum—is the gold standard for providing an accurate diagnosis of small-intestinal adenocarcinoma. However, differentiating adenocarcinoma from adenoma or reactive change is sometimes difficult and objective diagnostic markers would aid for definitive diagnosis. Detection of overexpression of p53 [24, 37], abnormal expression of β catenin [37], and loss of carbamoyl phosphate synthetase I expression [7]—which is present in normal small-intestinal epithelium [14]—may have potential utility in confirming a diagnosis of small-intestinal adenocarcinoma based on challenging biopsy specimens.

The insulin-like growth factor-II (IGF-II) mRNA-binding protein 3 (IMP3) is an oncofetal protein belonging to a conserved family of mRNA-binding proteins [22]. IMP3 was originally identified as a highly-expressed gene in pancreatic cancer [21], and is expressed at low or undetectable levels in adult tissues [22, 23]. However, IPM3 is expressed in developing epithelia, muscle, and the placenta during early stages of embryogenesis [22, 23]. Here IMP3 plays important roles in cell growth, cell migration and RNA trafficking and stabilization [22]. Importantly, the high levels of IMP3 expression in various malignant tumor cells promote tumor cell proliferation and invasion *in vitro* [17, 38]. Clinically, IMP3 is an independent prognostic factor for malignant neoplasms such as renal cell carcinoma [12], urothelial carcinoma of the urinary bladder [33], endometrial serous carcinoma [42], bile duct adenocarcinoma [29], pancreatic adenocarcinoma [30], pulmonary adenocarcinoma [5], colorectal adenocarcinoma [15], and gastric adenocarcinoma [25]. However, to the best of our knowledge, IMP3 expression has not been investigated in small-intestinal carcinomas.

We conducted an immunohistochemical IMP3 protein expression analysis of tissue from normal, inflamed, and dysplastic small-intestinal epithelium. This study examined the biological characteristics and roles of IMP3 protein in small-intestinal adenocarcinoma, and evaluated the diagnostic value of IMP3 immunostaining for interpreting small-intestinal epithelial lesions by histopathology.

II. Materials and Methods

Cell culture and western blot analysis

Anti-human IMP3 antibody (clone 69.1, Dako, Carpinteria, CA) was used for western blot analysis. Antibody specificity was confirmed using cell lysate from human lung carcinoma A549 cells (Japanese Cancer Research Bank, Tokyo, Japan). IMP3 is frequently expressed in pulmonary adenocarcinoma [5], and the specificity of this individual anti-human IMP3 antibody has been previously validated by western blot analysis of A549 lysate (DAKO internal study). A549 cells were maintained in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum. A549 cells were routinely cultured in a humidified incubator at 37°C under 5% CO_2. Adherent A549 cells were incubated in 0.05% trypsin for 10 min at 37°C to obtain a cell suspension. This cell suspension was centrifuged at 1000 rpm for 5 min and supernatant was discarded. The cell pellet was sonicated in 200–500 μL phosphate-buffered saline (PBS), followed by estimation of total protein concentrations using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Protein lysates were denatured in sample buffer containing 5% 2-mercaptoethanol, subjected to electrophoresis on an 8–16% SDS-PAGE mini gel (TEFCO, Tokyo, Japan), and transferred to a nitrocellulose membrane. The membrane was blocked in 5% non-fat dry milk dissolved in 0.1% Tween-20-PBS prior to incubation with mouse anti-IMP3 antibody (clone 69.1, Dako, Carpinteria, CA) at a 1:500 dilution. This antibody was generated following immunization of mice with purified recombinant IMP3 protein encoding amino acids 2–580 [41]. No immunoreactivity with this anti-IMP3 antibody was reported in samples of normal esophagus [20], duodenum [41], ampullary [41], bile duct [29, 41], or pancreatic duct [41] epithelium, while weak immunoreactivity was identified in limited cases of normal colon epithelium [15].

Following incubation with HRP-conjugated secondary antibody, signals were detected using ECL-PLUS (GE Healthcare, Amersham, UK).

Effect of fixation time on immunohistochemical analysis of IMP3

To determine the impact of the length of fixation in 10% buffered formalin on the expression of IMP3 by immunohistochemical analysis, we studied cell blocks of A549 cells and tissue samples from a normal term-placenta. This was because IMP3 is highly expressed in trophoblast cells of human placental villi [16]. A549 cell pellets were obtained as described above and was fixed with 10% buffered neutral formalin for 1, 3, 7, or 14 days. After the fixation periods, each cell sample was immediately processed into a paraffin cell-block using the sodium alginate cell block method [4]. Tissue samples from a normal term-placenta were fixed with 10% buffered neutral formalin for 1, 3, 7, or 14 days. After the fixation periods, each cell sample was immediately processed into paraffin.

Tissue samples

Samples from small-intestinal adenomas (low-grade adenoma: n=10; high-grade adenoma: n=13, including six cases of adenocarcinoma with high-grade adenoma), and adenocarcinomas of the duodenum (n=17), jejunum (n=2) and ileum (n=4), were retrieved from the pathology files at

Table 1. *IMP3 expression in neoplastic small intestinal tissues, and its association with clinicopathological parameters*

Variable	No. of patients (%)	IMP3 Summed Score	*P*
Adenocarcinoma (n=23)	20 (87)	6 (3.5, 6)*	<0.01[a] <0.01[b]
Sex			NS
Male	12 (52.2)	5.5 (2, 6)	
Female	11 (47.8)	6 (5, 6)	
Site			NS
Duodenum	17 (73.9)	6 (3, 6)	
Others	6 (26.1)	6 (5.25, 6)	
Jejunum	2		
Ileum	4		
pT-status			<0.01
pT1	5 (21.7)	2 (0, 3)	
Greater than pT1	18 (78.3)	6 (5.25, 6)	
pT2	0		
pT3	11		
pT4	7		
Nodal metastasis			NS
Negative	9 (39.1)	6 (4, 6)	
Positive	9 (39.1)	6 (6, 6)	
Not determined	5 (21.7)		
Histologic type			<0.05[c]
Well differentiated	11	5 (2.5, 3)	
Moderately differentiated	10	6 (6, 6)	
Poorly differentiated	1	6	
Signet ring cell carcinoma	1	0	
p53 overexpression			NS
Negative	14 (60.9)	5.5 (2.5, 6)	
Positive	9 (39.1)	6 (5, 6)	
Adenoma			<0.01
Low-grade (n=10)	0 (0)	0 (0, 0)	
High-grade (n=13)	7 (53.8)	2 (0, 3)	

IMP3 scoring: *Data are given as median score (interquartile range), the sum of the staining intensity, and the staining extent scores. [a]Adenocarcinoma vs. Adenoma, low-grade, [b]Adenocarcinoma vs. Adenoma, high-grade, [c]Well differentiated vs. Moderately differentiated.

the Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan, Department of Pathology (1997–2013), Aizawa Hospital, Matsumoto, Japan (2003–2013), Department of Pathology, Nagano Municipal Hospital, Japan (2010–2014), and Department of Pathology, Matsumoto Municipal Hospital, Japan (2012–2014). Table 1 provides clinicopathological details of these samples. All specimens of low-grade adenoma were obtained by endoscopic mucosal resection of tumors. Specimens of high-grade adenoma without adenocarcinoma were obtained by surgical resection (n=1) or endoscopic resection (n=6; 5 endoscopic mucosal resection and 1 endoscopic submucosal dissection) of tumors. Specimens of adenocarcinoma including adenocarcinoma with high-grade adenoma were obtained by surgical resection (n=21) or endoscopic mucosal resection (n=2). Nine adenocarcinoma patients had lymph node metastases (LNM), and seven of these were retrieved to compare IMP3 expression in LNM with that in primary tumor tissues. Additionally, six cases of preoperative endoscopic biopsy specimens from duodenal adenocarcinomas examined in this study were analyzed to investigate the utility of IMP3 immunostaining of biopsy specimens as a diagnostic tool for the assessment of small-intestinal adenocarcinomas.

Tumors located in the pylorus of the stomach and at the ileocecal valve were excluded. Tumors arising from the ampullary region were also excluded because of their heterogeneous histogenesis. Additionally, those patients with familial adenomatous polyposis and Crohn's disease were also excluded from adenoma and carcinoma cases examined in this study. Furthermore, regenerating mucosa of duodenal ulcer (n=6) and ileal active Crohn's disease (n=5) in separate cases were examined.

All materials were fixed in 10% neutral-buffered formalin for 1 to 12 days (mean: 3 days) before routine processing and embedding in paraffin. Pathologic diagnosis and histological grading of tumor specimens were performed according to the histological classification described in "General rules for clinical and pathological studies on cancer of the colon, rectum and anus Japanese society for cancer of the colon and rectum" [34] and the World Health Organization classification of tumors of the digestive system [32].

Hematoxylin and eosin-stained archive slides were reviewed. For immunohistochemical analysis, formalin-fixed and paraffin-embedded tissue blocks were selected to

include the tumor area best reflecting the general features of the tumor and normal-appearing non-neoplastic intestinal mucosa in the same blocks, if possible.

Immunohistochemical staining

Immunohistochemical studies were performed on 3-μm sections from formalin-fixed, paraffin-embedded tissue blocks. Briefly, tissue sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase. Following heat-induced epitope retrieval using a microwave (600 W) for 25 min with 0.01 M Tris-HCl buffer containing 1 mM EDTA-2Na (pH 8.6), slides were incubated with mouse monoclonal antibodies specific for either IMP3 (Dako) at a 1:200 dilution, Ki-67 (clone MIB-1, Dako) at a 1:50 dilution, or p53 (clone DO-7, Dako) at a 1:100 dilution overnight at 4°C. Sections were then incubated with the Histofine Simple Stain MAX PO Multi reagent (a polymer conjugated with goat-anti-mouse/anti-rabbit-Ig and horseradish peroxidase; Nichirei Biosciences, Tokyo, Japan) for 30 min, followed by addition of 3,3'-diaminobenzidine and hydrogen peroxide to produce the visible brown pigment. Sections were then counterstained with hematoxylin, dehydrated, and coverslips applied over permanent media. Sections of pancreatic adenocarcinoma known to be IMP3-positive and sections of colonic adenocarcinoma were used as positive controls for IMP3 and p53 staining, respectively. Additionally, staining of germinal centers in lymphoid follicles of small-intestinal tissue sections served as an internal positive control for IMP3 [13]. Negative control samples involved omission of the primary antibody.

Evaluation of immunostaining

Positive staining of IMP3, p53, and Ki-67 was defined as a dark-brown staining pattern in the cytoplasm (IMP3) or nucleus (p53 and Ki-67) of epithelial cells. Both p53 and Ki-67 were scored as positive only when a strong nuclear signal was detected. Staining of IMP3, p53, and Ki-67 was scored semi-quantitatively in accordance with the percentage of positively-staining tumor cells in the field evaluated: <5% (0, negative); 5–10% (1, sporadically positive); 11–50% (2, focally positive); and >50% (3, diffusely positive) [11, 37, 39]. The intensity of IMP3 staining was also scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong), when at least 5% of the cells were positive. For final IMP3 staining scores, the sum of the staining extent and the staining intensity scores was used [15]. For evaluation of IMP3 levels in biopsy specimens, all biopsy samples containing adenocarcinoma tissue were evaluated together in each case. Tumor cells with focal or diffuse positive staining for p53 were estimated as possessing p53 protein overexpression [37]. All samples were reviewed independently by two observers (S.D. and H.O.). To resolve intraobserver variation, all specimens were assessed on two separate occasions. If the scores differed between the different investigators, the slides were reevaluated on a multi-head microscope until consensus was achieved.

Staining scores are non-parametric and are thus expressed as a median score with the interquartile range rather than mean values. This study was approved by the ethics committee of Shinshu University, Japan.

Statistical analysis

The Mann–Whitney U test was used to compare the clinical data and staining scores between the groups. Spearman's correlation coefficient by rank test was used to analyze the correlation between the scores given for the immunoreactivity of anti-MIP3 and anti-Ki-67. Survival rates were calculated by the Kaplan–Meier method, and differences in survival curves were analyzed by means of the log rank test. Differences were considered significant when the *P* value was less than 0.05.

III. Results

Characterization of anti-IMP3 antibody

Western blot analysis

Western blot analysis of A549 cell lysate showed that the anti-IMP3 antibody detected a single band corresponding to the expected size with a molecular weight of approximately 70 kDa (Fig. 1a).

Effect of fixation time on immunohistochemical analysis of IMP3

All samples, regardless of whether they were fixed for 1, 3, 7, or 14 days, showed diffuse and strong cytoplasmic staining with anti-IMP3 antibody in A549 cells (Fig. 1b and 1c) and in syncytiotrophoblasts of placental villi (Fig. 1d and 1e). No significant staining difference was noted among the various fixation times (Fig. 1b–e).

Clinicopathological features of small-intestinal adenocarcinoma

The clinicopathological features of adenocarcinoma of the small intestine are summarized in Table 1. Patients with primary small-intestinal adenocarcinoma were 44–85 years of age (median, 65 years). Of these patients, 12 were men and 11 were women (M/F ratio: 1.1:1). At the time of resection, 5 cases were classified pT1, 11 pT3, and 7 pT4. Eleven cases were well differentiated, 10 were moderately differentiated, one was poorly differentiated, and one was signet ring cell carcinoma. None of the patients with adenocarcinoma received preoperative neoadjuvant therapy.

Immunohistochemical findings

Non-neoplastic tissues

All normal small-intestinal epithelial tissues adjacent to adenoma and adenocarcinoma were negative for IMP3 staining (Fig. 2a and 2b). Regenerative epithelium or inflamed small-intestinal mucosa in duodenal ulcers (Fig. 2c and 2d) and Crohn's disease (Fig. 2e and 2f) were

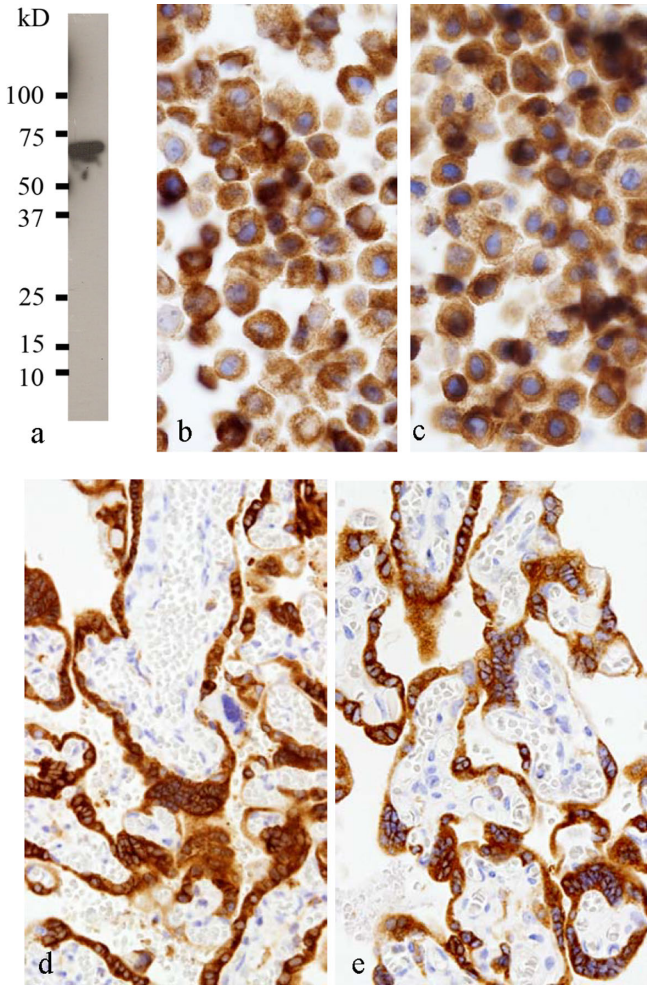

Fig. 1. Characterization of the anti-IMP3 antibody. Western blotting of A549 cell lysate using an anti-IMP3 antibody identifies a single band corresponding to the predicted molecular weight of approximately 70 kDa (**a**). Immunohistochemical staining of IMP3 in A549 cells and placental tissue fixed for 1 day (**b, d**) and 14 days (**c, e**). A549 cells (**b, c**) and syncytiotrophoblasts in placental villi (**d, e**) show diffuse and intense cytoplasmic staining of IMP3. (**b**)–(**e**): IMP3 immunohistochemistry.

weakly IMP3-positive, with rare small patchy or individual crypt staining regions.

Cells at the germinal center of lymphoid follicles in each specimen served as an internal positive control for IMP3 immunoreactivity (Fig. 2b).

Neoplastic tissues

Adenomas

IMP3 staining was absent from all low-grade adenomas (Fig. 3a and 3b), but present in 7/13 (53.8%) cases of high-grade adenomas (Table 1 and Fig. 3c and 3d). IMP3 staining in high-grade adenomas was sporadic in five (38.5%), focal in one (7.7%), and diffuse in one (7.7%) of 13 cases (Table 2). Additionally, the intensity of the staining reaction was weak in two (15.4%) and moderate in five (38.5%) of 13 cases of high-grade adenoma (Table 3). IMP3 expression in high-grade adenoma did not correlate with Ki-67 expression (Fig. 3d and 3e). Overexpression of p53 was observed in two (15.4%) of 13 cases of high-grade adenoma. In these 2 cases of high-grade adenoma with p53 overexpression, IMP3 expression was sporadic and of moderate intensity in one, and diffuse and of moderate intensity in the other. IMP3 expression in high-grade adenoma did not correlate with p53 expression (Fig. 3d and 3f).

Adenocarcinomas

We detected IMP3 staining in 20/23 (87%) small-intestinal adenocarcinoma samples (Table 1, Fig. 4a and 4b). The staining reaction was sporadic in 2 (8.7%), focal in 2 (8.7%), and diffuse in 16 (69.6%) cases (Table 2). The intensity of the staining reaction was weak in 3 (13%), moderate in 4 (17.4%), and strong in 13 (56.5%) (Table 3). Adenocarcinoma tissues expressing IMP3 were histologically indistinguishable from those that were IMP3-negative. IMP3 staining scores in adenocarcinoma samples were significantly higher than those of high-grade adenomas (Table 1). We assessed intramucosal lesions in 13 cases of invasive adenocarcinoma and observed decreased intensity of IMP3 staining in 5 cases, compared with the main tumor (Fig. 4a and 4b). A single case had higher expression of IMP3 relative to the main tumor, while no difference was detected in the remaining 7 cases. At the invading edges of invasive adenocarcinomas, 6/20 cases (30%) showed a higher intensity of IMP3 staining relative to the main tumor (Fig. 4a and 4b). An additional 12 instances of invasive adenocarcinoma exhibited the same staining intensity as the main tumor or displayed an identical level of heterogeneity, while a single case had decreased staining intensity.

IMP3 staining was negative in 3/23 small-intestinal adenocarcinoma samples (Table 1). In these IMP3-negative cases, the germinal centers in lymphoid follicles of small-intestinal tissue sections were positive for IMP3. These samples were all from duodenal adenocarcinomas. Specifically, they were an intramucosal welldifferentiated adenocarcinoma (fixed in formalin for 1 day), a welldifferentiated adenocarcinoma with submucosal invasion (fixed in formalin for 1 day), and a signet ring cell carcinoma with pancreatic invasion (fixed in formalin for 2 days).

Correlation between IMP3 protein expression and clinicopathological factors

IMP3 expression was higher in moderately differentiated adenocarcinomas compared with well-differentiated adenocarcinomas ($P<0.05$; Table 1). IMP3 expression in adenocarcinomas was associated with an advanced stage (pT1 vs. more advanced stages; $P<0.05$; Table 1), and weakly correlated with Ki-67 expression ($r=0.28$; Fig. 4b and 4c). No statistically significant differences in adenocarcinoma sample IMP3 immunoreactivity were observed between males and females, between duodenal and other (jejunal and ileal) adenocarcinomas, or between adeno-

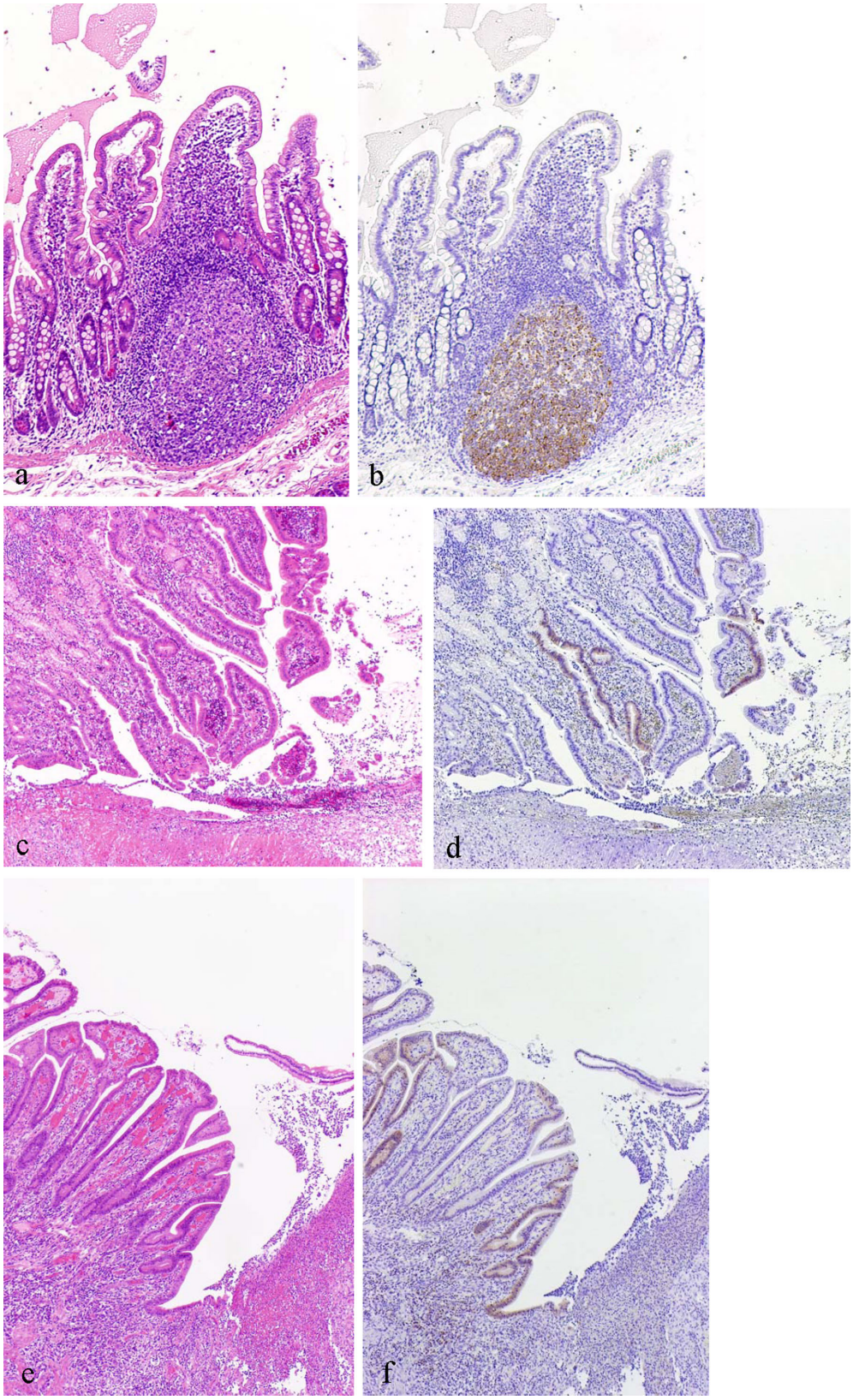

Fig. 2. Expression of IMP3 in non-neoplastic tissues. In normal duodenal mucosa (**a**), IMP-3 expression is restricted to the germinal center only (**b**). Regenerative epithelium from a duodenal ulcer (**c**) exhibite weak IMP3 expression defined as small patchy staining (**d**). Inflamed intestinal mucosa in Crohn's disease (**e**) exhibited weak IMP3 expression defined as individual crypt staining (**f**). (**a**) Hematoxylin and eosin stain (HE); (**b**) IMP3 immunohistochemistry; (**c**) HE; (**d**) IMP3 immunohistochemistry; (**e**) HE; (**f**) IMP3 immunohistochemistry.

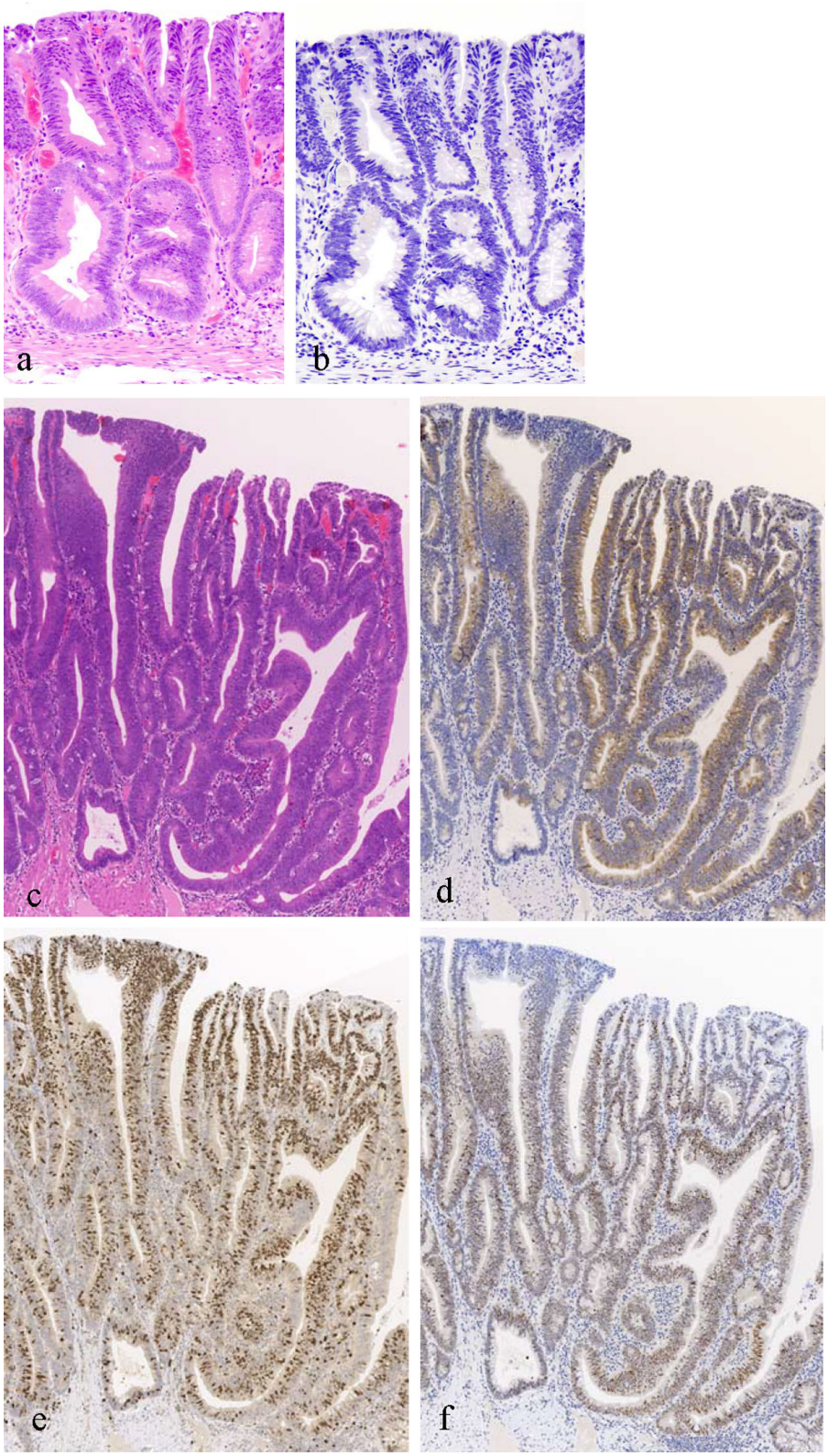

Fig. 3. Expression of IMP3 in adenoma. In low-grade duodenal adenoma (**a**), IMP3 staining is negative (**b**). High-grade adenoma (**c**) shows weak staining for IMP3 (**d**). IMP3 expression dose not correlate with Ki-67 expression (**e**). p53 is not overexpressed (**f**). (**a**) HE; (**b**) IMP3 immunohistochemistry; (**c**) HE; (**d**) IMP3 immunohistochemistry; (**e**) Ki-67 immunohistochemistry; (**f**) p53 immunohistochemistry.

Table 2. *Extent of IMP3 staining in small intestinal adenocarcinoma and adenoma tissues*

Categories	IMP3 Extent Score			
	0	1	2	3
Adenocarcinoma				
Primary sites (n=23)	3 (13)*	2 (8.7)	2 (8.7)	16 (69.6)
Lymph node metastases (n=7)	0 (0)	0 (0)	0 (0)	7 (100)
Biopsy samples (n=6)	1 (16.7)	1 (16.7)	0 (0)	4 (66.7)
Adenoma				
Low-grade (n=10)	10 (100)	0 (0)	0 (0)	0 (0)
High-grade (n=13)	6 (46.2)	5 (38.5)	1 (7.7)	1 (7.7)

IMP3 scoring: 0 (negative) when <5%; 1 (sporadically positive) when 5–10%; 2 (focally positive) when 11–50%, and 3 (diffusely positive) when >50% of the tumor cells were positive. * Data are given as number (percentage).

Table 3. *Intensity of IMP3 staining in small intestinal adenocarcinoma and adenoma tissues*

Categories	IMP3 Intensity Score			
	0	1	2	3
Adenocarcinoma				
Primary sites (n=23)	3 (13.0)*	3 (13.0)	4 (17.4)	13 (56.5)
Lymph node metastases (n=7)	0 (0)	0 (0)	0 (0)	7 (100)
Biopsy samples (n=6)	1 (16.7)	1 (16.7)	2 (33.3)	2 (33.3)
Adenoma				
Low-grade (n=10)	10 (100)	0 (0)	0 (0)	0 (0)
High-grade (n=13)	6 (46.2)	2 (15.4)	5 (38.5)	0 (0)

IMP3 scoring: 0=negative, 1=weak, 2=moderate, and 3=strong immunoreactivity. *Data are given as number (percentage).

carcinomas with lymphoid metastasis and those without lymphoid metastasis (Table 1).

Comparison of expression of IMP3 and p53

p53 was overexpressed in 9/23 (39.1%) adenocarcinomas (Table 1). All cases overexpressing p53 also possessed IMP3 expression (Fig. 4b and 4d). Additionally, IMP3 was expressed in 11/14 (78.6%) adenocarcinoma samples which did not overexpress p53. There was no statistically significant difference in the IMP3 staining score between adenocarcinomas with and without p53 overexpression (Table 1). A reciprocal relationship between p53 and IMP3 was not observed (Fig. 4b and 4d).

IMP3 expression in LNM

All seven cases of LNM analyzed had strong and diffuse IMP3 expression which was elevated compared with primary small intestinal adenocarcinomas ($P<0.05$) (Tables 2 and 3). IMP3 protein expression was elevated in two LNM samples compared with matched primary tumor samples.

IMP3 expression in biopsy samples

IMP3 staining was detected in 5/6 (83.3%) cases (Tables 2 and 3). IMP3 was negative in both the biopsy and the corresponding resected sample of one case of duodenal signet ring cell carcinoma. Of the positive cases, one sample was taken in two cases, three in one case, and four in two cases. Only one sample in a case in which four biopsy samples were taken was negative. The staining reaction was sporadic in one (16.7%) case and diffuse in four (66.7%) cases (Table 2). The intensity of the staining reaction was weak in one (16.7%), moderate in two (33.3%), and strong in two (33.3%) cases (Table 3). IMP3 expression was low in two biopsy samples compared with matched primary tumor samples.

Correlation between IMP3 protein expression and prognosis

Follow-up information was available for all but one case, with survival ranging 1–122 months (mean: 41.7). Patients with early-stage cancer (T1 tumors, n=5) were alive without disease (n=4) or dead of disease (n=1). Patients with advanced cancer (stage T3–4 tumors, n=18) were either alive without disease (n=9), alive with disease (n=3), dead of disease (n=5), or dead of other disease (n=1). To further confirm a role for IMP3 expression in small-intestinal adenocarcinoma progression, survival of the 17 patients with advanced small-intestinal adenocarcinoma (pT3–4 tumors) was analyzed using Kaplan-Meier curves. Patients with higher expression of IMP3 (summed score 6) had impaired 5-year disease-free survival compared with patients with lower IMP3 expression (summed score<6; $P<0.05$). However, there was no difference in overall survival.

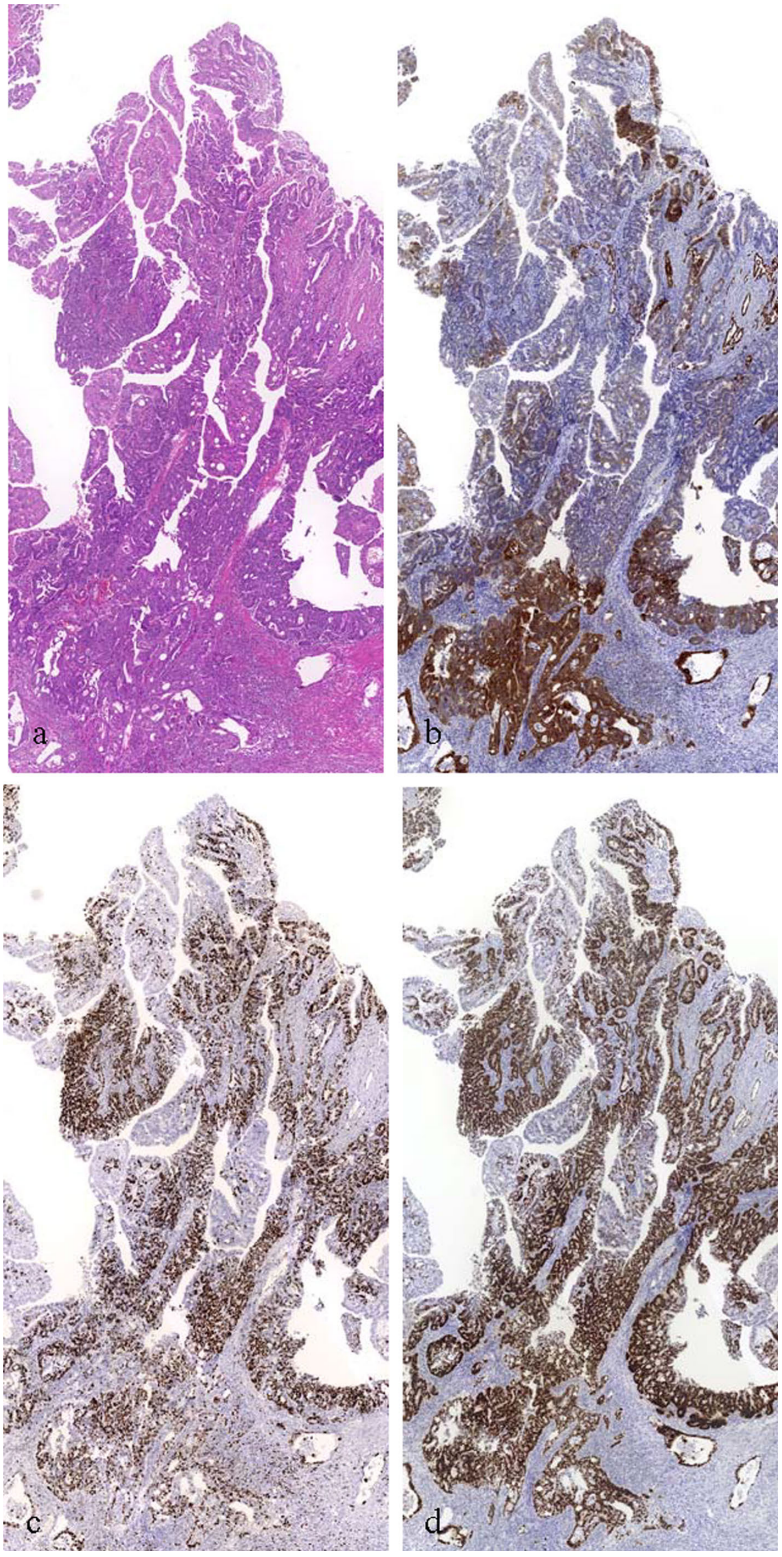

Fig. 4. Expression of IMP3, Ki67, and p53 in a jejunal adenocarcinoma. Adenocarcinoma (**a**) shows a distinctive pattern of increased IMP3 staining at the invasive front (**b**). Distribution of strong immunoreactivity for IMP3 is localized within densely packed regions of Ki-67 positive cells (**c**). p53 is diffusely expressed in adenocarcinoma (**d**). (**a**) HE; (**b**) IMP3 immunohistochemistry; (**c**) Ki-67 immunohistochemistry; (**d**) p53 immunohistochemistry.

IV. Discussion

We determined the immunohistochemical expression of IMP3 in non-neoplastic and neoplastic small-intestinal tissue and found that adenocarcinomas frequently exhibit focal to diffuse and moderate to strong expression of IMP3. Furthermore, this IMP3 expression is associated with tumor differentiation, an advanced stage, and Ki-67 expression. Additionally, high-grade adenoma frequently exhibits sporadic and mild to moderate IMP3 expression. In contrast to adenocarcinomas and high-grade adenomas, low-grade adenomas and normal mucosa were IMP3 negative. In reparative epithelium, IMP3 expression was weakly defined, being present as small patches or in individual crypts.

Considering that the immunohistochemical expression of IMP3 correlated with the degree of dysplasia in adenomas and the stage of adenocarcinomas, IMP3 expression appears to be a late event in small-intestinal carcinogenesis. Consistently, a prior *in vitro* study showed that IMP3 could promote tumor cell invasion by stabilizing CD44 mRNA [38]. Furthermore, some invasive small-intestinal adenocarcinoma samples we assessed showed increased intensity of IMP3 expression at the invading edges relative to the main tumors. This is in accordance with a previous immunohistochemical study of IMP3 expression in endometrial clear cell carcinoma [8]. Additionally, we observed increased expression of IMP3 in nodal metastases compared with expression in primary small-intestinal adenocarcinomas. These findings suggest that IMP3 may play a critical role in invasive behavior and the development of metastases.

We also observed increased IMP3 expression in moderately-differentiated adenocarcinoma compared with well-differentiated adenocarcinoma, consistent with a previous immunohistochemical study of IMP3 expression in pulmonary adenocarcinoma [9]. These findings support the utility of IMP3 immunohistochemical expression as a marker of increased biologic aggressiveness.

p53 mutation is suggested to be a late event during the development of small-intestinal adenocarcinoma [2]. Here, p53 mutation may promote tumor progression in a manner similar to its role in the classical adenoma–carcinoma progression sequence of colon cancer [3, 28]. We observed p53 overexpression in 9/23 (39.1%) small-intestinal adenocarcinoma samples, consistent with previous observations employing immunohistochemistry [3, 24, 28, 37, 40]. IMP3 expression in small-intestinal adenocarcinoma with p53 overexpression was not significantly different from IMP3 expression in adenocarcinoma lacking p53 overexpression, suggesting that both events may occur independently. Furthermore, IMP3 expression may serve as a complimentary immunohistochemical marker to aid diagnosis of small-intestinal adenocarcinoma, particularly when samples are negative for p53 immunostaining.

We detected a correlation between increased IMP3 levels and Ki-67 positivity in small-intestinal adenocarcinoma in accordance with other previous studies of bile duct adenocarcinoma [29] and colon adenocarcinoma [15]. Supporting this, *in vitro* studies, have shown that IMP3 promotes human leukemia cell [17] and glioblastoma cell [36] proliferation via up-regulation of IGF-II, which is a potent mitogenic factor involved in autocrine growth stimulation in a variety of cancer cells including human lung, breast, gastric, and colon cancer cells [10, 26]. Increased IMP3 [15] and IGF-II [35] levels have been reported in human colon cancers. Interestingly, IGF-II system is causally implicated in obesity [19], and obese individuals have increased risks of colon cancer and small-intestinal adenocarcinoma [6]. Taken together, these findings suggest that overexpression of IMP3 in small-intestinal adenocarcinoma may promote small-intestinal carcinogenesis in part through the IGF-II pathway. Further study is required to confirm this.

Clinical studies have demonstrated that aberrant IMP3 expression in tumor cells is associated with aggressive clinical behaviors in several malignant neoplasms, including renal cell carcinoma [12], urothelial carcinoma of the urinary bladder [33], endometrial serous carcinoma [42], bile duct adenocarcinoma [29], pancreatic adenocarcinoma [30], colorectal adenocarcinoma [15], gastric adenocarcinoma [25], and pulmonary adenocarcinoma [5]. Furthermore, we found that patients with high IMP3-expressing tumors had a lower disease-free survival rate compared with those expressing low levels of IMP3. Further study of a larger sample size is needed to properly elucidate the prognostic value of IMP3 in small-intestinal carcinomas.

A high level of IMP3 (focal to diffuse expression and moderate to strong intensity) is highly sensitive for identification of small-intestinal adenocarcinoma in resected samples. Therefore, histological detection of IMP3 expression may be a useful diagnostic tool for the assessment of small-intestinal tumor biopsy samples. When assessing IMP3 expression by immunohistochemical analysis of biopsy samples in which the amount of available material is limited, it should be recognized that IMP3 expression may be weaker in pT1 tumors compared with advanced tumors. Also, IMP3 expression may be weaker in the intramucosal lesions of invasive adenocarcinomas compared with the main tumor.

We are the first to report that IMP3 expression appears as a late event in small-intestinal carcinogenesis. Furthermore, we reveal that the IMP3 staining pattern can be a useful adjunct in distinguishing adenocarcinoma from high-grade adenoma, low-grade adenoma, or reactive atypia when used in conjunction with other histological criteria. IMP3 expression may therefore serve as a complementary immunohistochemical marker for diagnosing small-intestinal adenocarcinoma.

V. References

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