Original article

Tubular adenoma and intramucosal intestinal-type adenocarcinoma of the stomach: what are the pathobiological differences?

SATORU KASE, MITSUHIKO OSAKI, SOICHIRO HONJO, HIRONOBU ADACHI, and HISAO ITO

Division of Organ Pathology, Department of Microbiology and Pathology, Faculty of Medicine, Tottori University, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan

Abstract

Background. The concept of human early gastric cancer has discrepancies among pathologists worldwide. Recently, a novel morphological classification of gastrointestinal intramucosal neoplasias was proposed and conceptual differences have gradually decreased. However, there are few studies on the differences of pathobiological properties among the intraepithelial neoplasms. The aim of the present study was to clarify the clinicopathological significance of gastric intramucosal tumors from the pathobiological viewpoint.

Methods. This study analyzed 70 tubular adenomas (TAs) with mild or moderate atypia and 52 intramucosal intestinal-type adenocarcinomas (IMACs) of the stomach from the viewpoint of the apoptotic index (AI), Ki-67 labeling index (KI), and intratumoral microvessel density (IMVD), using immunohistochemistry and the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) method. We also examined the expression of P53 and thymidine phosphorylase (dThdPase).

Results. The mean AI was significantly higher and the mean KI and IMVD were significantly lower in the TAs than in the IMACs ($P < 0.05$, $P < 0.001$, and $P < 0.01$, respectively). AI and IMVD showed a negative correlation in TAs and IMACs (TAs; $P = 0.015$; $r = 0.28$; IMACs; $P = 0.006$; $r = 0.41$). dThdPase and nuclear P53 expression were significantly higher in the IMACs than in the TAs ($P < 0.05$, $P < 0.01$). Mean IMVD was significantly higher in dThdPase-positive tumors than in the negative ones ($P < 0.01$, $P < 0.01$) in both TAs and IMACs.

Conclusion. IMACs possess higher proliferative activity of tumor cells, with a vascular-rich microenvironment, than TAs. This might reflect the pathobiological differences between gastric IMACs and TAs, and could also provide useful information on indications for gastric endoscopic mucosal resection for IMACs as has been recommended by the Japanese Gastric Cancer Association.

Key words Apoptosis · Microvessel · Proliferation

Introduction

There have been significant differences in diagnostic criteria for gastric intestinal-type carcinoma between Japanese and Western pathologists [1]. Most Western pathologists have paid attention to evident invasion into the lamina propria or submucosa of neoplastic tubules as definitive criteria of carcinoma. They have suggested that Japanese pathologists “overdiagnose” gastric dysplasia as carcinoma. This might partly be due to the relatively small number of gastric forceps biopsy specimens used in routine diagnostic practice by Western pathologists. In Japan, mass screening programs for early gastric carcinoma and the enormous improvement and advances in endoscopic techniques have resulted in higher proportions of mucosal carcinomas among early gastric carcinomas, approximately 10%–30% of which are resected endoscopically. Stolte [2] reported that “anti-Japanese” commentators had overlooked that the diagnoses made on biopsy material had been subjected to “quality control” in later endoscopic mucosal resection specimens.

A total of 31 pathologists reviewed 35 gastric samples and discussed the findings on the occasion of the World Congress of Gastroenterology in September 1998 [3]. Diagnostic agreements between Japanese and Western pathologists appeared to be clearly improved, compared with the findings reported in *The Lancet* [1]. At this meeting, a novel morphological classification of gastrointestinal epithelial neoplasias, the so-called Vienna classification, was proposed [3]. These neoplasias were classified into five main categories; (1) negative for neoplasia/dysplasia, (2) indefinite for neoplasia/dysplasia, (3) noninvasive low-grade neoplasia, (4) noninvasive high-grade neoplasia, and (5) invasive

carcinoma. This classification appears to reduce the differences between Western and Japanese pathologists in the diagnostic classification of gastric epithelial neoplastic lesions. More recently, the Vienna classification was revised, and intramucosal carcinoma was added as a fourth subcategory of category 4 [4]. Gastric intramucosal neoplasias, including adenomas, however, have not been adequately analyzed with respect to specific pathobiological points, including cell proliferation, apoptosis, and intratumoral microvessels.

Angiogenesis is essential for tumor development and proliferation. In previous studies, the correlation between apoptosis and intratumoral microvessels or the expression of thymidine phosphorylase (dThdPase), one of the angiogenic factors [5–7], was examined in colorectal carcinoma [8], early and advanced gastric carcinoma [9,10], and esophageal squamous cell carcinoma [11]. Although we have previously reported the frequent occurrence of apoptosis in gastric adenoma [12], correlations between apoptosis and microvessels have not been sufficiently examined in gastric adenoma and intramucosal carcinoma.

The aim of the present study was to clarify the clinicopathological significance of gastric intramucosal tumors from the pathobiological viewpoint. As the first step, we analyzed two categories in the revised Vienna classification [4]: noninvasive low-grade neoplasia (low-grade adenoma/dysplasia) of category 3, and invasive carcinoma (intramucosal carcinoma) of subcategory 4.4. The former is almost synonymous with tubular adenoma (TA) with mild or moderate atypia in the Japanese classification [13]. The latter clearly includes intramucosal adenocarcinoma (IMAC), which is classified as intestinal-type carcinoma according to the Lauren classification [14]. In fact, the Vienna classification seems to be intended for the diagnosis of intestinal-type carcinomas, and the original and revised classifications showed only intestinal-type dysplasia/neoplasia in the published figures [3,4]. Diffuse-type adenocarcinomas do not give rise to conflicting diagnoses among pathologists [1].

Materials and methods

Specimens

Studies were conducted on 52 specimens of IMACs and 70 TAs, all of which were resected endoscopically or by partial gastrectomy. These specimens were extracted from files of the Department of Pathology, Faculty of Medicine, Tottori University, and its related teaching hospitals. Because papillary adenomas of the stomach may suggest malignant potential [15], and TAs with severe atypia are often difficult to diagnose and to distinguish from intestinal-type adenocarcinoma histo-

pathologically, we excluded them and selected only TAs with mild or moderate atypia. We selected intramucosal adenocarcinoma of the intestinal-type, because diffuse-type adenocarcinomas, including signet-ring cell carcinoma, do not give rise to conflicting diagnoses among Western and Japanese pathologists [1]. Routinely processed, formalin-fixed, paraffin-embedded tissue blocks containing the most tumor mass were selected. Sections 3- μ m-thick were examined by light microscopy, immunohistochemistry, and the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) procedure. The histological classifications were routinely made according to the criteria of the Japanese Gastric Cancer Association [13].

Immunohistochemistry

Dewaxed paraffin sections were immunostained using the streptavidin-biotin peroxidase complex (SAB) method [16]. The following primary antibodies were used: mouse monoclonal antibody raised against Ki-67 (MIB-1, diluted 1:50; Dako, Kyoto, Japan), CD34 (NU-4A1; Nichirei, Tokyo, Japan), dThdPase (1C6, diluted 1:1000; Nippon Roche Research Center, Kanagawa, Japan), and P53 (DO7, diluted 1:50; Novocastra Laboratories, Newcastle, UK). For Ki-67 and P53, all the sections were heated in a 10 mM citrate buffer, pH 6.0, in a microwave oven for 30 min at 94°C. Immunoreactions were visualized with diaminobenzidine (DAB), and the sections were counterstained with 3% methylgreen.

To examine the specificity of immunostaining, the primary antibody was replaced by mouse normal IgG at a 1:100 dilution, or Tris-buffered saline. Control slides were invariably negative for immunostaining. We immunostained appropriate control slides of gastric carcinoma, which were previously known to show positive immunoreactivity for Ki-67, CD34, dThdPase, and P53, at the same time, to obtain consistent findings [9,12]. In the present study, specimens were regarded as P53- or dThdPase-positive when more than 10% of the tumor cells showed positive signals.

TUNEL method

To detect DNA breaks in situ, TUNEL was performed according to the method of Gavrieli et al. [17], using an Apop Tag Plus in-situ apoptosis detection kit (Intergen, New York, NY, USA). Briefly, after deparaffinization and blocking of endogenous peroxidase with 2% hydrogen peroxidase (H_2O_2) in methanol for 30 min at room temperature, sections were incubated with 20 μ g/ml proteinase K (Boehringer Mannheim/Yamanouchi, Tokyo, Japan) for 20 min at 37°C. After prehybridization treatment, the sections were incubated with terminal deoxynucleotidyl transferase (TdT), digoxigenin-11-

dUTP, and dATP in a moist chamber for 90 min at 37°C. Incubation with anti-digoxigenin-antibody-peroxidase for 30 min at room temperature was employed for the detection of digoxigenin-11-dUTP labeling, followed by color development with a solution containing 3,3'-diaminobenzidine and H_2O_2 . Methylgreen was used for counterstaining.

To examine the specificity of the TUNEL procedure, slides were treated with TdT buffer solution not containing TdT or digoxigenin-11-dUTP as a negative control. At the same time, the TUNEL procedure was also conducted with control slides of gastric carcinoma, which were already known to show many apoptotic cells with TUNEL signals, to obtain consistent findings.

Evaluation of apoptotic index, Ki-67 labeling index, and intratumoral microvessel density

The apoptotic index (AI; percentage of apoptotic cells) in both categories of specimens was obtained as the number of TUNEL-positive tumor cells per 1000 tumor cells in the most frequently labeled area. The Ki-67 labeling index (KI) was determined in those tumor areas showing the highest density of cells with positively stained nuclei, regardless of the areas used for intratumoral microvessel density (IMVD) counting, and calculated under a 200-fold magnification. At least five areas were selected, and the KI of each gastric tumor was obtained as the number of Ki-67-positive tumor cells per 1000 tumor cells. For the determination of IMVD, using CD34, at least five of the most highly vascularized areas within a section were selected and counted under a light microscope with 200-fold magnification, as described by Weidner et al. [18]. The average numbers were recorded as the IMVD for each case. The

IMVD was counted by two authors (S.K. and M.O.) independently without knowledge of the clinical information, and then adjusted.

Statistical analyses

Correlations between AI and IMVD, as well as those between AI and KI, were analyzed using the Spearman rank correlation coefficient. The difference in the numerical data between the two groups was evaluated using the Mann-Whitney *U*-test. The level of significance was set at $P < 0.05$.

Results

Macroscopic and histologic appearance of TAs and IMACs

Most TAs formed slightly elevated lesions with a flat surface and the histologic picture frequently showed a characteristic two-layer structure with unchanged mucosa or cystic dilated glands in the subjacent part of the adenoma [19]. Goblet cells were variably intermingled in adenoma glands. Slender and hyperchromatic nuclei showed slight variation in size and shape. The nuclei of TAs localized in basal lamina and structures atypical of adenoma glands were not conspicuous (Fig. 1A). The mean diameter was 5.6 mm, ranging between 1.5 and 25 mm.

On the other hand, IMACs showed increased and/or invading atypical cells; however, the invasion of these cells did not expand the submucosal layer. Tumor glands showed an irregular arrangement, and had variable size and shape (Fig. 1B). Nuclei were rounded, of

Fig. 1. **A** Tubular adenomas (TAs). The glands have a relatively uniform shape and size. Slender and hyperchromatic nuclei show slight variation in size and shape. **B** Intramucosal intestinal-type adenocarcinomas (IMACs). The glands show an irregular arrangement, and have variable size and shape. Some are branching and some are very small. Cellular atypicities are more prominent. **A** and **B** H&E, $\times 50$

Fig. 2A,B. Intense terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) signals (arrows) are visible in the nuclei of apoptotic tubular adenoma (A) and intramucosal intestinal-type adenocarcinoma cells (B). A and B $\times 200$

Table 1. Apoptotic index (AI), Ki-67 labeling index (KI), intratumoral microvessel density (IMVD), and expression of thymidine phosphorylase (dThdPase) and P53 in the 70 tubular adenomas and 52 intramucosal intestinal-type adenocarcinomas

	AI	KI	IMVD	dThdPase	P53
Tubular adenomas ($n = 70$)	4.79 ± 0.38	39.5 ± 1.56	59.4 ± 2.47	25/70 (35.7%)	14/70 (20.0%)
Intramucosal intestinal-type adenocarcinomas ($n = 52$)	3.26 ± 0.27	53.6 ± 1.84	72.4 ± 3.49	30/52 (57.7%)	29/52 (55.8%)
<i>P</i> values	0.013	<0.001	0.008	0.016	<0.001

variable size, and had enlarged prominent nucleoli. In part, the lesions showed budding of glands, single neoplastic cells, and trabecular growth. The mean diameter was 7.0 mm, ranging between 1.0 and 24 mm. There was no significant difference in tumor size between TAs and IMACs ($P = 0.2$).

Correlation between AI, KI, IMVD, and P53

TUNEL-positive (Fig. 2A,B) and Ki-67 positive (Fig. 3A,B) tumor cells showed nuclear reactivity. Intratumoral microvessels were clearly demonstrated with CD34 immunostaining (Fig. 3C,D). Table 1 shows the mean AI, KI, and IMVD in the 70 gastric TAs and the 52 IMACs, the values being 4.79 ± 0.38 , 39.5 ± 1.56 , and 59.4 ± 2.47 in the 70 TAs, and 3.26 ± 0.27 , 53.6 ± 1.84 , and 72.4 ± 3.49 in the 52 IMACs, respectively. The mean AI was significantly higher in the TAs than in the IMACs ($P = 0.013$). On the other hand, the mean KI and IMVD were significantly higher in the IMACs than in the TAs ($P < 0.01$, $P < 0.01$, respectively). Thus, IMACs might be characterized as showing higher proliferative activity and a lower cell loss rate (apoptosis), compared with TAs.

Regression analysis of the Spearman rank correlation coefficient, on plots of AI versus IMVD on a per-case

basis, showed a significant inverse correlation between AI and IMVD in both types of lesions (TA, $P = 0.015$, $r = 0.28$; IMAC, $P = 0.006$; $r = 0.41$; Fig. 4). On the other hand, there was no significant correlation between KI and IMVD (Fig. 5).

Nuclear P53 expression was noted in 14 (20.0%) of the 70 TAs and in 29 (55.8%) of the 52 IMACs (Fig. 3E, Table 1). The frequency was significantly higher in the latter than in the former ($P < 0.01$). P53 expression did not correlate with AI, KI, or IMVD (Table 2).

dThdPase expression

dThdPase immunoreactivity was observed in both the cytoplasm and the nucleus of the TAs and IMACs, the immunoreaction being more predominant in the cytoplasm than the nucleus (Fig. 3F). The immunoreactivity was also noted in stromal lymphocytes, fibroblasts, and macrophages to varying degrees, as described previously in analyses of colonic carcinomas [8,20].

dThdPase expression was noted in 25 (35.7%) of the 70 TAs and in 30 (57.7%) of the 52 IMACs. The frequency of dThdPase-positive cases was significantly higher in the IMACs than in the TAs ($P < 0.05$) (Table 1).

Fig. 3A–F. Immunohistochemical positivity for: **A, B** Ki-67 (**A** tubular adenomas [TAs]; **B** intramucosal intestinal-type adenocarcinomas [IMACs]; **C, D** CD34 (**C** TAs; **D** IMACs; **E** P53 (IMACs); and **F** thymidine phosphorylase (dThdPase; IMACs). Several TA cells exhibit a positive nuclear reaction for Ki-67 (**A**). IMAC tubules show almost inverse positivity for Ki-67 (**B**) and P53 (**E**). Endothelial cells of intratumoral microvessels are detected by CD34 immunoreactivity (**C, D**). Cytoplasmic immunoreactivity for dThdPase is noted in IMACs (**F**). **A–F** $\times 100$

dThdPase expression and AI, KI, and IMVD

Table 3 shows the relation between dThdPase expression and the mean IMVD of the gastric adenomas and carcinomas. The mean IMVD was 69.4 ± 4.07 in the 25 dThdPase-positive TAs and 53.9 ± 2.82 in the 45 nega-

tive TAs. Similarly, the mean IMVD was 80.4 ± 4.37 in the 30 dThdPase-positive IMACs and 61.5 ± 4.90 in the 22 negative IMACs. The mean IMVD was significantly higher in the dThdPase-positive tumors than in the negative tumors in both the TAs and IMACs ($P < 0.01$, $P < 0.01$). However, there was no significant correla-

Fig. 4A,B. Spearman rank correlation coefficient tests for the correlation between the apoptotic index (*AI*) and intratumoral microvessel density (*IMVD*) for all cases. **A** Tubular adenomas (*TAs*), $r = 0.28$; $P = 0.015$; **B** intramucosal intestinal-type adenocarcinomas (*IMACs*), $r = 0.41$; $P = 0.006$. Closed circles, dThdPase-positive; open circles, dThdPase-negative

Fig. 5A,B. Spearman rank correlation coefficient tests for the correlation between the Ki-67 labeling index (*KI*) and intratumoral microvessel density (*IMVD*) for all cases. **A** Tubular adenomas (*TAs*), $r = 0.09$; $P = 0.92$; **B** intramucosal intestinal-type adenocarcinomas (*IMACs*), $r = 0.16$; $P = 0.36$. Closed and open circles, as in Fig. 4

tion between dThdPase expression and AI or KI. Thus, dThdPase expression was associated with IMVD, but not with AI and KI in gastric TAs and IMACs.

Discussion

In the present study, we initially selected gastric tubular adenomas (TAs) and intramucosal intestinal-type

adenocarcinomas (IMACs) from the Japanese criteria, and then examined the pathobiological properties of both types of lesions, from the viewpoint of cell proliferation, apoptosis, intratumoral microvessels, and the expression of dThdPase and P53. In the comparison of the two types of lesions, a significantly higher proliferative activity, a lower rate of cell loss, and a higher number of intratumoral microvessels were demonstrated in the IMACs than in the TAs. In other words, gastric TAs

Table 2. Correlation between P53 expression and intratumoral microvessel density (IMVD), apoptotic index (AI), and Ki-67 labeling index (KI) in tubular adenomas and intramucosal intestinal-type adenocarcinomas

	IMVD	AI	KI
Tubular adenomas			
P53-Positive (<i>n</i> = 14)	63.4 ± 5.25	4.72 ± 0.84	43.8 ± 4.93
-Negative (<i>n</i> = 56)	58.4 ± 2.81	4.81 ± 0.43	38.5 ± 1.51
Intramucosal intestinal-type adenocarcinomas			
P53-Positive (<i>n</i> = 29)	77.3 ± 5.01	3.05 ± 0.34	56.4 ± 2.31
-Negative (<i>n</i> = 23)	66.3 ± 4.41	3.53 ± 0.43	50.1 ± 2.86

Table 3. Correlation between thymidine phosphorylase (dThdPase) expression, intratumoral microvessel density (IMVD), apoptotic index (AI), and Ki-67 labeling index (KI) in tubular adenomas and intramucosal intestinal-type adenocarcinomas

	IMVD		AI	KI
Tubular adenomas				
dThdPase-Positive (<i>n</i> = 25)	69.4 ± 4.07	]*	4.23 ± 0.63	36.7 ± 1.86
-Negative (<i>n</i> = 45)	53.9 ± 2.82		5.10 ± 0.48	41.1 ± 2.18
Intramucosal intestinal-type adenocarcinomas				
dThdPase-Positive (<i>n</i> = 30)	80.4 ± 4.37	]*	3.15 ± 0.34	54.8 ± 2.35
-Negative (<i>n</i> = 22)	61.5 ± 4.90		3.42 ± 0.44	52.1 ± 2.99

* *P* < 0.01

showed a rather more static nature than IMACs. In fact, longterm follow-up studies of gastric TAs disclosed a lower incidence of malignant transformation, of 10% to 11%, than in IMACs [21,22]. These pathobiological properties may confirm the validity of the Japanese histopathological criteria for gastric intramucosal tumors and may also provide useful information for indications for gastric endoscopic mucosal resection (EMR). Recently, the Japanese Gastric Cancer Association reported the guideline for treatment for gastric cancer, in which IMACs less than 2.0cm in diameter are recommended to be treated by EMR [23]. Thus, histopathological differential diagnosis between TAs and IMACs is essential for deciding on the appropriate treatment.

Angiogenesis is essential for tumor development and proliferation. Prevascular tumors might remain dormant in situ (less than 3 mm in diameter) for months to years, and then a phenotypically transformed subclone of prevascular cells possessing angiogenic activity shows rapid growth, progression, and even metastasis [24,25]. Angiogenesis inhibitors can induce and sustain dormancy of experimental primary tumors and micrometastases by increasing tumor cell apoptosis. In the present study, we confirmed a significant inverse correlation between AI and IMVD in both gastric TAs and IMACs, implying a close relation between blood flow and the occurrence of apoptosis in the tumor cells. Moreover, the IMVD was significantly higher in the IMACs than in the TAs. dThdPase expression was significantly correlated with IMVD, but not with AI and

KI in both types of lesions. These results might suggest that dThdPase expression provides an advantage for the tumor growth of gastric TAs and IMACs by forming a favorable, vascular-rich intratumoral microenvironment. Although Matsuura et al. [8] confirmed a significant inverse correlation between dThdPase expression and AI in human advanced colorectal carcinomas, we could not confirm such an association in the gastric TAs and IMACs. This discrepancy might, partly, be due to differences in tumor stage. dThdPase expression might play a more crucial role in the proliferation and apoptosis of tumor cells in submucosal invasive or advanced gastric carcinomas. In fact, Osaki et al. [9] confirmed a significant correlation of dThdPase expression and high IMVD, which attenuated apoptosis in submucosal invasive early gastric carcinomas and advanced carcinomas, the AI being clearly lower in the latter than in the former. Similarly, Matsuura et al. [8] demonstrated a close correlation between dThdPase expression and KI in colorectal carcinomas of Dukes' C and D, but not in those of Dukes' A and B, implying crucial differences in the tumor stage. This might explain our finding of the lack of correlation between dThdPase expression and KI in the gastric TAs and IMACs.

It has been well demonstrated that *p53* gene mutation or P53 expression is clearly more frequent in gastric carcinomas than in TAs [26,27]. The present study also confirmed a significantly higher frequency of P53 expression in IMACs than in TAs. P53 protein has been shown to be required for the induction of the apoptotic

pathway triggered by oncogenic activation and the expression of cytotoxic genes [28,29]. The protein might sensitize damaged cells to apoptosis, acting to prevent the propagation of transforming mutations. Cells lacking a functional P53 protein would be refractory to this process of elimination and might proliferate more aggressively. We have demonstrated that the expression of a mutated *p53* gene attenuates apoptotic cell death in human advanced gastric carcinomas in vivo with marginal significance, implying that apoptosis occurs via both *p53*-dependent and -independent pathways [30]. However, the present study did not confirm a significant correlation between P53 expression and AI on IMVD in either TAs or IMACs. Thus, apoptosis might occur exclusively via a *p53*-independent pathway in gastric intramucosal tumors, including TAs and IMACs.

In conclusion, we have demonstrated the pathobiological properties of gastric TAs and IMACs, the latter of which are characterized as having higher proliferation and lower cell loss, this being related, in part, to IMVD. These findings might support the validity of the Japanese histopathological criteria for gastric intramucosal tumors, although no single parameter could differentiate between TAs and IMACs. To clarify more precisely the clinicopathological significance of gastric intramucosal tumors, further studies are required, e.g., from the viewpoints of gene alterations and epigenetic changes, in addition to the expression of various proteins, such as cell-cycle-related molecules.

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