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ORIGINAL ARTICLE

Depressed type of intramucosal differentiated-type gastric cancer has high cell proliferation and reduced apoptosis compared with the elevated type

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Abstract
Background The depressed type of early gastric cancer, in comparison to the elevated type, tends to invade the submucosal layer and metastasize to the lymph nodes. This study compared the differences in tumor cell proliferation and apoptosis between the elevated and depressed types of intramucosal differentiated gastric cancer.
Methods A total of 57 intramucosal differentiated gastric cancers were studied. Twenty samples were the elevated type and 37 were the depressed type. The tumor cells were analyzed by immunohistochemistry for Ki-67, Bcl-2, and Bax, and terminal deoxynucleotidyl transferase 2́-deoxyuridine, 5́-triphosphate (dUTP)-biotin nick end labeling was carried out to detect apoptotic cells.
Results (1) The Ki-67 labeling index (KI) was higher in the depressed type (median: 38.6) than in the elevated type (median: 21.2). (2) Immunopositivity for Bax and the apoptosis index (AI) were lower in the depressed type (median AI: 0.20) than the elevated type (median AI: 1.05). (3) The AI/KI was lower in the depressed type (median: 0.17) than in the elevated type (median: 5.57). (4) The AI in the tumors with a Bcl-2-negative and Bax-positive pattern (median: 2.0) was higher than that in the tumors with a Bcl-2-positive and Bax-negative pattern (median: 0.2).
Conclusion These results show that, regarding cell proliferation and apoptosis, the depressed type of intramucosal differentiated-type gastric cancer has high malignant potential in comparison to the elevated type.

Keywords Intramucosal differentiated-type gastric cancer · Elevated type · Depressed type · Ki-67 · Apoptosis

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Introduction

When endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) are performed for early gastric carcinoma, it is important to accurately evaluate the potential for lymph node metastasis from the macroscopic features, and there have been many investigations of the indications for EMR and ESD that take these features into account [1–5]. The rate of lymph node metastasis in intramucosal cancer is approximately 2 % but the rate is up to 20 % in submucosal carcinoma [1, 2, 6–9], and therefore it is important to predict submucosal invasion based on the macroscopic features. The depressed type of early gastric cancer tends to invade the submucosal layer and to metastasize to the lymph nodes even if it is smaller in size compared with the elevated type [7–12]. Furthermore, intramucosal undifferentiated gastric carcinoma sometimes spreads within the mucosa beyond the macroscopic margins [5] and intramucosal gastric cancer with lymph node metastasis often has an undifferentiated component [1, 6]. The clinical indications for EMR and ESD in

Japan are limited to intramucosal differentiated adenocarcinoma less than 2 cm in diameter without ulceration or scarring [13]. The extended indications for ESD recommended by clinical studies are limited to intramucosal differentiated adenocarcinoma more than 2 cm without ulceration or scarring, or less than 3 cm with ulceration or scarring, and intramucosal undifferentiated adenocarcinoma less than 2 cm without ulceration or scarring [13].

It is likely that the clinicopathological features of gastric cancer are closely associated with its growth pattern, determined by cell proliferation and apoptosis. Although many studies have shown that cell proliferation and apoptosis in gastric cancer are related to the clinicopathological features, this relationship has not been demonstrated in intramucosal differentiated-type gastric cancer.

This study investigated the difference in cell proliferation and apoptosis between the elevated type and depressed type of intramucosal differentiated-type gastric cancer, for which EMR and ESD are often applied.

Materials and methods

Tissues studied

Fifty-seven specimens of intramucosal differentiated-type gastric adenocarcinoma (category 4.2–4.4 of the revised Vienna classification [14, 15]), which had been surgically resected at Kyushu University Hospital and its affiliated hospitals, were selected for the present study; including undifferentiated component cases were excluded. Category 4.2 is "non-invasive carcinoma", category 4.3 is "suspicious for invasive carcinoma", and category 4.4 is "intramucosal carcinoma". Category 4.4 is equivalent to the Japanese classification Group 5. These lesions require endoscopic or local surgical treatment. Twenty samples were the elevated type and 37 were the depressed type (ulcer cases were not included). All the lesions were serially cut into slices of 3–4 mm, fixed in 10 % formalin solution, and embedded in paraffin.

Immunohistochemical staining

This study used immunohistochemical staining for the Ki-67 antigen (cell proliferation) and the apoptosis-related proteins, Bcl-2 (inhibits apoptosis) and Bax (induces apoptosis). Dewaxed paraffin sections were immunostained by the streptavidin–biotin–peroxidase complex (SAB; Histofine SAB-PO Kits; Nichirei, Tokyo, Japan) method, using the following primary antibodies: monoclonal antibodies against Ki-67 (MIB-1, diluted 1:100; DAKO, Glostrup, Denmark) and Bcl-2 (clone 124, diluted 1:100; DAKO, Glostrup, Denmark), and polyclonal antibody against Bax (P-19, diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). As pretreatment, microwave-based antigen retrieval was performed for both Ki-67 and Bcl-2. Trypsin pretreatment was performed for Bax. The sections were visualized with diaminobenzidine (DAB) and counterstained with Mayer's hematoxylin.

Terminal deoxynucleotidyl transferase 2́-deoxyuridine, 5́-triphosphate (dUTP)-biotin nick end labeling (TUNEL) staining method

Apoptotic cancer cells were detected by the TUNEL method. Apoptotic cells can be detected by hematoxylin and eosin (H&E) staining (Fig. 1a), but the TUNEL method detects apoptotic cells more clearly. The Takara in situ Apoptosis Detection Kit (Takara, Tokyo, Japan) was used according to the manufacturer's instructions.

Counting method

The Ki-67 labeling index (KI) and apoptosis index (AI) were defined as the percentage of tumor cells displaying immunoreactivity in 1000 tumor cells. The lesion was defined to be positive for Bcl-2 or Bax if more than 10 % of the tumor cells showed cytoplasmic or perinuclear immunostaining [16, 17]. The AI/KI ratio was also investigated. A low AI/KI ratio reveals a rapid growth pattern, and a high AI/KI ratio indicates a slow growth pattern [18].

Statistical analysis

Statistical analyses were performed using the χ^2test, the Mann–Whitney *U*-test, and the Kruskal–Wallis rank test. Values of $P < 0.05$ were considered to indicate statistical significance.

Results

Differences in clinicopathological characteristics between elevated type and depressed type

Table 1 lists the clinicopathological characteristics of the elevated and depressed types of lesions. Size was significantly larger in the elevated type (median: 3.6 cm) than in the depressed type (median: 1.5 cm). In regard to the Vienna classification, none of the elevated type showed category 4.2, but there was no significant difference between the elevated and the depressed types in categories 4.4 and 4.3. Other factors such as age, gender, and location showed no significant differences between the types.

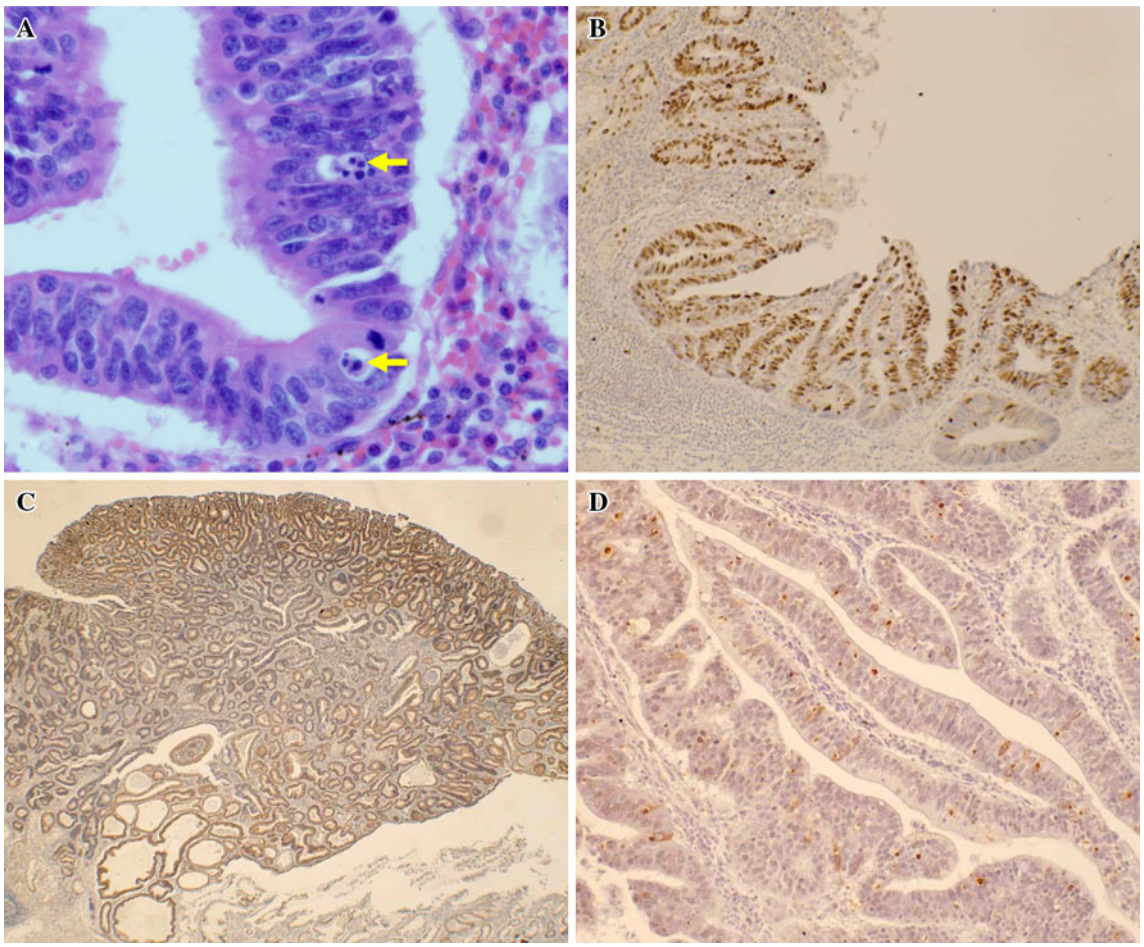

Fig. 1 **a** Apoptotic body characterized by shrinkage and nuclear fragmentation (*arrows*) (H&E, ×400). **b** Ki-67 was confined to the nucleus in depressed type (MIB-1, ×200). **c** Bax was confined to the cytoplasm in elevated type (P-19, ×30). **d** Terminal deoxynucleotidyl transferase 2́-deoxyuridine, 5́-triphosphate (dUTP)-biotin nick end labeling (TUNEL)-positive cells in elevated type reveal cell with apoptosis (×250)

Correlation between morphological features and proliferative activity and apoptosis

Table 2 shows the relationship between the morphological features and proliferative activity and apoptosis. Ki67 immunostaining was almost entirely confined to the nucleus. The KI was higher in the depressed type (median: 38.6) than in the elevated type (median: 21.2) (Fig. 1b).

Bcl-2 immunoreactivity was found throughout the cytoplasm in tumor cells. No significant differences in Bcl-2 immunoreactivity were observed between the elevated type (55 %) and the depressed type (68 %). Bax immunoreactivity was found throughout the cytoplasm in tumor cells. Bax-positive cells were observed more frequently in the elevated type (75 %) than in the depressed type (35 %) (Fig. 1c). TUNEL-positive cells were observed more frequently in the elevated type (median AI: 1.05) than in the depressed type (median AI: 0.20) (Fig. 1d).

The apoptosis index/Ki-67 labeling index (AI/KI) was significantly lower in the depressed type (median: 0.17) than in the elevated type (median: 5.57).

We further analyzed the Vienna classification category 4.4 and 4.3 groups (Table 3). In the 4.4 group, which is equavalent to the Japanese classification Group 5, the KI was higher in the depressed type (median: 41.2) than in the elevated type (median: 21.1). No significant differences in Bcl-2 immunoreactivity were observed between the elevated type (57 %) and the depressed type (46 %). Bax-positive cells were observed more frequently in the elevated type (78 %) than in the depressed type (27 %). The AI was higher in the elevated type (median: 1.25) than in the depressed type (median: 0.10). The AI/KI was also higher in the elevated type (median: 5.60) than in the depressed type (median: 0.41).

An analysis of the 4.3 group showed that the KI was higher in the depressed type (median: 40.3) than in the

elevated type (median: 21.2), but values for Bcl-2 and Bax immunoreactivity, and the AI and AI/KI were not significantly different between the elevated type and the depressed type.

Table 1 Clinicopathological characteristics

Factor	Elevated type ($n = 20$)	Depressed type ($n = 37$)	*P* value
Age (years), median (range)	68.0 (34–79)	64.5 (41–78)	0.326
Gender (male:female)	10:10	27:10	0.145
Size (cm), median (range)	3.6 (2.0–6.2)	1.5 (0.5–5.3)	<0.001
Location (U:M:L)[a]	0:11:9	0:14:23	0.268
Vienna classification			0.003
Category 4.2	0	13	
Category 4.3	6	13	
Category 4.4	14	11	
Lymphatic invasion	2	1	0.279
Venous invasion	0	0	–
Lymph node metastasis	0	0	–

[a] Upper, middle, and lower thirds of the stomach

Table 2 Relationship between morphological features and cell proliferation and apoptosis

Morphological type	Bcl-2	Bax	AI	KI	AI/KI
Elevated type ($n = 20$)	11 (55 %)	15 (75 %)	1.05	21.2	5.57
Depressed type ($n = 37$)	25 (68 %)	13 (35 %)	0.20	38.6	0.17
P value	0.512	0.009	0.002	<0.001	0.007

AI apoptosis index, *KI* Ki-67 labeling index

Table 3 Relationship between morphological features and cell proliferation and apoptosis in Vienna category 4.4 and 4.3 lesions

Morphological type	Bcl-2	Bax	AI	KI	AI/KI
Category 4.4					
Elevated type ($n = 14$)	8 (57 %)	11 (78 %)	1.25	21.1	5.60
Depressed type ($n = 11$)	5 (45 %)	3 (27 %)	0.10	41.2	0.41
P value	0.695	0.017	0.007	<0.001	0.005
Category 4.3					
Elevated type ($n = 6$)	3 (50 %)	4 (67 %)	0.55	21.2	1.92
Depressed type ($n = 13$)	12 (92 %)	6 (46 %)	0.30	40.3	0.78
P value	0.071	0.629	0.511	0.016	0.569

Correlation between the apoptosis index and immunopositivity for Bcl-2 and Bax

The correlation between the AI and immunopositivity for Bcl-2 and Bax is summarized in Fig. 2. The AI in Bcl-2-negative and Bax-positive pattern tumors (median: 2.0) was higher than that in Bcl-2-positive and Bax-negative pattern tumors (median: 0.2).

Discussion

This study investigated differences in the characteristics of cell proliferation and apoptosis between the elevated type and depressed type of intramucosal differentiated-type gastric cancer, which included subcategories 4.2–4.4 of the revised Vienna classification [14, 15]. These subcategories are treated in the same manner (endoscopic or surgical resection) because they are equivalent to Group 4 (strongly suspected of carcinoma) or Group 5 (definite carcinoma) in the Japanese group classification [13, 14, 15].

Many studies have evaluated the macroscopic invasion of early gastric cancer [1–3, 9–12]. Mitsunaga et al. [11] have reported that type IIa (superficial elevated type) shows no submucosal invasion with tumors less than 2 cm in diameter. On the other hand, the depressed type often tends to exhibit submucosal invasion even with lesions that are less than 1 cm in diameter. Oohara et al. [12] have reported that most minute gastric cancers less than 5 mm in diameter with submucosal invasion are the depressed type and none are the elevated type. Furthermore, Baba et al.

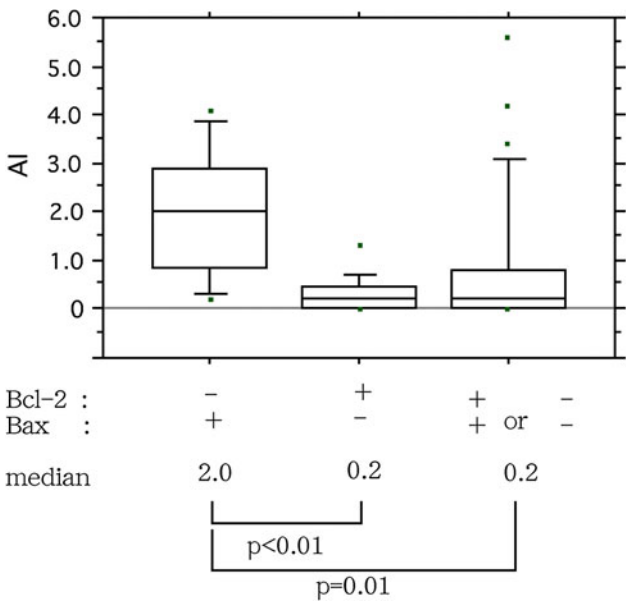

Fig. 2 Correlation of apoptosis index (*AI*) and immunopositivity for Bcl-2 and Bax

and Yamao et al. [7, 8] reported that the macroscopic depressed type was a risk factor for lymph node metastasis. The depressed type of early gastric cancer tends to show submucosal invasion or lymph node metastasis more frequently than the elevated type.

There have been many investigations of the relationship between Ki-67 and the clinicopathological features of gastric cancer, and many of these studies report that a high Ki-67 labeling index (KI) is closely correlated with tumor histology and lymph node metastasis [18, 19–21]. Kakeji et al. and Yonemura et al. [19, 20] also reported that a high KI was a risk factor for lymph node metastasis. Oya et al. [6] reported that the KI was higher in intramucosal well-differentiated gastric cancer with lymph node metastasis than in these cancers without lymph node metastasis. Muller et al. [21] reported that there was a positive correlation between a high KI at the tumor invasion front and the presence of blood or lymphatic vessel invasion. The present study found that the KI was higher in the depressed type than in the elevated type of intramucosal differentiated gastric cancer, a finding which suggests that the depressed type may have high malignant potential.

Studies have investigated the relationship between the apoptosis index (AI) and immunopositivity for Bcl-2 and Bax in gastric cancer. Koshida et al. [22] reported a correlation between the AI and immunopositivity for Bcl-2 and Bax, and they noted that Bcl-2 and Bax immunopositivity was distributed in opposite patterns in gastric cancer. The present study found that the AI was higher in the Bcl-2-negative and Bax-positive cases than in the Bcl-2-positive and Bax-negative cases, consistent with Koshida's study. These results indicate a close relationship between the AI and immunopositivity for Bcl-2 and Bax.

Many studies have evaluated the relationship between apoptosis and the clinicopathological features of gastric tumors [22–26]. Saegusa et al. [23] reported that the AI was higher in the advanced stage of gastric cancer than in the early type, and also that the AI was higher in differentiated carcinoma in comparison to the undifferentiated type. Pan et al. reported that carcinomas with decreased apoptosis were more likely to invade the deeper layer and to metastasize to the lymph nodes [24]. Kasagi et al. [25] have reported that a high frequency of apoptosis may be related to the slow-growing nature of well-differentiated gastric adenocarcinoma. In the present study, values for Bax and the AI in the depressed type were lower than the values in the elevated type, suggesting that the malignant potential of the depressed type may be higher than that of the elevated type. Ishii et al. [26] have reported that the combination of low apoptosis and high cell proliferation drives gastric cancer development. Shinohara et al. [18] reported that a high rate of apoptosis and a low rate of cell proliferation in well-differentiated carcinomas were factors in the slow rate of growth or progression of the cancers. In the present study, we found that the AI/KI ratio was much lower in the depressed type than in the elevated type, which suggests that the depressed type may have a rapid growth pattern.

In the present study we also investigated the Vienna category 4.4 and 4.3 groups. The depressed type in the 4.4 group showed high cell proliferation and low apoptosis, but there was no significant difference in apoptosis or in the AI/KI ratio between the elevated type and depressed type in the 4.3 group. In general, morphologically, the 4.3 group has a lower malignant potential than the 4.4 group, and therefore these results suggest that apoptosis or the growth speed in the 4.3 group may not show a substantial difference between the elevated type and the depressed type.

In the present study, the elevated-type lesions were larger than the depressed type in median size, but the elevated type showed lower cell proliferation and higher apoptosis than the depressed type. These results indicate that the elevated type has low malignant potential in comparison to the depressed type even if the size is larger.

In conclusion, the depressed type of early gastric cancer has high malignant potential, as indicated by its cell proliferation and apoptosis characteristics. Therefore, EMR or ESD for depressed-type early gastric cancer requires careful follow up for lymph node metastasis and local recurrence even if the tumor is the differentiated type of intramucosal carcinoma. Furthermore, EMR or ESD is recommended for the elevated type of early gastric cancer.

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