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Large Granule-aggregating Non-polypoid Colorectal Neoplasm: A Clinically-important Entity with Unique Cell Loss and Proliferation Kinetics

H. Eguchi[1, 2, 4], H. Hasegawa[4], H. Yamada[3, 4], H. Iino[3], H. Fujii[1, 3]

[1]Department of Research Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan
[2]Fijiyoshida Municipal Hospital, Fijiyoshida, Yamanashi, Japan
[3]University of Yamanashi Hospital, Surgery 1, Yamanashi, Japan
[4]Oral Pathology Unit, Graduate School of Oral Medicine, Matsumoto Dental University, Nagano, Japan

Abstract
The large non-polypoid colorectal neoplasm with a granule-aggregating appearance shows characteristic features clinicopathologically. The aim of this study was to investigate the developmental mechanisms of this unique lesion.

Among large non-polypoid tumours with a diameter of 10mm or more, 26 granule-aggregating tumours (GATs) were evaluated while using 19 polypoid tumours (PTs) as controls. Apoptosis and proliferation indices in the superficial and deeper portions of lesions were assessed by immunohistochemical staining with anti-ss-DNA and Ki-67 antibodies, respectively. These indices were also analyzed for clinicopathological conditions to clarify the developmental manner of GATs.

The apoptosis index (AI) of GATs was significantly higher than that of PTs ($p = 0.0003$). Especially in the deeper portion of the tumour, the AI value of GATs (4.54, SD: 1.86) was statistically more significant than that (0.34, SD: 0.48) of PTs ($p<0.001$). However, irrespective of dysplasia, a higher AI of GATs in the deeper portion was demonstrated in the early stage (10-19mm in diameter), in contrast to that of PTs ($P = 0.0001$). The Ki-67 labeling index as proliferation index (PI) of the deeper portion of GATs was 22.4 (SD: 7.8), which was significantly lower than that (33.4, SD: 11.7) of PTs ($p = 0.0016$). No significant differences in the superficial and the whole tumours were obtained comparing GATs and PTs. PI values of GATs increased with their size ($p = 0.0034$).

The current investigation clearly indicates that colorectal adenomas/tumours with granule-aggregating appearance have a higher apoptosis index in the deeper portion. This was demonstrated from an early stage of growth. These characteristic cell loss and cell proliferation kinetics give this tumour unique clinical features, that is, a laterally spreading manner or infrequent invasion even after malignant transformation.

Therefore, the importance of recognizing non-polypoid colorectal neoplasms with a granular feature as a new disease concept is emphasized.

Key words: non-polypoid colorectal neoplasms, granule-aggregating tumour, apoptosis, cell proliferation

Introduction

Colorectal lesions are of a polypoid or non-polypoid nature. The former is easily detected by colonoscopy while it is difficult for the latter. In Japan, advances in equipment and improvements in techniques have facilitated the detailed examination of superficial colorectal tumours. These changes increased the frequency of detection of non-polypoid colorectal lesions. Many of the non-polypoid colorectal adenomas less than 10 mm in diameter are superficial, flat, or depressed lesions [1-6]. In recent years, the importance of this type of tumour has gradually gained worldwide recognition [7-9].

Detection of large but flat colorectal tumours 10 mm or more in diameter is also difficult through an endoscopic examination [10]. However, numerous non-polypoid tumours have been reported in Japan [11, 12]. Most of these non-polypoid colorectal lesions develop laterally rather than vertically [13], and are known as laterally spreading tumours [4, 5, 11], nodular tumours [9], or granule-aggregating tumours (GATs) [14-16]. Large non-polypoid neoplasms designated as GATs in this study are uncommon in western countries, and are considered by the Japanese Society for Cancer of the Colon and Rectum to be a special type of superficial tumour [14, 15, 17]. On histological examinations, GATs rarely contain a carcinomatous component with submucosal invasion, even when the lesion is larger than 20 mm in diameter [4].

In the present study, the biological differences between polypoid tumours and non-polypoid GATs were examined in an effort to understand their differences in growth and spreading patterns.

Patients and Methods

Patients and Selected Cases

Twenty-six patients with endoscopically or surgically removed granule-aggregating tumours (GATs) and 19 patients with polypoid tumours (PTs) were retrospectively selected for this study at Yamanashi University Hospital and its branch hospitals from January 1995 to December 2005.

As in our previous reports [15, 16], GATs were defined by the following four characteristics:

1. Laterally spreading colorectal tumours with a granule-aggregating appearance composed of granules or small elevated lesions that project above the surrounding mucosa for 1-2 millimeters (Fig. 1)
2. No ulceration of the tumour surface
3. Tumour diameter of 10 mm or more
4. Histological appearance of tubular or tubulovillous adenomas with a villous component accounting for 0-24% or 25-74% of the tumours, respectively [18-23]

PTs were defined as protruding colorectal adenomas measuring 10 mm or more in diameter without a villous component and including pedunculated and sessile lesions accounting for 75% or more tumours. Exclusion criteria were a family history of familial adenomatous polyposis or hereditary non-polyposis colorectal cancer.

Clinicopathologically, in comparing GATs and PTs, the male : female ratios were 16 : 10 and 15 : 4; and the mean ages were 64.0(SD: 10.3) and 64.0(SD: 9.0). The size of GATs ranged from 12 to 62 mm with a mean of 28.0 (SD: 11.6) mm and that of PTs from 10 to 19 mm, with a mean of 13.4 (SD: 2.5) mm. The mean size of GATs was significantly larger than that of PTs ($P<0.0001$).

Histologically, selected GATs and PTs were divided into two groups of low grade atypia (LGA) showing mild to moderate dysplasia and high grade atypia (HGA) including severe dysplasia with or without focal carcinomatous components. The LGA : HGA ratio was 9 : 17 and 11 : 8 in GATs and PTs, respectively.

Tissue Preparation

All specimens were fixed in 10% buffered formalin, and routinely processed for histological examination of 3 μm-thick paraffin sections. The same pathologist (HH) examined all slides stained with haematoxylin and eosin. Investigators blinded to the clinicopathological data performed all of the assessments.

Immunohistochemical Staining

The sections were deparaffinized in xylene, dehydrated through a graded alcohol series, and washed in phosphate-buffered saline (PBS). To perform immunohistochemical detection of single-stranded (ss) DNA, tissues were digested with proteinase K (20 μg/ml in PBS; Wako Chemicals, Osaka, Japan) at room temperature (RT) for 6 min, followed by rinsing 3 times in PBS at RT for 5 min each. To perform immunohistochemistry for Ki-67, heat treatment for antigen retrieval was done in an autoclave (121 °C, 15 min) using 0.01 M sodium citrate buffer (pH 6.0). The sections were then incubated with a 3% H_2O_2 solution at RT for 10 min to inhibit intrinsic peroxidase activity. After washing with PBS, the sections were kept at RT in 10% normal bovine serum for 5 min, followed by overnight incubation at 4 °C with a rabbit polyclonal antibody for ssDNA (A4506, Dako, Glostrup, Denmark) at a 1 : 100 dilution and with an antibody for Ki-67 (clone: MM1, Novocastra Laboratories Ltd. Newcastle, UK) at a 1 : 50 dilution. After washing with PBS, incubated sections were reacted with Histofine Simple Stain MAX PO, Multi (Nichirei Co., Tokyo, Japan) at RT for 30 min, followed by rinsing 3 times in PBS at RT for 5 min each. After staining with 3-3'-diaminobenzidine tetrahydrochloride, the sections were counterstained with haematoxylin. Negative control slides were processed without the primary antibodies.

The apoptosis index (AI) and the proliferation index (PI) of the superficial portion and the deeper portion of each tumour were calculated as the number of stained tumour cells among 1,000 tumour cells in each region. At least five representative areas in each region were selected by light microscopy at 200 x magnification.

Statistical Analysis

Associations between the clinicopathological variables and the AI/PI values were assessed by the Mann-Whitney U test, the Wilcoxon signed-rank test, the Kruskal-Wallis test, Student's t-test, or Fisher's exact test using StatView-J 5.0 software. Probability (P) values<0.05 were considered to indicate significance.

Ethical Compliance

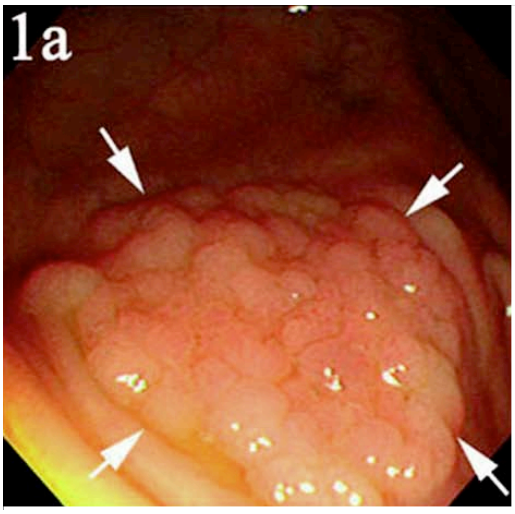
This study was performed in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all of the patients.

Results

Histologically, positive reactions to anti-ssDNA were frequently demonstrated in the deeper portion of the tumour in GATs (Fig. 2a) but in the superficial portion in PTs (Fig. 2b). Cells positive by anti-Ki-67 immunostaining were concentrated in the superficial portions of both GATs and PTs but with an uneven distribution (Figs. 3a and b).

The AI value of the whole area was 2.81 (SD: 1.32) in GATs and 1.59 (SD: 0.52) in PTs. This difference was statistically significant. ($p = 0.0003$). In the deeper portion, the AI of GATs (4.54, SD: 1.86) also showed a significantly higher value than that (0.34, SD: 0.48) of PTs ($p<0.0001$). However, in the superficial portion, the AI of GATs (1.21, SD: 1.47) was significantly lower than that (2.67, SD: 0.93) of PTs ($p<0.0001$). Similar AI-results comparing GATs and PTs were obtained in every portion of the LGA and HGA groups (Table 1A).

For PI, only the deeper portion of GATs (22.4, SD: 7.8) was significantly lower ($p = 0.0016$) than that (33.4, SD: 11.7) of PTs. The superficial and whole groups showed no differences of PI. PIs of the deeper portion in the LGA group (16.9, SD: 3.4 in GATs versus 27.9, SD: 11.7 in PTs, $p = 0.0185$) and in the HGA group(25.4, SD: 7.9 in GATs versus 40.9, SD: 6.9 in PTs, $p = 0.0005$) also demonstrated the same tendency for a strong significant difference. Additionally, in the HGA group, the PIs of the whole and superficial portion of GATs were also lower than that of



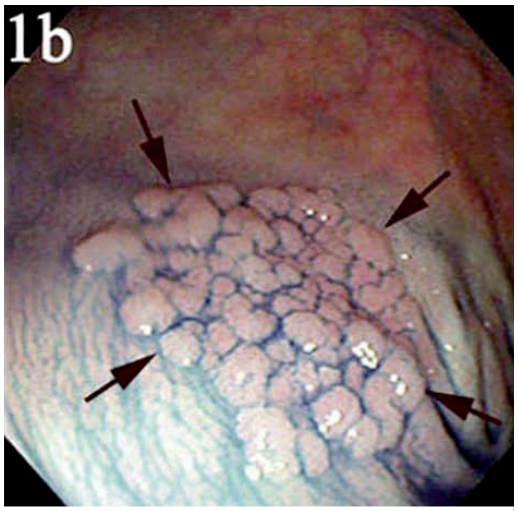
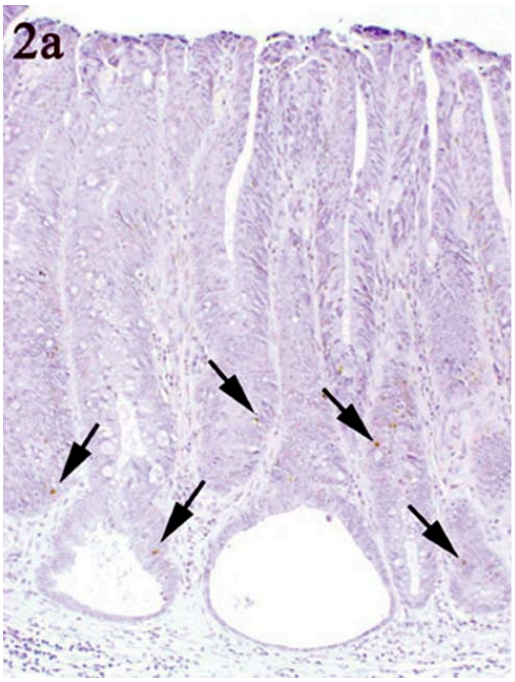


Fig. 1. Endoscopically, a typical laterally-spreading tumour (arrows) showed small granular surface (a) classified as granule-aggregating tumour (GAT), and its appearance was highlighted by dye spraying (b).



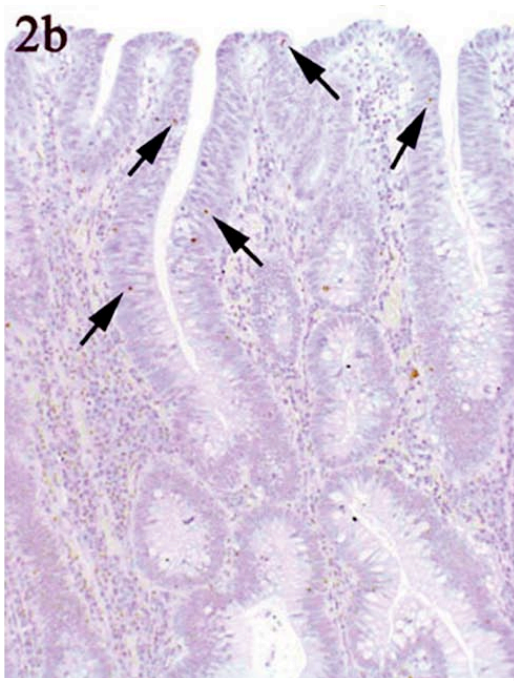
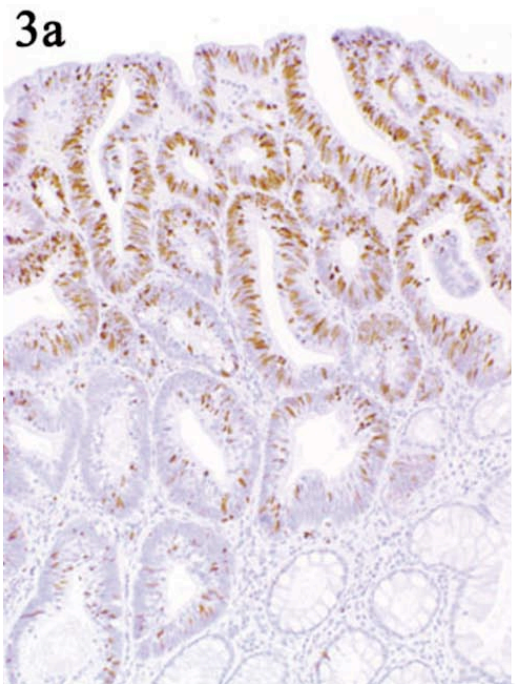


Fig. 2. Positive immuno-reactions (arrows) to anti-ssDNA antibody were mainly demonstrated in the deeper-half portion of laterally spreading tumours with granular features (a), but in the superficial-half of polypoid tubular adenomas (b). Original magnificagtion x50.



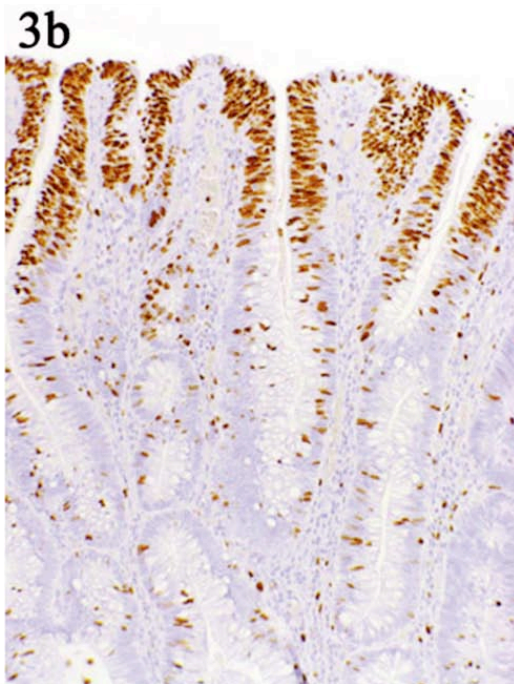


Fig. 3. Ki-67 positive cells were aggregated in the superficial portions of both laterally spreading and polypoid tumours (a, b). Original magnification x50.

Table 1. Comparison between granule-aggregating tumours and polypoid tumours in low and high grade groups for apoptosis index (A) and proliferation index (B)

A. Apoptotic index

	Total			LGA			HGA		
	GATs (n=26)	PTs (n=19)	p value	GATs (n=9)	PTs (n=11)	p	GATs (n=17)	PTs (n=8)	p value
superficial	1.21(1.47)	2.67 (0.93)*	<0.0001	0.40 (0.52)	2.65 (1.03)*	0.0003	1.64 (1.64)	2.69 (0.84)*	0.0198
deeper	4.54 (1.86)*	0.34 (0.48)	<0.0001	4.76 (2.25)*	0.28 (0.39)	0.0001	4.42 (1.68)*	0.43 (0.59)	<0.0001
whole	2.81 (1.32)*	1.59 (0.52)	0.0003	2.43 (0.93)*	1.56 (0.56)	0.0098	3.01 (1.47)*	1.63 (0.49)	0.0080

B. Proliferation index

	Total			LGA			HGA		
	GATs (n=26)	PTs (n=19)	p value	GATs (n=9)	PTs (n=11)	p value	GATs (n=17)	PTs (n=8)	p value
superficial	60.6 (14.1)	57.0 (16.6)	0.5051	49.5 (11.8)	44.2 (6.8)	0.2386	66.5 (11.7)	74.6 (5.3)	0.0546
deeper	22.4 (7.8)	33.4 (11.7)*	0.0016	16.9 (3.4)	27.9 (11.7)*	0.0185	25.4 (7.9)	40.9 (6.9)*	0.0005
whole	42.9 (9.8)	45.4 (12.9)	0.6791	34.4 (7.1)	35.9 (6.4)	0.7324	47.4 (7.9)	58.4 (5.9)*	0.0025

LGA=low grade atypia including mild and moderate dysplasia. HGA=high grade atypia including severe dysplasia and focal adenocarcinoma. GATs=granule-aggregating tumours. PTs=polypoid tumours. Values are the mean (SD). *p values (Mann-Whitney U test) for comparison between groups.

Table 2. Comparison between low and high grade groups of granule-aggregating tumours and polypoid tumours for apoptosis index (A) and proliferation index (B)

A. Apoptotoic index

	GATs (n=26)			PTs (n=19)		
	LGA (n=9)	HGA (n=17)	p value	LGA (n=11)	HGA (n=8)	p value
superficial	0.40 (0.52)	1.64 (1.64)	0.1097	2.65 (1.03)	2.69 (0.84)	0.9441
deeper	4.76 (2.25)	4.42 (1.68)	0.8590	0.28 (0.39)	0.43 (0.59)	0.5002
whole	2.43 (0.93)	3.01 (1.47)	0.3743	1.56 (0.56)	1.63 (0.49)	0.8886

B. Proliferation index

	GATs (n=26)			PTs (n=19)		
	LGA (n=9)	HGA (n=17)	p value	LGA (n=11)	HGA (n=8)	p value
superficial	49.5 (11.8)	66.5 (11.7)	0.1386	44.2 (6.8)	74.6 (5.3)*	0.0117
deeper	16.9 (3.4)	25.4 (7.9)	0.1731	27.9 (11.7)	40.9 (6.9)	0.0687
whole	34.4 (7.1)	47.4 (7.9)	0.0858	35.9 (6.4)	58.4 (5.9)*	0.0117

GATs=granule-aggregating tumours. PTs=polypoid tumours. LGA=low grade atypia including mild and moderate dysplasia. HGA=high grade atypia including severe dysplasia and focal adenocarcinoma. Values are the mean (SD). *p value (Wilcoxon signed-rank test) for comparison between the groups.

Table 3. Comparison between the tumour size and index for apoptosis (A) and proliferation (B) in granule-aggregating tumours

A. Apoptotic index

	10-19 mm (n=6)	20-29 mm (n=10)	>30 mm (n=10)	p value
superficial	0.53 (0.43)	0.94 (0.60)	1.89(2.15)	0.5081
deeper	4.42 (0.98)	4.83 (2.06)	4.32(2.17)	0.6534
whole	2.42 (0.69)	2.73 (0.84)	3.09(1.85)	0.8429

B. Proliferation index

	10-19 mm (n=6)	20-29 mm (n=10)	>30 mm (n=10)	p value
superficial*	42.6 (10.4)	63.8 (11.0)	68.2(9.1)	0.0039
deeper	16.4 (2.7)	21.5 (7.2)	27.0(8.0)	0.0325
whole*	31.2 (5.9)	43.5 (7.3)	49.3(7.6)	0.0031

Values are the mean (SD).*p value (Kruskal-Wallis test) for comparison between sizes.

Table 4. Comparison between granule-aggregating tumours and polypoid tumours measuring 10-19 mm in diameter for apoptosis index and proliferation index

	Apoptotic index			Proliferation index		
	GATs (n=6)	PTs (n=19)	p value	GATs (n=6)	PTs (n=19)	p value
superficail	0.53 (0.43)	2.67 (0.93)*	0.0004	42.6 (10.4)	57.0 (16.6)	0.0747
deeper	4.42 (0.98)*	0.34 (0.48)	0.0001	16.4 (2.7)	33.4 (11.7)*	0.0034
whole	2.42 (0.69)*	1.59 (0.52)	0.0156	31.2 (5.9)	45.4 (12.9)*	0.0186

GATs=granule-aggregating tumours. PTs=polypoid tumours. Values are the mean (SD). *p value (Mann-Whitney U test) for comparison between the groups.

PTs but at a weaker level of significance. In other cells, especially in the superficial PI values showed no significant differences even though their histological grades were considered (Table 1B).

Concerning histological grading, there were no significant differences between AI values of LGA and HGA groups in GATs and in PTs (Table 2A). The PI values of GATs also showed no significant difference between the LGA and HGA groups. The PI values of PTs however, were significantly higher in the HGA group than in the LGA group for the superficial and whole portions, as shown by the following: 74.6, SD: 5.3 versus 44.2, SD: 6.8, $p = 0.0117$ in the superficial portion and 58.4, SD: 5.9 versus 35.9, SD: 6.4 $P = 0.0117$ in the whole of tumour (Table 2B).

With respect to the size of GATs, the analysis revealed no significant differences of AIs among the three groups (superficial portion: $p = 0.5081$, deeper portion: $p = 0.6534$, whole tumour: $p = 0.8429$) (Table 3A). In contrast, the PI of GATs increased with tumour size in the superficial ($p = 0.0039$), the deeper ($p = 0.0325$) and the whole ($p = 0.0031$) portions (Table 3B).

The AI and PI values between GATs and PTs were analyzed by focusing only on the GATs of 10-19 mm in diameter versus PTs. When this was done, the AI values of the GATs were significantly higher than the PTs for the deeper (4.42, SD: 0.98 versus 0.34, SD: 0.48, $p<0.0001$) as well as the whole portions (2.42, SD: 0.69 versus 1.59, SD: 0.52, $p = 0.0156$). In the superficial portion however, the AI value for the GATs was significantly lower than in the PTs (0.53, SD: 0.43 versus 2.67, SD: 0.93, $p = 0.0004$). For the PI values, the deeper portion (16.4, SD: 2.7 versus 33.4, SD: 11.7, $p = 0.0034$) and whole portion (31.2, SD: 5.9 versus 45.4, SD: 12.9, $p = 0.0186$) of the GATs were significantly lower than the corresponding values for PTs (Table 4).

Discussion

In our series, as we previously reported, 19 tumours (73.1%) were asymptomatic and 11 of these 19 tumours (57.9%) were detected by faecal occult blood testing. Also, nine of the 26 patients with GATs (34.6%) had synchronous cancer at other sites [24-25]. GATs examined in this study showed strikingly unique features among colorectal neoplasms.

The present study revealed that the mean AI was higher in GATs than in PTs, despite the difference in the grade of atypia. On the other hand, it has been reported that the AI of villous adenomas was significantly lower than that of tubular adenomas [26]. Therefore, the AI values suggest that GATs are biologically as well as histologically and morphologically distinct from villous adenomas. Regarding the relationship between apoptosis and tumour growth, some authors have indicated that apoptosis is frequently noted in slowly growing tumours [27-29]. Despite GATs being larger, these tumours did not invade the submucosa [4], which indicates GATs are slow-growing tumours.

Neoplasms develop as a result of an imbalance between cell proliferation and cell loss. Thus, factors affecting this balance should influence the biological behavior of tumours. In a previous study, apoptotic cell loss in large non-polypoid adenomas (GATs) was examined by using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) method [30] and immunostaining for ssDNA. Apoptotic cells were strikingly observed in the deeper portion of the tumour compared with the superficial portion.

Considering this evidence, PTs were compared with GATs for the distribution or localization of apoptosis and cell proliferation in the superficial and deeper parenchyma, in order to investigate the developmental mechanism of GATs. The aim was to determine the association of GATs with AI or PI values and its morphological or clinical manifestations. As a result, GATs were clearly shown to possess significant apoptotic activity in the deeper portion of the tumour, while PI values were significantly lower in the deeper portion of GATs than PTs. These findings suggest that inhibition of cell proliferation is caused by the significantly greater prevalence of apoptosis in the deeper portion of GATs. Accordingly, a marked increase of apoptosis may inhibit invasion of cells with malignant transformation in the deeper portion of GATs. Consequently, the kinetics of cell proliferation and loss in GATs promote horizontal growth rather than vertical invasion, resulting in the lateral spread of this tumour.

The PI values tended to increase in both the superficial and deeper portions of GATs as an increasing atypical feature, but there was no significant relation between cell proliferation and the histological grading. On the other hand, PI values of GATs were higher in the superficial and deeper portions with increasing size, and were statistically significant. This suggests that GATs gradually increase in size, which is an important clinical factor characterizing this neoplasm.

The AI values of GATs were roughly constant in the superficial and deeper portions irrespective of tumour size, while apoptotic cells were always significantly more common in the deeper portion of tumour. Interestingly, in comparing the AI of GATs with that of PTs the difference in cell loss between GATs and PTs occurred in the early developmental stage of tumours at least 10mm in diameter. This suggests that neoplasms measuring 10mm or less in diameter and showing frequent apoptosis in the deeper portion are precursors of non-polypoid colorectal adenomas growing laterally and with granule-aggregating appearance. To confirm our hypothesis, it will be necessary to continue to discover the small colorectal adenomas with such biological characteristics and a diameter less than 10mm. Detailed electronic endoscopic observations at 1000x magnification may help to clarify the initial stages of this tumour [31-33].

GATs are clinically important because they can grow to a large size but infrequently invade the submucosa, even though undergoing malignant transformation. Thus, the biological behavior of GATs is different from that of PTs. Actually, three current cases of PTs over 20 mm in diameter (22 mm, 29 mm, and 36 mm, respectively) developed a component of adenocarcinoma invading the submucosa. Whereas there was no invasion of the submucosa by GATs in this

study, which included a patient with a tumour measuring 62 mm [34]. This raises the important question of the best treatment for GATs [35]. We previously investigated the clinical characteristics of the present series of GATs [15, 16], in which endoscopic and surgical treatments were performed in seven (26.9%) and 19 (73.1%) cases, respectively. Follow up data for 1 to 10 years after the removal of tumours have shown no recurrence in cases receiving either treatment. Therefore, it is clinically important to recognize the large non-polypoid neoplasm (GAT) as a new disease concept, not only to assist in the screening and prevention of colorectal tumours, but also to avoid excessive surgery [36, 37].

Awareness of the existence of GATs may have important implications for gastroenterology and for future investigations into the development of non-polypoid colorectal tumours.

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Address for correspondencer:
Hideo Eguchi
Fijiyosida Municipal Hospital
6350 Kamiyoshida Fujiyosida-shi
Yamanashi 403-0005
Japan
Phone: +81-555-22-4111
Fax: +81-555-22-6995
E-mail: byoin@city.fujiyoshida.lg.jp