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Clinicopathological and Molecular Features of Colorectal Serrated Neoplasias With Different Mucosal Crypt Patterns

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OBJECTIVES: **Endoscopic examination shows that serrated neoplasias (SNs), such as serrated adenomas and sessile serrated adenomas, exhibit different mucosal crypt patterns. However, it remains unclear whether advanced serrated polyps with different mucosal crypt patterns have different clinicopathological or molecular features.**

METHODS: **We classified the mucosal crypt patterns of 86 SNs into three types (hyperplastic, adenomatous, and mixed pattern) and evaluated their clinicopathological and molecular features.**

RESULTS: **We found significant differences in the proliferative activity status between SNs with mixed/adenomatous patterns and those with the hyperplastic patterns. SNs with the hyperplastic pattern were frequently located in the proximal colon and had a macroscopically superficial appearance, whereas SNs with the adenomatous pattern were often located in the distal colon and had a protruding appearance. Furthermore, a significant difference was observed in the frequency of the CpG island methylator phenotype (CIMP), involving the methylation of two or more CIMP-related genes (*MINT1*, *MINT2*, *MINT31*, *p16*, and *MLH1*), between SNs with the hyperplastic pattern and those with the mixed/adenomatous patterns (18/32 (56%) vs. 8/28 (29%) or 7/26 (27%); *P*=0.0309 or *P*=0.0249, respectively). Moreover, the prevalence of *KRAS* mutations was significantly higher in SNs with the adenomatous pattern than in those with the hyperplastic pattern (7/26 (27%) vs. 1/32 (3%); *P*=0.0173). In comparison with other patterns, the mixed pattern was detected more frequently in mixed serrated polyps (MSPs), which contain separate histological components. Some MSPs exhibited concordant molecular alterations among the different histological components.**

CONCLUSIONS: **The clinicopathological and molecular features of SNs correlated strongly with their mucosal crypt patterns, which were observed using chromoendoscopy.**

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INTRODUCTION

Colorectal cancers (CRCs) are one of the cancers diagnosed most commonly in Western countries (1). Recently, there appears to have been an increase in the incidence of CRCs in Eastern populations. Most CRCs develop over a long period via a multistep process through the adenoma-carcinoma sequence (2). The process often begins with the inactivation of the adenomatous polyposis coli (APC)/β-catenin signaling pathway. Subsequently, the accumulation of specific genetic and epigenetic changes results in disease progression via three distinct clinicopathological pathways, which involve DNA methylation, microsatellite instability (MSI), and epigenetic/genetic interactions leading to mutations of the KRAS or BRAF oncogenes and of the p53 tumor-suppressor genes (3–5).

Previous studies have suggested that the risk of CRC could be reduced by endoscopic removal of precursor lesions (6).

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Accordingly, the guidelines for CRC screening recommend that average-risk patients should undergo colonoscopy for cancer prevention, even if they are asymptomatic (7). However, a significant number of CRCs will still develop, despite these screening strategies. Although interval cancers may be missed during endoscopy, inappropriate recognition of their precursors may result in cancer development.

Hyperplastic polyps (HPs) are detected often in the colorectum, especially in the sigmoid colon and rectum, of elderly patients (8,9). Histological examination of HPs reveals an architecturally serrated configuration without neoplastic features. For many years, HPs have been considered as nonneoplastic lesions without malignant potential; thus, they have been dismissed as innocuous. However, some HPs show molecular features similar to those of CRCs (10,11). Moreover, several subtypes of serrated polyps, such as sessile serrated adenoma (SSA) (12,13), traditional serrated adenoma (TSA) (14), and mixed polyp (15), have been proposed to be the precursors of CRCs. SSA, which is often a large and proximal lesion with abundant mucin secretion, exaggerated serration, and atypical architecture, was reported as a variant of HP. Furthermore, TSAs, which exhibit a combination of the dysplastic features of an adenoma and serrated architecture, are thought to be a subtype of adenoma. Mixed polyps are described as the presence of at least two separate serrated or nonserrated components combined within a single polyp. These advanced serrated polyps exhibit BRAF and KRAS mutations, MSI, and widespread DNA methylation (15–18). Thus, the serrated pathway appears to be an alternative pathway for the development of CRCs, which is involved in the formation of ~10% of CRCs (19).

Several articles have reported the endoscopic features of TSA. Matsumoto *et al.* (20) suggested that the mucosal crypt patterns of TSAs can be classified into three types, i.e., hyperplastic, cerebriform, and combined pattern, based on chromoendoscopic findings. The presence of a combined hyperplastic–cerebriform pattern is a surface-specific feature of TSAs. However, Boparai *et al.* (21) have reported a low diagnostic accuracy of any of the novel endoscopic imaging techniques for differentiating SSAs from HPs. In contrast, advanced serrated polyps follow different pathways for progression to cancer. We hypothesized that there are biological or molecular differences among the advanced serrated polyps with different mucosal crypt patterns and that these features are associated with their different pathways of progression to CRC. To test this hypothesis, we evaluated the methylation and mutation status in patients with advanced serrated polyps.

METHODS

Patients and samples

We examined 86 serrated neoplasias (SNs; 56 TSAs, 15 SSAs, and 15 mixed serrated polyps (MSPs)) from 74 patients who underwent endoscopic resection at the Showa University Hospital between April 2005 and December 2008. We excluded patients with a familial predisposition to cancers such as familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer. Tissue collection was approved by the ethics committee of the Showa University School of Medicine.

Endoscopic evaluation

All patients were prepared for the procedure via administration of 1.8 l of an oral electrolyte lavage solution. Colonoscopists with extensive experience performed all examinations using a high-resolution video colonoscope (CF-260AI; Olympus Optical, Tokyo, Japan). We followed a procedure reported previously for the observation of mucosal crypt patterns on the surface of a lesion (22). The mucosal crypt pattern of each lesion was determined according to the modified classification of Kudo *et al.* (23). Briefly, this system classifies mucosal crypt patterns into three types: hyperplastic pattern, type II (stellar or papillary pits) (**Figure 1b**); adenomatous pattern, type III (tubular or small roundish pits) or type IV (branch-like or gyrus-like pits) (**Figure 1c**); and mixed pattern (**Figure 1d**), which is a combination of the two patterns described above (hyperplastic and adenomatous patterns). The hyperplastic and adenomatous patterns were observed in a clearly distinctive area. Our preliminary data of the mucosal crypt pattern showed that the interobserver and intraobserver agreement of the experienced colonoscopists was $\kappa = 0.76$ and $\kappa = 0.80$, respectively.

Lesions with adenomatous or mixed patterns that were >5 mm in diameter were removed using coagulation biopsy ("hot biopsy") or endoscopic resection (i.e., endoscopic mucosal resection or polypectomy). However, we used the following criteria to decide whether lesions that exhibited a hyperplastic pattern on their surface should be removed endoscopically: (i) typical location of HPs in the rectosigmoid area, and (ii) small size (usually ≤5 mm in diameter) and a symmetrical and uniform shape. If a colonoscopist established a diagnosis of HP, no further endoscopic treatment was performed.

Tissue samples and histological evaluation

Serial sections (3 μm) were obtained from paraffin blocks and prepared for hematoxylin and eosin staining and immunostaining. All hematoxylin and eosin-stained slides were reviewed by a senior pathologist (T.Y.), who was blinded to the endoscopic findings. The histological diagnoses of TSA and SSA were based on the definition established by Longacre *et al.* (14) and Torlakovic *et al.* (13), respectively. Serrated lesions that exhibited more than two different histological components (e.g., HP or conventional adenoma (CAD)) were defined as MSPs. A total of 93 SNs were diagnosed histologically as SNs. These SNs were 4.1% of colorectal adenomas removed endoscopically during the above period. However, seven SNs were excluded from this study because of poor endoscopic observation or amounts of sample material that were insufficient for analysis. Finally, 86 SNs were included in this study.

To extract genomic DNA, we used 67 formalin-fixed, paraffin-embedded samples and 19 frozen tissue samples. The frozen tissue samples were obtained using colonoscopic biopsy and stored at −80 °C. Before obtaining colonic biopsy specimens from the patients, the pit patterns were classified to distinguish between the neoplastic and nonneoplastic areas in the lesion accurately, based on chromoendoscopic findings (22,23). DNA was extracted from the frozen tissue samples using the standard proteinase K/phenol/chloroform methods.

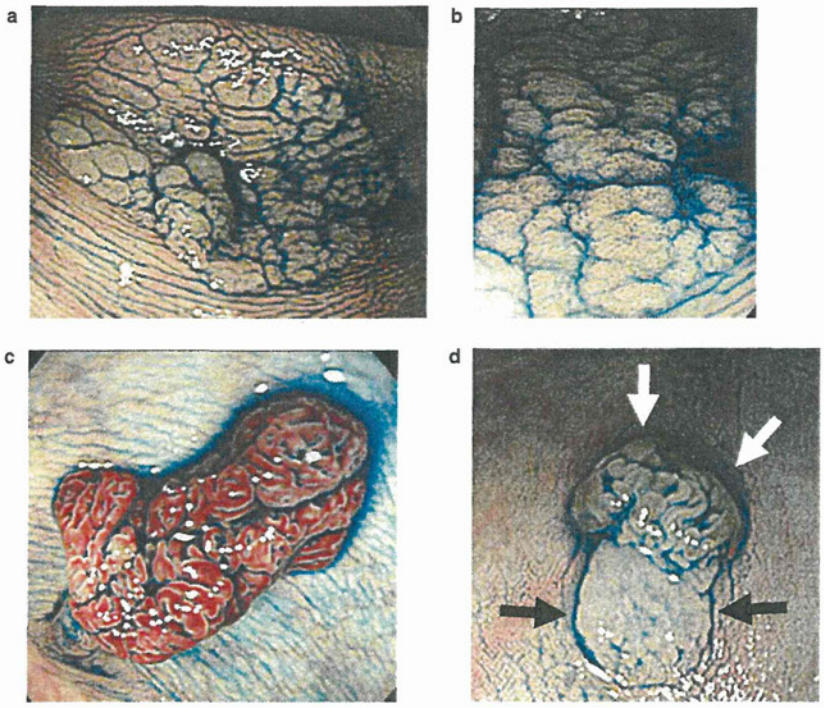

Figure 1. The macroscopic appearance and mucosal crypt pattern of serrated neoplasias. Chromoendoscopic images show (**a**) superficial, (**c**) protruded, and (**d**) mixed (combination of superficial and protruded types) macroscopic appearances of serrated neoplasias. The mucosal crypt patterns were classified into three types according to Kudo's classification: (**b**) hyperplastic pattern (magnifying view of lesion **a**), type II (stellar or papillary pits); (**c**) adenomatous pattern, type III (tubular or small roundish pits) or IV (branch-like or gyrus-like pits); and (**d**) mixed pattern, a combination of the two patterns (hyperplastic (black arrows) and adenomatous (white arrows) patterns.

Serial slides were obtained from the archival blocks of formalin-fixed, paraffin-embedded tumor tissues. One slide was stained with hematoxylin and eosin for microdissection. After microdissection, DNA was extracted using the QIAamp DNA mini kit (QIAGEN, Valencia, CA).

Immunohistochemical staining of Ki-67

The protocol for Ki-67 immunohistochemical staining was performed as described previously (18). Immunostained sections were evaluated by our pathologist (T.Y.), who was blinded to any clinical and molecular findings. To evaluate the Ki-67 staining distribution, we divided neoplastic glands into three equal crypt zones (upper, middle, or lower zones). The Ki-67-positive rates were determined as the number of positive cells/500 cells in each zone, of the total of 1,500 cells counted, in randomly selected crypts.

Bisulfite PCR and pyrosequencing analysis of DNA methylation

Bisulfite treatment was performed as previously described (24). Bisulfite-treated DNA (2 or 3μl) was used as the template for PCR. The primers and PCR conditions used for the amplification of specific DNA fragments of various target genes were set according to those described in our previous report (25). The protocol for pyrosequencing, which is a quantitative tool for methylation density, was described in detail previously (25). Pyrosequencing measures the methylation status of several CpG sites in a given promoter. Usually, these different sites show highly concordant methylation. For each gene, we averaged the methylation percentage of all CpGs measured.

Methylation-related genes and definition of the CpG island methylator phenotype (CIMP)

It has been reported that sporadic CRCs can be classified into two groups, CIMP positive and CIMP negative, according to the frequency of methylation of the CpG islands in the promoter of five genes (*MINT1*, *MINT2*, *MINT31*, *p16*, and *MLH1*). The determination of CIMP status required a quantitative tool, and methylation positivity was defined as a methylation density > 15%. A tumor was considered CIMP positive if two or more of the CIMP markers were methylated as described previously (26). All others were defined as CIMP negative.

KRAS and *BRAF* gene mutations and MSI

We used direct DNA sequencing to analyze samples for the presence of activating mutations in codons 12 and 13 of *KRAS* and in codon 600 of *BRAF*, as described previously (18,27). MSI analysis was previously reported for this set of samples (18).

Data analysis and statistics

Continuous variables (i.e., age and tumor size) were analyzed using the Mann–Whitney test. Categorical variables were compared between tumor groups using the χ^2 test or Fisher's exact test

when testing small samples. All tests were two sided, and $P<0.05$ was considered statistically significant.

RESULTS

The mucosal crypt patterns of the 86 SNs were classified as 32 hyperplastic patterns, 28 mixed patterns, and 26 adenomatous patterns, based on the chromoendoscopic examination. The correlation between the mucosal crypt patterns and the histological type of the SNs is shown in **Table 1**.

Immunohistochemical staining for Ki-67 in the mucosal crypt patterns of SNs

The results of the comparison of Ki-67 immunostaining among the different mucosal crypt patterns are shown in **Table 2**. In the lower crypt zone, the values for the cell proliferative index were significantly higher for lesions with the hyperplastic pattern than for those with the mixed and adenomatous patterns ($P=0.0003$ and $P<0.0001$, respectively). However, similar values were observed for lesions with mixed and adenomatous patterns. In the upper zone, the cell proliferative indices for lesions with the hyperplastic pattern were significantly lower than those observed for lesions with the mixed and adenomatous patterns ($P<0.0001$). However, there was no significant difference between the values obtained for mixed and adenomatous patterns. In the middle zone, we observed no significant difference in the cell proliferative indices among the three mucosal crypt patterns.

Comparison of mucosal crypt patterns with the clinicopathological or molecular characteristics of SNs

Table 1 shows the comparison of endoscopic findings with the clinicopathological or molecular characteristics of the studied patients with SNs. SNs with the hyperplastic pattern were found more frequently in proximal than in distal tumors, whereas SNs with the adenomatous and mixed patterns were detected often in distal lesions. These differences were statistically significant ($P<0.0001$). Macroscopically, ~40% of lesions with the hyperplastic pattern were superficial (**Figure 1a**), whereas >90% of lesions with the adenomatous pattern were protruded (**Figure 1c**; $P=0.0125$). Only lesions with the mixed pattern exhibited a macroscopically mixed appearance (**Figure 1d**). We observed significant differences in the frequency of the mixed-macroscopic type between SNs with the mixed pattern and SNs with the hyperplastic/adenomatous pattern ($P=0.0075$ and $P=0.0235$, respectively). Histological evaluation indicated that all SSAs had a hyperplastic pattern and that 96% of lesions with the adenomatous pattern were TSAs. The frequency of MSPs was higher in lesions with the mixed pattern than in lesions with the hyperplastic and adenomatous patterns (32 vs. 16 and 4%, respectively). We found that five SNs with high-grade dysplasia showed either a mixed pattern or an adenomatous pattern.

We classified tumors as CIMP positive or CIMP negative based on the methylation of two or more CIMP-related genes (MINT1, *MINT2*, *MINT31*, *p16*, and *MLH1*). We observed a significant difference in the frequency of CIMP between lesions with the

Table 1. Comparison of endoscopic findings with clinicopathological or molecular features in serrated neoplasias

	Endoscopic mucosal crypt pattern		
	Hyperplastic (*N*=32)	Mixed (*N*=28)	Adenomatous (*N*=26)
Gender			
Male	20	14	18
Female	6	12	8
Age (years) (range)	59.1 (36–77)	60.2 (23–85)	57.6 (38–82)
Tumor location[a]			
Proximal	25	5	4
Distal	7	23	22
Size (mm) (range)	11.2 (5–30)	11.3 (5–35)	11.8 (4–24)
Macroscopic[b]			
Protruded	20	19	24
Superficial	12	3	2
Mixed	0	6	0
Histology[c]			
TSA	12	19	25[d]
SSA	15	0	0
MSP	5	9[d]	1[d]
KRAS[e]			
Mut+	1	5	7
Mut−	31	23	19
BRAF			
Mut+	17	16	14
Mut−	15	12	12
CIMP[f]			
Positive	18	8	7
Negative	14	20	19
MSI-L			
Positive	3	3	2
Negative	29	25	24
MSI-H			
Positive	0	2	2
Negative	32	26	24

CIMP, CpG island methylator phenotype; MSI, microsatellite instability; MSI-H, high-frequency MSI; MSI-L, low-frequency MSI; MSP, mixed serrated polyp; Mut+, presence of mutation; Mut−, absence of mutation; SSA, sessile serrated adenoma; TSA, traditional serrated adenoma.

[a]Hyperplastic vs. mixed and hyperplastic vs. adenomatous, $P<0.0001$.

[b]Hyperplastic vs. mixed, $P=0.0037$; hyperplastic vs. adenomatous, $P=0.0125$; mixed vs. adenomatous, $P=0.0348$.

[c]Hyperplastic vs. mixed, $P=0.0037$; hyperplastic vs. adenomatous, $P=0.0119$; mixed vs. adenomatous, $P<0.0001$.

[d]One mixed pattern (MSP) and four adenomatous patterns (3 TSAs and 1 MSP) showed high-grade dysplasia.

[e]Hyperplastic vs. mixed, $P=0.0884$; hyperplastic vs. adenomatous, $P=0.0173$.

[f]Hyperplastic vs. mixed, $P=0.0309$; hyperplastic vs. adenomatous, $P=0.0249$.

Proximal means cecum, ascending and transverse colon; distal means descending and sigmoid colon and rectum.

Table 2. Comparison of Ki-67 expression in serrated neoplasias

	Endoscopic mucosal crypt pattern		
	Hyperplastic (*N*=32)	Mixed (*N*=28)	Adenomatous (*N*=26)
Upper zone of crypts	4.9* (3.6–6.3)	14.6 (9.9–19.2)	16.4 (12.6–20.2)
Middle zone of crypts	22.3 (17–27.5)	25.7 (18.9–32.5)	27.7 (20.7–34.6)
Lower zone of crypts	65.6* (60.6–70.6)	45.3 (37.3–53.3)	36.7 (27.7–45.8)

*Significantly different from mixed/adenomatous pattern lesions (see *P* value in the Results section of the text).
Data show mean value in percent (95% CI).

hyperplastic pattern and lesions with the mixed or adenomatous patterns (18/32 (56%) vs. 8/28 (29%) or 7/26 (27%); $P=0.0309$ or $P=0.0249$, respectively). The frequency of *KRAS* mutations was significantly higher in lesions with the adenomatous pattern than in lesions with the hyperplastic pattern (7/26 (27%) vs. 1/32 (3%); $P=0.0173$). However, we found no significant differences in the frequency of BRAF mutations, low-frequency MSI (MSI-L), and high-frequency MSI (MSI-H) among lesions exhibiting the three patterns. All remaining tumors but one had either *BRAF* or *KRAS* mutations.

Comparison of mucosal crypt patterns with the clinicopathological or molecular characteristics of TSAs

TSAs with the hyperplastic pattern were located more frequently in the proximal colon compared with TSAs with the mixed or adenomatous patterns ($P<0.0001$; **Table 3**). The frequency of CIMP was significantly higher in TSAs with the hyperplastic pattern than in TSAs with the adenomatous pattern ($P=0.0274$). However, the frequency of KRAS mutations was higher in TSAs with the adenomatous pattern compared with TSAs with the hyperplastic pattern, although this difference did not reach statistical significance ($P=0.0721$). There was no significant difference in gender, age, frequency of BRAF mutations, MSI-L, and MSI-H among TSAs with the three mucosal crypt patterns.

Clinicopathological and molecular features of MSPs

We examined the clinical and molecular features of two histological components of eight MSPs (**Table 4**). Microdissection allowed the separate examination of KRAS and BRAF mutations, CIMP, and MSI status in different histological areas of MSPs. Although TSA accompanied with HP showed heterogeneity of genetic or epigenetic changes, concordant changes were observed in mixed-TSA/SSA and TSA/tubular adenoma.

Table 3. Comparison of endoscopic findings with clinicopathological or molecular features in traditional serrated adenomas

	Endoscopic mucosal crypt pattern		
	Hyperplastic (*N*=12)	Mixed (*N*=19)	Adenomatous (*N*=25)
Gender			
Male	10	7	16
Female	2	9	8
Age (years) (range)	58.8 (37–77)	59.5 (23–85)	56.8 (38–82)
Tumor location[a]			
Proximal	11	2	3
Distal	1	17	22
Size (mm) (range)	8.8 (5–12)	10.4 (5–35)	11.8 (4–24)
Macroscopic[b]			
Protruded	9	14	23
Superficial	3	1	2
Mixed	0	4	0
KRAS[c]			
Mut+	0	3	7
Mut–	12	16	18
BRAF			
Mut+	9	11	13
Mut–	3	8	12
CIMP[d]			
Positive	8	5	6
Negative	4	14	19
MSI-L			
Positive	0	2	2
Negative	12	17	23
MSI-H			
Positive	0	2	2
Negative	12	17	23

CIMP, CpG island methylator phenotype; MSI, microsatellite instability; MSI-H, high-frequency MSI; MSI-L, low-frequency MSI; Mut+, presence of mutation; Mut–, absence of mutation.
[a]Hyperplastic vs. mixed and hyperplastic vs. adenomatous, $P<0.0001$.
[b]Mixed vs. adenomatous, $P=0.0547$.
[c]Hyperplastic vs. adenomatous, $P=0.0721$.
[d]Hyperplastic vs. mixed, $P=0.0596$; hyperplastic vs. adenomatous, $P=0.0274$.
Proximal means cecum, ascending and transverse colon; distal means descending and sigmoid colon and rectum.

DISCUSSION

We evaluated the cell proliferation among three mucosal crypt patterns using the Ki-67 antibody, which stains the nuclei of proliferating cells in all cell cycle phases, with the exception of G0 (28). The proliferating cells of SNs with the hyperplastic pattern were located more frequently in the lower zone crypts, compared with SNs with the mixed/adenomatous pattern. However, the cellular proliferative activity in the upper zone of SNs with the mixed/adenomatous pattern was significantly higher

Table 4. Clinicopathological and molecular features of mixed serrated polyps

No.	Endoscopy	Gender	Age	Location	Macroscopic type	Size (mm)	Histology	KRAS	BRAF	CIMP	MSI
1	Mixed	F	57	D	Mixed	7	TSA	Wild	Wild	–	MSS
							HP	Wild	Wild	+	MSS
2	Mixed	M	70	P	Mixed	20	TSA	Wild	Wild	+	MSI-L
							HP	Wild	V600E	–	MSI-L
3	Adenomatous	F	58	D	Protruded	15	TSA	Wild	Wild	–	MSS
							HP	Wild	Wild	–	MSS
4	Mixed	M	39	D	Protruded	9	TSA	Wild	V600E	–	MSS
							SSA	Wild	V600E	–	MSS
5	Hyperplastic	M	65	P	Protruded	8	TSA	Wild	V600E	+	MSS
							SSA	Wild	V600E	+	MSS
6	Mixed	M	53	D	Protruded	9	TSA	Wild	V600E	–	MSS
							SSA	Wild	V600E	–	MSS
7	Mixed	M	71	P	Superficial	20	TSA with HGD	Wild	Wild	+	MSS
							SSA	Wild	Wild	+	MSS
8	Mixed	F	57	D	Protruded	11	TSA	G12C	Wild	–	MSS
							T	G12C	Wild	–	MSS

CIMP, CpG island methylator phenotype; D, distal; F, female; HGD, high-grade dysplasia; HP, hyperplastic polyp; M, male; MSI, microsatellite instability; MSI-L, low-frequency MSI; MSS, microsatellite stable; P, proximal; SSA, sessile serrated adenoma; T, tubular adenoma; TSA, traditional serrated adenoma.

than that observed for SNs with the hyperplastic pattern. These immunohistochemical findings indicate the presence of different biological features between SNs with the hyperplastic and mixed/adenomatous patterns.

Endoscopic examination revealed that SSAs are superficial lesions with irregular borders and that they were often covered with mucus (21,29). As mentioned above, chromoendoscopic studies have shown that their mucosal crypt patterns are similar to those of HPs. However, our analysis of SNs that appeared as Kudo's type II (hyperplastic pattern) revealed the presence of various serrated histological types. Although one-third of SNs with the hyperplastic pattern were SSAs, the remaining lesions included TSAs and MSPs. Moreover, SNs with the hyperplastic pattern showed a proximal location and a superficial-macroscopic appearance significantly more frequently compared with the other patterns, which suggests that SNs with the hyperplastic pattern have clinicopathological features of SSAs. Mäkinen (30) suggested that SSAs represent a morphological intermediate between HP and TSA, as there are certain differences in their molecular backgrounds, including the frequency of *BRAF* mutations and DNA methylation. We also found that CIMP was found more frequently in SNs with the hyperplastic pattern than in SNs with the mixed/adenomatous pattern and that half of the SNs with the hyperplastic pattern exhibited BRAF mutations. Moreover, the evaluation of TSAs exclusively revealed that TSAs with the hyperplastic pattern had a predilection for the proximal colon, higher prevalence of CIMP, and lower frequency of *KRAS* mutations compared with TSAs with the adenomatous pattern. These observations indicate that SNs with the hyperplastic pattern, especially those located in the proximal colon, may be implicated in the sessile serrated pathway.

In contrast to SNs with the hyperplastic pattern, SNs with the adenomatous pattern were more likely to have a distal location, be of the protruded type, and have a higher frequency of KRAS mutations. The TSAs with the adenomatous pattern also showed these characteristics. Previous studies have reported that TSAs have a predilection for the distal colon and rectum and that they are more likely to have a pedunculated or polypoid growth pattern than SSAs (10,31). Genetically, *KRAS* mutations are more common in TSAs than in SSAs (10,18). These results suggest that SNs with the adenomatous pattern are more likely to be associated with the traditional serrated pathway.

The interpretation of the appearance of the mixed polyps should be considered. Serrated polyps rarely include two separate components. One component is usually nondysplastic (e.g., HP or SSA), whereas the second dysplastic component is either CAD or TSA. Sometimes, both components include different types of dysplasias (i.e., TSA/CAD). It has been speculated that mixed polyps represent either a collision tumor or that they are present during the progression phase of serrated tumorigenesis. Mäkinen *et al.* (32) have reported that a serrated adenomatous component adjacent to a serrated adenocarcinoma harbored areas of CAD in one-third of cases. We identified 15 MSPs in this series and found that the mixed pattern was detected more frequently in MSPs than in the other patterns (60 vs. 40%). Furthermore, the macroscopically mixed type (mixed-protruded and superficial type) was a specific feature of MSP. In contrast, 8 of the 15 MSPs were examined

successfully in detail regarding their molecular features. Although three TSA/HP lesions showed discordant molecular findings, the remaining five MSPs exhibited concordant molecular findings. Moreover, two SSA/TSAs and one TSA/CAD tumor, respectively, exhibited CIMP and *KRAS* mutations in both components. These clinicopathological and molecular findings support the hypothesis that MSPs are present in the progression phase of serrated tumorigenesis rather than in collision tumors.

Serrated adenocarcinoma has been recognized as a distinct entity among CRCs, accounting for 7.5% of these (19). A majority of serrated adenocarcinomas appear as MSS or MSI-L and originate from TSAs, whereas ~20% of serrated adenocarcinomas may derive from SSAs, frequently presenting MSI-H (19,33,34). Most MSI-H cancers, which are found in the proximal colon, exhibit CIMP and are frequently accompanied by methylation in the MLH1 promoter region. In this series, proximal SNs exhibited CIMP more often, whereas the frequency of MSI-H and MLH1 methylation was quite low. One explanation for this very low frequency of MLH1 methylation is the design of the primers. MLH1 methylation occurs more frequently in the upstream than downstream promoter region and an assay for the latter region revealed the presence of DNA methylation in the normal colon (35). However, our previous study using the same assay for MLH1 found that the frequency of MLH1 methylation was consistent with MSI-H and that no MLH1 methylation was detected in the mucosa of normal colon (27). Although the small number of SNs with high-grade dysplasia is a limitation of our study, we suggest that MLH promoter methylation followed by MSI is a late event in serrated tumorigenesis. This possibility is supported by the report of O'Brien *et al.* (36).

Recently, the Australia Cohort Study group reported an ethnic difference in the risk of *BRAF*/CIMP-related CRC between populations of southern European and Anglo-Celtic origins (37). Given that these cancers arise along the serrated pathway, it is possible that there are some ethnic differences in the clinicopathological or molecular changes of advanced serrated polyps. Previous studies indicate that Japanese patients with TSA are 5 years younger than Western patients with TSA (mean, 60 vs. 65 years of age, respectively) (38–40). In our study, the average age of the patients with TSAs was 58 years (**Supplementary Table S1** online). In contrast, serrated polyps and CADs share the same risk factors such as alcohol intake, smoking, high body mass index, and low fiber intake. Clarification of these issues might help determine the appropriate management and prevention strategies for SNs.

The correlation between the endoscopic crypt patterns and the clinicopathological or molecular features of SNs has important implications for the differential diagnosis of serrated polyps. As noted in the "Introduction" section, it is difficult to distinguish between SNs with the hyperplastic pattern and HPs. However, our data indicate that SNs with the hyperplastic pattern are more likely to have proximal predominance and a superficial-macroscopic appearance. In addition, SNs with the hyperplastic pattern showed a higher frequency of CIMP compared with the other patterns, which suggests that these SNs have a potential risk for precursor lesions of CIMP-positive CRCs. Thus, colonoscopists should consider the endoscopic removal of colon polyps >5 mm that exhibit a hyperplastic pattern, proximal location, and superficial-macroscopic type on colonoscopy. Regarding the clinical application of the mucosal crypt patterns of SNs, however, there is a need to identify more specific characteristics of SNs before proceeding to clinical trials on the differential diagnosis of serrated polyps.

In summary, our results indicate the presence of distinct clinicopathological and molecular characteristics among the three different mucosal crypt patterns of SNs. We suggest that the development of proximal and distal SNs occurs via the sessile and traditional serrated pathways, respectively.

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CONFLICT OF INTEREST

Guarantor of the article: Kazuo Konishi, MD, PhD.

Specific author contributions: K. Konishi, A. Katagiri, K. Kaneko, and N. Yoshikawa performed the clinical research; Y. Yano, H. Nozawa, H. Suzuki, M. Toyota, Y. Kubota, T. Muramoto, Y. Kobayashi, M. Tojo, K. Konda, and R. Makino performed the molecular research; T. Yamochi and H. Ota performed the pathological research; Y. Yano, K. Konishi, and M. Imawari wrote the paper.

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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Serrated neoplasias, such as serrated adenomas and sessile serrated adenomas, are implicated in colorectal carcinogenesis.
- ✓ Chromoendoscopic examination of serrated neoplasias shows several mucosal crypt patterns.

WHAT IS NEW HERE

- ✓ There were distinct clinicopathological and molecular characteristics among serrated neoplasias with different mucosal crypt patterns.
- ✓ This has important implications for endoscopic resection of colon polyps.

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Review

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Role of DNA Methylation in the Development of Diffuse-Type Gastric Cancer

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Key Words
Diffuse-type gastric cancer · *Helicobacter pylori* · Epstein-Barr virus · DNA methylation

Abstract
Cancer cells exhibit two opposing methylation abnormalities: genome-wide hypomethylation and gene promoter hypermethylation. Downregulation of E-cadherin (CDH1) plays a key role in the development of diffuse-type gastric cancer, and DNA methylation is a major cause of the gene's silencing. Hereditary diffuse gastric cancer is caused by germline mutation of CDH1 gene, and DNA methylation frequently serves as the second hit completely inactivating the gene. In sporadic diffuse-type gastric cancer, methylation of CDH1 is more prevalent than mutation of the gene. Epstein-Barr virus (EBV)-associated gastric carcinoma (EBV-associated GC) is characterized by concurrent methylation of multiple genes, and diffuse-type gastric cancer is frequently seen among EBV-associated GCs. Patients with pangastritis or enlarged-fold gastritis, which are both caused by *Helicobacter pylori* infection, reportedly have an increased risk for diffuse-type gastric cancer. Notably, the gastric mucosa of enlarged-fold gastritis patients exhibits CDH1 hypermethylation and genome-wide hypomethylation. These data suggest that aberrant DNA methylation is an essential promoter of carcinogenesis in individuals at high risk for diffuse-type gastric cancer.

Introduction

Gastric cancer is one of the most commonly occurring malignant neoplasms, worldwide, and remains a leading cause of cancer death in Asia and some European countries [1]. It is clear that the major etiologic risk factor for gastric cancer is *Helicobacter pylori* infection [2]; however, only a small proportion of individuals infected with *H. pylori* develop gastric cancer, and it is difficult for physicians to conduct proper early detection and prevention of the disease. Consequently, the development of appropriate biomarkers that reflect an individual's cancer risk is essential to reduce mortality from gastric cancer.

Gastric cancers are divided into two distinct histological groups, intestinal and diffuse [3]. Differences in the clinicopathological characteristics between these two types indicate that they develop via distinct molecular pathways. Intestinal-type cancers are histologically differentiated and are thought to be derived from gastric

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mucosa cells. It is believed that *H. pylori* infection plays a pivotal role in the development of intestinal-type gastric cancer, which arises from chronic gastritis, atrophy and intestinal metaplasia. Indeed, it is well known that patients with high-grade atrophic gastritis and intestinal metaplasia are at high risk of developing gastric cancer. On the other hand, the sequence of events via which histologically undifferentiated diffuse-type gastric cancers develop is poorly understood, though it is thought that a subset of diffuse-type gastric cancers develop independently of atrophic gastritis or intestinal metaplasia. In contrast to hereditary diffuse gastric cancer (HDGC), *H. pylori* and/or Epstein-Barr virus (EBV) infections reportedly play essential roles in the development of sporadic diffuse-type gastric cancers. In particular, patients with pangastritis and enlarged-fold gastritis, the cause of which is *H. pylori* infection, are reportedly at increased risk of developing diffuse-type gastric cancer [2, 4, 5].

Cancer is thought to arise through the accumulation of multiple genetic alterations, leading to activation of oncogenes and loss of function of tumor-suppressor genes. With respect to the latter, mutation of p53 gene is seen in approximately 40% of intestinal-type gastric cancers, but it is rare in diffuse-type gastric cancers [6, 7]. CDH1, which encodes E-cadherin, is frequently mutated in sporadic diffuse-type gastric cancers [8], and germline mutations of CDH1 are detected in a subset of HDGC patients [9]. In addition, activation of Wnt signaling through mutation of APC is a common feature of colorectal cancer, and APC mutation is also frequently seen in gastric adenoma. By contrast, APC mutation is not often seen in either intestinal- and diffuse-type gastric cancers [6, 7, 10]. With respect to oncogenes, activating mutation of CTNNB1, which encodes β-catenin, is seen in approximately 20% of intestinal-type gastric cancers [11], while the frequency of KRAS mutation is low in both histological types [10]. Taken together, these data suggest that, as compared to other cancer types (e.g. colorectal cancers), genetic mutations are relatively infrequent in gastric cancer [12].

A growing body of evidence now suggests that, in addition to genetic alterations, epigenetic changes, including DNA methylation and histone modification, also play important roles in the development and progression of human malignancies [13–16]. Epigenetics are inherited factors that influence gene activity but do not alter primary DNA sequences; among them, DNA methylation is a key event that silences gene expression. It has been hypothesized that DNA methylation initially evolved as a defense mechanism against viruses and other DNA pathogens. Under normal physiological conditions, DNA methylation plays a role in genome imprinting, X-chromosome inactivation and inactivation of repetitive sequences. In cancer, however, two contradicting epigenetic events coexist, namely global hypomethylation, which is mainly observed in repetitive sequences within the genome, and regional hypermethylation, which is frequently associated with CpG islands in gene promoters. Global hypomethylation is thought to be associated with proto-oncogene activation and chromosomal instability, whereas regional hypermethylation leads to inactivation of tumor-suppressor genes.

A number of studies provide evidence that both genetic and epigenetic alterations play critical roles in gastric tumorigenesis. For example, approximately 20% of intestinal-type gastric cancers show microsatellite instability that is closely associated with hypermethylation of MLH1 gene [17, 18]. A number of tumor-suppressor and tumor-related genes, including APC, CDH1 (E-cadherin), CHFR, DAPK, GSTP1, p16 and RUNX3, are known to be silenced by hypermethylation in gastric cancer [15, 16, 19]. Moreover, such methylation is frequently observed at premalignant stages of gastric cancer (e.g. with chronic gastritis and intestinal metaplasia), suggesting that aberrant methylation occurs early during the multistep process of gastric carcinogenesis [20–23]. Accumulation of aberrant methylation is thought to promote carcinogenesis through activation of common cancer pathways. For instance, although genetic mutation of APC or CTNNB1 is relatively infrequent in gastric cancer, a number of negative regulators of Wnt signaling, including SFRP1, SFRP2, DKK2, DKK3 and WIF1, are frequently methylated in gastric cancer [24–26]. In addition, methylation of RASSF family genes is thought to serve as an alternative to KRAS mutation in the signaling pathway leading to activation the Ras [27].

Mounting evidence suggests that diffuse-type gastric cancer is strongly associated with aberrant DNA methylation. A subset of cancers that exhibit concurrent hypermethylation of multiple genes is thought to represent a CpG island methylator phenotype (CIMP) [28]. In colorectal cancer, CIMP is strongly associated with MLH1 methylation and microsatellite instability [28]. In gastric cancer, however, CIMP is frequently observed in diffuse-type cancers in which MLH1 methylation and microsatellite instability are less frequent [29]. In this review, we will highlight the contribution made by DNA methylation to the development of diffuse-type gastric cancer, and its clinical application as a potential biomarker.

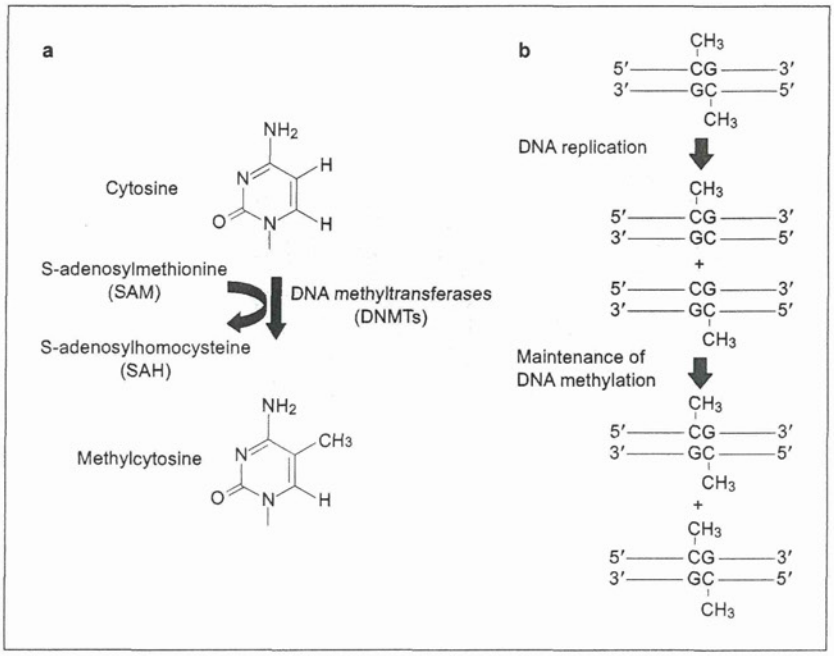

Fig. 1. a DNA methyltransferases (DNMTs) catalyze the methylation of cytosine using S-adenosylmethionine (SAM) as the methyl donor. **b** DNA methylation is maintained after DNA replication by a maintenance DNA methyltransferase, DNMT1.

Hereditary Diffuse Gastric Cancer

According to the International Gastric Cancer Linkage Consortium, HDGC is clinically characterized by either (1) two or more documented cases of diffuse gastric cancer in first- or second-degree relatives with at least one diagnosed before age 50 years, or (2) three or more cases of documented diffuse gastric cancer in first- or second-degree relatives, independent of the age of onset [30]. HDGC is an autosomal-dominant inherent cancer syndrome, and approximately 30% of individuals with a clinical diagnosis of HDGC harbor germline mutation of CDH1 [31, 32]. Among individuals with CDH1 germline mutations, the cumulative risk of advanced gastric cancer by age 80 years is 69% in men and 83% in women [33].

CDH1 is located on chromosome 16q22.1 and encodes E-cadherin, which is a transmembrane homodimeric protein that is central to calcium-dependent adhesion of epithelial cells. The mature protein is comprised of three major domains: a large extracellular domain and smaller transmembrane and cytoplasmic domains. The N-terminal ends of the large extracellular domains of the dimers interact with similar E-cadherin dimers on opposing cell surfaces, while the C-terminal ends of the cytoplasmic domains associate with the actin cytoskeleton via a complex that includes α-catenin, β-catenin and γ-catenin. And by competing with APC for binding to β-catenin, E-cadherin also modulates activity in the intracellular β-catenin signaling pathway [34]. Loss of E-cadherin is believed to lead to loss of cell adhesion, which would promote invasion and metastasis. Among all the reported CDH1 germline mutations, approximately 80% are predicted to generate truncated E-cadherin transcripts (nonsense, splice-site and frameshift mutations), while the remaining 20% are missense mutations [31, 35].

In addition to genetic mutation or allelic loss, epigenetic gene silencing associated with DNA methylation is now recognized as an alternative mechanism by which tumor-suppressor genes are inactivated. Within the mammalian genome, DNA methylation occurs only at cytosine bases located 5′ to a guanosine in a CpG dinucleotide (fig. 1). This dinucleotide is actually underrepresented in much of the genome, but short regions (>500 bp in length) known as CpG islands are rich in CpG dinucleotides [36]. The majority of CpG islands are found in the 5′ end regions of approximately half of the genes in the human genome, and are generally unmethylated in normal cells. By contrast, a number of tumor-suppressor and tumor-associated genes are hypermethylated and transcriptionally inactivated in cancer cells (fig. 2). CDH1 promoter methylation and intragenic deletions in the wild-type allele are frequently observed in HDGC and

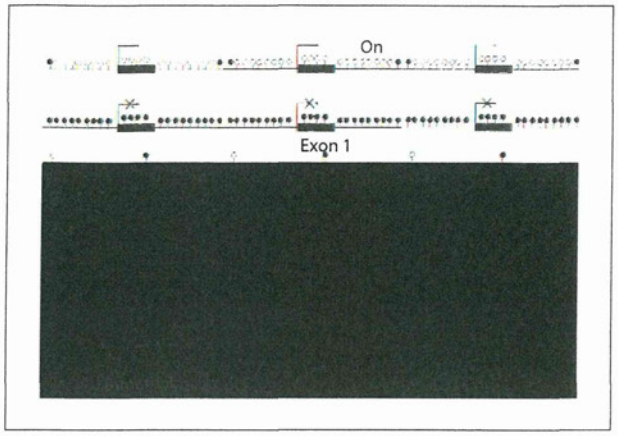

Fig. 2. Methylation of a CpG island within a gene promoter and transcriptional silencing. **a** In normal cells, most CpG islands are unmethylated, and gene transcription is active. **b** In cancer cells, the CpG islands of a number of genes are hypermethylated, which results in transcriptional silencing of the genes.

are thought to serve as a second hit that completely inactivates the gene [37, 38], although the specific mechanisms by which DNA methylation is altered in cancer remain unclear. Recently, the use of endogastric capsules has enabled successful detection of CDH1 methylation in DNA from the gastric juice of diffuse-type gastric cancer patients [39]. Because methylation is one of the major mechanisms involved in the second hit in CDH1 germline mutation carriers, assessing the methylation of CDH1 in gastric juice could be a non-invasive way of detecting individuals carrying mutations that put them at greater risk of developing HDGC.

Epstein-Barr Virus-Associated Gastric Carcinoma

EBV is a ubiquitous human herpes virus that was first identified in Burkitt's lymphoma cells. EBV is transmitted from host to host mainly via saliva, and has been established as a persistent infection of B-cells in over 90% of the world's adult population [40]. EBV does not usually replicate in B-cells, but instead establishes a latent infection characterized by limited expression of a subset of latent virus genes. Although the vast majority of EBV infections remain asymptomatic throughout one's entire life, a small portion of infected individuals develop tumors, and EBV has been implicated in the oncogenesis of lymphoproliferative diseases such as Burkitt's lymphoma and Hodgkin lymphoma. EBV has also been detected in certain epithelial tumors, including carcinomas of the nasopharynx and stomach.

EBV-associated gastric carcinoma (EBV-associated GC) is defined by the presence of EBV within tumor cells [41–43]. EBV, or its small non-coding RNA (EBER1 and EBER2), has been identified in nearly all neoplastic cells in EBV-associated GC tissue samples. Burke et al. [44] first reported detecting the EBV genome in lymphoepithelioma-like carcinoma, which accounts for approximately 1% of gastric cancers. Lymphoepithelioma-like carcinomas are almost always EBV-positive, and are characterized as a poorly differentiated carcinoma with dense infiltration of lymphocytes. EBV is also detected in about 5–10% of ordinary types of gastric cancer. EBV-associated GCs are observed among all types of gastric adenocarcinoma, but they are slightly more common in moderately differentiated tubular types and poorly differentiated solid types. EBV-associated GCs are also more likely in males than females, and are less likely to be found in the gastric antrum than in the cardia or body.

Evidence suggests that epigenetic abnormalities play pivotal roles in the development of EBV-associated GC. Although p53 mutations are found in about 30–40% of gastric cancers, p53 is mutated in less than 10% of EBV-associated GCs [42, 43]. In contrast, hypermethylation of tumor-suppressor genes such as CDH1, p14, p15 and p16 is frequently observed in EBV-associated GC [43, 45]. In particular, simultaneous hypermethylation of the promoters of multiple genes (i.e. CIMP) is a characteristic abnormality in EBV-associated GC [29, 46–48]. CIMP was originally defined in the context of gastric cancer using methylation of five CIMP marker loci (MINT1, 2, 12, 25 and 31) identified by Toyota et al. [49]. Tumors showing methylation of 4 or 5 of the markers were defined as CIMP-high (CIMP-H), while those with 1–3 markers were CIMP-low (CIMP-L), and those with no methylation were CIMP-negative (CIMP-N) [29]. To further characterize CIMP in gastric cancer, we assessed the methylation status of 12 other genes (BNIP3, CHFR, CSPG2, p16, HLTF, HRK, PAX5β, SLC5A8, p57, MLH1, SOCS-3, TIG1) and compared it with the tumors' CIMP status [29]. We found that 24% of gastric cancers are CIMP-H, as are all EBV-positive cancers, which accounts for approximately half of the CIMP-H tumors. As compared to the CIMP-L/CIMP-N group, CIMP-H tumors show frequent methylation of the aforementioned 12 genes, and are positively associated

with upper stomach localization and diffuse-type of histology. And when Chang et al. [47] used five different genes (LOX, HRASLS, FLNc, HAND1 and TM) as CIMP markers and defined CIMP using the same criteria as Toyota et al. [49] – i.e., tumors with >4 markers were defined as CIMP-H, those with 1–3 marker were CIMP-intermediate (CIMP-I), and those without methylation were CIMP-N – they too found that 24% of gastric cancers are CIMP-H, as are nearly all cases of EBV-associated GC.

Interestingly, EBV-negative CIMP-H gastric cancers are strongly associated with MLH1 methylation and microsatellite instability, whereas EBV-positive CIMP-H gastric cancers are strongly associated with diffuse-type of histology and rarely show MLH1 methylation [29, 46–48]. It thus appears that EBV-positive and -negative cancers represent distinct subclasses among CIMP-H gastric cancers. Given that DNA methylation is utilized as a host defense mechanism to suppress the expression of viral genes, it is plausible that EBV may activate a methylation pathway that affects multiple genes during gastric carcinogenesis, although the molecular mechanism underlying EBV-associated aberrant methylation is currently unknown.

Synchronous multicentric cancers are frequently reported in EBV-associated GC patients, suggesting that, with EBV infection, the gastric mucosa is at high risk of carcinogenesis. A marked degree of atrophy and a moderate to marked degree of lymphocytic infiltration were observed in the mucosa surrounding EBV-associated GC, but not EBV-negative GC. It is plausible that *H. pylori* infection is associated with the atrophy and inflammation in the gastric mucosa surrounding EBV-associated GC, although it is still controversial whether the rate of *H. pylori* infection is higher among EBV-positive individuals than among those that are not infected. In situ hybridization analysis revealed that EBERs are rarely expressed in non-neoplastic epithelia adjacent to gastric cancers, but are present in a small portion of infiltrating lymphocytes [50, 51]. In addition, p14, p16 and p73 are commonly methylated in EBV-associated GC, whereas methylation was less frequently detected in surrounding non-neoplastic mucosa [52]. To date, there have been no studies examining CDH1 methylation in the gastric mucosa surrounding EBV-associated GC. Further analysis of aberrant DNA methylation in both EBV-associated GC and the adjacent gastric mucosa may lead to identification of new molecular markers for risk prediction and early diagnosis.

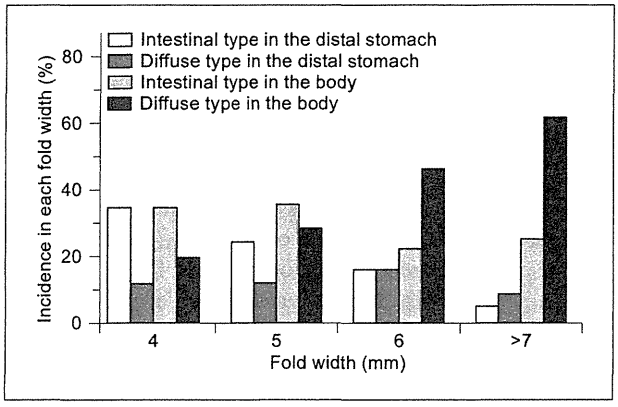

Fig. 3. Association between fold width, histological type and location of gastric cancers. The results are derived from Nishibayashi et al. [5].

H. pylori-Induced Gastritis and Diffuse-Type Gastric Cancer

In gastric cancers, epigenetic alteration of tumor-related genes reportedly occurs much more frequently than genetic alteration. The frequencies of APC, KRAS and p53 mutations, which play important roles in colorectal multistep carcinogenesis, are especially low in diffuse-type gastric cancer [12]. Although CDH1 mutations are detected in approximately half of sporadic diffuse-type gastric cancers [53, 54], they are rarely found in early diffuse-type gastric cancers and are generally associated with the progression from differentiated to undifferentiated cancer [7, 19]. By contrast, hypermethylation of CDH1 is observed in more than 50% of early diffuse-type gastric cancers [7, 12, 19]. Hypermethylation of gene promoters is strongly associated with age and chronic inflammation [55, 56]. In the stomach, methylation of tumor-suppressor or tumor-related genes, including CDH1, increases with age [55, 57]. In addition, a number of studies have reported the presence of gene promoter methylation in the non-neoplastic gastric mucosa of *H. pylori*-infected individuals [58–62]. As higher methylation levels in the gastric mucosa are associated with an increased risk of gastric cancer [61–63], *H. pylori* infection is thought to promote stomach carcinogenesis through induction of aberrant DNA methylation.

H. pylori-related active gastritis has been divided into three groups, antrum-predominant gastritis, pangastritis and corpus-predominant gastritis. Patients with pangastritis are reportedly at high risk of diffuse-type gastric

cancer [2]. It is also well known that nodularity in the gastric antrum and enlarged fold in the gastric body initiated by *H. pylori* infection are indicators of a high risk of diffuse-type gastric cancer [2, 4, 5]. Moreover, the prevalence of diffuse-type gastric cancer in the gastric body region increases with increasing fold width (fig. 3). Taken together, these findings suggest that enlarged-fold gastritis puts one at high risk of developing diffuse-type gastric cancer. Further study of aberrant DNA methylation in pangastritis, nodular gastritis, enlarged-fold gastritis and in the non-cancerous gastric mucosa of diffuse-type gastric cancer patients should help to clarify the pathogenesis of diffuse-type gastric cancer and lead to identification of molecular markers to predict cancer risk.

Aberrant DNA Methylation in *H. pylori*-Related Enlarged-Fold Gastritis

Enlarged gastric folds are associated with a variety of diseases, including hypertrophic gastritis, Ménétrier disease, Zollinger-Ellison syndrome, primary gastrin cell hyperplasia, gastric cancer and lymphoma. The accumulated evidence suggests *H. pylori*-induced gastritis is also a possible cause of enlarged gastric folds [64–66]. *H. pylori* reportedly causes the enlarged-fold gastritis (fold width >5 mm) that accompanies foveolar hyperplasia, massive infiltration of inflammatory cells, and increased production of interleukin-1β (IL-1β) and hepatocyte growth factor (HGF) in the corpus mucosa. The prevalence of enlarged-fold gastritis is higher in middle-aged (40- to 59-year-old) males than in men in other age groups or in females. It has also been reported that the prevalence of diffuse-type gastric cancer in the gastric body region increases with increasing fold width, suggesting enlarged-fold gastritis is a major risk factor for diffuse-type gastric cancer. In addition, the mutagenicity of gastric juice and mucosal levels of 8-hydroxydeoxyguanidine, an indicator of reactive oxygen species-induced DNA damage, in the body regions of the stomach in patients with enlarged-fold gastritis were significantly higher than in either *H. pylori*-negative controls or *H. pylori*-positive patients without enlarged folds [5]. Increased production of IL-1β and HGF, increased serum gastrin levels, and decreased acid secretion are all associated with enlarged-fold gastritis and are thought to promote gastric tumorigenesis [64, 67].

Aberrant DNA methylation is frequently observed in enlarged-fold gastritis. CDH1 methylation is detected in almost all cases of *H. pylori*-induced enlarged-fold gastritis, and quantitative methylation analysis revealed that levels of CDH1 methylation are much higher in enlarged-fold gastritis than in *H. pylori*-positive gastritis without enlarged folds [68, 69]. Detailed methylation analysis using bisulfite sequencing revealed that CDH1 is densely methylated in enlarged-fold gastritis [69], and that expression of E-cadherin is downregulated in the gastric mucosa of enlarged-fold gastritis [68]. Furthermore, a significant reduction in the level of CDH1 methylation is seen after *H. pylori* eradication [68]. These results strongly suggest that hypermethylation of CDH1 is a major contributor to the development of diffuse-type gastric cancer. It has also been reported that treatment of the MKN-1 gastric cancer cell line with transforming growth factor-α, epidermal growth factor (EGF) or reactive oxygen species induces CDH1 methylation, and that EGF treatment upregulated DNA methyltransferase activity [68]. Thus, inflammatory cytokines, growth factors and reactive oxygen species induced by *H. pylori* infection are likely involved in CDH1 methylation. This suggests CDH1 methylation could be a molecular marker predicting the development of diffuse-type gastric cancer risk.

More than 40% of the human genome is composed of repetitive sequences, including long interspersed nuclear element (LINE) and short interspersed nuclear element, and the methylation level of the former has been used as a surrogate for global methylation levels [70]. LINE-1 hypomethylation is known to occur during the development of various human malignancies, and it is reportedly associated with tumor malignancy and a poor prognosis [71–75]. One recent study revealed that levels of LINE-1 methylation are significantly reduced in the mucosa from patients with enlarged-fold gastritis [69]. The role of hypomethylation in tumorigenesis is not fully understood, but it is thought to induce activation of proto-oncogenes, endogenous retroviruses or transposable elements, as well as chromosomal instability. A link between LINE-1 hypomethylation and gene promoter hypermethylation (CDH1, CDH13 and PGP9.5) was also found in enlarged-fold gastritis [69]. CDH13 encodes H-cadherin, which is involved in suppressing cell growth, invasion and metastasis, is frequently methylated in gastric cancer [76]. In addition, PGP9.5 was identified in a screening for epigenetically silenced genes in diffuse-type gastric cancer, and it has been shown to serve as a tumor suppressor in gastric cancer [77]. Thus, gene hypermethylation and global hypomethylation both appear to be forces driving the development of diffuse-type cancer in enlarged-fold gastritis (fig. 4). Further analysis of aberrant DNA meth-

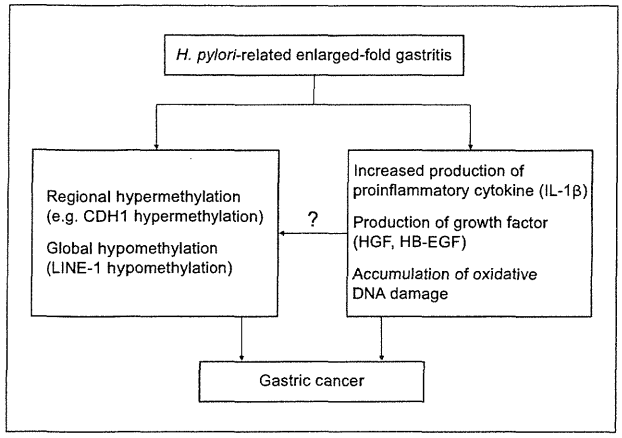

Fig. 4. Hypothesis for the progression of gastric cancer in *H. pylori*-induced enlarged-fold gastritis.

ylation in enlarged-fold gastritis should shed light on the molecular mechanisms underlying gastric tumorigenesis.

In patients with enlarged-fold gastritis, inflammatory infiltrates, cytokine release, foveolar thickness and fold width are all significantly reduced after eradication of *H. pylori* [66]. Eradication of *H. pylori* also restores acid secretion and decreases serum gastrin concentrations [64]. Reduction of CDH1 methylation after *H. pylori* eradication in chronic gastritis has also been reported [78–80]. It is thus possible that eradication of *H. pylori* reduces the risk of diffuse-type gastric cancer; however, eradication does not completely restore normal DNA methylation, and individuals with sustained alteration of DNA methylation are thought to be at higher risk, even after *H. pylori* eradication. Evaluation of DNA methylation after eradication also may serve as a useful diagnostic tool for predicting cancer development, although further study is needed to clarify the relationship between residual methylation and cancer risk after eradication.

Prospects

From the available evidence, it is clear that both genetic and epigenetic alterations play an important role in the development of diffuse-type gastric cancer. *H. pylori* infection induces aberrant DNA methylation of multiple genes, including CDH1, in gastric mucosa and enlarged-fold gastritis, which puts the patient at high risk for diffuse-type gastric cancer. In addition, global hypomethylation is also commonly observed in enlarged-fold gastritis. Further study of DNA methylation in high-risk individuals should not only clarify the mechanism underlying gastric carcinogenesis, but may also lead to the development of new molecular markers for risk prediction and early detection of gastric cancer.

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Aberrant DNA methylation associated with aggressiveness of gastrointestinal stromal tumour

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ABSTRACT

Background and aims The majority of gastrointestinal stromal tumors (GISTs) have *KIT* mutations; however, epigenetic abnormalities that could conceivably potentiate the aggressiveness of GISTs are largely unidentified. Our aim was to establish epigenetic profiles associated with the malignant transformation of GISTs.

Methods Methylation of four tumor suppressor genes, *RASSF1A, p16, CDH1, and MGMT* was analyzed in GISTs. Additionally, genome-wide DNA methylation profiles were compared between small, malignant-prone, and malignant GISTs using methylated GpG island amplification microarrays (MCAM) in a training set (n=40). Relationships between the methylation status of genes identified by MCAM and clinical features of the disease were tested in a validation set (n=75).

Results Methylation of *RASSF1A* progressively increased from small to malignant GISTs. *p16* was specifically methylated in malignant-prone and malignant GISTs. MCAM analysis showed that more genes were methylated in advanced than in small GISTs (average of 473 genes vs 360 genes, respectively, $P=0.012$). Interestingly, the methylation profile of malignant GISTs was prominently affected by their location. Two genes, *REC8* and *PAX3*, which were newly-identified via MCAM analysis, were differentially methylated in small and malignant GISTs in the training and validation sets. Patients with methylation of at least *REC8*, *PAX3*, or *p16* had a significantly poorer prognosis ($P=0.034$).

Conclusion Our results suggest that GIST is not, in epigenetic terms, a uniform disease and that DNA methylation in a set of genes is associated with aggressive clinical behavior and unfavorable prognosis. The genes identified may potentially serve as biomarkers for predicting aggressive GISTs with poor survivability.

Significance of this study

What is already known about this subject?

- Approximately 90% of gastrointestinal stromal tumours (GIST) have activating mutations in the *KIT* gene (~85%) or *platelet-derived growth factor receptor alpha* gene (~5%), which were detected even in small GIST (<10 mm), suggesting that these mutations are early initiation steps in GIST formation.
- Although two key prognostic features (tumour size and mitotic index) of primary GIST appear to be of use for risk stratification, a persistent problem is that a subset of cases classified as low risk nonetheless occasionally metastasize.
- Location may also be considered as a factor for the risk assessment of GIST, because a small intestinal GIST is more aggressive than a gastric GIST of equal size.
- Elucidating the underlying mechanism that potentiates the aggressiveness of GIST is key to facilitating the development of a new strategy for the treatment of malignant GIST.

What are the new findings?

- Genome-wide DNA methylation analysis revealed that more genes were methylated in advanced GIST than small GIST, suggesting a link between the accumulation of DNA methylation and disease progression.
- Among the hypermethylated genes, we identified potent methylation markers, *REC8*, *PAX3* and *p16*, of which DNA methylation status is significantly correlated with a worse prognosis of GIST.
- The DNA methylation profile of malignant GIST was prominently affected by its location in the gastrointestinal system; gastric and small-intestinal GIST displayed distinctive DNA methylation profiles, supporting the idea that the anatomical location of GIST affects the clinical significance of the respective disease.

Gastrointestinal stromal tumours (GIST) are the most common mesenchymal tumours of the digestive tract. Risk assessment at initial diagnosis is recognised as quite important, because a subset of GIST relapses after surgery.[1 2] Two key prognostic features of a primary GIST are its size and mitotic index.[3] These two features appeared to be practically useful for a consensus approach to risk stratification (low, intermediate, or high) of GIST.[4] However, a persistent problem is that a subset of cases exist that, having low mitotic rates and being classified as low risk, occasionally metastasise to or invade other organs. In addition to these two prognostic features, location may be considered as a factor for the risk assessment of a primary tumour, because a small intestinal GIST is more aggressive than a gastric GIST of equal size.[1]

Significance of this study

How might it impact on clinical practice in the foreseeable future?

- Our findings provide a better understanding of the role of aberrant DNA methylation in GIST, and may provide new molecular diagnostic tools for this disease subtype.
- Examination of three methylation markers could be informative for diagnostic and prognostic assessment of GIST and could be elucidated using samples obtained from minimally invasive procedures, such as biopsy specimens obtained by the endoscopic ultrasound-guided fine-needle aspiration technique.

GIST are known to originate from interstitial cells of Cajal (ICC) or their precursors. It has been suggested that the acquisition of *KIT* and platelet-derived growth factor receptor alpha (*PDGFRA*) mutations in ICC is a possible early initiation step in GIST tumorigenesis.[3 5 6] In addition to *KIT* and *PDGFRA* mutations, the majority of GIST also display other genetic abnormalities as they progress to a malignant phenotype.[7] One particular study demonstrated that other genetic abnormalities, such as losses of 13q, 10q and 22q, contribute to the progression and malignant transformation of GIST.[8] Inactivation of the cell cycle regulators p16 and/or p27 has also been shown to correlate with malignancy in GIST.[9 10] However, these genetic abnormalities do not completely explain the underlying mechanisms that potentiate the aggressiveness of this tumour type.

Aberrant DNA methylation has been observed in many human malignancies, sometimes correlated with potentiated aggressiveness of the tumour.[11 12] As DNA methylation of particular genes is known to be associated with patient outcome, the detection of aberrant DNA methylation in clinical specimens could be a useful biomarker for malignancies.[13 14] Recent advances in technologies for high-throughput genome-wide DNA methylation analyses have facilitated epigenetic profiling of human malignancies.[15] To investigate epigenetic events in GIST, we performed genome-wide screening for genes with aberrant DNA methylation using a methylated CpG island amplification microarray (MCAM) approach, which provides reproducible results with a high validation rate.[16–18] We further evaluated correlations between DNA methylation status and clinicopathological features of GIST including gene mutations.

MATERIALS AND METHODS

Tissue samples

We collected 115 GIST samples in accordance with institutional policy from patients who underwent surgical resection at the Aichi Cancer Center Central Hospital, Nagoya, at the Osaka University Hospital, Osaka, at the Osaka Police Hospital, Osaka, or at Sapporo Medical University Hospital, Sapporo in Japan. Specimens showing a high proportion of tumour cells (>80%) without definite evidence of necrosis were analysed. We divided GIST samples into two sets, a training set (n=40) and a validation set (n=75) without any bias (table 1). GIST were classified into three groups: small GIST (n=33), malignant-prone GIST (n=49), and malignant GIST (n=33), based on the modification of 2002 consensus criteria (low, intermediate and high-risk groups; table 1).[3] Small GIST refers to small tumours (≤5 cm) with low mitotic frequency (≤5 mitosis per 50 high-power fields) and without any evidence of metastasis or invasion (low-risk group). Malignant-prone GIST refers to larger tumours (>5 cm) or those with more than five mitoses per 50 high-power fields and without any evidence of metastasis or invasion (intermediate and high-risk groups). Malignant GIST refers to tumours from patients with a history of metastasis or invasion. Metastatic disease has been diagnosed using imaging studies and clinical analysis when primary tumours were surgically treated. Patients in the training set and in the validation set had similar features, except more patients in the validation cohort were classified as having malignant-prone GIST (table 1). We also collected 18 muscle layers from six stomachs, six small intestines and six colons. Samples and clinical data were collected after the approval of the institutional review board of Aichi Cancer Center was received and written informed consent had been obtained from all patients.

Microscopic dissection of ICC

ICC were identified as c-Kit-positive cells among the intestinal cells. A higher density of ICC is observed at the level of the myenteric plexus between circular and longitudinal muscle layers in the small intestine.[19] To obtain ICC as a normal counterpart of GIST cells, we prepared serial sections (10 μm thick), one of which was stained with anti-c-Kit (A4502; DAKO, Glostrup, Denmark) as an indicator, and we carefully dissected an unstained section under stereoscopic microscope observation (see supplementary figure 1, available online only).

Bisulfite-pyrosequencing for DNA methylation analysis

We performed bisulfite treatment on genomic DNA as previously described.[18] DNA methylation levels were measured by

Table 1 Clinical features of the GIST in this study

Feature	Total (n=115) No of patients (%)	Training set (n=40) No of patients (%)	Validation set (n=75) No of patients (%)	p Value
Sex				
Male	62 (54)	19 (48)	43 (57)	0.314
Female	53 (46)	21 (52)	32 (43)	
Age, years				
<60	41 (35)	19 (48)	22 (29)	0.067
≥60	74 (65)	21 (52)	53 (71)	
Tumour origin				
Stomach	66 (57)	23 (57)	43 (57)	0.801
Small intestine	40 (35)	15 (38)	25 (33)	
Rectum	8 (7)	2 (5)	6 (8)	
Oesophagus	1 (1)	0 (0)	1 (2)	
Size, cm*				
<5	46 (41)	15 (40)	31 (41)	0.216
≥5	67 (59)	23 (60)	44 (59)	
Mitotic index/50 HPF*				
<5	60 (59)	18 (56)	42 (60)	0.139
≥5	42 (41)	14 (44)	28 (40)	
Classification				
Small				
Low risk†	33 (29)	9 (23)	24 (32)	0.006
Malignant-prone				
Intermediate risk†	21 (18)	9 (23)	12 (16)	
High risk†	28 (24)	4 (10)	24 (32)	
Malignant				
Metastasis	33 (29)	18 (44)	15 (20)	

*Clinical data of some patients were unavailable.
†Low risk, size less than 5 cm and mitotic index less than 5/50 high-power fields (HPF); intermediate risk, size less than 5 cm and mitotic index 5–10/50 HPF, or size 5–10 cm and mitotic index less than 5/50 HPF; high risk, size 5–10 cm and mitotic index 5–10/50HPF, or size 10 cm or greater or mitotic index 10/50 HPF or greater.
GIST, gastrointestinal stromal tumour.