SPANDIDOS PUBLICATIONS

Expression of Fhit, Mlh1, p16^{INK4A} and E-cadherin in early gastric neoplasia: Correlation with histological grade and gastric phenotype

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Abstract. An increasing number of tumor suppressor genes (TSGs) that are inactivated by hypermethylation of CpG islands in the promoter have been reported in gastric carcinomas. The aim of this study is to evaluate the clinical significance of TSG protein expression, which correlates with the promoter status, methylated or not, during the early stages of gastric carcinogenesis and to examine its relationship with mucin phenotype. The protein expression of 4 TSGs including Fhit, Mlh1, p16^{INK4A} and E-cadherin was examined using immunohistochemical methods in 103 early gastric neoplasias, comprising 41 adenomas and 62 intramucosal carcinomas, obtained by endoscopic mucosal resection. In addition, phenotypic expression patterns (gastric-, intestinal- and mixed-phenotypes) were also examined. The expression of Fhit, Mlh1, p16 and E-cadherin was lost or reduced in 7.3, 12.2, 12.2 and 9.8% of the adenomas and in 35.5, 29.0, 29.0 and 32.3% of the intramucosal carcinomas, respectively. The absent expression of p16 was significantly associated with the degree of dysplasia in the adenomas (p=0.038). The average number of proteins among the 4 TSGs, whose expression was lost or reduced per sample, was significantly higher in the intramucosal carcinomas (1.35) than in the adenomas (0.41) (p=0.00013). Similarly, the average number was significantly higher in the gastric-type tumors (2.05) than in the intestinal-type tumors (0.49) (p=0.0000019). We demonstrated an increase in the number of TSG proteins whose expression is reduced or lost in the early stages of gastric tumorigenesis, and that this increase is associated with histological grade and gastric phenotype.

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Abbreviations: FHIT, fragile histidine triad; TSG, tumor suppressor gene

Key words: gastric neoplasia, mucin phenotype, Fhit, Mlh1, p16, E-cadherin

Introduction

A variety of genetic and epigenetic alterations are associated with gastric carcinoma (1,2). Aberrant methylation of promoter CpG islands, a well-known epigenetic change, is now recognized as an important mechanism for gene inactivation as an alternative to gene mutation or deletion in tumorigenesis (3-5).

Tumor suppressor genes (TSGs) have been identified by demonstrating a close link between the methylation of CpG islands of a specific gene and loss of mRNA or protein. An increasing number of TSGs that are inactivated by the hypermethylation of CpG islands have been reported in gastric cancer, but their role in early neoplastic lesions is not well understood. On the other hand, gastric adenomas might be considered to be precancerous lesions, but are clinically heterogeneous, since some may develop into adenocarcinomas, whereas others persist unchanged for long periods. Therefore, identifying adenomas with a progressive nature is important, since intervention (e.g., endoscopic mucosal resection) is mandatory (6,7).

In gastric carcinomas, *p16^{INK4A}*, *hMLH1* and *E-cadherin* have been demonstrated to be inactivated through hypermethylation of their promoters (8-14). These genes could be inactivated by a combination of genetic or epigenetic alterations of two alleles. However, epigenetic change seems to be the predominant mechanism (major pathway) associated with the loss of *p16*, *hMLH1* and *E-cadherin* function in sporadic gastric carcinomas (8-14).

A candidate tumor suppressor gene, fragile histidine triad (*FHIT*), was identified at chromosome 3p14.2 spanning the FRA3B common fragile site (15). Alterations of the *FHIT* gene and its expression have been reported not only in gastric cancer but also in premalignant lesions (16,17). Aberrant methylation of the 5'CpG island of *FHIT* has been reported to be closely associated with transcriptional inactivation in lung, breast, bladder, cervical and esophageal carcinomas,

and methylation of the *FHIT* promotor methylation is now considered a major cause of loss of Fhit expression (18-20).

Gastric carcinomas have been divided into two histological types: intestinal and diffuse, according to Lauren (21), or differentiated and undifferentiated, according to Nakamura *et al* (22). However, mucin-based histochemical and immunohistochemical examinations have recently demonstrated that gastric and intestinal phenotypic cell markers are widely expressed in gastric carcinomas, irrespective of histological type (23-25). Moreover, it has generally been reported that gastric carcinomas with a predominantly gastric phenotype have a pronounced tendency toward invasion, metastasis and poor prognosis compared with gastric carcinomas that have intestinal phenotypic expression (24,26-29). Therefore, a phenotypic subclassification would be useful in understanding the biologic behavior of carcinomas and selecting a suitable therapeutic method.

We evaluated the clinical significance of TSG protein expression, which correlates with the status, methylated or not, of the promoter, during the early stages of gastric carcinogenesis. The protein expression of 4 TSGs (Fhit, Mlh1, p16 and E-cadherin), was examined immunohistochemically in 103 early gastric neoplasias, comprising 41 adenomas and 62 intramucosal carcinomas, removed by endoscopic mucosal resection. In addition, we also compared TSG protein expression with the phenotypic expression.

Table I. Clinicopathological features in early gastric neoplasias

	Adenoma (n=41)		Intramucosal carcinoma (n=62)	
Gender (M/F)	17/24		44/18	
Age (mean ± SD; years)	72.2±6.6		71.2±8.0	
Histologic type (Grade)	Mild	1	Tub1	56
	Moderate	26	Tub2	5
	Severe	14	Pap	1
Location				
Upper	4		7	
Middle	20		19	
Lower	17		36	
Gross classification				
Elevated	41		52	
Flat or depressed	0		10	

Tub1, well-differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma; Pap, papillary adenocarcinoma.

Materials and methods

Patient and tissue samples. Tumor specimens were obtained from 103 patients (61 males and 42 females) who had undergone endoscopic mucosal resection at Tottori University Hospital between 1994 and 2002. Based on a histological examination, the 103 tumors were classified as 41 adenomas and 62 intramucosal carcinomas (Table I). Macroscopic and histological evaluations were made according to the classification established by the Japanese Research Society for Gastric Cancer (1993) (30). The macroscopic features were divided into two major types: elevated, and flat or depressed. The depth of invasion and histological grade were classified according to the predominant features. In this study, adenoma samples correspond to low or high grade adenoma/dysplasia, and carcinoma samples correspond to non-invasive carcinoma or intramucosal carcinoma in the Vienna classification system (31). All intramucosal carcinomas were histologically differentiated carcinomas. Pathological diagnoses were verified by two experienced pathologists (H.A. and H.I.). All the cases were analyzed anonymously, i.e., all the specimens were assigned a new number without any personal information. Institutional Review Board approval was obtained.

Immunohistochemical staining. Paraffin-embedded, 4-μm sections were immunohistochemically stained with anti-Fhit rabbit polyclonal antibody (IBL, Gunma, Japan; dilution 1:100), anti-Mlh1 mouse monoclonal antibody (G168-15, PharMingen, San Diego, CA, USA; dilution 1:50), $p16^{INK4A}$ anti-mouse monoclonal antibody (16P07, NeoMarkers, Inc., Westinghouse, CA, USA; dilution 1:50), E-cadherin anti-mouse monoclonal antibody (HECD-1, Takara, Bio Inc., Shiga, Japan; dilution 1:50), anti-human gastric mucin (HGM) mouse monoclonal antibody (45M1, Novocastra Laboratories, Ltd., Newcastle, UK; dilution 1:50), anti-MUC2 mouse monoclonal antibody (Ccp58, Novocastra, Newcastle, UK; dilution 1:100) and anti-CD10 mouse monoclonal antibody (56C6, Novocastra, Newcastle, UK; dilution 1:50) using the avidin-biotin-peroxidase complex technique.

Immunohistochemical staining was performed as described below. In brief, after deparaffinizing in xylene and rehydrating in ethanol, the sections were immersed in a citrate buffer (0.01 M, pH 6.0) and heated in a microwave oven for 20-30 min to retrieve antigens. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2, then incubation with the primary antibody was performed overnight at 4˚C. As a negative control, the primary antibody was replaced with normal serum IgG at a similar dilution. The detection reaction followed the protocol of Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen, and haematoxylin was used as a counterstain. The sections were incubated with biotinylated anti-rabbit or mouse IgG and avidin-biotin-peroxidase and visualized using diaminobenzidine tetrahydrochloride.

The protein expression was evaluated by two independent observers (H.A. and K.Y.). Immunohistochemical analysis was performed in a blinded manner with respect to the clinical information.

Assessment of Fhit, Mlh1, $p16^{INK4A}$ and E-cadherin immunostaining (Fig. 1). The expression of Fhit was graded for both the extent and intensity of immunopositivity as described previously (32). The extent of positivity was scored as follows:

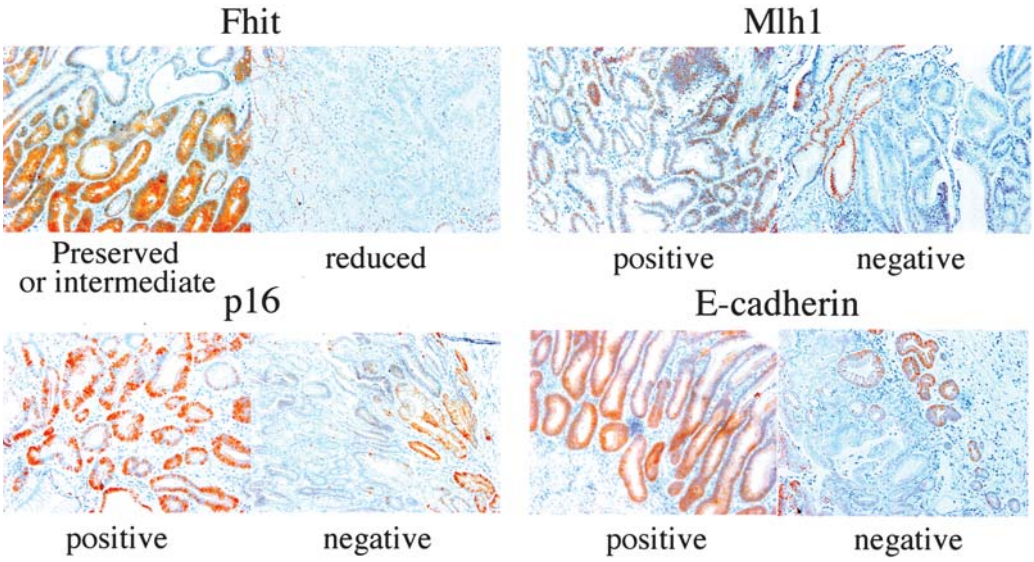

Figure 1. Immunohistochemical staining of Fhit, Mlh1, p16 and E-cadherin protein in early gastric neoplasias. Fhit, Mlh1, p16 and E-cadherin immunostaining in human gastric non-neoplastic and neoplastic tissues. Tumor cells show positive immunostaining (left) and reduced or negative immunostaining (right). Negative immunostaining of an intramucosal carcinoma and positive immunostaining of an adenomatous and non-neoplastic epithelium.

0, <5%; 1, 5-25%; 2, 25-50%; 3, 50-75%; and 4, >75% of the gastric epithelial cells in the respective lesions. The intensity was scored as follows: 0, negative; 1+, weak; 2+, moderate; and 3+, as strong as normal mucosa. The final score was obtained by multiplying the extent of positivity and intensity scores, producing a range from 0 to 12. Scores 9-12 were defined as a preserved or strong staining pattern, scores 5-8 were defined as an intermediate staining pattern, and scores 0-4 were defined as markedly reduced or lost expression. The evaluation of Mlh1 and p16 expression was classified as normal or decreased. Cases with definite nuclear staining in <30% of the tumor cells were categorized as decreased. In the evaluation of E-cadherin, cases with definite membrane staining in >30% of the tumor cells were categorized as normal, while those with such staining in <30% of the tumor cells or with a complete absence of membrane staining were categorized as decreased.

Assessment of HGM, MUC2 and CD10 immunostaining and classification of the phenotypes. HGM staining was seen in the cytoplasm of the gastric foveolar epithelium and mucous neck cells. MUC2 staining was seen in the cytoplasm around the nuclei of the goblet cells. CD10 staining was seen along the brush border of the luminal surface of the epithelium. Although CD10 can also be expressed in the apical portion of the cytoplasm of normal gastric mucosa, only the expression of CD10 on the brush border was studied. The results of staining were categorized into two groups: positive expression and negative expression. Staining of >10% of the adenoma and carcinoma cells was classified as positive, and <10%, as negative. The phenotypes were classified into four categories according to the combination of the expression of CD10, MUC2 and HGM (24,28). The intestinal phenotype (I-type) was positive for MUC2 and/or CD10, but negative for HGM. The gastric and intestinal mixed phenotype (GI-type) was positive for MUC2 and/or CD10, and positive for HGM. The gastric phenotype (G-type) was positive for HGM, but negative for both MUC2 and CD10. The unclassified phenotype (UC-type) was negative for MUC2, CD10 and HGM.

Table II. TSG (Fhit, Mlh1, p16 and E-cadherin) expression in early gastric neoplasias.

Absent or reduced expression	Adenoma (n=41) (%)	Intramucosal carcinoma (n=62) (%)	P-value
Fhit	3 (7.3)	22 (35.5)	0.0000029
Mlh1	5 (12.2)	18 (29.0)	0.07
p16	5 (12.2)	18 (29.0)	0.07
E-cadherin	4 (9.8)	20 (32.3)	0.0003

Rate of absent or reduced Fhit and E-cadherin expression was significantly higher in the intramucosal carcinomas than in the adenomas, p=0.0000029 and p=0.0003, respectively.

Statistical analysis. The statistical analysis was performed with the χ^2 test, Fisher's test, and the Mann-Whitney test (U-test). P<0.05 was considered significant.

Results

The 4 TSGs (Fhit, Mlh1, p16^{INK4A} and E-cadherin) expressions in early gastric neoplasias. The expression of Fhit, Mlh1, p16 and E-cadherin was either lost or reduced in 3 (7.3%), 5 (12.2%), 5 (12.5%) and 4 (9.8%) of the 41 adenomas and in 22 (35.5%), 18 (29.0%), 18 (29.0%) and 22 (32.3%) of the 62 intramucosal carcinomas, respectively (Table II). The incidence of absent or reduced Fhit and E-cadherin expression

Table III. TSG (Fhit, Mlh1, p16 and E-cadherin) expression in gastric adenomas.

Absent or reduced expression	Adenoma (n=1)		
	Mild, moderate (n=27) (%)	Severe (n=14) (%)	P-value
Fhit	2 (7.4)	1 (7.1)	1
Mlh1	2 (7.4)	2 (14.3)	0.28
p16	1 (3.7)	4 (28.6)	0.038
E-cadherin	2 (7.4)	2 (14.3)	0.28

Rate of absent expression of p16 was significantly associated with the degree of dysplasia in adenomas, p=0.038.

Table IV. Phenotypic expression in early gastric neoplasias.

Phenotype	No.	Adenoma (n=41) (%)	Intramucosal carcinoma (n=62) (%)	P-value
G-type	19	1 (2.4)	18 (29.0)	0.0004
GI-type	26	9 (22.0)	17 (27.4)	0.69
I-type	51	29 (70.7)	22 (35.5)	0.00046
UC-type	7	2 (4.9)	5 (8.1)	0.69

Rate of G-type is higher in carcinomas than adenomas, p=0.0004. Rate of I-type is lower in carcinomas than adenomas, p=0.00046.

was significantly higher in the intramucosal carcinomas than in the adenomas (p=0.0000029 and 0.0003, respectively, Fisher's test). The rate of absent Mlh1 and p16 expression also tended to be higher in the intramucosal carcinomas than in the adenomas (Table II).

When the adenomas were histologically divided into mild/moderate and severe types, the reduced levels of Fhit, Mlh1 and E-cadherin did not differ between the groups. The loss of expression of p16 was significantly associated with the degree of dysplasia in adenomas (p=0.038, Fisher's test) (Table III).

In this study, aberrant TSG expression in early gastric neoplasias had no correlation with clinical parameters, including size, age, gender and location (data not shown).

Distribution of phenotypes in early gastric neoplasias. The distribution of phenotypes in the 41 adenomas and 62 intramucosal carcinomas was as follows; 1 (2.4%) adenoma and 18 (29.0%) intramucosal carcinomas for the G-type tumors, 9 (22.0%) and 17 (27.4%) for the GI-type tumors, and 29 (70.7%) and 22 (35.5%) for the I-type tumors, respectively. The frequency of the G-type in carcinomas (p=0.0004, Fisher's test) and the I-type in adenomas (p=0.00046, χ^2 test) was significantly high (Table IV).

Relationship between phenotype and the expression of the 4 TSG proteins in early gastric neoplasias. Absent or reduced expression of Fhit, Mlh1, p16 and E-cadherin was observed in 52.6, 42.1, 57.9 and 36.8% of G-type, 30.8, 23.1, 23.1 and 30.8% of GI-type, and 11.8, 13.7, 5.9 and 15.7% of I-type early gastric neoplasia, respectively. Rates of absent or reduced Fhit, Mlh1 and p16 expression were significantly higher in G-type tumors than in the others (p=0.0029, 0.0469 and 0.0084, χ^2 test). Rates of absent E-cadherin expression tended to higher in G and GI types than in I-type tumors (Table V).

Correlation of the number of TSG proteins whose expression is lost or reduced with phenotype and histological grade in early gastric neoplasias. Forty-three (69.4%) of the intramucosal carcinomas and 13 (31.7%) of the adenomas had at

Table V. Relationship between phenotype and Fhit, Mlh1, p16 and E-cadherin expression in early gastric neoplasias.

Phenotype	No.	Absent or reduced expression			
		Fhit (%)	Mlh1 (%)	p16 (%)	E-cadherin (%)
G-type	19	10 (52.6)	8 (42.1)	11 (57.9)	7 (36.8)
GI-type	26	8 (30.8)	6 (23.1)	6 (23.1)	8 (30.8)
I-type	51	6 (11.8)	7 (13.7)	3 (5.9)	8 (15.7)
UC-type	7	1 (14.3)	2 (28.6)	3 (42.9)	1 (14.3)

Rate of reduced Fhit expression is higher in G-type tumors than in the others, *p=0.0029. Rate of absent Mlh1 expression is higher in G-type tumors than in the others, **p=0.0469. Rate of absent p16 expression is higher in G-type tumors than in the others, †p=0.0084. Rate of absent E-cadherin expression tended to be higher in G-, GI-type than I-type tumors, ††p=0.0747.

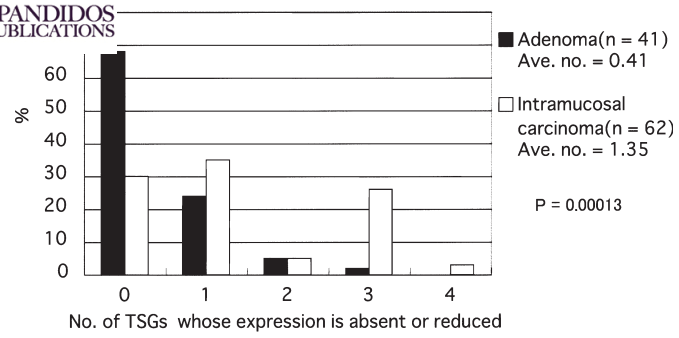

Figure 2. The average number of proteins among Fhit, hMlh1, p16 and E-cadherin whose expression was absent or reduced per sample was significantly higher in intramucosal carcinomas (1.35) than in adenomas (0.41) (p=0.00013). Twenty-one (33.9%) intramucosal carcinomas demonstrated absent or reduced expression of ≥2 (50%) of the TSG proteins examined. Only 3 (7.3%) adenoma samples had absent or reduced expression of ≥2 TSG proteins.

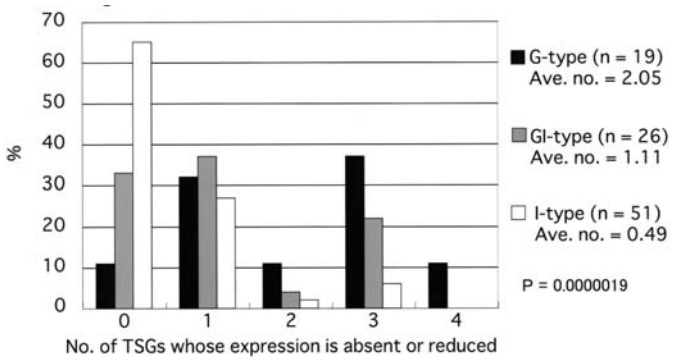

Figure 3. The average number of TSGs was significantly higher in gastric-type tumors (2.05) than in intestinal-type tumors (0.49) (p=0.0000019). Eleven (57.9%) G-type tumors demonstrated absent or reduced expression of ≥2 (50%) of the TSG proteins examined. However, only 4 (7.8%) I-type tumors had absent or reduced expression of ≥2 TSG proteins.

least 1 TSG whose expression was lost or reduced. Twenty-one (33.9%) of the intramucosal carcinomas demonstrated absent or reduced expression of ≥2 (50%) of the TSG proteins examined. Only 3 (7.3%) of the adenoma samples, on the other hand, had absent or reduced expression of ≥2 TSG proteins. The number of TSG proteins whose expression was lost or reduced was significantly higher in intramucosal carcinomas than in adenomas (mean 1.35 vs. 0.41, p=0.00013, Mann-Whitney test; Fig. 2).

Eleven (57.9%) G-type tumors demonstrated an aberrant expression of ≥2 (50%) of the TSG proteins examined. However, only 4 (7.8%) I-type tumors had absent or reduced expression of ≥2 TSG proteins. The number of TSG proteins with absent or reduced expression was significantly higher in G-type tumors than in I-type tumors (mean 1.35 vs. 0.41, p=0.0000019, Mann-Whitney test; Fig. 3).

Discussion

Epigenetic changes have recently emerged as an important cause of tumorigenesis. Of particular interest is that the hypermethylation of the CpG island of a tumor-suppressor gene can result in transcriptional silencing of the gene with subsequent loss of protein expression (1,3-5). Studies have shown that CpG islands are frequently hypermethylated in gastric cancer. These reports indicated that the DNA methylation status of 4 TSGs (*FHIT*, *MLH1*, *p16* and *E-cadherin*) could be simply detected by conducting an immunohistochemical analysis of tumor specimens (3-5,8-14,18-20).

Not all gastric adenomas are precursors for gastric carcinomas. Only some adenomas develop into carcinomas (6,7). However, the process of carcinogenesis has not been revealed. Most previous studies focused on the methylation of these genes in gastric cancer samples only, and did not involve a study of the status of the genes in early neoplastic lesions. In the present study, the expression of all 4 TSGs was lost or reduced in gastric adenomas and intramucosal carcinomas. The expression of these 4 TSGs showed a tendency to be reduced or lost with the progression from gastric adenoma to intramucosal carcinoma. Moreover, the average number of proteins among the 4 TSGs whose expression was absent or reduced per sample in intramucosal carcinomas (1.35) was significantly higher than in adenomas (0.41) (p=0.00013). Oue *et al* (33) reported that the accumulation of DNA methylation of tumor-related genes is associated with tumor progression in gastric cancer. In addition, the cumulative loss of expression of tumor-related genes has been reported to be associated with tumor stage in gastric cancer (34). Thus, a continuous increase in the number of TSGs whose expression is reduced or lost may be an important pathogenetic mechanism, not only for the progression but also for the early development of gastric cancer.

Furthermore, unlike the expression of Fhit, Mlh1 and E-cadherin, the reduced expression of p16 was correlated with the histological grade of adenoma (p=0.038). A recent study on precursor lesions of human gastric carcinoma suggested that *p16* methylation might be an early event in the development of gastric carcinoma (35). Sun *et al* (36) reported that *p16* was methylated in all gastric carcinomas that developed from the *p16* methylated human gastric dysplasia lesions (during 5 years of follow-up). Taken together, these results indicate that the inactivation of *p16* might play an important role in the early stages of gastric carcinogenesis and immunohistochemical detection of p16 status in adenoma may be predictive of malignant transformation.

There are obvious differences in the biological behavior of gastric phenotype versus intestinal phenotype carcinomas (23-25). Generally, gastric phenotype carcinomas are considered to have greater invasiveness and metastatic potential than intestinal phenotype carcinomas (24,26-29). In this study, gastric phenotype tumors were significantly associated with absent or reduced expression of Fhit, Mlh1 and p16. DNA methylation of hMLH1 occurred more frequently in G-type gastric carcinomas (37,38). Therefore, DNA methylation may occur frequently in G-type gastric carcinomas.

Moreover, the average number of TSG proteins (among Fhit, Mlh1, p16 and E-cadherin) whose expression was absent or reduced per sample was significantly higher in G-type tumors (2.05) than in I-type tumors (0.49) (p<0.01). Kang *et al* (3) reported that methylation of TSGs correlated with the progression of gastric carcinomas and a higher histo-

logical grade. Therefore, combined analyses of phenotypic expression and TSG expression using immunohistochemistry could be useful for evaluating the malignant potential of gastric neoplasias. Furthermore, we indicated that the incidence of the gastric phenotype was significantly lower in gastric adenomas than intramucosal carcinomas. This result was consistent with a previous report (39).

In conclusion, we examined the absent or reduced expression of TSGs in the early stages of gastric tumorigenesis, and found that the accumulation was associated with histological grade and gastric phenotype. A better understanding of TSG and phenotypic expression will provide new insights into gastric carcinogenesis, cancer treatment and feasible chemopreventive pathways.

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