AID, p53 and MLH1 expression in early gastric neoplasms and the correlation with the background mucosa

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Abstract. A number of tumor-associated genes have been associated with gastric cancer development. The present study evaluated differences in tumor-associated protein expression and phenotype among early gastric neoplasms, and correlated these data with those of the background mucosa. The expression of activation-induced cytidine deaminase (AID), p53 and MLH1 in 151 early gastric neoplasms [22 gastric adenomas, 92 intramucosal carcinomas (MCs), and 37 submucosal carcinomas (SMCs)] was examined immunohistochemically and compared with that of the corresponding background mucosal condition. The cellular phenotypes of the neoplasms and the corresponding background intestinal metaplasia were also determined. Aberrant AID, p53 and MLH1 expression was detected in 36.4, 0 and 0% of the adenomas, in 35.9, 32.6 and 16.3% of the MCs, and in 56.8, 62.2 and 21.6% of the SMCs, respectively. The frequency of aberrant AID and p53 expression in the SMCs was significantly increased compared with that in the MCs (AID, $P<0.05$; p53, $P<0.01$). Aberrant AID expression was significantly associated with p53 overexpression in the SMCs ($P<0.01$), but not in the adenomas or MCs. In addition, AID expression was associated with the severity of mononuclear cell activity in the non-cancerous mucosa adjacent to the tumor ($P<0.05$), particularly in the SMC cases. The percentage of MCs (34.8%) and SMCs (24.3%) that were of the gastric phenotype was higher compared with the percentage of adenomas (18.2%). These results indicated that p53 and MLH1 expression and a gastric phenotype may be important for carcinogenesis, and that chronic inflammation and AID and p53 expression are associated with submucosal progression.

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Introduction

Despite the worldwide reduction in gastric cancer mortality in the last decade, the disease remains a notable health problem (1). *Helicobacter pylori* (HP) infection results in gastric cancer through the slow progression of the premalignant stages of intestinal metaplasia (IM), atrophic gastritis and adenoma/dysplasia (2). In addition, a number of previous studies have indicated the possibility that highly active inflammation induced by HP is involved in cancer development (3,4). However, the molecular mechanisms that underlie gastric cancer development through the progression of HP-associated chronic gastritis have not been well studied.

Gastric cancer results from the accumulation of genetic and epigenetic alterations (5). In sporadic gastric cancer, the frequency of *p53* gene mutations is 25-50% (5,6) and the frequency of DNA methylation of the DNA mismatch repair gene mutL homolog 1 *(MLH1)* gene is 20-30% (5,7). Hypermethylation of the *MLH1* promoter region is the primary cause of microsatellite instability in primary gastric cancer (8). Previous studies have also demonstrated that activation-induced cytidine deaminase (AID) is important in HP-associated gastric cancer development (9). AID is an enzyme that edits DNA and RNA, and was originally identified to induce somatic hypermutations and class-switch recombination in immunoglobulin genes (10). Previous studies have indicated that AID transgenic mice develop malignant T-cell lymphomas and lung adenomas. These findings indicate that aberrant AID expression results in tumor-associated gene mutations and may be a cause of human malignancy (11). Cag-pathogenicity island (cag-PAI)-positive HP infection leads to the activation of nuclear factor (NF)-κB in the gastric epithelium. As a result, AID is transcriptionally upregulated in gastric epithelial cells, and may contribute to the accumulation of *p53* and other gene mutations, leading to gastric carcinogenesis (12). However, the histological association between AID expression, gastric tumors and the background mucosa is not well understood.

Gastric cancer has been divided into two histological types: Intestinal and diffuse [Lauren's classification (13)] or differentiated and undifferentiated [Nakamura *et al* (14)]. Previous studies using mucin-based histochemical and immunohistochemical analyses indicated that gastric and intestinal

phenotypic cell markers are widely expressed in gastric cancer, irrespective of the histological type (15-17).

In the present study, to improve the understanding of the mechanisms underlying malignant transformation in gastric cancer and its association with developmental processes, the expression of AID, p53 and MLH1, and the phenotypic expression in early gastric neoplasms were evaluated and compared with the background mucosal condition.

Patients and methods

Patient and tissue samples. Gastric tumors were obtained from 151 patients (109 males and 42 females) who had undergone endoscopic or surgical tumor resection at the Tottori University Hospital between 2003 and 2009, and who possessed histopathologically diagnosed tubular adenomas (n=22; males, 14; females, 8; average age, 72.5 years), intramucosal carcinomas (MCs; n=92; males, 67; females, 25; average age, 70.2 years), and submucosal carcinomas (SMCs; n=37; males, 28; females, 9; average age, 66.8 years), according to the Japanese Classification of Gastric Carcinoma (Table I) (18). All tubular adenomas corresponded to category 3, all MCs were classified as category 4 and all SMCs as category 5 of the Vienna classification (19). The mean age was statistically younger in the SMC group compared with the adenoma and MC groups. A younger age, larger tumor size and depressed type tumors were more frequently observed in the patients with SMCs compared with those with adenomas and MCs.

All patients were classified as having HP infections based on endoscopic atrophic changes or by testing seropositive for HP-immunoglobulin G (IgG). It was further confirmed that these patients had no HP eradication history. All specimens were assigned a novel number without the inclusion of personal information in order to maintain anonymity. This study was approved by the Institutional Ethics Committee of Tottori University (no. 314) and complies with the Declaration of Helsinki.

Evaluation of the tumor-surrounding mucosa. HP-associated gastritis adjacent to the tumor was evaluated by using the updated Sydney system (USS) (20). Using this system, gastritis was scored from 0 (absent) to 3 (marked). Glandular atrophy, IM and mononuclear cell infiltration (chronic inflammation) were examined. IM adjacent to the tumor was absent in 1 adenoma, 1 MC and 6 SMC cases.

Immunohistochemical staining. Paraffin-embedded sections (4-μm thick) were immunohistochemically stained with the indicated mouse monoclonal antibodies against the following proteins: p53 (DO-7; Dako, Glostrup, Denmark; dilution, 1:50), MutL homolog 1 (MLH1; G168-15; BD Pharmingen, San Diego, CA, USA; dilution, 1:50), mucin 5AC, oligomeric mucus/gel-forming (MUC5AC; 45M1; Leica Biosystems, Ltd., Newcastle, UK; dilution, 1:50), mucin 2, oligomeric mucus/gel-form (MUC2; Ccp58; Leica Biosystems, Ltd.; dilution, 1:100) and cluster of differentiation 10 (CD10; 56C6; Leica Biosystems, Ltd.; dilution, 1:50) or with a rat monoclonal anti-AID antibody (EK2 5G9; Cell Signaling Technology, Inc., Danvers, CA,

USA; dilution 1:400), using the avidin-biotin-peroxidase complex (ABC) technique.

Immunohistochemical staining was performed in a blinded manner with respect to the clinical information. The sections were deparaffinized in xylene and rehydrated in ethanol. The sections were then immersed in a citrate buffer (0.01 mol/l; pH 6.0) and heated in a microwave oven for 20-30 min to retrieve antigens. Endogenous tissue peroxidase activity was blocked by incubation with 3% H_2O_2. The sections were subsequently incubated with primary antibody overnight at 4°C. As a negative control, the primary antibody was replaced with normal serum IgG at a similar dilution. Signal detection followed the Vectastain Elite ABC kit instructions (Vector Laboratories, Inc., Burlingame, CA, USA) using diaminobenzidine as the chromogen. The sections were counterstained with hematoxylin, and were subsequently incubated with biotinylated anti-rat or anti-mouse IgG and avidin-biotin-peroxidase (included with Vectastatin Elite ABC Kit). The sections were subsequently visualized using diaminobenzidine tetrahydrochloride. Two independent observers evaluated protein expression using an optical microscope (BX50; Olympus Corporation, Tokyo, Japan).

Assessment of AID immunostaining. The internal positive controls were lymphocytes of germinal centers in lymphoid follicles (Fig. 1A). The follicles contain activated B cells and all specimens stained markedly positive for AID. The cytoplasm was scored as positive when >30% of the tumor cells stained as markedly as the germinal centers. Aberrant AID expression refers to AID overexpression.

Assessment of p53 immunostaining. The tumors were scored as positive for p53 when a distinct nuclear immunoreaction occurred in >25% of the tumor cells (21), as presented in Fig. 1B. Positive staining for p53 is considered aberrant p53 expression (overexpression).

Assessment of MLH1 immunostaining. MLH1 expression was classified as either normal or reduced (aberrant). Tissue specimens with definite nuclear staining in <30% of the tumor cells were categorized as having reduced staining (22).

Assessment of MUC5AC, MUC2 and CD10 immunostaining, and classification of phenotypes. Staining of >10% of the adenoma and carcinoma cells for MUC5AC, MUC2 or CD10 was classified as positive for that marker, and staining of <10% was classified as negative. The tumor phenotypes were classified into 3 categories according to the combination of the expression of CD10, MUC2 and MUC5AC. The intestinal phenotype (I-type) was positive for MUC2 and/or CD10, but negative for MUC5AC. The gastric and intestinal mixed phenotype (GI-type) was positive for MUC2 and/or CD10, and positive for MUC5AC. The gastric phenotype (G-type) was positive for MUC5AC, but negative for MUC2 and CD10. IM was also classified into GI- and I-types using these markers (23).

Statistical analysis. All data were statistically analyzed using the χ^2 test with Yates' correction, Fisher's test and Student's *t*-test using Stat View software, version 5.0 (SAS Institute,

Table I. Clinicopathological characteristics of early gastric neoplasias.

Characteristic	Adenoma (n=22)	MC (n=92)	SMC (n=37)
Age, years (mean ± SD)	72.5±9.2	70.2±7.4	66.8±12.2
Gender, n			
Male	14	67	28
Female	8	25	9
Size, mm (mean ± SD)	11.6±7.2	10.0±4.5	25.3±14.9
Site, n			
Upper third	4	19	6
Middle third	6	38	20
Lower third	12	35	11
Macroscopic type, n			
Elevated	11	31	4
Flat or depressed	11	61	33
Histology, n			
Differentiated		91	23
Mixed	NA	1	5
Undifferentiated		0	9

SD, standard deviation; NA, not applicable; MC, intramucosal carcinoma; SMC, submucosal carcinoma. 'Differentiated' includes tubular and papillary types. 'Undifferentiated' consists of signet-ring cell and/or poorly-differentiated types. 'Mixed' is composed of 'Differentiated' and 'Undifferentiated'.

Table II. Percentages of early gastric neoplasias showing aberrant expression of AID, p53 and MLH1.

	Aberrant expression, n/total n (%)		
Protein	Adenoma (n=22)	MC (n=92)	SMC (n=37)
AID	8/22 (36.4)	33/92 (35.9)[a]	21/37 (56.8)[a]
p53	0/22 (0.0)	30/92 (32.6)[b]	23/37 (62.2)[b]
MLH1	0/22 (0.0)	15/92 (16.3)	8/37 (21.6)

[a]$P=0.048$, [b]$P=0.004$. AID, activation-induced cytidine deaminase; MLH1, MutL homolog 1; MC, intramucosal carcinoma; SMC, submucosal carcinoma.

Cary, NC, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Frequency of aberrant AID, p53 and MLH1 expression. The protein expression of AID, p53 and MLH1 was immunohistochemically determined in early gastric neoplasms (Table II). Aberrant AID, p53 and MLH1 expression (overexpression, overexpression and loss of expression, respectively) was detected in 36.4% (8/22), 0% (0/22) and 0% (0/22) of the adenomas, in 35.9% (33/92), 32.6% (30/92) and 16.3% (15/92) of the MCs, and in 56.8% (21/37), 62.2% (23/37) and 21.6% (8/37) of the SMCs, respectively. p53 overexpression and loss of MLH1 expression was not detected in the adenomas. Overexpression of AID and the *p53* gene product was significantly more frequent in the SMCs compared with the MCs (AID, $P<0.05$;

p53, $P<0.01$). There were no significant differences in the loss of MLH1 between the MCs and SMCs. In addition, there were no significant correlations between the expression levels of these proteins and other clinicopathological data, including age, gender, location or macroscopic type of tumor (data not shown).

Association between AID, p53 and MLH1 expression. Aberrant AID expression was significantly associated with p53 overexpression in the SMCs ($P<0.01$), but not in the MCs (Table III). p53 overexpression was significantly and inversely associated with the loss of MLH1 expression, particularly in the MCs ($P<0.05$). The percentage of SMCs that demonstrated aberrant AID and p53 expression (45.9%) was significantly increased compared with that of the MCs (10.9%) ($P<0.0001$). When the expression of aberrant AID and p53 was used for the diagnosis of submucosal invasion, the sensitivity was 45.9%, the

Table III. Association between AID and p53 expression in early gastric cancers.

	AID expression					
	MC (n=92)			SMC (n=37)		
p53 expression	Positive, n	Negative, n	P-value	Positive, n	Negative, n	P-value
Positive	10	20		17	6	
Negative	23	39	P=0.724	4	10	P=0.0069

AID, activation-induced cytidine deaminase; MC, intramucosal carcinoma; SMC, submucosal carcinoma.

Table IV. Association between AID and p53 expression and submucosal invasion in early gastric cancers.

Aberrant expression	MCs + SMCs	MC (n=92)	SMC (n=37)	P-value
AID(+)/p53(+), n	27	10	17	
Other, n	102	82	20	P<0.0001

Sensitivity, 45.9% (17/37); specificity, 89.1% (82/92); positive predictive value, 63.0% (17/27); negative predictive value, 80.4% (82/102). AID, activation-induced cytidine deaminase; MC, intramucosal carcinoma; SMC, submucosal carcinoma.

Table V. Updated Sydney system score of the background mucosa of early gastric neoplasias.

Parameter	Adenoma (n=22)	MC (n=92)	SMC (n=37)
Atrophy	2.22[a]	1.83	1.89
IM	2.31[b]	1.90	1.78
Chronic inflammation	1.77	1.89	2.24[c]

[a,b]Mean scores of glandular atrophy and IM surrounding the adenomas were significantly higher than those surrounding the MCs and SMCs (P<0.05). [c]The mean score of chronic inflammation surrounding the SMCs was higher than those surrounding the adenomas and MCs (P<0.01). MC, intramucosal carcinoma; SMC, submucosal carcinoma; IM, intestinal metaplasia.
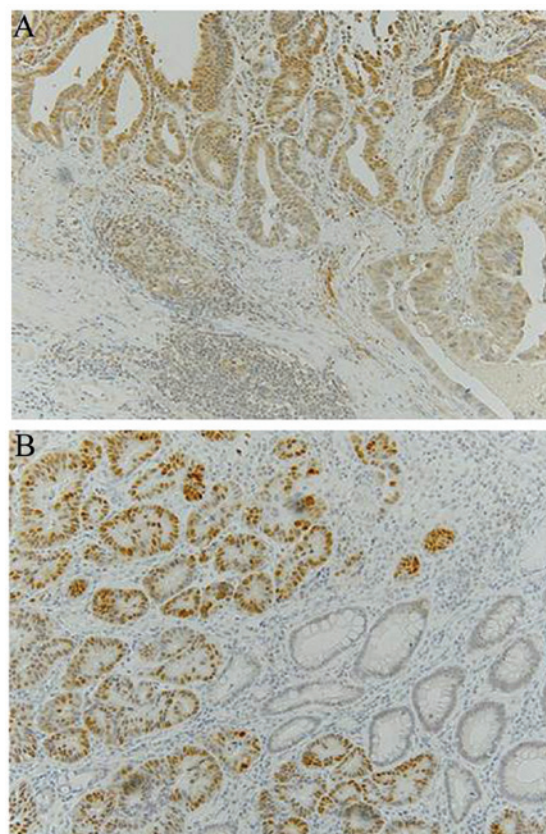

Figure 1. Representative images of AID and p53 immunostaining in an early gastric neoplasm. (A) Positive AID immunostaining in the cytoplasm of intramucosal carcinoma. (B) Overexpression of p53 in intramucosal carcinoma. AID, activation-induced cytidine deaminase.

specificity was 89.1%, the positive predictive value was 63.0% and the negative predictive value was 80.4% (Table IV).

Association between AID, p53 and MLH1 expression levels, and the background mucosa. Next, the gastritis in the background mucosa of the tumors was analyzed by evaluating the glandular atrophy, IM and chronic inflammation (mononuclear cell infiltration). Table V shows the mean USS scores for these parameters. The mean scores of gastritis surrounding the adenomas, MCs and SMCs were as follows: Glandular atrophy, 2.22, 1.83 and 1.89; chronic inflammation, 1.77, 1.89 and 2.24; and IM, 2.31, 1.90 and 1.78, respectively. A significant increase was noted in the mean scores of glandular atrophy and IM surrounding the adenomas compared with those surrounding the MCs and SMCs (P<0.05), and the mean score of chronic inflammation surrounding the SMCs was significantly increased compared with those surrounding the adenomas and MCs (P<0.01). The mean score of chronic inflammation in the AID-positive neoplasms was significantly increased compared with that in the AID-negative neoplasms, particularly in the SMC cases (P<0.05) (Fig. 2A). In addition, the mean score of chronic inflammation in the MLH1-negative neoplasms was reduced compared with the MLH1-positive neoplasms (P<0.05; Fig. 2B). The mean score of IM adjacent to the p53-positive SMCs was increased compared with that adjacent to the p53-negative SMCs (Fig. 2C).

Table VI. Phenotype of early gastric neoplasias and their surrounding IM.

Parameter	Adenoma (n=22)	MC (n=92)	SMC (n=37)
Phenotype of tumor, n (%)			
G-type	4 (18.2)	32 (34.8)	9 (24.3)
GI-type	6 (27.3)	33 (35.9)	16 (43.2)
I-type	12 (54.5)	27 (29.3)	12 (32.4)
Phenotype of surrounding IM, n (%)[a]			
GI-type	15 (71.4)	66 (72.5)	17 (54.8)
I-type	6 (28.6)	25 (27.5)	14 (45.2)

[a]IM adjacent to the tumor was absent in 1 adenoma, 1 MC and 6 SMC cases. IM, intestinal metaplasia; MC, intramucosal carcinoma; SMC, submucosal carcinoma; G-type, gastric phenotype; GI-type, gastric and intestinal mixed phenotype; I-type, intestinal phenotype.

Phenotype of tumors and their surrounding IM. The gastric/intestinal phenotypes of the tumors and their surrounding IM were analyzed (Table VI). A high percentage of MCs (34.8%) and SMCs (24.3%) were of the G-type, and a high percentage of adenomas (54.5%) were of the I-type (P<0.05). Using the same phenotypic markers, the tumor-surrounding IM was divided into two major types: A GI-type and a solely I-type. The phenotype of the IM surrounding SMCs was largely classified as the I-type (45.2%), and the phenotypes of the IMs surrounding the adenomas and the majority of the MCs were classified as the GI-type (71.4 and 72.5%, respectively; P=0.063). The combined data indicated that the background IM phenotype was similar for the adenomas and MCs, but differed for the SMCs. In addition, the cellular phenotype in tumors and the background mucosa were not correlated with tumor-associated protein expression or with the mean scores of the USS classification (data not shown).

Discussion

The present study examined AID, p53 and MLH1 expression, and the cellular phenotype of early gastric neoplastic samples, and then these results were compared with patient clinicopathological characteristics and the condition of the mucosa surrounding or adjacent to the tumor. The expression levels of p53 and MLH1, and a gastric phenotype were significantly associated with carcinogenesis. In addition, the features of the background mucosa of the adenomas and MCs were similar. Notably, chronic inflammation and AID and p53 expression were significantly associated with submucosal invasion.

Previous studies have demonstrated important roles for AID, a cellular genome mutator, in HP-associated gastric cancer development (9). Infection with HP triggers the aberrant expression of AID within the gastric epithelium, which results in the accumulation of altered nucleotides in the *p53* gene (12,24,25). The percentage of adenomas (36.4%), MCs (35.9%) and SMCs (56.8%) that demonstrated aberrant AID expression in the present study was slightly increased compared with the percentage of GCs that demonstrated aberrant AID expression in two previous studies (26.9 and 22.5%) (25,26). Notably, in the present study, the percentage of adenomas and MCs that demonstrated aberrant AID
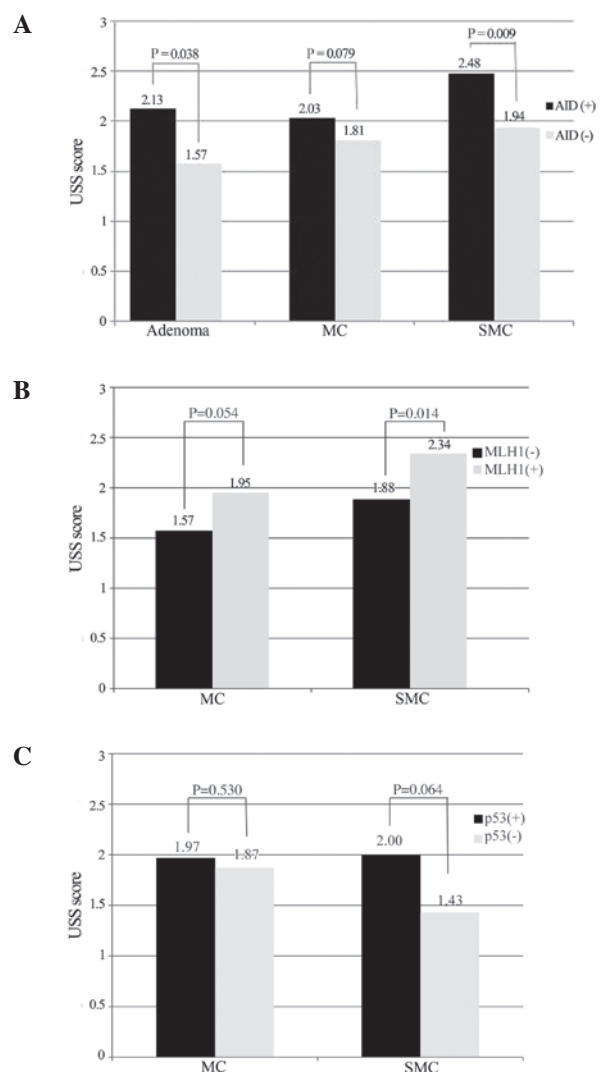

Figure 2. Expression of (A) AID, (B) MLH1 and (C) p53 in early gastric neoplasias and USS scores of (A and B) chronic inflammation and (C) IM in their background mucosa. (A) The mean score of chronic inflammation in the AID-positive neoplasms was significantly increased compared with that in the AID-negative neoplasms, particularly in the SMC cases (P<0.05). (B) The mean score of chronic inflammation in MLH1-negative neoplasms tended to be low compared to MLH1-positive neoplasms (P<0.05). (C) The mean score of IM adjacent to p53-positive SMCs tended to be high compared with that adjacent to p53-negative SMCs. AID, activation-induced cytidine deaminase; MLH1, MutL homolog 1; USS, updated Sydney system; MC, intramucosal carcinoma; SMC, submucosal carcinoma; IM, intestinal metaplasia.

expression was the same, whereas the percentage of SMCs demonstrating aberrant AID expression was significantly increased compared with that of MCs. The variability in the findings may be a result of the differences in the stage of carcinoma progression and in the degree of tumor differentiation.

Kim *et al* (25) observed a significant association between the aberrant expression of AID and the nuclear overexpression of p53 in a range of gastric cancer types. However, our previous study did not demonstrate an association between aberrant AID expression and p53 overexpression in early differentiated gastric cancer (26). Similarly, Goto *et al* (27) observed no correlation between AID and p53 expression in early differentiated and poorly-differentiated gastric cancer. In the present study, aberrant AID expression was significantly associated with p53 overexpression in the SMCs (P<0.01), but not in the MCs or adenomas. Therefore, mutations in *p53* as a result of aberrant AID expression may increase with tumor progression. However, the p53 protein may also become altered through cigarette smoking, as occurs in lung and esophageal carcinogenesis (28,29). AID is a well-described molecule that is involved in DNA mutations in the human genome. The typical pattern of nucleotide alteration induced by its enzymatic activity is cytosine/guanine (C/G) to thymine/adenine (T/A) transition (9). Tobacco smoking also induces G/C to T/A transversion (30). Further investigation is required to clarify the correlation between the expression of p53 and aberrant AID expression.

The expression of AID in gastric epithelial cells may be altered by the direct action of HP macromolecules through the type IV secretion system encoded by cag-PAI (31). Additionally, in human gastric epithelial cells, AID expression is induced by HP-associated inflammatory mediators, including tumor necrosis factor α and interleukin-1β via activation of NF-κB (12). Furthermore, the expression of AID in tumors, including colon cancer, cholangiocarcinoma and hepatocellular carcinoma, is also mediated by stimulation of proinflammatory cytokines via NF-κB (32-34). Aberrant AID expression correlates with chronic active inflammation, glandular atrophy and IM in non-neoplastic gastric mucosa (26). The present study demonstrated that aberrant AID expression in tumors correlated with mononuclear cell activity in the mucosa surrounding the tumor, particularly in SMC cases, which supports the aforementioned mechanism of AID expression and is consistent with previous studies (26,27). Nagata *et al* (35) demonstrated that the enhanced expression of AID in human gastric mucosa infected by HP is reduced following HP eradication. In addition, Kang *et al* (36) reported that rebamipide suppressed HP-induced AID expression and also suppressed invasion of gastric cancer cells through the downregulation of phospholipase D1 (PLD1), via inhibition of the HP cagA-NFκB-PLD1 signaling pathway. Therefore, HP eradication and a drug that results in AID suppression via inhibition of HP-mediated PLD1 may prevent submucosal invasion in gastric cancer.

Submucosal invasion in gastric cancer has also been associated with aberrant AID and p53 expression. Sugai *et al* (37) reported that gastric cancer with p53 overexpression is associated with a high risk of submucosal invasion. These data indicate that AID is important in the progression of gastritis to gastric cancer, particularly in SMC. The results of the present

study indicated that tumor progression may be associated with *p53* mutations that arise due to aberrant AID expression. Also, combined aberrant AID expression and p53 overexpression may provide valuable information for the prediction of aggressive tumor behavior.

In the present study, the percentage of the MCs and SMCs that demonstrated loss of MLH1 expression was 16.3 and 21.6%, respectively, and this loss of MLH1 expression was inversely associated with p53 overexpression in the MCs. These findings are consistent with previous studies (5). Aberrant DNA methylation of promoter CpG islands appears to be the predominant mechanism associated with the loss of *hMLH1* function in chronic inflammation-associated sporadic gastric carcinomas (8). In addition, previous studies have demonstrated that AID is crucial in inducing *p53* mutations and DNA demethylation (38). This mechanism may explain this association. In addition, in the present study there was no difference in MLH1 expression between MCs and SMCs, indicating that loss of MLH1 expression appears to be independent of the degree of invasion, which is consistent with previous study findings (37).

Marked differences between the biological behavior of gastric phenotype carcinomas and intestinal phenotype carcinomas have previously been reported (39). In the present study, the percentage of the MCs (34.8%) and SMCs (24.3%) that were of the gastric phenotype was significantly increased compared with that of the adenomas (18.2%), and the percentage of the adenomas that were of the intestinal phenotype (54.5%) was increased compared with that of the MCs and SMCs (29.3 and 32.4%). Based on these data, the gastric phenotype may be important for malignant transformation. This result is consistent with previous studies (40). The current study further demonstrated that the phenotype of the background IM of the SMCs was largely classified as the intestinal type (45.2%), whereas the majority of the phenotypes of the background IMs of the adenomas and MCs were classified as the mixed type (71.4 and 72.5% respectively). The combined data indicated that the background IM phenotype of the adenomas and MCs was similar, but that the phenotype of the SMCs was different. Previous observations in animal models indicated that the phenotype of IM changed from the GI mixed-type to type I during tumor progression (41). This may indicate a phenotypic shift in expression from the gastric to intestinal phenotype during tumor progression.

In conclusion, p53 and MLH1 expression, and a gastric phenotype may be important for carcinogenesis, and adenomas and MCs may arise from a similar background mucosa. Furthermore, chronic inflammation and AID and p53 expression may affect submucosal invasion. These findings may provide valuable information for the prediction of aggressive tumor behavior and may aid in the decision between endoscopic therapies and surgical resection. However, additional extensive future studies are required to confirm the results of the present study.

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