

Expression of AID, P53, and Mlh1 proteins in endoscopically resected differentiated-type early gastric cancer

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Abstract

AIM: To analyze the expression of the tumor-related proteins in differentiated-type early gastric carcinoma (DEGC) samples.

METHODS: Tumor specimens were obtained from 102 patients (75 males and 27 females) who had received an endoscopic tumor resection at Tottori University Hospital between 2007 and 2009. Ninety-one cancer samples corresponded to noninvasive or intramucosal carcinoma according to the Vienna classification system, and 11 samples were submucosal invasive carcinomas. All of the EGCs were histologically differentiated carcinomas. All patients were classified as having *Helicobacter pylori* (*H. pylori*) infections by endoscopic atrophic changes or by testing seropositive for *H. pylori* IgG. All of the samples were histopathologically classified as either tubular or papillary adenocarcinoma according to their structure. The immunohistochemical staining was performed in a blinded manner with

respect to the clinical information. Two independent observers evaluated protein expression. All data were statistically analyzed then.

RESULTS: The rates of aberrant activation-induced cytidine deaminase (AID) expression and P53 overexpression were both 34.3% in DEGCs. The expression of Mlh1 was lost in 18.6% of DEGCs. Aberrant AID expression was not significantly associated with P53 overexpression in DEGCs. However, AID expression was associated with the severity of mononuclear cell activity in the non-cancerous mucosa adjacent to the tumor ($P = 0.064$). The rate of P53 expression was significantly greater in flat or depressed tumors than in elevated tumors. The frequency of Mlh1 loss was significantly increased in distal tumors, elevated gross-type tumors, papillary histological-type tumors, and tumors with a severe degree of endoscopic atrophic gastritis ($P < 0.05$).

CONCLUSION: Aberrant AID expression, P53 overexpression, and the loss of Mlh1 were all associated with clinicopathological features and gastric mucosal alterations in DEGCs. The aberrant expression of AID protein may partly contribute to the induction of nuclear P53 expression.

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Key words: Gastric cancer; Activation-induced cytidine deaminase; P53; Mlh1; Endoscopic resection

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INTRODUCTION

Gastric cancer (GC) is the second leading cause of cancer death and the fourth most common malignant tumor in the world^[1]. The mortality rate associated with the disease is high, with a 5-year survival rate of approximately 20% being observed worldwide^[2]. The 5-year survival rate for GC is over 50% in Japan^[3]. One of the main factors limiting the survival rate is late tumor detection. Therefore, a better understanding of the clinicopathological characteristics in early GC (EGC) is critical. Infection with *Helicobacter pylori* (*H. pylori*), especially when “cag” pathogenicity island (cag PAI) positive, increases the risk of developing GC by more than 6-fold. Therefore, cag PAI is considered an important carcinogenic trigger^[4]. Almost all *H. pylori* strains in Japan are cag PAI-positive^[5]. Infection with *H. pylori* causes chronic inflammation of the gastric mucosa, which slowly progresses through the premalignant stages of atrophic gastritis, intestinal metaplasia and adenoma/dysplasia to GC^[6]. The Japanese Research Society for Gastric Cancer has proposed that GC is divided into differentiated and undifferentiated types according to the degree of glandular formation by the tumor cells^[7]. Additionally, each type of cancer might follow different genetic pathways during carcinogenesis^[8]. The frequency of differentiated-type carcinomas among total EGC is approximately 60%. Therefore, differentiated-type early gastric carcinoma (DEGC) is considered to represent the initial phase of GC^[9].

Gastric carcinoma results from the accumulation of genetic and epigenetic alterations^[8]. The frequency of *MLH1* DNA methylation is 20%-30%^[8,10] and the frequency of *P53* gene mutations is 25%-50%^[8,11] in sporadic GC. *MLH1* is a DNA mismatch repair gene. Hypermethylation of the *MLH1* promoter region is the main cause of microsatellite instability (MSI) in primary GCs^[12]. Activation-induced cytidine deaminase (AID) is a DNA- and RNA-editing enzyme that was originally identified as an inducer of somatic hypermutation and class-switch recombination in the immunoglobulin genes^[13]. Previous reports indicate that AID transgenic mice develop malignant T-cell lymphomas and lung adenomas. This finding suggests that aberrant AID expression results in tumor-related gene mutations and might be a cause of human malignancy^[14]. It has been reported that cag PAI-positive *H. pylori* infection causes the aberrant expression of AID in the gastric epithelium. Aberrant AID expression leads to the accumulation of nucleotide alterations in the *P53* gene^[15]. Although the relationship between AID and Mlh1 is currently unclear, the expression of P53 has been reported to be inversely associated with Mlh1 loss in GC^[8]. Elucidation of the relationship between the clinicopathological characteristics and the

molecular events in EGC might improve the early detection, treatment, and surveillance of GC.

In this study, we evaluated AID, P53, and Mlh1 expression in endoscopically resected DEGCs and investigated their relationships with clinicopathological characteristics and background mucosa.

MATERIALS AND METHODS

Patient and tissue samples

Tumor specimens were obtained from 102 patients (75 males and 27 females) who had received an endoscopic tumor resection at Tottori University Hospital between 2007 and 2009 (Table 1). The mean age (\pm SD) was 70.6 ± 7.8 years (range: 55-92 years). The male patients were statistically younger than the female patients (69.4 ± 7.9 vs 74.2 ± 6.6 , $P = 0.006$). We classified the DEGCs based on the Japanese classification of GC, 13th edition (7) according to location, macroscopic, and morphological types. The tumor location was defined as the upper third, middle third, or lower third of the tissue. The macroscopic type of DEGC was determined as elevated, depressed, or flat. All of the samples were histopathologically classified as either tubular or papillary adenocarcinoma according to their structure.

Ninety-one cancer samples corresponded to noninvasive or intramucosal carcinoma according to the Vienna classification system^[16], and 11 samples were submucosal invasive carcinomas. All of the EGCs were histologically differentiated carcinomas. All patients were classified as having *H. pylori* infections by endoscopic atrophic changes or by testing seropositive for *H. pylori* IgG. Two experienced pathologists (Yashima K and Ito H) verified the pathological diagnoses. Moreover, we confirmed that these patients had no *H. pylori* eradication history. All specimens were assigned a new number without personal information to maintain anonymity. This study was approved by the institutional ethics committee of Tottori University (No. 314).

Evaluation of endoscopic gastric atrophy

All endoscopic examinations were performed using video scopes (model GIF-Q260; Olympus, Tokyo, Japan) and two endoscopists (Takeda Y and Yashima K) evaluated gastric atrophy according to the location of the atrophic border as described by Kimura *et al.*^[17]. A difference in the color and height of the gastric mucosa defines the border between the pyloric and fundic gland regions. We scored endoscopic gastric atrophy as marked (O2-O3), moderate (C3-O1) or mild (C1-C2). Previously, Takao *et al.*^[18] reported a significant correlation between endoscopic gastric atrophy (Kimura-Takemoto classification^[17]) and the histological gastritis (updated Sydney system^[19]). This suggests that the degree of endoscopic gastric atrophy can be considered as the grade of atrophic gastritis.

Evaluation of surrounding mucosal inflammation

We evaluated mononuclear cell activity in the non-can-

Table 1 Patients and tissue samples *n* (%)

	Total (<i>n</i> = 102)	Male (<i>n</i> = 75)	Female (<i>n</i> = 27)	Gender difference
Age (yr, mean ± SD)	70.6 ± 7.8	69.4 ± 7.9	74.2 ± 6.6	<i>P</i> = 0.006
Tumor size (cm)				
< 2.0	76 (74.5)	54 (72.0)	22 (81.5)	<i>P</i> = 0.332
≥ 2.0	26 (25.5)	21 (28.0)	5 (18.5)	
Tumor location				
Upper third	23 (22.5)	19 (25.3)	4 (14.8)	<i>P</i> = 0.095
Middle third	40 (39.2)	32 (41.6)	8 (29.6)	
Lower third	39 (38.2)	24 (32.0)	15 (55.6)	
Gross tumor appearance				
Flat/depressed	63 (61.8)	47 (62.7)	16 (59.3)	<i>P</i> = 0.755
Elevated	39 (38.2)	28 (37.3)	11 (40.7)	
Histological type				
Tubular	88 (86.3)	66 (88.0)	22 (81.5)	<i>P</i> = 0.605
Papillary	14 (13.7)	9 (12.0)	5 (18.5)	
Depth of invasion				
Mucosa	91 (89.2)	66 (88.0)	25 (92.6)	<i>P</i> = 0.509
Submucosa	11 (10.8)	9 (12.0)	2 (7.4)	

cerous mucosa adjacent to a tumor and scored it as mild, moderate or marked according to the updated Sydney system^[19].

Immunohistochemical staining

Paraffin-embedded sections (4 μm) were immunohistochemically stained with an anti-AID rat monoclonal antibody (EK2 5G9, Cell Signaling TECHNOLOGY, Danvers, CA, USA; dilution 1:400), an anti-P53 mouse monoclonal antibody (DO-7, Dakopatts, Copenhagen, Denmark; dilution 1:50), and an anti-Mlh1 mouse monoclonal antibody (G168-15, PharMingen, San Diego, CA, USA; dilution 1:50) using the avidin-biotin-peroxidase complex technique.

The 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. The endogenous tissue peroxidase activity was blocked by incubation with 3% H₂O₂. 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. The detection reaction followed the Vectastain Elite ABC kit protocol (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine as the chromogen. The sections were counterstained with hematoxylin. The sections were incubated with biotinylated anti-rat or anti-mouse IgG and avidin-biotin-peroxidase. The sections were subsequently visualized using diaminobenzidine tetrahydrochloride. Two independent observers (Takeda Y and Yashima K) evaluated protein expression.

Assessment of AID immunostaining

The internal positive controls were lymphocytes of germinal centers in lymphoid follicles (Figure 1A). The follicles contain activated B cells and intensely stained posi-

tive for AID in all specimens. The cytoplasm was scored as positive when > 30% of tumor cells were stained as strongly as the germinal centers.

Assessment of P53 immunostaining

The tumors were scored as positive for P53 when a distinct nuclear immunoreaction occurred in > 25% of tumor cells^[20] as shown in Figure 1B.

Assessment of Mlh1 immunostaining

The evaluation of Mlh1 expression was classified as being either normal or decreased (Figure 1C). Tissue specimens with definite nuclear staining in < 30% of the tumor cells were categorized as having decreased staining^[21].

Statistical analysis

All data were statistically analyzed by the χ^2 test with Yates' correction, Fisher's test and the Mann-Whitney test (*U*-test) using Stat View 5.0 software (SAS Institute, Cary, NC, USA). Statistical significance was established at *P* < 0.05.

RESULTS

Frequency of aberrant AID, P53, and Mlh1 expression

Aberrant AID expression and P53 overexpression in DEGCs were detected in 35 (34.3%) cases. The loss of Mlh1 expression was observed in 19 (18.6%) cases. Among elderly patients (≥ 65 years old), the loss of Mlh1 expression in DEGCs was significantly higher in female patients than in male patients [10/26 (38.5%) *vs* 6/54 (11.1%), *P* = 0.004] (Table 2).

Relationships between AID, P53 and Mlh1 expression

The overexpression of P53 was significantly more frequent in patients with Mlh1-positive tumors than Mlh1-negative tumors [33/83(39.7%) *vs* 2/19(10.5%), *P* = 0.015] (Table 3). The overexpression of P53 was not associated with aberrant AID expression (*P* = 0.657).

Table 2 Frequency of aberrant activation-induced cytidine deaminase, p53 and Mlh1 expression

	Total	Age < 65 yr (n = 22)			Age ≥ 65 yr (n = 80)		
		Male	Female		Male	Female	
AID							
+	35	6	1	<i>P</i> = 0.689	16	12	<i>P</i> = 0.147
-	67	15	0		38	14	
P53							
+	35	11	0	<i>P</i> = 1.000	20	4	<i>P</i> = 0.086
-	67	10	1		34	22	
Mlh1							
+	83	18	1	<i>P</i> = 0.278	48	16	<i>P</i> = 0.004
-	19	3	0		6	10	-

AID: Activation-induced cytidine deaminase.

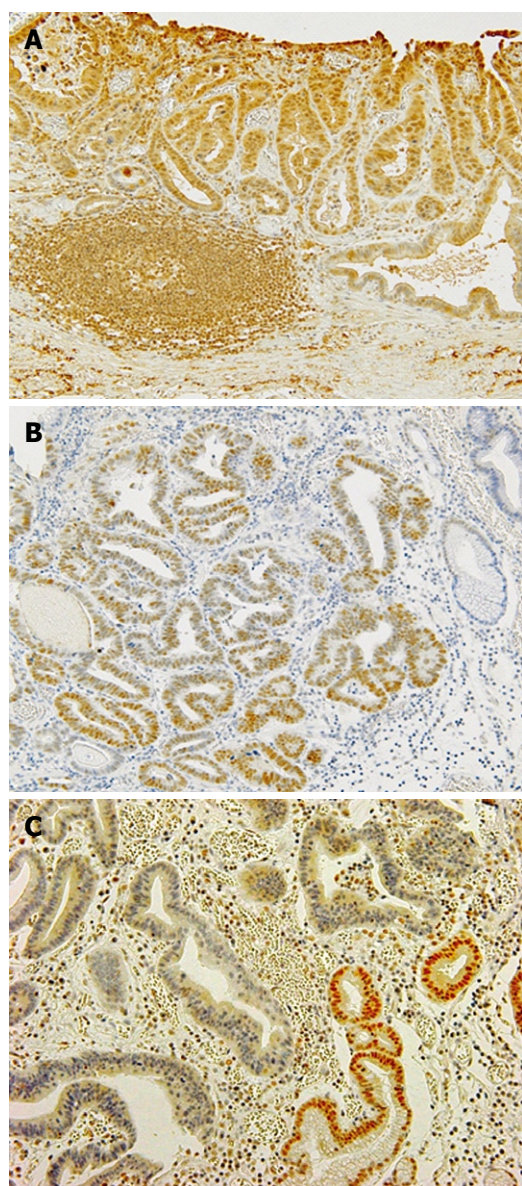


Figure 1 Representative findings of activation-induced cytidine deaminase, P53 and Mlh1 immunohistological stain in differentiated-type early gastric carcinoma. A: Positive activation-induced cytidine deaminase immunostaining in cytoplasm of differentiated-type early gastric carcinoma (DEGC); B: Overexpression of P53 in DEGC; C: No nuclear immunoreactivity for Mlh1 protein in DEGC.

Table 3 Relationships among activation-induced cytidine deaminase, P53 and Mlh1 expression

	AID			Mlh1		
	+	-		+	-	
P53						
+	11	24	<i>P</i> = 0.657	33	2	<i>P</i> = 0.015
-	24	43		50	17	

AID: Activation-induced cytidine deaminase.

Relationship of AID, P53, and Mlh1 expression with tumor features

The aberrant AID expression frequency was correlated with the location of DEGCs. However, there was no correlation between AID expression and tumor growth or histological type. The incidence of P53 overexpression in DEGCs was significantly more frequent in flat or depressed tumors than in elevated type tumors [28/64 (43.8%) *vs* 7/38 (18.4%), *P* = 0.009] (Table 4). The overexpression of P53 was found more often in tubular tumors than in papillary adenocarcinoma [34/88 (38.6%) *vs* 1/14 (7.1%), *P* = 0.045]. A loss of Mlh1 expression was closely associated with distal location (*P* = 0.027), elevated gross type (*P* = 0.039) and papillary histological type (*P* = 0.033).

Relationships of AID, P53, and Mlh1 expression with background mucosa

Although aberrant AID expression was not related to gastric atrophy, mononuclear cell activity tended to be marked in the surrounding mucosa adjacent to DEGCs with aberrant AID expression (*P* = 0.064). The P53 expression in DEGCs was not associated with gastric atrophy and mononuclear cell activity in the surrounding mucosa. The loss of Mlh1 expression in DEGCs was associated with marked endoscopic gastric atrophy (*P* = 0.020) and mild mononuclear cell activity (*P* = 0.053) (Table 5).

DISCUSSION

The present study examined AID, P53 and Mlh1 expres-

Table 4 Relationships of activation-induced cytidine deaminase, P53 and Mlh1 expression with tumor features

	AID		<i>P</i>	P53		<i>P</i>	Mlh1		<i>P</i>
	+	-		+	-		+	-	
Tumor location									
Upper third	4	19	<i>P</i> = 0.067	8	15	<i>P</i> = 0.543	22	1	<i>P</i> = 0.027
Middle third	13	27		16	24		34	6	
Lower third	18	21		11	28		27	12	
Tumor growth									
Flat/depressed	19	45	<i>P</i> = 0.202	28	36	<i>P</i> = 0.009	56	8	<i>P</i> = 0.039
Elevated	16	22		7	31		27	11	
Histological type									
Tubular	29	59	<i>P</i> = 0.673	34	54	<i>P</i> = 0.045	75	13	<i>P</i> = 0.033
Papillary	6	8		1	13		8	6	

AID: Activation-induced cytidine deaminase.

Table 5 Relationships of activation-induced cytidine deaminase, P53 and Mlh1 expression with background mucosa

	AID		<i>P</i>	P53		<i>P</i>	Mlh1		<i>P</i>
	+	-		+	-		+	-	
Endoscopic gastric atrophy									
Mild	8	17	<i>P</i> = 0.540	9	16	<i>P</i> = 0.376	20	5	<i>P</i> = 0.020
Moderate	19	29		19	29		44	4	
Marked	8	21		7	22		19	10	
Mononuclear cell activity									
Mild	5	17	<i>P</i> = 0.064	4	17	<i>P</i> = 0.232	14	8	<i>P</i> = 0.053
Moderate	24	47		28	44		61	10	
Marked	6	3		3	6		8	1	

AID: Activation-induced cytidine deaminase.

sion in endoscopically resected DEGCs, and these results were compared with the clinicopathological characteristics and the surrounding mucosa. Aberrant AID expression in endoscopically resected DEGCs significantly correlated with marked mononuclear cell activity in tumor background mucosa but not with P53 overexpression. In addition, P53 expression significantly correlated with flat or depressed types of gross tumor appearance. The loss of Mlh1 expression correlated with elevated type, papillary type histology, distal location and severe endoscopic atrophic gastritis.

Infection with *H. pylori* triggers aberrant AID expression in the gastric epithelium, which leads to the accumulation of altered nucleotides in the *P53* gene^[15,22,23]. The rate of aberrant AID expression in DEGC (34.3%) was slightly higher than the 26.9% and 22.5% described in two previous reports^[23,24]. The variability in the findings may be caused by differences in the stage of carcinoma progression and the degree of tumor differentiation. All of our data were obtained from endoscopically resected, well-differentiated early carcinomas.

Previously, Kim *et al.*^[23] found a significant association between aberrant AID expression and the nuclear overexpression of P53 in various types of GCs. However, we did not find a relationship between aberrant AID expression and P53 overexpression in DEGCs. Similarly, Goto *et al.*^[24] found no correlation between AID and P53 in early differentiated and poorly differentiated GCs. There are

several possible explanations for these different findings. One explanation is that nonsense mutations were considered to be false-negative. Additionally, P53 protein could accumulate to repair damaged DNA in false-positive cells without *P53* mutations^[25]. The rate of P53 expression might also increase with tumor progression^[26]. Moreover, P53 protein might become altered through cigarette smoking, as in lung and esophageal carcinogenesis^[27,28]. Further investigation is needed to clarify the correlation between the expression of P53 and aberrant AID expression.

The expression of AID in gastric epithelial cells could be altered by the direct action of *H. pylori* macromolecules through the type IV secretion system encoded by *cag* PAI^[29]. Additionally, *H. pylori* infection is associated with inflammatory cytokines, such as tumor necrosis factor α , that are produced during gastric inflammation^[15]. Furthermore, AID expression in tumors such as hepatocellular carcinoma, cholangiocarcinoma and colon cancer is also mediated by proinflammatory cytokine stimulation *via* nuclear factor κ B^[30-32]. Aberrant AID expression correlates with chronic active inflammation, glandular atrophy and intestinal metaplasia in the non-neoplastic gastric mucosa^[24]. The present study found that aberrant AID expression in tumors correlated with mononuclear cell activity in the mucosa surrounding the tumor, which would support the mechanisms of AID expression.

The 33.7% frequency of P53 overexpression in the

DEGC was consistent with previous findings^[8]. The expression of P53 was associated with flat or depressed macroscopic tumor features but not with the other clinicopathological features of age, gender, or location and tumor size. In agreement with our results, Sasaki *et al*^[9] also demonstrated that P53 overexpression is more frequent in depressed-type differentiated GCs.

Several epigenetic alterations in GC have been described^[10,16]. DNA methylation of *MLH1* promoter region CpG islands is closely associated with a loss of Mlh1 expression in GCs that exhibit MSI^[16]. *MLH1* hypermethylation is evident in 20%-28% of differentiated carcinomas^[10,33]. The reported frequency of negative Mlh1 expression in both early and sporadic GC ranges from 13%-20%^[34,35]. In the present study, the frequency of lost Mlh1 expression in DEGCs was 18.6%. Our study and previous studies have shown that GCs with reduced Mlh1 expression are statistically more prevalent among elderly women. Previous reports have suggested that^[36], high-frequency MSI (MSI-H) GCs are characterized by an antral location and proliferation. A loss of Mlh1 expression was associated with the lower third of the stomach and elevated gross type in our study. Additionally, our findings were consistent with the Guos report^[37], which showed a higher prevalence of MSI-H in papillary type GC than in early well-differentiated carcinoma.

Chronic gastritis induced by *H. pylori* infection usually progresses to atrophic gastritis, which is an established risk factor for GC. The risk increases with the degree and the extent of atrophic gastritis. However, no clinicopathological studies regarding the relationship between molecular events and the degree of endoscopic atrophy in patients with GC have been published. Factors such as aging, dietary habits, alcohol consumption, cigarette smoking and autoimmunity promote atrophic gastritis^[38,39]. The frequency of Mlh1 loss increases in tumors that cause a severe degree of endoscopic atrophic gastritis. Our results suggest that several factors are involved in the gastric atrophic changes found in patients with DEGC accompanied by aberrant Mlh1 expression. However, more studies are required to identify the mechanism of this association. Moreover, significantly less mononuclear cell infiltration was evident in patients with DEGC that had lost Mlh1. These results might be a consequence of a reduction in *H. pylori* density accompanied with severe glandular atrophy, which might contribute to reduced inflammatory infiltration.

In conclusion, we investigated the relationships between AID, P53 and Mlh1 expression, clinicopathological characteristics, and mucosal alterations. Our results suggest that aberrant AID expression may partly contributes to P53 overexpression.

COMMENTS

Background

Helicobacter pylori infection causes aberrant activation-induced cytidine deaminase (AID) expression in gastric epithelial tissues, which results in alterations in various tumor-related genes. However, the precise molecular mechanisms

underlying gastric carcinogenesis are not fully understood, particularly in endoscopically resected early gastric tumors.

Research frontiers

In this study, the authors analyzed the expression of the tumor-related proteins: AID, P53, and Mlh1 in 102 differentiated-type early gastric carcinoma (DEGC) samples obtained by endoscopic resection.

Innovations and breakthroughs

The authors found that the rates of aberrant AID expression and P53 overexpression were both 34.3% in DEGCs. The expression of Mlh1 was lost in 18.6% of DEGCs. Aberrant AID expression was not significantly associated with P53 overexpression in DEGCs. However, AID expression was associated with the severity of mononuclear cell activity in the non-cancerous mucosa adjacent to the tumor. The rate of P53 expression was significantly greater in flat or depressed tumors than in elevated tumors. The frequency of Mlh1 loss was significantly increased in distal tumors, elevated gross type tumors, papillary histological type tumors, and tumors with a severe degree of endoscopic atrophic gastritis.

Applications

Collectively, the data of this study suggested that aberrant AID expression, P53 overexpression, and the loss of Mlh1 were all associated with clinicopathological features and gastric mucosal alterations in DEGCs. Furthermore, the aberrant expression of AID protein may partly contribute to the induction of nuclear P53 expression.

Peer review

Takeda *et al* analyzed expression of the tumor-related proteins: AID, P53, and Mlh1 in 102 DEGC samples obtained by endoscopic resection and found aberrant AID expression, P53 overexpression and the loss of Mlh1 expression were associated with some of the clinicopathological features and gastric mucosal alterations in DEGCs, and that the aberrant expression of AID protein might partly contribute to the induction of nuclear P53 expression.

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