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Original Article

Predictive Significance of Promoter DNA Methylation of *Cysteine Dioxygenase Type 1 (CDO1)* in Metachronous Gastric Cancer

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ABSTRACT

Purpose: Promoter DNA methylation of various genes has been associated with metachronous gastric cancer (MGC). The cancer-specific methylation gene, *cysteine dioxygenase type 1 (CDO1)*, has been implicated in the occurrence of residual gastric cancer. We evaluated whether DNA methylation of *CDO1* could be a predictive biomarker of MGC using specimens of MGC developing on scars after endoscopic submucosal dissection (ESD).
Materials and Methods: *CDO1* methylation values (TaqMeth values) were compared between 33 patients with early gastric cancer (EGC) with no confirmed metachronous lesions at >3 years after ESD (non-MGC: nMGC group) and 11 patients with MGC developing on scars after ESD (MGCSE groups: EGC at the first ESD [MGCSE-1 group], EGC at the second ESD for treating MGC developing on scars after ESD [MGCSE-2 group]). Each EGC specimen was measured at five locations (at tumor [T] and the 4-point tumor-adjacent noncancerous mucosa [TAM]).
Results: In the nMGC group, the TaqMeth values for T were significantly higher than that for TAM (P=0.0006). In the MGCSE groups, TAM (MGCSE-1) exhibited significantly higher TaqMeth values than TAM (nMGC) (P<0.0001) and TAM (MGCSE-2) (P=0.0041), suggesting that TAM (MGCSE-1) exhibited *CDO1* hypermethylation similar to T (P=0.3638). The area under the curve for discriminating the highest TaqMeth value of TAM (MGCSE-1) from that of TAM (nMGC) was 0.81, and using the cut-off value of 43.4, *CDO1* hypermethylation effectively enriched the MGCSE groups (P<0.0001).
Conclusions: *CDO1* hypermethylation has been implicated in the occurrence of MGC, suggesting its potential as a promising MGC predictor.

Keywords: Cysteine dioxygenase; Early gastric cancer; Endoscopic submucosal dissection; Methylation

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Author Contributions
Conceptualization: K.Y., T.S., Y.K.; Data curation: F.Y., S.T., F.Y., W.T., W.A., I.K., K.C.; Formal analysis: Y.K.; Funding acquisition: K.Y.; Investigation: K.Y.; Methodology: A.M., H.K., F.Y., S.T., Y.K.; Project administration: A.M.; Resources: I.K.; Software: F.Y., S.T.; Supervision: T.S., Y.K., K.W., K.C.; Validation: K.Y.; Visualization: K.Y., Y.K.; Writing - original draft: K.Y.; Writing - review & editing: T.S., A.M., Y.K., K.C.

Conflict of Interest
No potential conflict of interest relevant to this article was reported.

INTRODUCTION

Presently, gastric cancer ranks as the sixth most common type of malignant tumor and is the third leading cause of mortality in men worldwide [1]. Due to the eradication efforts for *Helicobacter pylori*, the incidence of gastric cancer has decreased in recent years in Japan; however, it remains the third leading cause of mortality among malignant tumors [2]. With the development and increasingly common use of endoscopic submucosal dissection (ESD), ESD has become the established standard treatment for early gastric cancer (EGC) [3]. With the widespread treatment of EGC by ESD, it has become well known that metachronous gastric cancer (MGC) can develop after ESD, and surveillance endoscopy for MGC is recommended after ESD in these patients [4]. There are certain cases in which MGC develops on scars after curative ESD. The incidence of MGC after ESD has been reported to range from 4.0% to 13.0% [5-7].

Various genetic abnormalities such as genomic gain, genomic loss, and genomic mutations are involved in gastric cancer development [8]. Furthermore, epigenetic abnormalities, including DNA methylation, are more dominant during gastric carcinogenesis [9] and are affected by epigenetic field cancerization in the tumor-adjacent noncancerous mucosa (TAM), where DNA methylation abnormalities in various genes increase because of chronic inflammation due to *H. pylori* infection in the gastric mucosa [10-12].

Promoter DNA methylation, which is associated with gastric carcinogenesis, has recently attracted attention as a cancer-specific biomarker [13]. Among them, *cysteine dioxygenase type 1 (CDO1)* has been recognized as a novel tumor suppressor gene candidate in human cancers [14,15]. Previous reports have described the excellent diagnostic performance and prognostic relevance of *CDO1* promoter hypermethylation in various gastrointestinal cancers, including gastric cancer [16–21]. However, there have been no reports on *CDO1* promoter hypermethylation as a predictor of MGC after ESD. Therefore, we investigated the association between the subsequent development of MGC after ESD and epigenetic abnormalities using specimens of MGC developing on scars after curative ESD.

MATERIALS AND METHODS

Patients and materials

A total of 2,055 patients underwent ESD for EGC at Kitasato University Hospital and Kitasato University East Hospital between September 2002 and December 2016 (**Fig. 1**). A total of 33 patients with the latest consecutive EGC with no confirmed evidence of metachronous lesions at >3 years after curative ESD were selected as controls from 1,896 patients with no metachronous lesions (non-MGC: nMGC group). All 33 patients tested positive for *H. pylori* infection (current infection or after eradication). Among the 2,055 patients, 11 (0.5%) exhibited the development of MGC on scars after curative ESD (MGCSE groups) (**Fig. 2**), all 11 tested positive for *H. pylori* (current infection or after eradication). All patients were investigated for *H. pylori* status at the time of the initial medical examination. Among the MGCSE groups, we also examined patients who underwent the first ESD for EGC (MGCSE-1 group) and those who underwent a second ESD to treat MGC developing on scars after curative ESD (MGCSE-2 group). Both groups (MGCSE-1 and MGCSE-2) were investigated in the same patients. Furthermore, in the MGCSE-2 group, no cases of new MGCSE occurred during the observation period after the second ESD.

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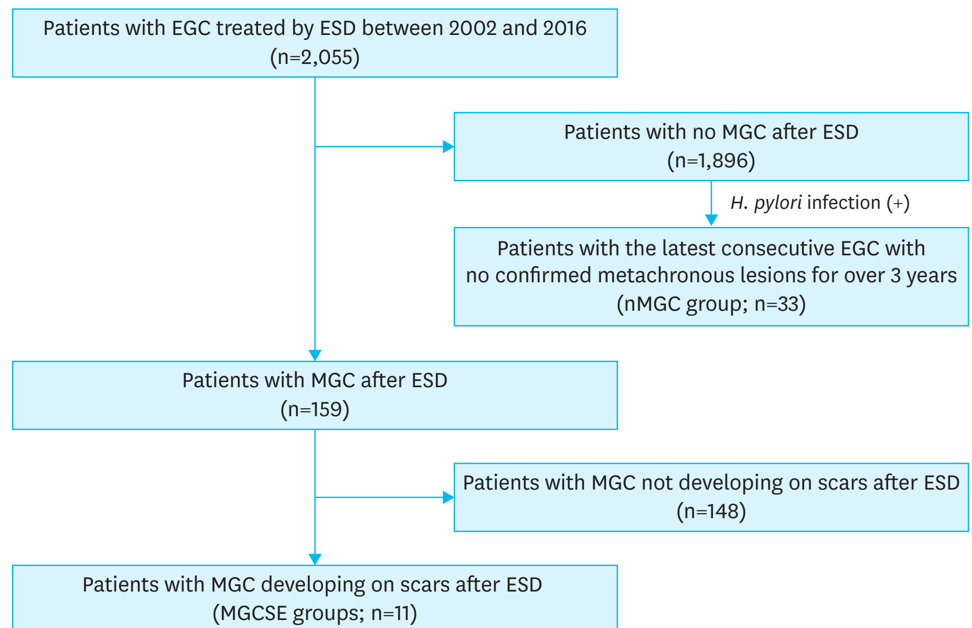

Fig. 1. Flowchart of study participants treated for early gastric cancer by endoscopic submucosal dissection.
EGC = early gastric cancer; ESD = endoscopic submucosal dissection; MGC = metachronous gastric cancer.

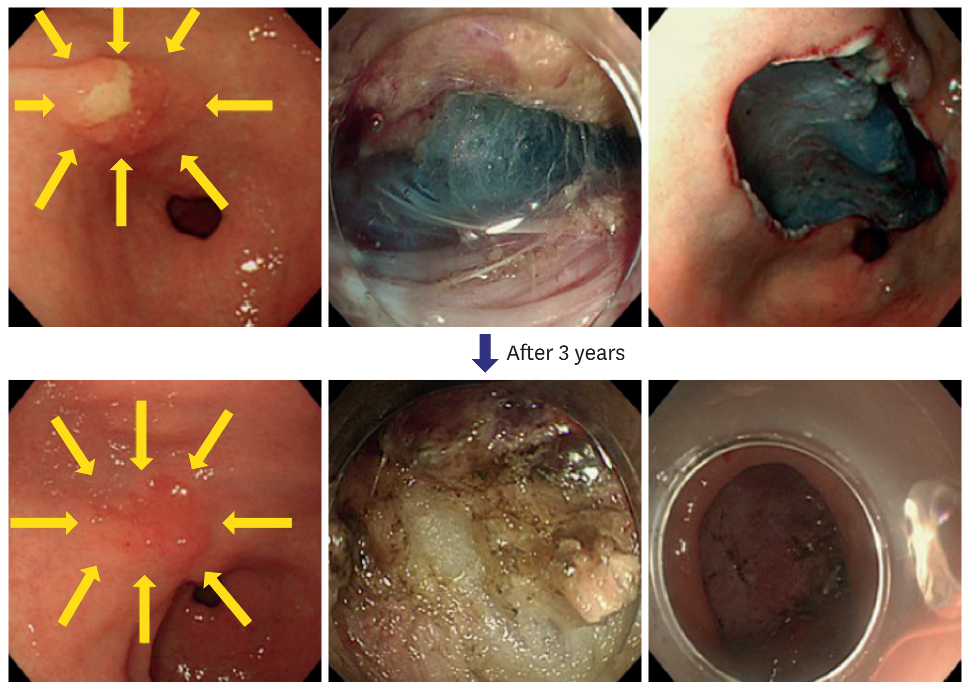

Fig. 2. Representative cases in the MGCSE groups. Representative case from MGCSE group. Three years after the first ESD (the upper left panel shows the lesion (yellow arrowhead) before ESD (MGCSE-1) and the upper right panel shows after ESD), we diagnosed a new lesion (lower left panel) categorized as MGCSE-2 (yellow arrowhead). The lower-right panel shows the lesion after the second ESD. While performing the first ESD, we could recognize the submucosal layer with a blue area (indigo carmine) (upper middle panel). On the second ESD, it was difficult to recognize the border between the submucosal and muscular layers due to fibrosis (white area), and it was difficult to treat (lower middle panel).
MGCSE = metachronous gastric cancer developing on scars after curative ESD; ESD = endoscopic submucosal dissection; MGCSE-1 = early gastric cancer at the first ESD; MGCSE-2 = EGC at the second ESD performed for treating MGC developing on scars after curative ESD.

Journal of Gastric Cancer

This study was conducted in accordance with the ethical guidelines outlined in the Declaration of Helsinki and was approved by the Kitasato University Hospital Ethics Committee (no. B18-036). All patients provided informed consent prior to enrollment.

Cell lines

The hepatocellular carcinoma cell line HepG2 and colorectal cancer cell line DLD1 were used as positive and negative controls for *CDO1* methylation, respectively, as previously described [22]. The DLD1 cells were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), whereas HepG2 cells were purchased from the RIKEN BioResource Research Center (Ibaraki, Japan). DLD1 cells were maintained in Roswell Park Memorial Institute-1640 medium (GIBCO, Carlsbad, CA, USA). HepG2 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine serum and penicillin-streptomycin (GIBCO).

Genomic DNA extraction and bisulfite treatment

The locations of the tumor (T) and TAM were identified in EGC specimens pathologically diagnosed using hematoxylin-eosin staining after ESD. Formalin-fixed paraffin-embedded tissues of excised specimens of the T and TAM were cut into 20 10-µm-thick slices. After deparaffinization, genomic DNA was extracted from the T and the separated TAMs (four points: oral TAM, anal TAM, right TAM, and left TAM) tissues using a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) (**Fig. 3**). TAMs were extracted from noncancerous mucosa at a distance of more than 2 mm from the T site to prevent contamination of the tumor components. The extracted genomic DNA (2 µg) was chemically converted by bisulfite treatment using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA). The bisulfite-treated DNA was subsequently amplified as a template via quantitative methylation-specific polymerase chain reaction (Q-MSP).

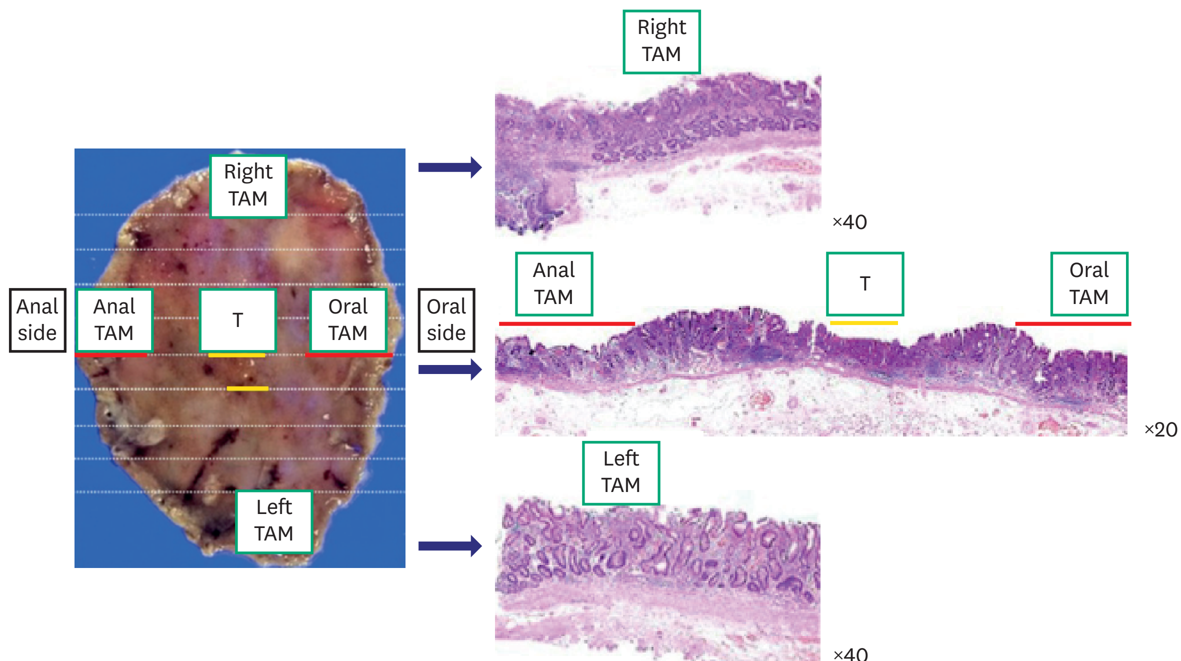

Fig. 3. Definition of sample locations for DNA extraction. Sample locations for DNA (left panel) and genomic DNA extraction from the T and separate TAMs (four points: oral TAM, anal TAM, right TAM, and left TAM) tissues. Corresponding pathological findings are shown in the right panels (hematoxylin and eosin staining, ×20, ×40). TAMs were extracted from noncancerous mucosa at a distance of more than 2 mm from the T site to prevent contamination of the tumor components.
T = tumor; TAM = tumor-adjacent noncancerous mucosa.

Q-MSP

Q-MSP for *CDO1* was performed using a C1000 Touch™ thermal cycler with a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Q-MSP was conducted at 95°C for 3 minutes, followed by 40 cycles at 95°C for 20 s, annealing temperature for 30 seconds, and 72°C for 30 seconds, in a 25-μL reaction volume containing 1 μL of bisulfite-treated genomic DNA, 300 nmol/L of each primer, 200 nmol/L of a fluorescent probe, and 12.5 μL of iQ Supermix (Bio-Rad). PCR conditions and primer and probe sequences were designed as previously described [22]. All reactions were performed in triplicate. The *CDO1* methylation value was defined as the ratio of the amplified signal value of methylated *CDO1* to the value of β-actin, which was subsequently multiplied by 100. The *CDO1* methylation value was designated as the TaqMeth value throughout the text.

Statistical analysis

Continuous and categorical variables were analyzed using the Student's t-test and χ^2 test, respectively. Univariate analyses of factors for the clinicopathological characteristics of EGC were performed using the log-rank method. The observation period was defined as the time from the first ESD to the day of the final upper gastrointestinal endoscopy (median, 59 months; range, 37–179 months). The median observation period was 59 months (37–66 months) in the nMGC group and 70 months (44–179 months) in the MGCSE group. The occurrence period for MGC was defined as the time from the day of the first ESD to the day of the second ESD (median, 22 months; range, 14–49 months). Statistical analyses were performed using the JMP 11 software (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at $P<0.05$.

RESULTS

Clinicopathological characteristics

Table 1 summarizes the clinicopathological characteristics of the 44 patients with EGC (nMGC group, n=33; MGCSE-1 group, n=11). With respect to *H. pylori* status at the time of ESD treatment, all 44 patients had been infected (current infection, n=16; after eradication, n=28; P=1.0000). Atrophic gastric mucosa was defined according to the Kimura-Takemoto classification [23-25]; all 44 patients (nMGC and MGCSE-1 group) had atrophic gastric mucosa (closed type: n=4, open type: n=40) (P=1.0000). With regard to histological type,

Table 1. Clinicopathological characteristics of the 44 patients with early gastric cancer

Variables		EGC (n=44)	nMGC group (n=33)	MGCSE-1 group (n=11)	P-value
Age	Median (range)	73 (58–85)	73 (58–85)	74 (65–84)	0.4319
Sex	Male	32 (72.7%)	25 (75.8%)	7 (63.6%)	0.4569
	Female	12 (27.3%)	8 (24.2%)	4 (36.4%)	
H. pylori status (during ESD)	Current infection	16 (36.4%)	12 (36.4%)	4 (36.4%)	1.0000
	After eradication	28 (63.6%)	21 (63.6%)	7 (63.6%)	
Atrophy	Closed type	4 (9.1%)	3 (9.1%)	1 (9.1%)	1.0000
	Open type	40 (90.9%)	30 (90.9%)	10 (90.9%)	
Location	Middle	26 (59.1%)	19 (57.6%)	7 (63.6%)	1.0000
	Low	18 (40.9%)	14 (42.4%)	4 (36.4%)	
Macroscopic type	Flat and elevated	16 (36.4%)	10 (30.3%)	6 (54.4%)	0.1694
	Flat and depressed	28 (63.6%)	23 (69.7%)	5 (45.6%)	
Histological type	Well-differentiated	39 (88.6%)	29 (87.9%)	10 (90.9%)	1.0000
	Moderately differentiated	5 (11.4%)	4 (12.1%)	1 (9.1%)	

EGC = early gastric cancer; nMGC = EGC with no confirmed evidence of metachronous lesions >3 years after curative ESD; MGC = metachronous gastric cancer; MGCSE = MGC developing on scars after curative ESD; MGCSE-1 = the EGC at the first ESD; ESD = endoscopic submucosal dissection.

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39 lesions were well-differentiated adenocarcinomas, whereas five lesions were moderately differentiated adenocarcinomas; however, the histological types were not significantly different among all patients (P=1.0000). Furthermore, among the MGCSE groups, the histological type of the lesion in the MGCSE-1 and MGCSE-2 groups was the same in 9 of 11 patients (**Supplementary Table 1**).

Quantification of *CDO1* promoter DNA methylation in the nMGC group

In the nMGC group, the *CDO1* TaqMeth values significantly differed between the T (n=33; median, 26.0; range, 3.1–81.2) and the TAM (n=33×4=132; median, 18.3; range, 0.0–65.8) tissues (P=0.0006; **Fig. 4A**). The *CDO1* TaqMeth values for TAM were significantly lower than those for T. In addition, our recent study on *CDO1* methylation status demonstrated that the methylation value was nearly zero (median, 0; range, 0.0–3.4) in 160 samples of the corresponding non-cancerous pancreas (non-CP) among patients with pancreatic ductal adenocarcinoma (PDAC) [26]. Therefore, considering the results of PDAC, the TaqMeth values for TAM in this study were surprisingly higher than expected. Thereafter, we quantified values in each of the TAMs separately (four points: oral TAM, anal TAM, right TAM, and left TAM) compared to the corresponding T value and found significant differences (P=0.0111, P=0.0451, P=0.0201, and P=0.0220, respectively; **Fig. 4B**).

Quantification of *CDO1* promoter DNA methylation in the MGCSE-1 group

In the MGCSE-1 group, no significant difference in *CDO1* TaqMeth values was identified between the tissue from the T (n=11; median, 40.1; range, 16.2–85.3) and TAM (n=11×4=44; median, 33.2; range, 7.1–100.6) (P=0.3914; **Fig. 4C**). Thereafter, we quantified the TAM values separately (four points: oral TAM, anal TAM, right TAM, and left TAM) in comparison with the corresponding T value, and found no significant differences (P=0.3606, P=0.9999, P=0.2535, and P=0.5242, respectively; **Fig. 4D**).

Quantification of *CDO1* promoter DNA methylation in the MGCSE-2 group

In the MGCSE-2 group, no significant difference in *CDO1* TaqMeth values was identified between the tissue from the T (n=11; median, 24.0; range, 8.6–47.2) and TAM (n=11×4=44; median, 24.1; range, 0.0–56.2) (P=0.5677; **Fig. 4E**). We subsequently quantified the TAM values separately (four points: oral TAM, anal TAM, right TAM, and left TAM) in comparison with the corresponding T value and found no significant differences (P=0.8779, P=0.3751, P=0.1948, and P=0.8386, respectively; **Fig. 4F**).

Unique characteristics of the TAM in the MGCSE-1 group

In all the groups (nMGC, MGCSE-1, and MGCSE-2 groups), we separately analyzed the values of T (**Fig. 5A**) and TAM samples (**Fig. 5B**). The median *CDO1* TaqMeth value for the T was 26.0 (range, 3.1–81.2) in the nMGC group (n=33), 40.1 (range, 16.2–85.3) in the MGCSE-1 group (n=11), and 24.0 (range, 8.6–47.2) in the MGCSE-2 group (n=11), albeit without significant differences (P=0.1096, P=0.6180, P=0.6314, respectively; **Fig. 5A**). The median *CDO1* TaqMeth value for the TAM was 18.3 (range, 0.0–65.8) in the nMGC group (n=132), 33.2 (range, 7.1–100.6) in the MGCSE-1 group (n=44), and 24.1 (range, 0.0–56.2) in the MGCSE-2 group (n=44), with significant differences between the MGCSE-1 group and the nMGC and MGCSE-2 groups (P<0.0001 and P=0.0041, respectively; **Fig. 5B**), and there was no significant difference between the nMGC and MGCSE-2 groups (P=0.0560). Intriguingly, no significant difference was detected between the TAM values in the MGCSE-1 group and the T value for the 55 cases pooled from all groups (P=0.3638; **Fig. 5C**).

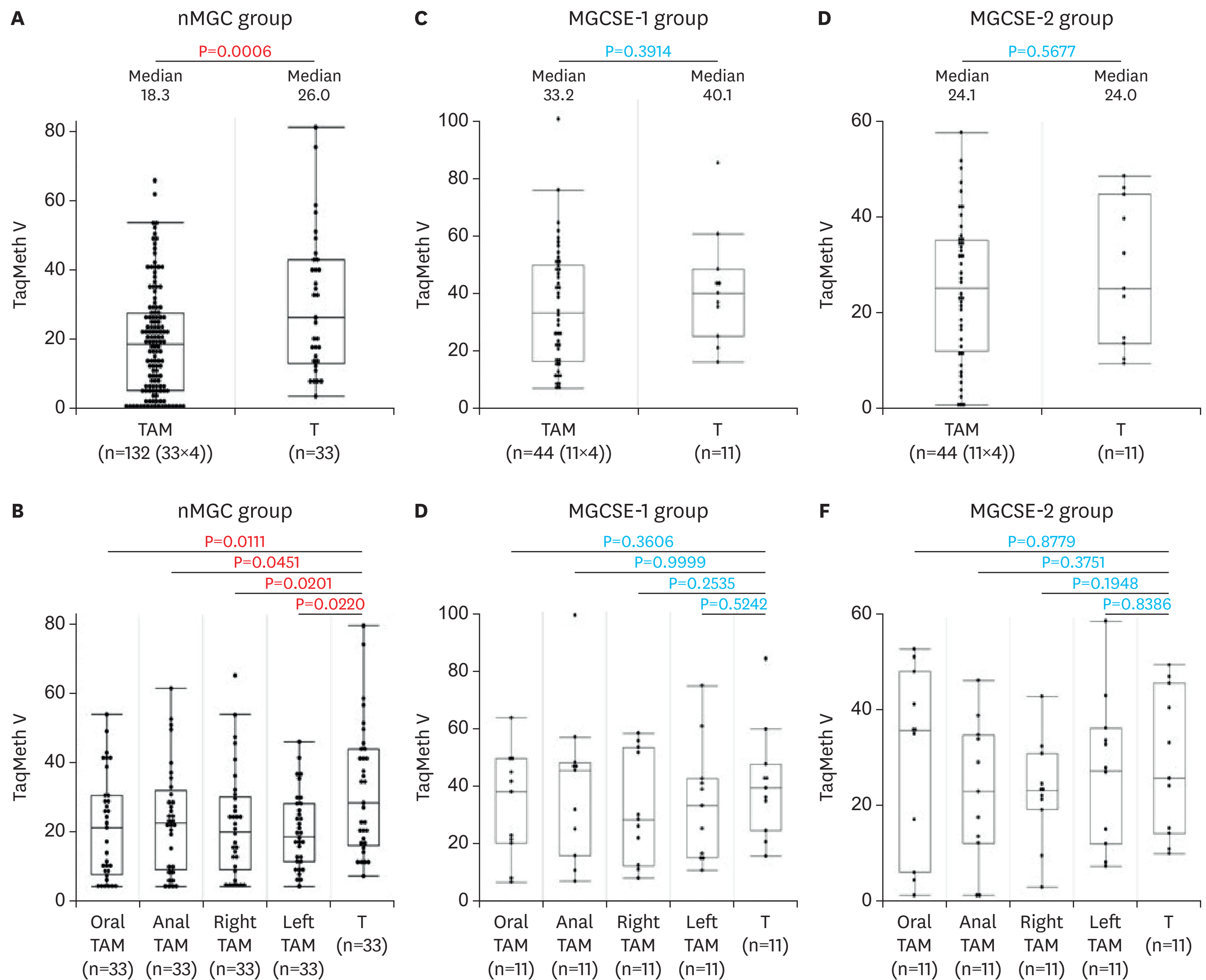

Fig. 4. Quantitative methylation-specific polymerase chain reaction for *cysteine dioxygenase type 1* in the T and the TAM in EGC. (A) In the nMGC group, there was a highly significant difference in the TaqMeth values of *CDO1* between the T and TAM tissues (P=0.0006). (B) TaqMeth values of *CDO1* for the T and the separate TAMs (four points: oral TAM, anal TAM, right TAM, and left TAM) in the nMGC group. Each comparison is significantly different (P=0.0111, P=0.0451, P=0.0201, and P=0.0220, respectively). (C) In the MGCSE-1 group, there was no significant difference in the TaqMeth values of *CDO1* between the T and TAM tissues (P=0.3914). (D) TaqMeth values of *CDO1* for the T and the separate TAMs (oral TAM, anal TAM, right TAM, and left TAM) in the MGCSE-1 group. None of the comparisons were significantly different (P=0.3606, P=0.9999, P=0.2535, and P=0.5242, respectively). (E) In the MGCSE-2 group, there was no significant difference in the TaqMeth values of *CDO1* between the T and TAM tissues (P=0.5677). (F) TaqMeth values of *CDO1* for T and the separate TAMs (oral TAM, anal TAM, right TAM, and left TAM) in the MGCSE-2 group. None of the comparisons were significantly different (P=0.8779, P=0.3751, P=0.1948, and P=0.8386, respectively).
TaqMeth values = methylation values; nMGC = EGC with no confirmed evidence of metachronous lesions >3 years after curative ESD; EGC = early gastric cancer; ESD = endoscopic submucosal dissection; TAM = tumor-adjacent noncancerous mucosa; T = tumor; MGCSE = metachronous gastric cancer developing on scars after curative ESD; MGCSE-1 = EGC at the first ESD; MGCSE-2 = EGC at the second ESD performed for treating MGC developing on scars after curative ESD.

Prediction according to the *CDO1* TaqMeth values in MGC

The optimal cut-off value for the prediction of MGC was analyzed from the TAM data in the nMGC and MGCSE-1 groups using receiver operating characteristic curves. The optimal TaqMeth value for all TAMs (n=176) in both groups was 29.1 (area under the curve [AUC], 0.74; P<0.0001; sensitivity, 56.8%; specificity, 79.6%; **Fig. 6A**). Furthermore, when the highest value for the separate four-point TAMs in both groups was extracted, the optimal TaqMeth value was 43.4 (AUC, 0.81; P<0.0001; sensitivity, 81.8%; specificity, 78.8%; **Fig. 6B**).

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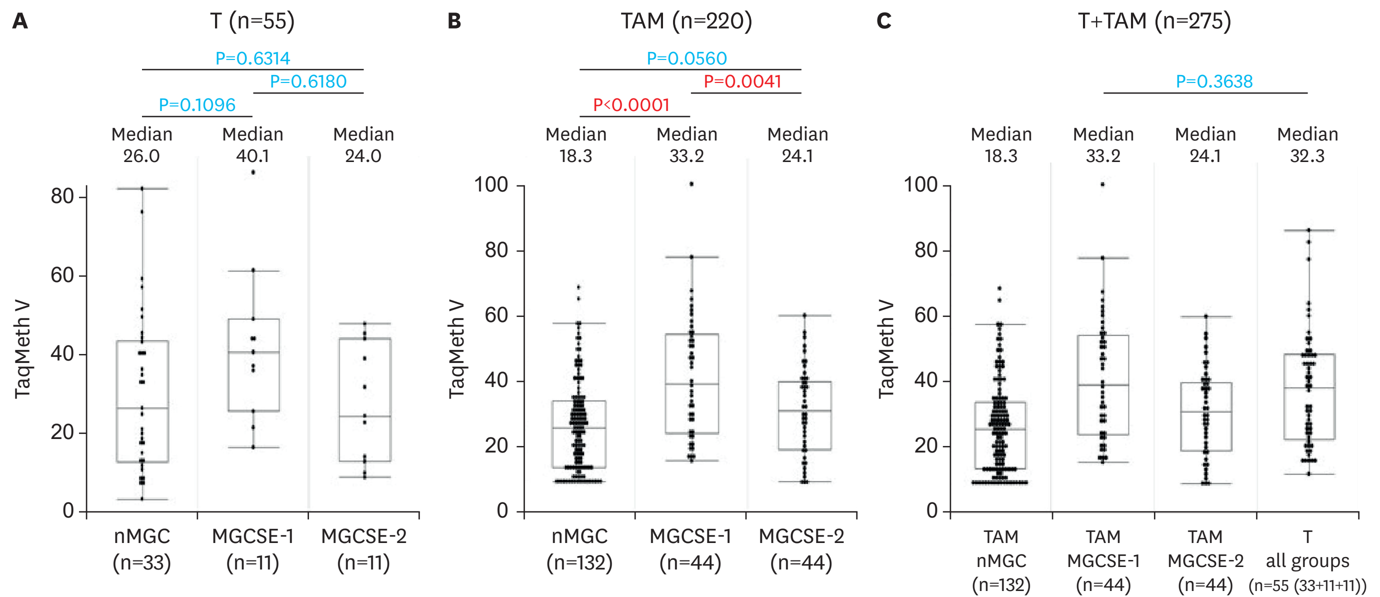

Fig. 5. Quantitative methylation-specific polymerase chain reaction for *CDO1* among the nMGC, MGCSE-1, and MGCSE-2 groups. (A) In the T tissue, there was no significant difference in the TaqMeth value of *CDO1* among the nMGC, MGCSE-1, and MGCSE-2 groups (P=0.1096, P=0.6180, P=0.6314, respectively). (B) In the TAM tissue, the TaqMeth value of *CDO1* in the MGCSE-1 group was significantly higher than that in the nMGC and MGCSE-2 groups (P<0.0001 and P=0.0041, respectively), although there was no significant difference between the nMGC and MGCSE-2 groups (P=0.0560). (C) In the T and TAM tissues, there was no significant difference in the TaqMeth values of *CDO1* between the pooled T tissue from all groups and the TAM in the MGCSE-1 group (P=0.3638).
T = tumor; TAM = tumor-adjacent noncancerous mucosa; TaqMeth values = methylation values; nMGC = EGC with no confirmed evidence of metachronous lesions >3 years after curative ESD; EGC = early gastric cancer; ESD = endoscopic submucosal dissection; MGCSE = MGC developing on scars after curative ESD; MGCSE-1 = EGC at the first ESD; MGCSE-2 = EGC at the second ESD for treating MGC developing on scars after curative ESD; MGC = metachronous gastric cancer; *CDO1 = cysteine dioxygenase type 1*.

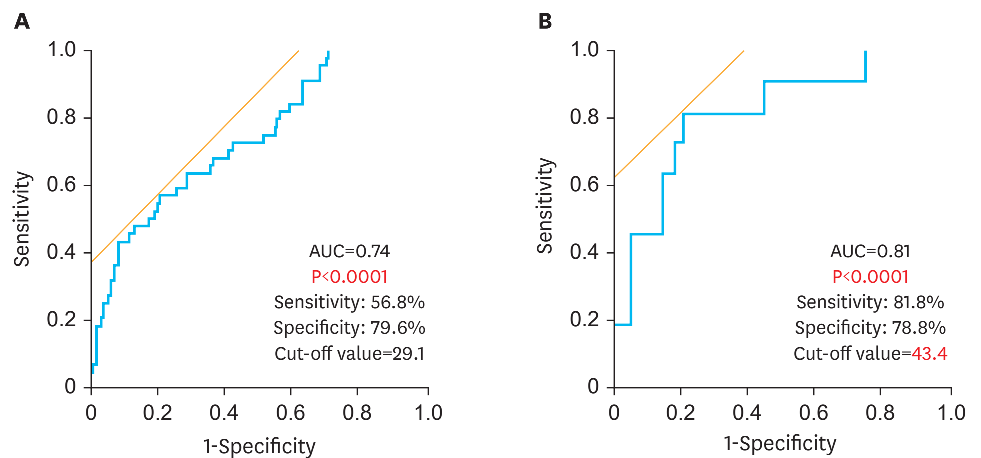

Fig. 6. Quantitative methylation-specific polymerase chain reaction for *cysteine dioxygenase type 1* at all points and the optimal cut-off value for MGC occurrence. (A) ROC curve for all TAMs (nMGC and MGCSE-1 groups). The AUC was 0.74, and there was a significant difference (P<0.0001). (B) ROC curve with the highest value for the separate four-point TAMs (nMGC and MGCSE-1 groups). The AUC was 0.81, and there was a significant difference (P<0.0001).
AUC = area under the curve; MGC = metachronous gastric cancer; ROC = receiver operating characteristic; TAM = tumor-adjacent noncancerous mucosa; nMGC = EGC with no confirmed evidence of metachronous lesions >3 years after curative ESD; MGCSE = MGC developing on scars after curative ESD; MGCSE-1 = EGC at the first ESD; EGC = early gastric cancer; ESD = endoscopic submucosal dissection.

The *CDO1* TaqMeth values for all cases are presented in **Supplementary Tables 2-4**. In seven cases (21.2%), the highest value for the TAM was equal to or greater than the cut-off value (43.4) among the 33 patients in the nMGC group. In contrast, in nine cases (81.8%), the

highest value for the TAM was equal to or greater than the cut-off value (43.4) among the 11 patients in the MGCSE-1 group. The values significantly differed between the MGCSE-1 and nMGC groups (P<0.0001) (**Supplementary Table 5**). In three cases (27.2%), the highest value for the TAM was equal to or greater than the cut-off value (43.4) among the 11 patients in the MGCSE-2 group.

DISCUSSION

This is the first study to report on the molecular alterations associated with MGC using specimens of MGC developing on scars after curative resection by ESD. MGCSE was detected in only 0.5% of all ESD cases. The median duration for the development of new atypical EGC on scars after ESD was 22 months (range, 14–49 months). The MGCSE-1 group was compared to the nMGC group, and no clear clinicopathological differences were identified them (**Table 1**). This result suggests that MGCSE might have formed because of molecular changes in the background atrophic mucosa. These changes may play a critical role in carcinogenesis among epigenetic factors. This is also the first report of the clinicopathological features of MGCSE.

The carcinogenic process in the gastric mucosa with atrophy is mainly attributable to epigenetic field abnormalities [10]. Atrophy related to old age and *H. pylori* infection is caused by irritation due to chronic inflammation [10,27]. Chronic inflammation leads to DNA and epigenetic abnormalities in the gastric mucosa [11,28]. Particularly, epigenetic alterations involved in gastric carcinogenesis are considered to contribute to a two-fold higher risk than that associated with esophageal squamous cell carcinoma [9], which may be due to so-called "field cancerization," in which epigenetic changes responsible for altering gene expression have already occurred in the background gastric mucosa [12]. Although the histological types of the MGCSE-1 and MGCSE-2 groups were almost equivalent in our study, the ESD results of all MGCSE groups showed that the lateral and horizontal margins were negative, and all cases achieved curative resection (R0 resection). Therefore, we suggest that MGCSE-1 was pathologically confirmed after curative resection by the first ESD and that MGCSE-2 was not caused by residual cancer. However, considering the presence of newly developed cancers in the same area, it is strongly suggested that the TAM in the MGCSE-1 group already harbored cancer-like changes. Furthermore, by measuring TAMs at four points rather than one, it is possible to confirm whether epigenetic changes have occurred in the entire TAM.

We focused on *CDO1*, a hypermethylated gene with particularly high specificity in human cancers because *CDO1* is the most promising candidate gene for evaluating cancer-specific epigenetic changes. *CDO1* is an enzyme that converts cysteine to cysteine sulfinic acid in cells, leading to an increase in SO_3^{2-} mediated by aspartate aminotransferase (GOT1) and replenishing cystine (CYS2) in the extracellular compartment [29]. xCT mediates the transport of cystine from the extracellular space to intracellular compartments, facilitating cancer stemness [29]. Its overexpression is accompanied by the production of nuclear factor erythroid 2-related factor 2 (NRF2), a central redox sensor, and results in the generation of reactive oxygen species, thereby promoting apoptosis [30].

Among the cancer-specific methylation genes in gastric cancer, *CDO1* has the highest AUC (0.95) for distinguishing cancerous from noncancerous tissues [14]. This finding suggests that *CDO1* methylation in cancer cells may be strongly associated with carcinogenesis. This study revealed that the TAM tissues in the nMGC group were hypermethylated, although

Journal of Gastric Cancer

not as much as the T tissue (**Fig. 4A**). Our previous study on *CDO1* methylation in PDAC showed that 90% of cancerous tissues were methylated, whereas non-CP tissues exhibited no methylation [26]. In addition, in remnant gastric cancer, *CDO1* was shown to be more highly methylated in cancerous tissue than in the noncancerous mucosa far from the tumor; however, its methylation level was detectable in the noncancerous mucosa [31]. Furthermore, *CDO1* methylation has been reported to be significantly higher in precancerous lesions of other cancers, such as small bowel cancer, colorectal cancer, and intraductal papillary mucinous neoplasm, than in noncancerous tissues [20,21,32]. This study demonstrated for the first time that *CDO1* hypermethylation in the TAM was more frequently detected in the MGCSE-1 group than in the nMGC group. In addition, the *CDO1* methylation values for the TAM in the MGCSE-1 group were significantly higher than that in the MGCSE-2 group (**Fig. 5B**). Moreover, there was no significant difference in the *CDO1* methylation values for the TAM between the MGCSE-2 and nMGC groups (**Fig. 5B**). Since there were no confirmed cases of a new MGCSE after the second ESD in the MGCSE-2 group, we propose that *CDO1* hypermethylation is involved in carcinogenesis. Moreover, using the optimal *CDO1* methylation value (43.4), we identified 9 out of 11 cases in the MGCSE-1 group. Maeda et al. reported an association between the development of MGC and hypermethylation of three genes (*miR-124a-3*, *EMX1*, and *NKX6-1*) in the gastric mucosa [33]. In this study, although the target genes were different, the TAM of the MGCSE group exhibited *CDO1* hypermethylation, indicating a high risk of carcinogenesis even after curative ESD.

Furthermore, MGCSE groups are considered useful as models for investigating the carcinogenesis of gastric cancer. The results of this study are essential, as the results in the MGCSE groups reflect epigenetic abnormalities that had already occurred. In predicting MGC, Asada et al. [34] examined gene methylation at a fixed point in the antrum (the lesser curvature, 2 cm from the pyloric ring) and reported a 2.3-fold increased risk of MGC. However, identifying the site of development of gastric cancer is difficult and cannot be accurately determined by prior biopsy. In contrast, in the MGCSE groups, MGC developed in the same location (MGCSE-2) as that of the first ESD, and the resection was curative (MGCSE-1). Furthermore, in the MGCSE-2 group, there was no development of MGCSE after the second ESD. Therefore, measuring *CDO1* methylation of the T and TAM in nMGC, MGCSE-1, and MGCSE-2 specimens may predict the occurrence of new cancers in the same location in which the first ESD was performed for EGC. Therefore, MGCSE may be an important model for predicting the development of EGC.

Our study has several important limitations. First, DNA methylation does not reflect a change that occurs in only a single gene; there is a strong association between methylated genes [35]. We have recently reported that when combined with *HOPX/Reprimo/CDH1*, *CDO1* methylation can predict future occurrences of remnant gastric cancer [31]. It has been shown that the analysis of methylation of only a single gene, *CDO1*, may predict MGC; however, other methylated genes should be considered in combination with *CDO1* for the prediction of MGC and in clinical applications. Second, we did not examine the case of MGC that did not develop scars after ESD in this study. We suggest that *CDO1* hypermethylation may be useful in predicting MGC compared to nMGC and MGCSE groups. However, since MGCSE is relatively rare, a comparison with MGC not developing on scars after ESD should be performed in the future to improve the accuracy of predicting MGC in *CDO1* hypermethylation. Furthermore, in this study, using the nMGC group as a control, the optimal methylation value of *CDO1* (43.4) was used to identify nine of the 11 cases in the MGCSE-1 group. However, among the nMGC group, we found seven cases in which the highest value for the TAM exceeded the

cut-off value (43.4). These cases need to be closely monitored because of the possibility of developing metachronous lesions.

In conclusion, MGCSE specimens can be used to evaluate *CDO1* DNA methylation. The results showed that *CDO1* hypermethylation of the TAM in the MGCSE-1 group was comparable to that of T. Thus, *CDO1* promoter DNA methylation was implicated in the occurrence of MGC and may be an important biomarker for predicting MGC.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Histological type in MGCSE groups (MGCSE-1 and MGCSE-2 groups)

Click here to view

Supplementary Table 2

CDO1 TaqMeth values in the nMGC group

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Supplementary Table 3

CDO1 TaqMeth values in the MGCSE-1 group

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Supplementary Table 4

CDO1 TaqMeth values in the MGCSE-2 group

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Supplementary Table 5

Comparison of the highest value TAM and the cut-off value (43.4) in the nMGC and MGCSE-1 groups

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REFERENCES

1. Fitzmaurice C, Abate D, Abbasi N, Abbastabar H, Abd-Allah F, Abdel-Rahman O, et al. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-

adjusted life-years for 29 cancer groups, 1990 to 2017: a systematic analysis for the Global Burden of Disease Study. JAMA Oncol 2019;5:1749-1768.
PUBMED | CROSSREF

2. Hori M, Matsuda T, Shibata A, Katanoda K, Sobue T, Nishimoto H, et al. Cancer incidence and incidence rates in Japan in 2009: a study of 32 population-based cancer registries for the Monitoring of Cancer Incidence in Japan (MCIJ) project. Jpn J Clin Oncol 2015;45:884-891.
PUBMED | CROSSREF
3. Japanese Gastric Cancer Association. Japanese gastric cancer treatment guidelines 2018 (5th edition). Gastric Cancer 2021;24:1-21.
PUBMED | CROSSREF
4. Nakajima T, Oda I, Gotoda T, Hamanaka H, Eguchi T, Yokoi C, et al. Metachronous gastric cancers after endoscopic resection: how effective is annual endoscopic surveillance? Gastric Cancer 2006;9:93-98.
PUBMED | CROSSREF
5. Fukase K, Kato M, Kikuchi S, Inoue K, Uemura N, Okamoto S, et al. Effect of *Helicobacter pylori* eradication on the incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomized controlled trial. Lancet 2008;372:392-397.
PUBMED | CROSSREF
6. Kamada T, Hata J, Sugiu K, Kusunoki H, Ito M, Tanaka S, et al. Clinical features of gastric cancer discovered after successful eradication of *Helicobacter pylori*: results from a 9-year prospective follow-up study in Japan. Aliment Pharmacol Ther 2005;21:1121-1126.
PUBMED | CROSSREF
7. Mori G, Nakajima T, Asada K, Shimazu T, Yamamichi N, Maekita T, et al. Incidence of and risk factors for metachronous gastric cancer after endoscopic resection and successful *Helicobacter pylori* eradication: results of a large-scale, multicenter cohort study in Japan. Gastric Cancer 2016;19:911-918.
PUBMED | CROSSREF
8. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature 2014;513:202-209.
PUBMED | CROSSREF
9. Yamashita S, Kishino T, Takahashi T, Shimazu T, Charvat H, Kakugawa Y, et al. Genetic and epigenetic alterations in normal tissues have differential impacts on cancer risk among tissues. Proc Natl Acad Sci U S A 2018;115:1328-1333.
PUBMED | CROSSREF
10. Niwa T, Tsukamoto T, Toyoda T, Mori A, Tanaka H, Maekita T, et al. Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells. Cancer Res 2010;70:1430-1440.
PUBMED | CROSSREF
11. Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, et al. High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. Clin Cancer Res 2006;12:989-995.
PUBMED | CROSSREF
12. Ushijima T, Hattori N. Molecular pathways: involvement of *Helicobacter pylori*-triggered inflammation in the formation of an epigenetic field defect, and its usefulness as cancer risk and exposure markers. Clin Cancer Res 2012;18:923-929.
PUBMED | CROSSREF
13. Ushijima T, Asada K. Aberrant DNA methylation in contrast with mutations. Cancer Sci 2010;101:300-305.
PUBMED | CROSSREF
14. Yamashita K, Hosoda K, Nishizawa N, Katoh H, Watanabe M. Epigenetic biomarkers of promoter DNA methylation in the new era of cancer treatment. Cancer Sci 2018;109:3695-3706.
PUBMED | CROSSREF
15. Brait M, Ling S, Nagpal JK, Chang X, Park HL, Lee J, et al. Cysteine dioxygenase 1 is a tumor suppressor gene silenced by promoter methylation in multiple human cancers. PLoS One 2012;7:e44951.
PUBMED | CROSSREF
16. Harada H, Hosoda K, Moriya H, Mieno H, Ema A, Ushiku H, et al. Cancer-specific promoter DNA methylation of Cysteine dioxygenase type 1 (CDO1) gene as an important prognostic biomarker of gastric cancer. PLoS One 2019;14:e0214872.
PUBMED | CROSSREF
17. Ushiku H, Yamashita K, Ema A, Minatani N, Kikuchi M, Kojo K, et al. DNA diagnosis of peritoneal fluid cytology test by CDO1 promoter DNA hypermethylation in gastric cancer. Gastric Cancer 2017;20:784-792.
PUBMED | CROSSREF

18. Ushiku H, Yamashita K, Katoh H, Ema A, Minatani N, Kikuchi M, et al. Promoter DNA methylation of CDO1 gene and its clinical significance in esophageal squamous cell carcinoma. Dis Esophagus 2017;30:1-9.
PUBMED

19. Kojima K, Yamashita K, Ushiku H, Katoh H, Ishii S, Tanaka T, et al. The clinical significance of cysteine dioxygenase type 1 methylation in Barrett esophagus adenocarcinoma. Dis Esophagus 2017;30:1-9.
PUBMED | CROSSREF

20. Kojima K, Nakamura T, Ooizumi Y, Igarashi K, Tanaka T, Yokoi K, et al. Clinical significance of cancer specific methylation of the CDO1 gene in small bowel cancer. PLoS One 2019;14:e0211108.
PUBMED | CROSSREF

21. Kojima K, Nakamura T, Ohbu M, Katoh H, Ooizumi Y, Igarashi K, et al. *Cysteine dioxygenase type 1 (CDO1)* gene promoter methylation during the adenoma-carcinoma sequence in colorectal cancer. PLoS One 2018;13:e0194785.
PUBMED | CROSSREF

22. Yamashita K, Waraya M, Kim MS, Sidransky D, Katada N, Sato T, et al. Detection of methylated CDO1 in plasma of colorectal cancer; a PCR study. PLoS One 2014;9:e113546.
PUBMED | CROSSREF

23. Kimura K, Takemoto T. An endoscopic recognition of the atrophic border and its significance in chronic gastritis. Endoscopy 1969;1:87-97.
CROSSREF

24. Kimura K, Satoh K, Ido K, Taniguchi Y, Takimoto T, Takemoto T. Gastritis in the Japanese stomach. Scand J Gastroenterol Suppl 1996;31:17-20.
PUBMED | CROSSREF

25. Quach DT, Hiyama T. Assessment of endoscopic gastric atrophy according to the Kimura-Takemoto classification and its potential application in daily practice. Clin Endosc 2019;52:321-327.
PUBMED | CROSSREF

26. Nishizawa N, Harada H, Kumamoto Y, Kaizu T, Katoh H, Tajima H, et al. Diagnostic potential of hypermethylation of the cysteine dioxygenase 1 gene (CDO1) promoter DNA in pancreatic cancer. Cancer Sci 2019;110:2846-2855.
PUBMED | CROSSREF

27. Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nat Genet 1994;7:536-540.
PUBMED | CROSSREF

28. Ando T, Yoshida T, Enomoto S, Asada K, Tatematsu M, Ichinose M, et al. DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. Int J Cancer 2009;124:2367-2374.
PUBMED | CROSSREF

29. Ishimoto T, Nagano O, Yae T, Tamada M, Motohara T, Oshima H, et al. CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc(-) and thereby promotes tumor growth. Cancer Cell 2011;19:387-400.
PUBMED | CROSSREF

30. Kang YP, Torrente L, Falzone A, Elkins CM, Liu M, Asara JM, et al. Cysteine dioxygenase 1 is a metabolic liability for non-small cell lung cancer. eLife 2019;8:e45572.
PUBMED | CROSSREF

31. Kojima K, Minatani N, Ushiku H, Ishii S, Tanaka T, Yokoi K, et al. Prediction of onset of remnant gastric cancer by promoter DNA methylation of *CDO1/HOPX/Reprimo/E-cadherin*. Oncotarget 2019;10:2423-2434.
PUBMED | CROSSREF

32. Fujiyama Y, Kumamoto Y, Nishizawa N, Nakamoto S, Harada H, Yokota K, et al. Promoter DNA hypermethylation of the *cysteine dioxygenase 1 (CDO1)* gene in intraductal papillary mucinous neoplasm (IPMN). Ann Surg Oncol 2020;27:4007-4016.
PUBMED | CROSSREF

33. Maeda M, Nakajima T, Oda I, Shimazu T, Yamamichi N, Maekita T, et al. High impact of methylation accumulation on metachronous gastric cancer: 5-year follow-up of a multicentre prospective cohort study. Gut 2017;66:1721-1723.
PUBMED | CROSSREF

34. Asada K, Nakajima T, Shimazu T, Yamamichi N, Maekita T, Yokoi C, et al. Demonstration of the usefulness of epigenetic cancer risk prediction by a multicentre prospective cohort study. Gut 2015;64:388-396.
PUBMED | CROSSREF

35. Toyota M, Ahuja N, Suzuki H, Itoh F, Ohe-Toyota M, Imai K, et al. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. Cancer Res 1999;59:5438-5442.
PUBMED