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Prospective study to validate the clinical utility of DNA diagnosis of peritoneal fluid cytology test in gastric cancer

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Abstract

The clinical efficacy of DNA cytology test (CY) in gastric cancer (GC) has been retrospectively proposed using cancer-specific methylation of *cysteine dioxygenase type 1* (*CDO1*). We confirmed the clinical utility of DNA CY in a prospective cohort. Four hundred GC samples were prospectively collected for washing cytology (UMIN000026191), and detection of the DNA methylation of *CDO1* was assessed by quantitative methylation-specific PCR in the sediments. Endpoint was defined as the match rate between conventional CY1 and DNA CY1 (diagnostic sensitivity), and the DNA CY0 rate (diagnostic specificity) in pStage IA. DNA CY1 was detected in 45 cases (12.5%), while CY1 was seen in 31 cases (8.6%) of 361 chemotherapy-naïve samples, where the sensitivity and specificity of the DNA CY in the peritoneal solutions were 74.2% and 96.5%, respectively. The DNA CY was positive for 3.5/0/4.9/11.4/58.8% in pStage IA/IB/II/III/IV, respectively ($P < .01$). In the multivariate analysis, DNA CY1 was independently correlated with pathological tumor depth (pT) ($P = .0012$), female gender ($P = .0099$), CY1 ($P = .0135$), P1 ($P = .019$), and carcinoembryonic antigen (CEA) ($P = .036$). The combination of DNA CY1 and P factor nearly all covered the potential peritoneal dissemination (P1 and/or CY1 and/or DNA CY1) (58/61:95.1%). DNA CY1 had a significantly poorer prognosis than DNA CY0 in GC patients ($P < .0001$). DNA CY1 detected by *CDO1* promoter DNA methylation has a great value to detect minimal residual disease of the peritoneum in GC clinics, representing poor prognosis as a novel single DNA marker.

KEYWORDS

Cysteine dioxygenase type 1, gastric cancer, methylation-specific PCR, peritoneal dissemination, washing cytology test

1 | INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy (1 033 371 cases in 2018) and the second leading cause of cancer-related death (782 685 deaths in 2018) worldwide.[1] Advanced GC defined as pathological tumor depth (pT) 2 or beyond has still poor survival outcomes despite progress in multidisciplinary therapy.[2-5] Among advanced GCs, tumors with macroscopic features of type III and type IV exhibited dismal prognosis as compared with type I/II/V tumors predominantly due to peritoneal recurrence including microscopic peritoneal dissemination representing positive peritoneal cytology test (CY1).[6] CY1 was recently acknowledged as a stage IV factor from a prognostic point of view in both the eastern and western countries.[7-9] Despite pathological CY0 confirmation after curative surgery, many tumors with macroscopic features of type III and type IV encountered peritoneal recurrences, suggesting that pathological CY0 is not sufficient to guarantee microscopic tumor–free status in GC clinics.

In fact, cancer-derived nucleotides from the tumor cells were actually detected by sensitive PCR test using carcinoembryonic antigen (CEA) mRNA, a specific marker for the epithelium-derived cells even in CY0 GC, and such tumors showed dismal prognosis.[10] However, mRNA is fragile, and clinical tests using mRNA may not be suitable as routine clinical tests and seem to have an obstacle for widespread prevalence. A DNA marker that can be used in cancer detection has been highly sought after due to the extraordinarily stable features of DNA. Obtaining such a DNA marker has great potential for general acceptance.

Promoter DNA methylation has an excellent potential if its frequency in primary cancer tissues is high; but such frequent methylation gene is rare,[11] and there have been no established DNA biomarkers for peritoneal metastasis of GC. Rigorous explorative studies searching for tumor-specific and frequent DNA methylation finally discovered *the cysteine dioxygenase type 1* (*CDO1*) gene as an ideal DNA marker with excellent performance capable of detecting minimal residual disease in GC.[11,12] Moreover, we previously showed the clinical potential of *CDO1* DNA methylation to detect minimal residual peritoneal disease of GC; however, the pilot study included few early GC cases.[13] *CDO1* DNA methylation as a single DNA marker was actually shown to be very promising (diagnostic accuracy was beyond 90%) as a cancer detection tool in human body fluids such as bile[14] and pancreatic juice[15] to diagnose hepatopancreato-biliary cancer. Here, a prospective study was conducted to validate the clinical utility of *CDO1* DNA methylation in a sufficient number of GC patients using intraperitoneal washing solution.

2 | MATERIALS AND METHODS

2.1 | Patients, tissue samples and clinicopathological factors

Between October 24, 2016 and January 18, 2019, a total of 400 samples (357 patients) with histologically confirmed primary GC underwent peritoneal lavage CY test at the Department of Surgery, Kitasato University School of Medicine, Sagamihara, Japan. Among the 31 CY1 GC samples, preoperative biopsy samples were available in 27.

Pathological tumor depth (pT) and pathological lymph node metastasis (pN) were classified according to the Japan Gastric Cancer Association (JGCA) Staging System, 15th edition.[16] The results of cytological examinations of either ascites or peritoneal lavage fluid were evaluated according to the JGCA and were classified as CY1, CY0, and CYX (not assessed by CY test).

Other clinicopathological factors included age, gender, macroscopic feature, surgical approach (open vs laparoscopic vs robotic method), surgical procedures (gastrectomy vs staging surgery), adjuvant chemotherapy, neoadjuvant chemotherapy, lymphatic permeation, vascular permeation, pretreatment value of serum CEA, and serum carbohydrate antigen 19-9 (CA19-9). Macroscopic features were based on Borrmann classification (type I: polypoid; type II: fungating, ulcerated with sharp raised margins; type III: ulcerated with poorly defined infiltrative margins; type IV: infiltrative, predominantly intramural lesion, poorly demarcated; type V: unclassified feature).

The stage diagnosed by staging laparotomy is pStage IV if CY1 or peritoneal dissemination is positive despite deficient information of pT/pN (pTX/pNX).

2.2 | Ethics approval and consent to participate

This study was approved by the Kitasato University Ethics Committee (number B16-58) and registered under UMIN000026191. This trial followed the ethical principles of the Declaration of Helsinki and the Japanese Ethical Guidelines for Clinical Research. All patients provided written informed consent. Eligibility criteria included pathologically proven primary gastric adenocarcinoma, with written informed consent before surgery, and a minimum age of 20 years.

2.3 | Endpoints

The primary endpoint was the concordance rate between conventional CY test and DNA-CY test (diagnostic sensitivity), and the DNA CY0 rate in early GC defined by pStage IA (diagnostic specificity). Moreover, the contribution of the DNA CY to prognosis was the secondary endpoint.

2.4 | Genomic DNA extraction and bisulfite treatment

A total of 200 ml normal saline was put into the upper abdominal cavity, and 50 mL peritoneal washing fluids were collected for both conventional CY test and DNA CY test, respectively.

Genomic DNA from the 400 peritoneal CY sediments after two spin protocols (2 262 ***g*** for 20 minutes and 14 256 ***g*** for

15 minutes) was extracted using QIAamp DNA Mini Kit (QIAGEN Sciences). Formalin-fixed paraffin-embedded tissue from preoperative biopsy samples were cut into six 10-μm-thick slices before genomic DNA extraction using the QIAamp DNA FFPE Tissue Kit (Qiagen).

Bisulfite treatment was done by using an EZ DNA Methylation-Gold Kit (Zymo Research), and the bisulfite-treated DNA was subjected to PCR.

2.5 | Quantitative methylation-specific PCR (Q-MSP)

For Q-MSP of *CDO1*, we performed real-time PCR using iQ Supermix (Bio-Rad) and CFX96 real-time systems. The TaqMeth V was defined as the quantity of fluorescence intensity derived from promoter amplification of *CDO1* divided by fluorescence intensity from *β-actin* amplification and multiplied by 100. This ratio represents the relative level of methylated DNA in samples.[12] All reactions were performed in triplicate.

2.6 | Statistical analyses

All statistical analyses were performed using JMP 14 software (SAS Institute Inc). Frequency tables were analyzed using the χ^2 test, with likelihood ratio or Fisher's exact test, to determine the significance of differences between categorical variables. Differences between the results of comparative tests were considered significant if the two-sided *P*-value was less than .05. Survival was calculated by the Kaplan-Meier method. Univariate analyses of prognostic factors for overall survival (OS) and peritoneal dissemination–free survival (PDFS) were performed using the log-rank method. PDFS was defined as time from surgery to death or recurrence of peritoneal dissemination. The median follow-up was 26 months (range 1-43 months).

3 | RESULTS

3.1 | Conventional peritoneal lavage CY test in GC according to tumor progression

Our total samples of the conventional CY test in GC were 400 from 357 patients. Among them, naïve samples with no prior chemotherapy were 361 from 346 patients. The fifteen redundant patients were largely examined for both staging laparotomy at the first-time operation and subsequent laparotomy with curative intent at the second-time operation (n = 12) (Table S1), among which the duplicate preoperative staging determined by the conventional CY test was nearly all consistent (n = 11). Other three redundant cases were examined for primary tumor resection at the first-time operation and subsequent second-time operations for suspected recurrence by staging laparotomy (n = 1), positive stump resection (n = 1), and small bowel obstruction (n = 1).

The 361 samples included 25 samples (from 25 patients, see Figure S1) who had staging laparotomy before chemotherapy, and the cumulative total number of the 361 samples were composed of 171 in pStage IA, 23 in pStage IB, 61 in pStage II, 35 in pStage III, 20 in pStage IV with CY0, 31 pStage IV with CY1, and 20 in pStage X. Among the 361 GC samples, CY1 was recognized in 8.6% (31/361). The conventional CY1 was never confirmed in GC with pT1 to pT3 or in that with pN0 to pN1. CY1 was seen in 13.2% of pT4 and 35.1% in pTX (Figure 1A), or in 9.1% in pN2, 12.5% in pN3, and 35.6% in pNX (Figure 1B).

3.2 | Peritoneal lavage DNA CY test in GC according to tumor progression

Among the 361 GCs, DNA CY1 was recognized in 12.5% (45/361), and this frequency was higher than the conventional CY1 (8.6%: 31/361) ($P = .09$). TaqMeth values of *CDO1* in the peritoneal fluids of the 361 GC samples ranged from 0 to 43.47 (average 0.78) (Figure 2A upper panel). The 45 DNA CY1 samples are magnified in the lower panel of Figure 2A. We showed concrete Q-MSP curves of the representative samples (*CDO1* TaqMeth V, 39.8, 2.7, 1.2, 0.3, 0.003/sample numbers, 202, 156, 123, 74, 145) (Figure 2B upper and lower panel). DNA CY1 was recognized in 3.1% (6/194) of pStage I, 4.9% (3/61) of pStage II, 11.4% of pStage III (4/35), in 35% (7/20) of pStage IV with CY0, and 74.2% (23/31) of pStage IV with CY1, and this increase is statistically significant ($P < .01$ excluding pStage X, Figure 1C). DNA CY1 was seen in 35% of pStage IV with CY0 (n = 7), and intriguingly all the seven cases had macroscopic peritoneal metastasis (CY0P1).

On the other hand, among the 31 CY1 samples, DNA CY1 was positive in 23 (74.2%), and the remaining eight samples were negative in the DNA CY test. This positive rate (diagnostic sensitivity) of 74.2% is one of the primary endpoints of the DNA CY test in this prospective study. The diagnostic specificity of the alternative primary endpoint was proven to be 96.5%, as 165 of the 171 pStage IA samples were confirmed to be negative for DNA cytology test.

3.3 | Clinicopathological relevance of DNA CY1 in GC

DNA CY1 was then assessed according to various clinicopathological factors (Table 1). In the univariate analysis, DNA CY1 was significantly associated with gender ($P = .026$), preoperative serum CEA ($P < .0001$), preoperative serum CA19-9 ($P < .0001$), surgical procedure (staging laparotomy) ($P < .0001$), tumor location ($P < .0001$), morphological type ($P < .0001$), histological type ($P < .0001$), synchronous multiple GC ($P < .0001$), pT ($P < .0001$), pN ($P < .0001$), distant metastasis ($P < .0001$), peritoneal dissemination ($P < .0001$), CY1

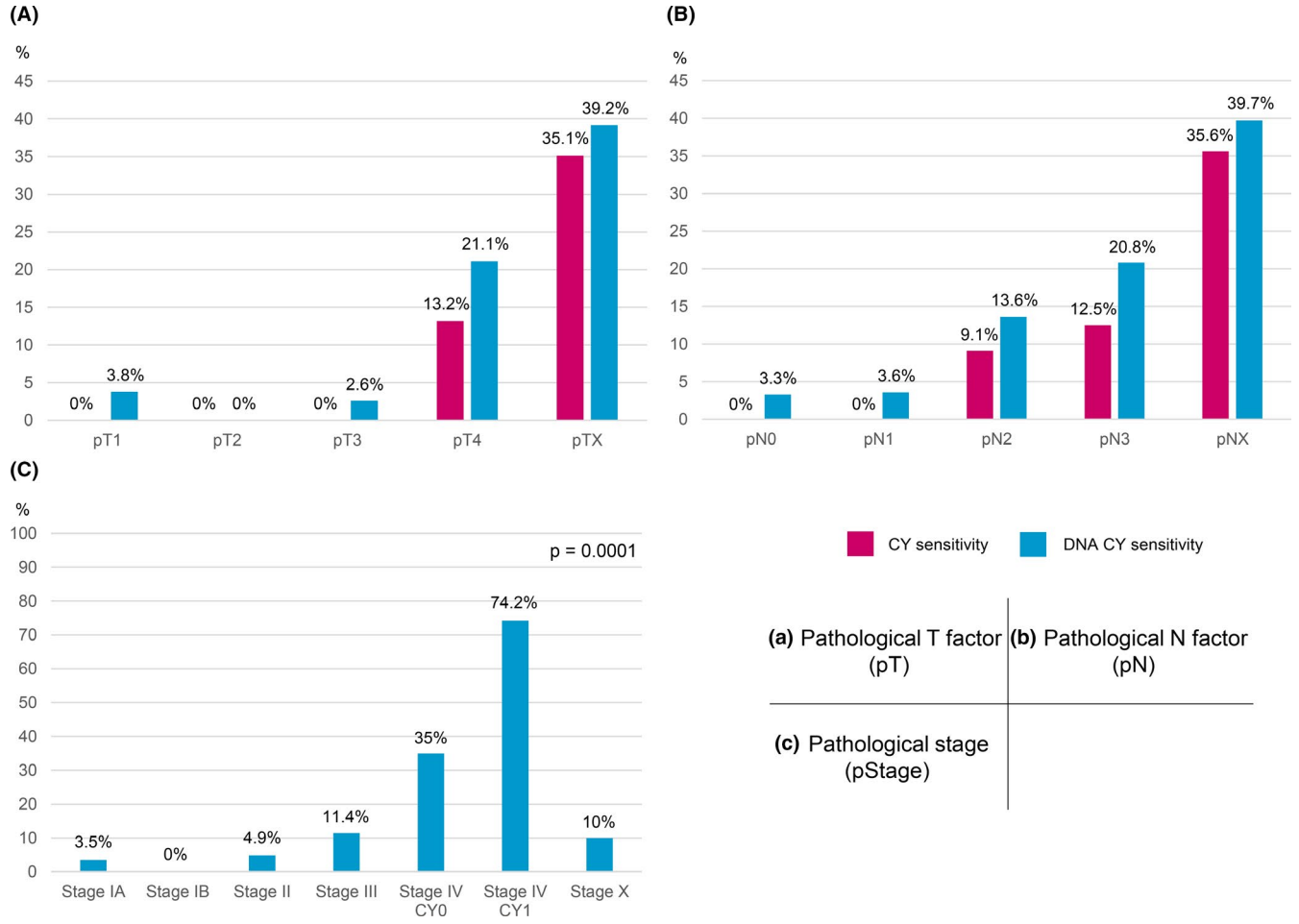

FIGURE 1 Bar graphs representing diagnostic sensitivity of the conventional CY1 (red bars) and the DNA CY1 (blue bars) according to pathological factor of gastric cancer (GC). A, Pathological T factor. B, Pathological N factor. C, Pathological stage

($P < .0001$), lymphatic permeation ($P < .0001$), vascular permeation ($P < .0001$), and pStage ($P < .0001$).

Multivariate regression analysis for DNA CY1 identified pT ($P = .0012$, LogWorth = 2.937), gender ($P = .0099$, LogWorth = 2.004), CY1 ($P = .0135$, LogWorth = 1.869), peritoneal dissemination ($P = .0193$, LogWorth = 1.714), and preoperative serum CEA ($P = .0359$, LogWorth = 1.445).

3.4 | Prognostic analysis of CY and DNA CY in GC patients

As shown in Table S1, of the 361 samples who did not receive chemotherapy prior to surgery, 15 patients had surgery twice, 346 naive patients were included in the analysis (Table S2). Prognostic analysis of the 346 GC patients showed that the 3-year OS of DNA CY1 was 36.2%, while that of DNA CY0 was 84.3%, so DNA CY1 had a significantly poorer prognosis than DNA CY0 ($P < .0001$) (Figure 3A). In 165 patients excluding pT1, the 3-year OS of DNA CY1 was 24%, and that of DNA CY0 was 71.9%, so DNA CY1 had a significantly poorer prognosis than DNA CY0 ($P < .0001$) (Figure 3B). Prognostic analysis was performed by dividing the 346 GC patients into three groups: DNA CY0 (n = 303), DNA CY1/CY0 (n = 21), and DNA CY1/CY1 (n = 22). The 3-year OS was 84.3%, 43.6%, and 28.6%, respectively; the DNA CY1/CY1 group had the poorest prognosis with regard to OS ($P < .0001$) and PDFS ($P < .0001$), respectively (Figure 3C, D), suggesting that DNA CY1 can reinforce the prognostic relevance of the peritoneal CY test.

The prognosis was compared in GC with DNA CY1/CY0 (n = 21), DNA CY0/CY1 (n = 8), and DNA CY1/CY1 (n = 22) to assess the prognostic significance of false positive cases with DNA CY1 defined using *CDO1* methylation. There was no significant difference in the OS and PDFS of the three groups ($P = .4996$, $P = .3666$) (Figure 3E, F). Further, we also examined the natural history of DNA CY1/CY0 (n = 21), which had seven cases with P1. At present, three cases of the seven P1 cases died from progression of peritoneal dissemination, whereas seven cases of the remaining 14 cases of P0 have already died, four cases of whom died due to progression of GC metastasis such as peritoneal dissemination, lymph node metastasis, and liver metastasis.

Multivariate prognosis analysis was performed for advanced GC excluding pT1, and DNA CY1 was an independent poor prognostic factor (HR 2.28, 95% CI 1.04-4.93, $P = .0395$) different from the conventional CY (HR 1.47, 95% CI 0.62-3.49, $P = .3813$) (Table S3).

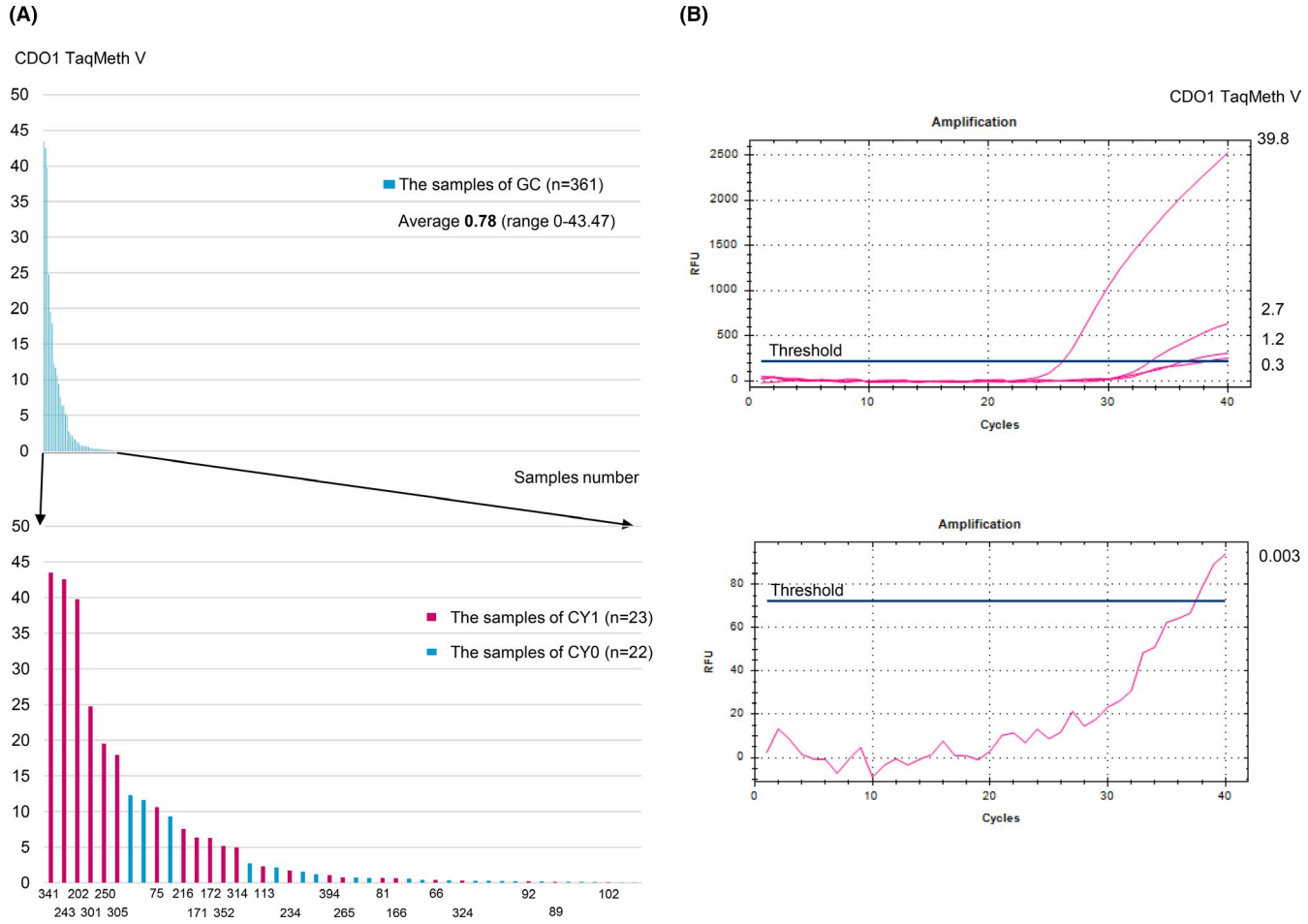

FIGURE 2 Quantitative methylation-specific PCR (Q-MSP) by using *CDO1* DNA methylation. A, TaqMeth values of *CDO1* in the peritoneal fluid samples of the 361 gastric cancers (GCs) (upper panel). The quantified outcomes of the DNA CY1 are magnified (lower panel). In the lower panel, red bar graphs represent conventional CY1, while blue graphs indicate conventional CY0. B, Q-MSP curves of the representative cases. *CDO1* TaqMeth values (Vs) were definitely represented as 39.8, 2.7, 1.2, and 0.3 in sample numbers 202, 156, 123, and 74, respectively (upper panel), while the minimum *CDO1* TaqMeth V 0.003 among DNA CY1 cases was judged as positive in sample number 145

3.5 | *CDO1* methylation status of preoperative tumor biopsy samples in GC with CY1

CDO1 methylation status of preoperative tumor biopsy samples was then assessed in GC with CY1 because some of the patients underwent preoperative chemotherapy, and resected tumors were considered to be modified by chemotherapy. Among the 31 GCs with CY1, 27 cases were available for preoperative tumor biopsy samples, and assessed by Q-MSP. Among the 27 cases, 19 showed DNA CY1 (70.4%) (Figure 4), among whom 18 exhibited definite hypermethylation of *CDO1* in primary tumors from preoperative biopsy samples. From the eight DNA CY0 cases, seven (cases 275, 296, 340, 289, 332, 76 and 50; see red circle) showed clear hypermethylation of *CDO1* in biopsy samples from primary tumor tissues, and only one patient (case 265) did not exhibit *CDO1* hypermethylation. These findings indicated that DNA CY0 in CY1 GC samples was not mainly explained by its deficient methylation of *CDO1* in the primary tumors.

3.6 | Clinical course follow-up of DNA CY test in GC patients who underwent surgery with prior chemotherapy

The cumulative total number of the 361 patients included 25 patients who underwent staging laparotomy (Figure S1). After combining the 25 samples (from 25 patients) with 39 samples (from 30 patients), 40 samples from 18 patients were informative for clinical course of the follow-up data of GC with preoperative chemotherapy followed by operation (Figure S1, and Table 2). Among the 18 patients who underwent staging laparotomy and subsequent chemotherapy (neoadjuvant chemotherapy, n = 12 and conversion surgery, n = 6), DNA CY1 was seen in five patients who were initially diagnosed as CY1P0 (n = 2, conversion surgery), CY1P1 (n = 1, conversion surgery), CY0P1 (n = 1, conversion surgery), and CY0P0 (n = 1, neoadjuvant chemotherapy).

Among the five cases with DNA CY1, all cases became DNA CY0/CY0 at radical gastrectomy after chemotherapy, suggesting

TABLE 1 Clinicopathological characteristics of DNA CY in 361 naïve samples with no prior chemotherapy

	Univariate analysis			Multivariate analysis	
Variable	**DNA CY0 (n = 316)**	**DNA CY1 (n = 45)**	***P*-value**	**LogWorth**	***P*-value**
Age (years)			.5534		
<75/>74	231/85	31/14			
Gender			.026	2.004	.0099
Male/female	227/89	25/20			
Serum CEA			<.0001	1.445	.0359
<5/>4	275/41	26/19			
Serum CA19-9			<.0001	0.482	.3296
No detection/<37/>36	3/281/32	0/30/15			
ASA-PS			.878		
ASA-PS 1/2/3	49/244/23	6/35/4			
Surgical approach			.0652		
OS/LS/RS	72/205/39	15/29/1			
Surgical procedure			<.0001	0.001	.9981
Gastrectomy/staging surgery	272/44	16/29			
Tumor location			<.0001	0.53	.2949
X/U/M/L/R	1/88/113/101/13	4/14/9/14/2			
Morphological type			<.0001	1.194	.064
Type X/0/1/2/3/4/5	45/184/8/28/32/7/12	29/5/0/3/6/1/1			
Histological type			<.0001	1.234	.0584
Type X/Ind/Int/Diff/Mix	44/4/90/67/111	29/0/7/1/8			
Synchronous multiple gastric cancer			<.0001	0.172	.6722
X/presence/absence	44/34/238	1/15/2029			
Depth of invasion (pT)			<.0001	2.937	.0012
pTX/1a/1b/2/3/4a/4b	45/80/95/29/37/29/1	29/1/6/0/1/8/0			
Lymph node metastasis (pN)			<.0001	0.143	.7193
pNX/0/1/2/3a/3b	44/207/27/19/9/10	29/7/1/3/2/3			
Distant metastasis (M)			<.0001	0.101	.7928
M0/1	295/21	15/30			
Peritoneal dissemination (P)			<.0001	1.714	.0193
P0/1	303/13	21/24			
Peritoneal cytological test (CY)			<.0001	1.869	.0135
CY0/1	308/8	22/23			
Lymphatic permeation (Ly)			<.0001	0.21	.6166
LyX/0/1a/1b/1c	45/190/36/23/22	29/4/3/3/6			
Vascular permeation (V)			<0.0001	0.22	.6023
VX/0/1a/1b/1c	45/170/43/40/18	29/5/2/3/6			
Pathological stage (pStage)			<.0001		
pStage X/IA/IB/II/III/IVCY0/IVCY1	18/165/23/58/31/13/8	2/6/0/3/4/7/23			

Abbreviations: ASA-PS, American Society of Anesthesiologists physical status; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; Diff, diffuse type; Ind, indeterminate; Int, intestinal type; L, lower site; LS, laparoscopic surgery; M, middle site; Mix, mixed type; OS, open surgery; R, residual stomach; RS, robotic surgery; U, upper site.

that downstage by preoperative treatments was successful. In the 18 GC patients with preoperative chemotherapy, DNA CY1 was significantly associated with CY1 ($P = .0022$), while it was marginally correlated with peritoneal dissemination ($P = .0995$) (Table S4). These findings suggested that DNA CY test could be supplemented with conventional CY test to increase diagnostic accuracy.

Of the five DNA CY1 cases before chemotherapy in Table 2, four had either CY1 or P1, whereas the remaining one case was CY0/P0,

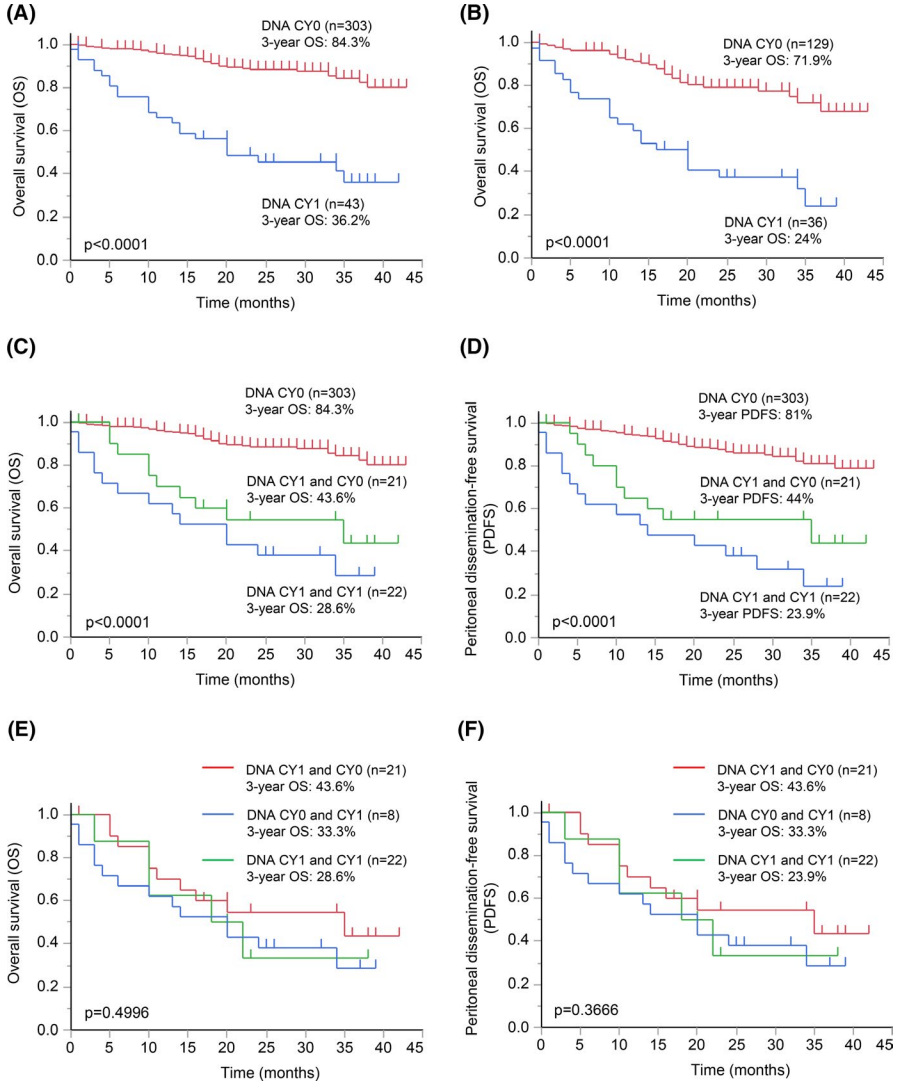

FIGURE 3 Prognosis analysis in DNA CY and CY. A, Overall survival of 346 gastric cancer (GC) patients associated with DNA CY. B, Overall survival of 165 advanced GC patients associated with DNA CY. C, Overall survival of 346 GC patients stratified according to the results of DNA CY and CY. D, Peritoneal dissemination–free survival of 346 GC patients stratified according to the results of DNA CY and CY. E, Overall survival of 51 GC patients with DNA CY and/or CY positive. F, Peritoneal dissemination–free survival of 51 GC patients with DNA CY and/or CY positive

which could have been false positive, but this patient died of the progression of GC 35 months after the start of treatment. DNA CY1 before chemotherapy, which might include false positive, showed aggressive clinical behaviors (Table 2).

4 | DISCUSSION

Our previous pilot study elucidated that DNA CY1 by *CDO1* methylation in Q-MSP was recognized in 100% (8/8) of CY1 GCs, and 0% (0/5) of pStage I GCs, with a DNA CY1 detection rate (17.6%: 18/102) superior to the detection rate of conventional CY1 (8%: 8/102) in GC.[13] Such diagnostic sensitivity and specificity are primary endpoints of our current study, as the previous pilot study exhibited highly promising clinical utility (100% sensitivity and 100% specificity) of DNA CY1 assessed by the signal DNA maker to predict peritoneal micrometastasis in GC clinics.

So, a prospective study has been conducted for validation in the current research, where the diagnostic sensitivity and specificity of DNA CY1 were 74.2% (23/31) and 96.5% (165/171), respectively; the diagnostic specificity was again confirmed as excellent. Moreover, DNA CY1 was detected in 12.5% (45/361), which is superior to the conventional CY1 (8.6%: 31/361). If limited to advanced GC (excluding pT1 GC), DNA CY1 was detected in 21.2% (38/179), which is consistent to our early report (18.6%: 18/97).[13] These results suggested that DNA CY test using the single-gene methylation is highly reproducible with regard to peritoneal diagnosis, especially in advanced GC.

This excellent sensitivity by the single-gene analysis is comparable with the previous report of the multiplex methylation analysis of three genes (*CHFR/E cadherin/BNIP3*) (sensitivity 57%)[17] and seven genes (*BNIP3/CHFR/CYP1B1/MINT25/SFRP2/RASSF2*) (sensitivity 75%)[18] in GC. In addition, multiple methylation analysis (*CDH1/p16/MGMT/APC*) in colorectal cancer reported that 25% of stage IV colorectal cancers that were positive for methylation in peritoneal lavage had peritoneal dissemination.[19] In our current study, DNA CY1 was confirmed in 35% (seven cases) of stage IV GCs with CY0, and all of the seven cases had macroscopic peritoneal dissemination (CY0P1). We recently reported that the methylation value of *CDO1* was significantly higher in patients who had

FIGURE 4 Detailed assessment for the conventional CY1 cases (n = 27). In quantitative methylation-specific PCR (Q-MSP), *CDO1* TaqMeth values (Vs) of the preoperative tumor biopsy samples (blue bars) were compared with those of the intraperitoneal fluid samples (red bars). Left scale represents *CDO1* TaqMeth V in Q-MSP. The red-circled samples were judged to be negative in Q-MSP because *CDO1* TaqMeth V was 0

primary GC tumors with macroscopic peritoneal dissemination than in others ($P < .0001$).[12] This finding indicates that *CDO1* methylation may be prone to identifying microscopic dissemination in GC with macroscopic peritoneal dissemination. These extraordinarily high performances of the single-gene analysis to detect peritoneal dissemination including micrometastasis of GC is due to its excellent performance in methylation profiles of primary GC tumors in contrast to noncancerous mucosa tissues (Area under the curve = 0.95),[11,20] where there have been few cancer tissues with no promoter DNA methylation of *CDO1*.

There are many reports using *CEA* mRNA for the detection of microscopic cancer cells in GC peritoneal lavage. A recent report found that 72% of CY positive cases were *CEA* mRNA positive and 85.4% of pT1 cases were *CEA* mRNA negative. This result was as sensitive as in this study (74.2%). DNA CY0 in pT1 cases was 96.2%, which was higher in this study.[21] Inferring from the diagnostic accuracy of *CEA* mRNA, the accuracy of this study (DNA CY) using DNA, which is more stable than mRNA, may contribute to the improvement of peritoneal dissemination diagnosis in the future.

The multivariate logistic regression analysis for DNA CY1 elucidated macroscopic peritoneal dissemination and conventional CY1 as independent predictors (Table 1), proposing a close association of the three factors P1, CY1, and DNA CY1 (see Figure S2A). As P1 and CY1 are both definitive peritoneal diseases of GC predicting future recurrences, DNA CY1 detected by *CDO1* methylation must also represent potential peritoneal disease rather than false positive. Interestingly, P1 together with DNA CY1 covers almost all potential peritoneal disseminations (95.1%: 58/61) (light blue bar in Figure S2B). The diagnostic accuracy of peritoneal dissemination was supposed to be increased by addition of DNA CY1 (n = 13) to the current clinics. According to our current study, DNA CY1 could elevate the diagnostic potential of micrometastasis in the peritoneum by 22% (Figure S2).

Our current study has also clarified the prognostic potential of DNA CY test for GC. DNA CY1 had a significantly poorer prognosis than DNA CY0, with similar results in advanced GC without pT1 (Figure 3B). In addition, from this current study, the data suggested that DNA CY can predict prognosis more precisely than conventional CY tests. However, the observation period was still short, and further follow-up period will be required for the final confirmation (Table S3). Importantly, the significant association between DNA CY1 and CY1P1 (Figure S2) may suggest that DNA CY1 has a bona fide potential utility as a clinical prognostic biomarker in GC patients. On the other hand, prospective studies in stage II/III GC are greatly needed with regard to

TABLE 2 Clinical course follow-up of DNA CY test in 18 gastric cancer patients with prior chemotherapy

Patients number	Chemotherapy	1st			2nd			3rd			Radical surgery			
		DNA CY	CY	P	DNA CY	CY	P	DNA CY	CY	P	DNA CY	CY	ypStage	TE
1	DOS	0	0	0							0	0	II	1
2	DOS	0	0	0							0	0	IA	2
3	S1 + leucovorin + oxaliplatin	1	1	0							0	0	II	2
4	DCS	0	0	0							0	0	II	2
5	SP + trastuzumab	0	0	0							0	0	IVCY0	1
6	DCS	0	0	0							0	0	III	1
7	DCS	1	1	0	0	0	0				0	0	III	2
8	DCS	1	1	1							0	0	II	1
9	DCS	0	0	0							0	0	III	1
10	DCS	0	0	0							0	0	II	2
11	DCS	0	0	1	0	1	1	0	1	0	0	0	III	1
12	DOS	0	0	0							0	0	II	1
13	DOS	0	0	0							0	0	IA	2
14	XP + trastuzumab	1	0	1							0	0	IA	2
15	SOX	0	0	0							0	0	II	1
16	DCS	0	0	0							0	0	III	1
17	DCS	0	0	0							0	0	II	2
18	DOS	1	0	0							0	0	II	1

Abbreviations: CY, washing cytology test; DCS, docetaxel + cisplatin + S1; DOS, docetaxel + oxaliplatin+S1; P, peritoneal dissemination; SOX, S1 + oxaliplatin; SP, S1 + cisplatin; TE, therapeutic effect; XP, capecitabine + cisplatin.

DNA CY test because the result would affect therapeutic strategies including postoperative adjuvant chemotherapy. In this current study, however, our primary endpoint is to know diagnostic specificity rather than such a clinical utility including therapeutic strategy, as early-stage GC is predominant, and our patient cohort is not appropriate for prognostic analysis of stage II/III GC patients. We propose a more highly warranted clinical study in the near future.

CDO1 methylation in primary GC tissues was significantly higher in CY1 than in CY0 ($P = .0002$),[12] and our current study also showed that the methylation value of *CDO1* in nearly all biopsy samples from the primary tumor tissues with CY1 was high (see Figure 4). Twenty-five (92.6%) from the 27 primary tumors showed definite methylation in primary tumors. That is why false negative case was considered below 10%. Of the 31 cases of pStage IV with CY1, eight cases were DNA CY0 and 23 cases were DNA CY1. As shown in Table S5, fewer cancer cells were confirmed in the peritoneal washing fluid of the 23 DNA CY0 patients than in that of the eight DNA CY1 patients ($P = .0029$). The amount of cancer cells was classified based on the reports submitted by pathologists. In the report description, small amount was described as "isolated" and large amount was described as "conglomerated." Interestingly, comparing both groups with clinical pathological factors, the DNA CY0 group has more cases with normal serum CEA ($P = .0312$) and a higher number of cancer cells, which may represent the results of the prognostic analysis (Table S5).

We additionally speculated that false negative cases (DNA CY0 among CY1 cases) can be due to unfair separation of the peritoneal fluids of the DNA CY test as compared with the conventional CY test, and smaller amounts of DNA were not amplified as appropriate. Actually, our previous study examining smaller amounts of DNA proved less sensitivity than examining larger amounts of DNA in the PCR dynamics in Q-MSP.[22]

Specificity was defined by negative diagnosis of pStage IA in the DNA CY test because our previous study proved that 431 consecutive GC patients with pStage I did not have recurrence at the peritoneum at all.[23] As a result, the specificity of the DNA CY test was proved to be 96.5% in our current prospective study, where six GC patients with pStage IA still had DNA CY1. Previous literature describing peritoneal recurrences after resection of early (pT1) GC reported that recurrences were seen in patients with lymph node metastasis (pStage IB to IIB).[24] These findings supported the notion that pStage I could be a gold standard indicating that residual peritoneal disease has no recurrence potential in GC.

Emerging promising complements have been reported by the use of monoclonal antibodies to tumor-associated antigen in immunocytochemical assays,[25,26] PCR amplification assays of tumor-associated aberrations of nucleotides (mRNA for CEA),[10] and flow cytometry using CD45 and EpCAM.[27] As compared with such promising diagnostic tools for micrometastasis of peritoneal dissemination in GC,

our current method is solely a DNA marker and has been prospectively validated.

The Cancer Genome Atlas (TCGA) molecularly classified GC phenotypes into four categories such as Epstein-Barr virus (EBV), microsatellite instability (MSI), genomically stable (GS), and chromosomal instability (CIN).[28] We have data of the EBV and MSI status in 136 GC tumor tissues; however, data of somatic copy number aberrations (SCNAs) status is not available by single nucleotide polymorphism array in this current study to further divide EBV (–)/MSI-H (–) GC cases into GS and CIN. Therefore, we initially defined EBV and MSI-H GC, and the remaining cases were analyzed as another group.

Intriguingly, *CDO1* methylation is higher in primary GC with MSI-H than in that with MSI-L/MSS ($P = .0005$, Figure S3). As CIN and GS phenotypes were reported to be uniquely characterized predominantly by intestinal and diffuse-type histology, respectively, in EBV (–)/MSI-H (–) GC, we then compared EBV (+), MSI-H (+), EBV (–)/MSI-H (–) intestinal type, and EBV (–)/MSI-H (–) diffuse type (Figure S4), and GC with MSI-H still showed the highest methylation of *CDO1* in GC ($P = .0065$). Nevertheless, DNA methylation of *CDO1* is not observed exclusively in MSI-H, and a given amount of methylation is observed in other cases. Therefore, DNA methylation of *CDO1* was considered quantifiable in primary GC regardless of GC subtype.

In conclusion, diagnostic sensitivity and specificity of DNA CY1 test using CDO1 methylation for peritoneal dissemination of GC is high, and it is very promising as an addition to the conventional diagnosis of microscopic peritoneal disease of GC.

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CONFLICT OF INTEREST

The authors have no competing interest to declare.

COMPLIANCE WITH ETHICAL STANDARDS

All the procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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