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Extensive methylation analysis of circulating tumor DNA in plasma of patients with gastric cancer

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DNA methylation is known to be involved in tumor progression. This is the first study to perform an extensive methylation analysis of plasma circulating tumor DNA (ctDNA) using targeted bisulfite sequencing in gastric cancer (GC) patients to evaluate the usefulness of ctDNA methylation as a new biomarker. Sixteen patients who received chemotherapy for recurrent GC were included. After confirmation of the methylation status of 63 genes using the Cancer Genome Atlas (TCGA) dataset, the methylation status in paired tumor and non-tumor tissues and plasma were investigated using targeted bisulfite sequencing in these genes. Forty-four of the 63 genes were significantly hypermethylated in GC patients in the TCGA cohort. Of these 44 genes, hierarchical clustering showed that five (*SPG20*, *FBN1*, *SDC2*, *TFPI2*, *SEPT9*) were particularly hypermethylated in tumor compared to non-tumor tissues in our GC cohort. In plasma methylation analysis, patients with high methylation of these genes had significantly worse overall survival than those with low methylation (log-rank P = 0.009). In a patient who underwent blood sampling at multiple points, the methylation levels of these five genes varied closely with clinical tumor status. The plasma ctDNA methylation levels of these five genes could be useful as a noninvasive prognostic biomarker for GC.

Keywords DNA methylation, Gastric cancer, Target bisulfite sequencing, Biomarker, Liquid biopsy

Globally, surgery has been the standard curative treatment for gastric cancer (GC), but many patients experience postoperative recurrence, and the prognosis is still poor despite various chemotherapies have been developed[1–6]. Several approaches thus far have been used to evaluate GC progression, including imaging examinations and the measurement of serum tumor markers[7]. However, the sensitivity of tumor markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) is only about 40–50%, even in stage IV patients[8,9]. In addition, frequent use of computed tomography is not suitable for tumor status evaluation because of its low sensitivity for the detection of peritoneal metastases and the risk of radiation exposure[10,11]. Therefore, there is an urgent need to develop minimally invasive, accurate biomarkers for GC that can be used in real time.

Liquid biopsy is broadly defined as the analysis of tumor material that has been obtained in a minimally invasive manner through the sampling of blood or other bodily fluids[12,13]. Circulating tumor DNA (ctDNA), which is tumor-derived DNA that has been released into the blood through tumor cell necrosis or apoptosis has been well studied. The amount of ctDNA in the blood has been reported to correlate with disease progression status in various cancer types[14–17]. In a previous study, we performed targeted deep sequencing of plasma cell–free DNA in GC patients and demonstrated a correlation between tumor status and mutations of *TP53*-ctDNA[18]. However, *TP53* mutations are found in only 56% of GC patients[19]; therefore, ctDNA analysis targeting specific gene mutations may be impractical for clinical use.

To overcome the limitations of ctDNA analysis in terms of identifying specific gene mutations, we focused on the detection of DNA methylation as a regulatory mechanism for epigenetic gene modification in oncogenesis[20]. Although several studies have explored the potential use of DNA methylation as a biomarker for prognostic purposes, the tissue methylation levels in these studies were mainly determined using invasive surgical methods[21,22]. Other studies analyzed ctDNA methylation in plasma from GC patients using methylation-specific PCR (MSP) or digital PCR, but they only evaluated a limited range of CpG sites in a small number of genes[23–25]. Here, with the aim of identifying new biomarkers for monitoring tumor status and predicting prognosis, we

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investigated the methylation status in paired tumor and non-tumor (T/N) tissues and plasma of patients with recurrent GC using target bisulfite sequencing of a broad range of CpG sites in 63 genes.

Results

Methylation status of the 63 genes in the TCGA cohort

The methylation status of the 63 genes in GC patients was analyzed using the TCGA-STAD dataset. After downloading methylation β-value data of the 63 genes from the Illumina Human Methylation 27 and/or 450 platforms, we confirmed that 44 of these genes were significantly hypermethylated in tumor tissues compared with non-tumor tissues (Supplementary table S1), and we analyzed these 44 genes in this study.

Methylation status of the 44 TCGA-derived genes in our cohort

We conducted methylation status profiling of the 44 genes selected from the TCGA cohort using targeted bisulfite sequencing of DNA samples from 10 paired T/N tissues in our cohort. Figure 1 shows a heatmap of the ratio of the methylation β-value in tumor tissues to that in non-tumor tissues (T/N ratio) for the 44 genes in our cohort. Hierarchical clustering analysis of all 44 genes revealed four major clusters (A, B, C, and D). The cluster-A genes (*SPG20*, *FBN1*, *SDC2*, *TFPI2*, *SEPT9*) showed the highest mean T/N ratio (cluster-A, 3.424; cluster-B, 1.693; cluster-C, 1.827; cluster-D, 1.120).

Methylation status analysis in plasma

We next performed methylation analysis of the 44 genes using targeted bisulfite sequencing of DNA samples from the plasma of 16 patients with postoperative recurrent GC. Figure 2 shows a heatmap of the methylation β-values of these 44 genes. In hierarchical clustering analysis of these genes, the 16 patients were divided into highly and poorly methylated groups, in which the mean β-values of the 44 genes were 0.1599 and 0.0710, respectively. Hierarchical clustering analysis of the five genes in cluster-A (*SPG20*, *FBN1*, *SDC2*, *TFPI2*, *SEPT9*)

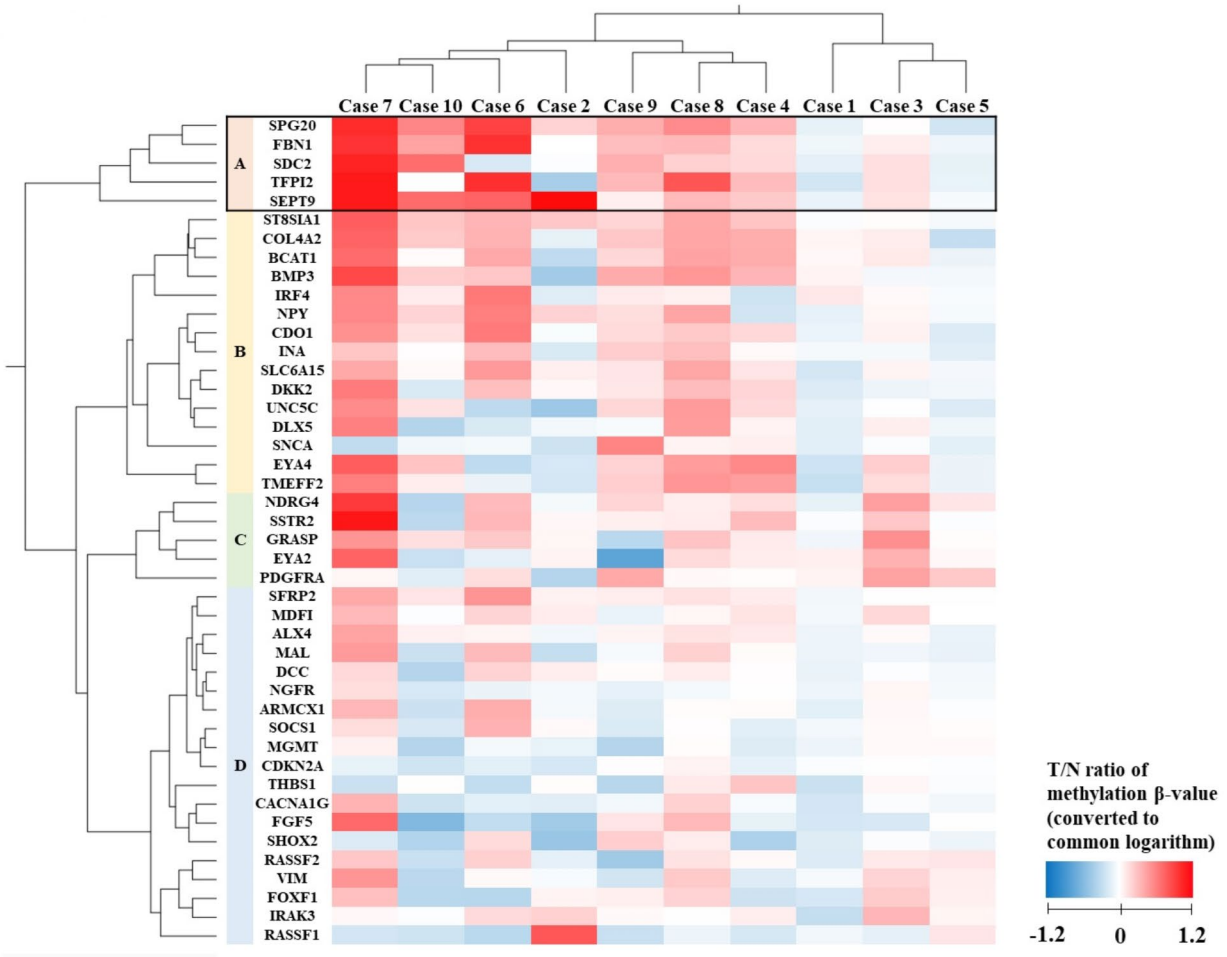

Fig. 1. Heatmap of the methylation β-value ratio between tumor and non-tumor tissues for the 44 genes in our cohort. Heatmap of the methylation β-value ratio between tumor and non-tumor tissues (T/N ratio) for 44 genes that were determined to be specifically hypermethylated in GC tumors in the TCGA cohort. The T/N ratio is expressed as − 1.2 to 1.2 by ordinary logarithmic conversion.

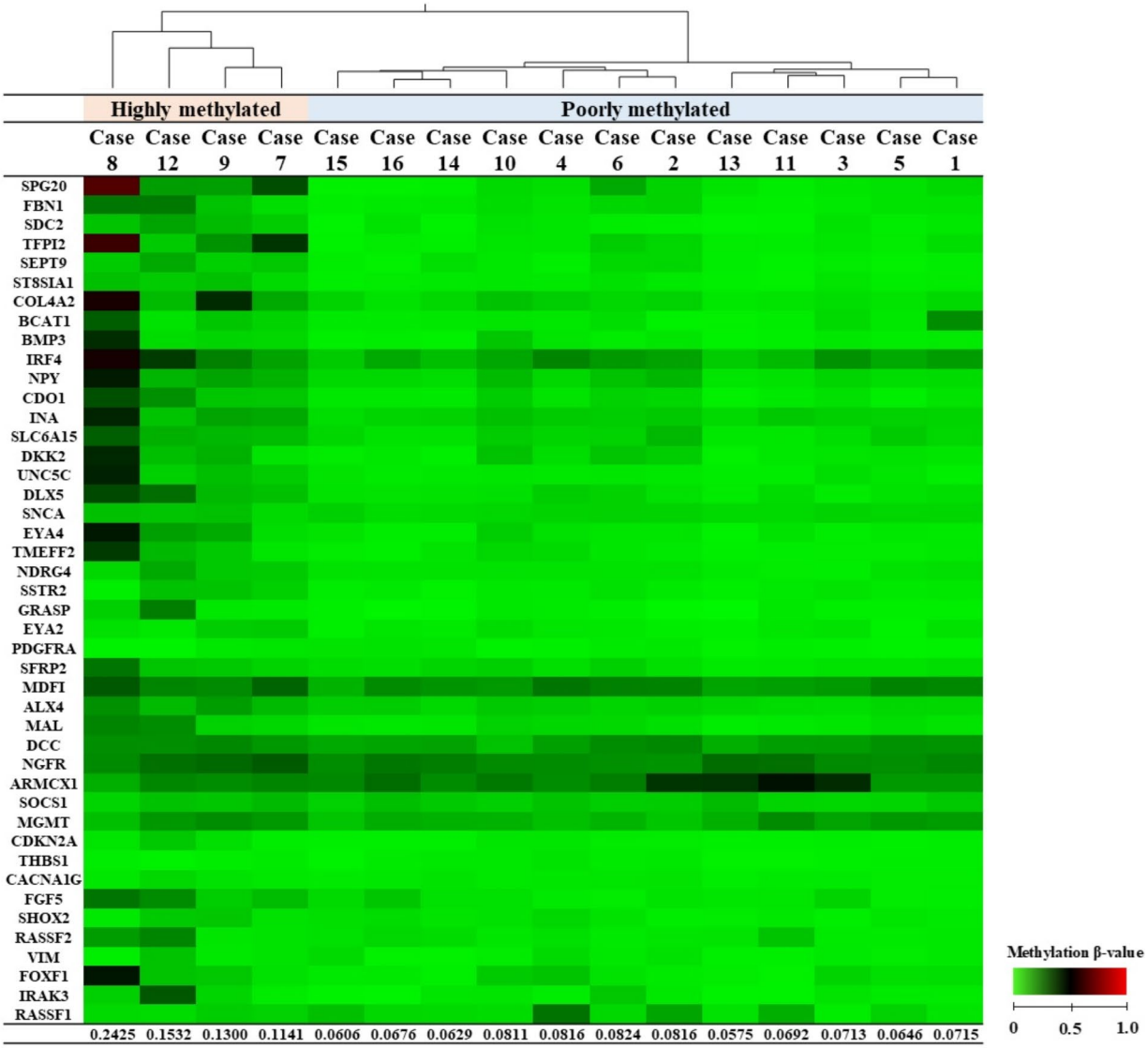

Fig. 2. Heatmap of the methylation β-values of 44 genes in the plasma of 16 GC cases at the time of postoperative recurrence.

resulted in categorization of the patients into the same two groups, with mean β-values of the five genes of 0.2159 and 0.0418, respectively (Fig. 3). Even when the 16 patients were divided into two groups using the overall mean of 0.0854 as the cutoff, the same patients were included in the highly methylated group (Supplementary Table S2).

Prediction of OS by plasma methylation status

We used the Kaplan–Meier method to assess the groups obtained in the hierarchical clustering analysis of the five cluster-A genes. The highly methylated group showed significantly worse OS from the date of blood sampling than the poorly methylated group (log-rank P = 0.009) (Fig. 4a). On the other hand, when the cases were divided into two groups by the serum CEA or CA19-9 level measured at the same time as ctDNA blood sampling, with a cutoff of twice the upper normal limit, the high CEA or CA19-9 group and the normal CEA or CA19-9 group showed no statistical difference in OS (log-rank P = 0.208) (Fig. 4b).

Relationship between methylation status and clinical tumor status

The relationships between the methylation levels of the five genes and various points in the clinical course in Case No. 3 are presented in Fig. 5. This patient had clinical stage III GC with lymph node metastasis and was treated with neoadjuvant chemotherapy followed by total gastrectomy with R0 resection. Adjuvant chemotherapy was administered, but a CT scan performed 1 year after surgery revealed peritoneal and bilateral ovarian recurrences.

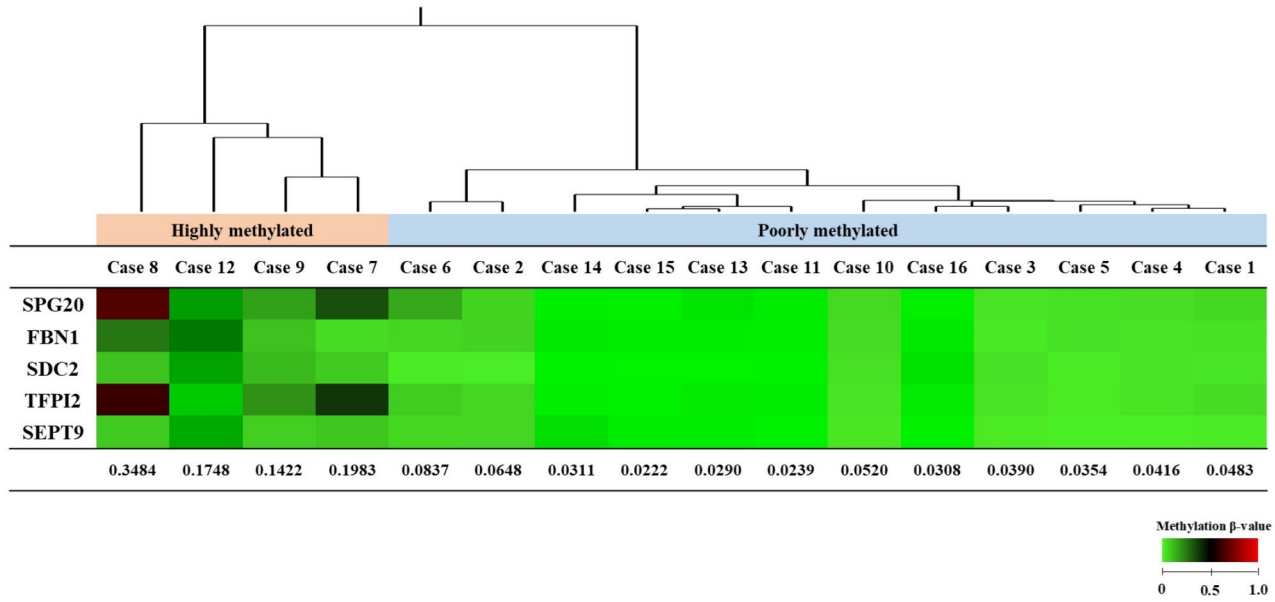

Fig. 3. Heatmap of cluster-A gene (*SPG20*, *FBN1*, *SDC2*, *TFPI2*, and *SEPT9*) methylation levels in the plasma of 16 GC cases at the time of postoperative recurrence. Mean methylation levels of the five cluster-A genes (*SPG20*, *FBN1*, *SDC2*, *TFPI2*, and *SEPT9*) are shown as a heatmap. Methylation levels are expressed as β-values, representing continuous measurements from 0 (completely unmethylated, shown in green) to 1 (completely methylated, shown in red).

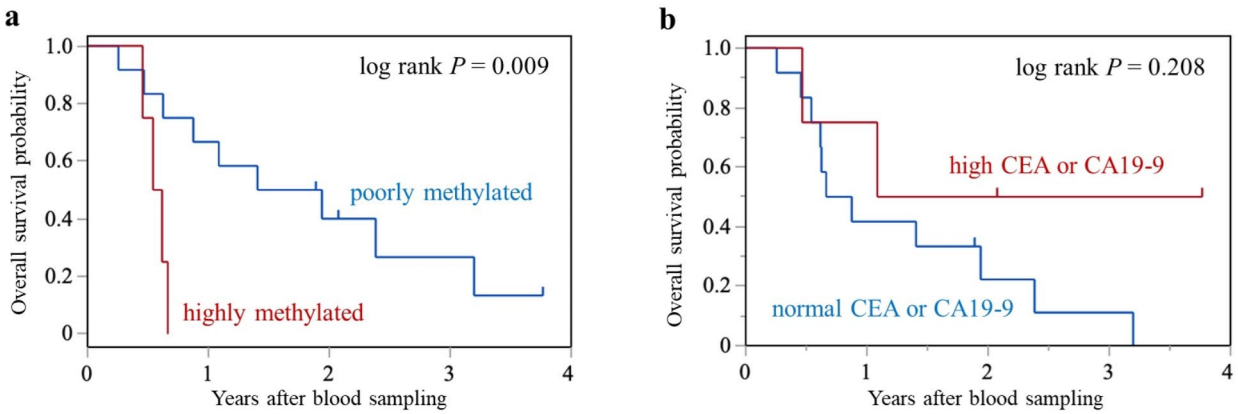

Fig. 4. Kaplan–Meier OS curves for the highly and poorly methylated groups (**a**) and the high and normal CEA or CA19-9 groups (**b**).

In this patient, blood sampling was performed at five time points, as described in the 'Patients and samples' subsection of the Methods section. Plasma before preoperative chemotherapy had the highest mean β-value of the five significant genes, at 0.054, but this value decreased to 0.026 after preoperative chemotherapy, and remained stable during postoperative chemotherapy. However, at the time of recurrence, the methylation level again increased to 0.039. In this patient, neither CEA nor CA19-9 showed elevation above the upper normal limit at any time point.

Discussion

This study demonstrated that five hypermethylated genes (*SPG20*, *FBN1*, *SDC2*, *TFPI2*, *SEPT9*) were particularly hypermethylated in advanced GC tissues compared with non-tumor tissues in both the TCGA dataset and our cohort. Additionally, the patients with higher ctDNA methylation levels of these five genes had a significantly worse prognosis. Although the number of cases studied was small, the results suggest that ctDNA methylation

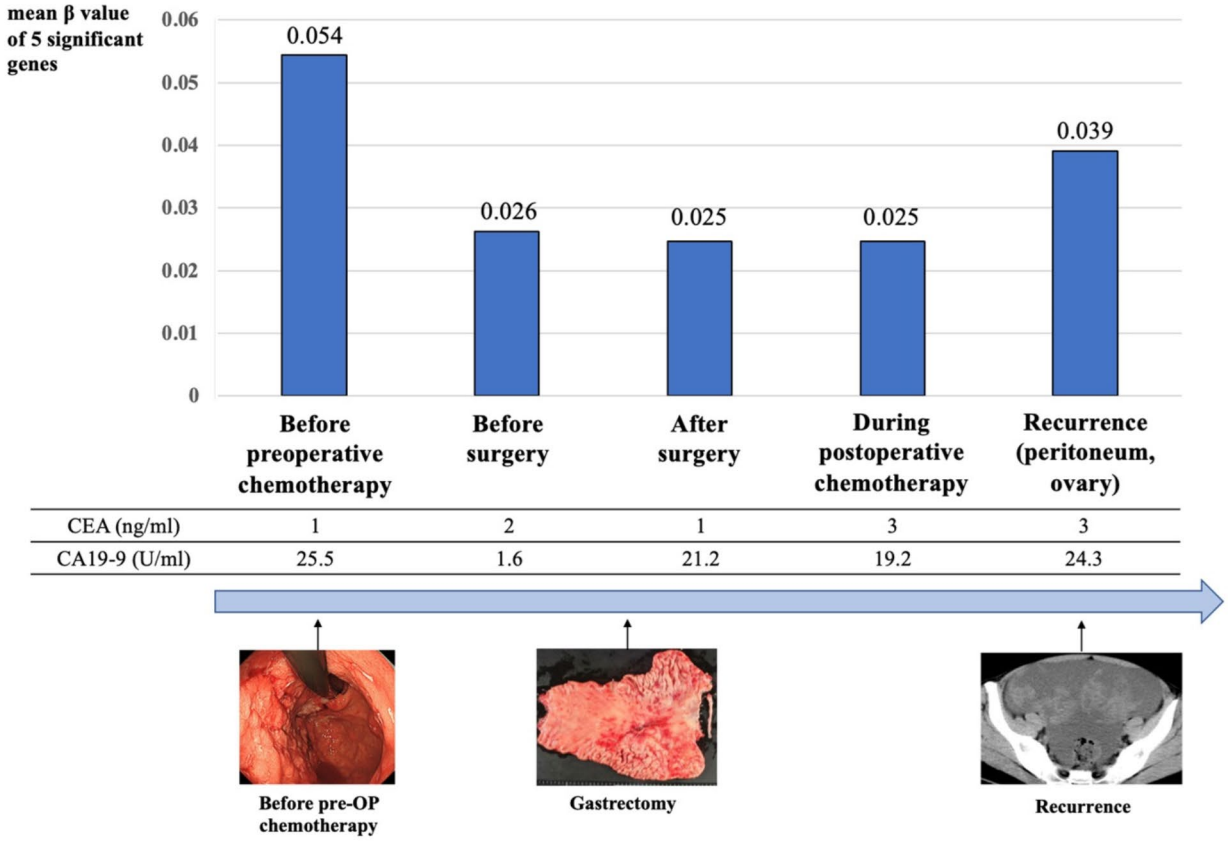

Fig. 5. Relationship between ctDNA methylation and disease progression in Case No. 3. Blue bars show the mean β-values of five cluster-A genes (*SPG20*, *FBN1*, *SDC2*, *TFPI2*, and *SEPT9*).

levels of these five genes in plasma closely correlate with clinical tumor status. Considering that two currently available tumor markers, CEA and CA19-9, did not clearly indicate prognosis, the plasma ctDNA methylation status of these five genes could be a more sensitive biomarker for monitoring tumor status.

Since the 2010s, we have conducted liquid biopsy research on gastrointestinal malignancies using ctDNA obtained from peripheral blood. We initially analyzed *TP53*-ctDNA using targeted deep sequencing of plasma cell–free DNA in GC patients, and found that the *TP53*-ctDNA mutant fraction correlated well with tumor disease status[18]. In a second study, we investigated secondary C-KIT mutations in the plasma of patients with imatinib-resistant gastrointestinal stromal tumors, and the ctDNA fraction changed with tumor status[26]. However, the major problem with the methods used in these two studies was that low concentrations of ctDNA could not be detected. To achieve more sensitive and versatile ctDNA detection, our next study employed NGS with molecular barcodes (MB)[27] and targeted *TP53*-ctDNA of esophageal squamous cell carcinoma. The maximum frequency of background errors decreased from 3.22% without MB to 0.08% with MB, indicating marked improvement in the sensitivity of ctDNA detection. *TP53* mutations are found in 90% of patients with esophageal squamous cell carcinoma, but in only 24% of patients with GC[18]; this suggests that the detection of ctDNA mutations may be impractical for malignancies like GC in which gene mutations are not very specific. Therefore, in the current study we focused on ctDNA methylation status as a regulatory mechanism for epigenetic gene modification in oncogenesis.

Studies of liquid biopsy and ctDNA methylation have recently been gaining attention in several cancers, but thus far not in GC[28–30]. Yan et al. used digital PCR technologies to examine plasma from 148 GC patients who underwent chemotherapy and found that hypermethylation of SFRP2 was associated with worse survival[23]. Hu et al. reported that the methylation status of THBS1 detected with quantitative MSP of preoperative peripheral blood was significantly associated with adverse prognosis[24]. Although the digital PCR and MSP techniques used in these previous studies were simple and inexpensive, they allow for a very limited number of genes and CpG sites to be examined. Therefore, we utilized the QIAseq Targeted DNA panels as the method for a larger number of CpG sites and cancer-specific genes related to DNA methylation. Currently, sequencers using bisulfite conversion and microarrays are available for the detection of DNA methylation status in the human genome on a single-nucleotide basis. While microarrays are inexpensive and can analyze a large number of samples, only certain methylated CpG sites can be measured[31]. On the other hand, targeted bisulfite sequencing has the

advantage of evaluating a wider range of methylated CpG sites and are reported no difference in the performance compared to microarrays[32]. Although in this study we analyzed 63 genes that were included in the QIAseq Targeted DNA panels, many of these genes were also found to be associated with GC, and in most of them the hypermethylated status was confirmed in the TCGA cohort[20,21].

In this study, we highlighted five particularly hypermethylated genes. *SPG20* encodes a multifunctional protein that is involved in intracellular epidermal growth factor receptor trafficking[33]. Furthermore, downregulation of *SPG20* may cause carcinogenesis[34], and methylation-mediated gene silencing of *SPG20* has been demonstrated previously in GC[33,35]. *FBN1* is involved in the production of the extracellular matrix component fibrillin-1, and its blood and stool methylation status has been reported as a potential biomarker in colorectal cancer (CRC)[36,37]. *SDC2* is a transmembrane protein involved in cell proliferation, cell migration and cell–matrix interactions[38]. In GC cells, *SDC2* plays a regulatory role in migration and invasion[39]. Two studies reported that plasma and stool tests of *SDC2* methylation were useful in screening for GC and CRC[40,41]. Regarding *TFPI2*, some reports suggested that hypermethylation of its promoter CpG island was more prevalent in primary GC tissues than normal tissues[42,43]. In addition, tissue and plasma *TFPI2* methylation was reported to be a prognostic marker in GC patients[44,45]. TFPI2 is also one of the top-performing biomarkers in GC methylation studies with a large number of cases[46]. *SEPT9* is an important component of the cytoskeleton and influences cell polarization, intracellular material transport, cell cycle regulation, and cell apoptosis[47,48]. Many biomarker studies using *SEPT9* methylation have been reported previously in various tumor types[49–51]. In GC, Lee et al. showed that patients with plasma *SEPT9* methylation before treatment were more likely to develop distant metastasis and to have worse disease-free survival than those without such methylation[52]. In addition, plasma *SEPT9* methylation was shown to be a useful diagnostic biomarker even in early-stage GC[53]. The literature reports on the five aforementioned genes strongly support the findings in this study indicating that the blood methylation levels of these genes could become important biomarkers for GC. Their relevance to the mechanism of tumor progression remains largely unexplored and requires further study.

This study also analyzed ctDNA methylation in a series of GC patients before and after preoperative chemotherapy, before and after surgery, and at postoperative recurrence, and showed that the methylation levels of these five genes were closely correlated with disease status. In various tumors, quantifying ctDNA through the methylation analysis of some genes correlates with total tumor burden and can therefore be used for the prognosis and disease monitoring biomarker[30]. Some studies have investigated the relationship between ctDNA methylation and clinical disease course in CRC[30,54–56], but few have examined this issue in GC patients. Regarding the efficacy of chemotherapy in metastatic CRC patients, a prospective study evaluated *NPY* methylation demonstrated that the rate of change in ctDNA methylation before and after chemotherapy was associated with the early prediction of treatment benefit[30,54]. In a study evaluating changes in ctDNA methylation of *GRI4A* before and after curative resection in CRC patients, the methylation rate decreased by 4.5 fold in metastatic CRC patients[55]. Our present report suggests that ctDNA methylation may reflect tumor disease status in GC patients as well as in those with CRC. Furthermore, several studies reported that ctDNA methylation biomarkers are more effective for predicting recurrence than existing CEA or imaging tests in CRC patients[56]. In our case No. 3, the ctDNA methylation of the five genes showed a correlation with tumor disease progression although CEA and CA19-9 did not change. We consider that our research suggests the possibility of ctDNA methylation as a marker for predicting recurrence in GC patients, but further research would be needed to compare to existing modalities.

There are several limitations to this study. First, the number of examined cases was small. However, there are still few methylation-related biomarker studies targeting a broad range of CpG sites specifically in patients with recurrent GC, and this study provides new insights despite the limited sample size. Second, we analyzed the methylation status of only 63 genes, and other genes may be suitable as biomarkers. Further studies on DNA methylation in GC are needed to understand the roles of other genes and their mechanisms in cancer progression. Third, the patient population is very heterogeneous, with different treatment regimens as well as varying metastasis. However, we believe that ctDNA methylation is not influenced by the patient's metastatic sites or treatment regimens. Indeed, previous methylation-related biomarker research has also included patients across various stages and metastatic levels[56–58]. In addition, patients with GC present with different recurrence patterns, and chemotherapy regimens are diverse as well. For these reasons, we comprehensively included these patients and demonstrated that those with higher ctDNA methylation levels of these five genes had a significantly worse prognosis, regardless of the metastatic regions or treatment regimens. Finally, targeted bisulfite sequencing is very costly and time consuming, making it difficult to implement in clinical practice. As a solution to these problems, we plan to use digital PCR in a larger number of cases to study the methylation status of the five genes selected in this study and to demonstrate their usefulness as biomarkers.

In conclusion, we demonstrated that the methylation status of five particularly hypermethylated genes (*SPG20*, *FBN1*, *SDC2*, *TFPI2*, *SEPT9*) in plasma reflected tumor status and prognosis in recurrent GC. This is the first study to perform an extensive methylation analysis of plasma ctDNA in patients with GC using targeted bisulfite sequencing to identify potential biomarkers.

Methods

Patients and samples

This study included 16 patients who received chemotherapy for recurrent GC at Osaka University Hospital between July 2019 and August 2021 (Table 1). Plasma samples were collected from all 16 patients (Cases No. 1 – No. 16). In Case No. 3, blood samples were obtained and stored at five time points, as follows: before neoadjuvant chemotherapy, before and after surgery, during adjuvant chemotherapy, and at the time of recurrence. Clinical staging was based on the 15th edition of the Japanese Classification of Gastric Carcinoma. Written informed

Case No	Sex	Age (year)	Histological type	Tumor recurrence site	Status*	Chemotherapy regimen*
1	M	67	por	Liver	Before 2nd line Cx	PTX + RAM
2	F	68	por	Liver	Before 1st line Cx	-
3	F	35	mod	Peritoneum, Ovary	Before 1st line Cx	-
4	M	80	mod	Peritoneum	Before 2nd line Cx	PTX + RAM
5	M	52	por	Peritoneum	Before 1st line Cx	-
6	M	65	por	Liver	During 5th line Cx	Nivolumab
7	M	81	mod	Liver	Before 3rd line Cx	PTX + RAM
8	M	78	por	Liver	Before 2nd line Cx	PTX + RAM
9	M	82	por	Liver	Before 3rd line Cx	PTX + RAM
10	M	72	por	Peritoneum	During 2nd line Cx	PTX + RAM
11	F	68	por	Peritoneum	Before 2nd line Cx	PTX + RAM
12	M	71	wel	Para-aortic lymph node	During 4th line Cx	IRI
13	F	41	por	Peritoneum	Before 4th line Cx	S-1 + DTX
14	M	78	por	Para-aortic lymph node	Before 9th line Cx	IRI + Trastuzumab
15	M	57	mod	Para-aortic lymph node	Before 4th line Cx	Nivolumab
16	M	73	por	Liver, Peritoneum	During 4th line Cx	IRI + RAM

Table 1. Characteristics of the 16 patients. *Status and chemotherapy regimen at the time of blood sample collection. M, male; F, female; por, poorly differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; wel, well differentiated adenocarcinoma; Cx, chemotherapy; PTX, paclitaxel; RAM, ramucirumab; IRI, irinotecan; DTX; docetaxel.

consent was obtained from all patients before sampling. This study was performed in accordance with the Declaration of Helsinki and approved by the Osaka University Research Ethics Committee (No. 894).

DNA preparation

Paired T/N tissues were obtained from formalin-fixed, paraffin-embedded (FFPE) slides of stomach specimens resected during the primary surgery from 10 patients (Cases No. 1 – No. 10), in which a sufficient amount of DNA for analysis could be isolated. DNA from tissue samples was isolated using the GeneRead DNA FFPE Kit (QIAGEN, Hilden, Germany). Blood samples were collected in 10-mL ethylenediaminetetraacetic acid disodium salt, 2-hydrate (EDTA-2Na) vacutainer tubes. Plasma was separated from blood cells via centrifugation at 1600 g for 10 min at 4 °C, followed by re-centrifugation at 16,000 g for 10 min at 4 °C. The centrifugation was conducted within 2 h and the plasma samples were stored at − 80 °C until DNA extraction. DNA from plasma samples was isolated using the Apostle MiniMax™ High Efficiency cfDNA Isolation Kit (Beckman Coulter, CA, USA).

Methylation panel and the *Cancer* Genome Atlas (TCGA) cohort

The QIAseq Targeted DNA panels (DHS-002Z, QIAGEN, Hilden, Germany) targets thousands of CpG sites in cancer-specific 63 genes (Supplementary table S3). To confirm the methylation status of these 63 genes in GC, we used TCGA-Stomach Adenocarcinoma (STAD) dataset and the UCSC Xena browser (https://xena.ucsc.edu). Gene methylation levels were scored using a β-value between 0 (completely unmethylated) and 1 (completely methylated). The 63 genes were analyzed by downloading the methylation β-value data of each case from the Illumina Human Methylation 27 and 450 platforms (https://xenabrowser.net). The mean methylation β-value was calculated for each of the 63 genes. Since no specific selection criteria exist for identifying genes with higher methylation in tumor tissues compared to non-tumor tissues using the β-value, we used the T/N ratio to analyze the differences. To broadly narrow down the list of higher methylated genes in GC, genes in the TCGA cohort were selected on the basis of the following: (i) the ratio of the β-value in tumor tissues to that in non-tumor tissues (β-value T/N ratio) > 1, and (ii) a P value < 0.05 as determined by the Wilcoxon rank sum test comparing tumor and non-tumor tissues.

Bisulfite conversion and next-generation sequencing (NGS)

Briefly, 5 ng plasma DNA and 200 ng FFPE DNA were bisulfite-converted with the EPITECT Fast Bisulfite Sequencing conversion kit (QIAGEN). The bisulfite-converted DNA was used as the input template to create targeted libraries as per the QIAseq Targeted Methyl Panel protocol. Sequencing was then performed on an Illumina Miseq 2 × 150 bp with V2 chemistry (Illumina, CA, USA). Paired-end reads were mapped to the human genome reference (hg19). Data were analyzed by the GeneGlobe Data Analysis Center.

Methylation β-value

For each CpG site, the DNA methylation level, defined as a β-value, was calculated as the methylated coverage (the number of reads with evidence of methylation at that position) divided by the context coverage (the number of reads conforming to the selected methylation context)[32]. The region involved in protein coding was selected as the gene type. The analysis area was ± 1,500 base pairs upstream and downstream from the transcription start site (TSS). Methylation values were defined as missing when the context coverage was less than 10X. Finally,

the β-value of each gene was calculated as the mean β-value of the included CpG sites. Hypermethylated genes in GC were assessed by comparing the β-values in tumor and non-tumor tissues. Selection of particularly hypermethylated genes was based on hierarchical clustering in the heatmap of the methylation β-value T/N ratio.

Statistics

Hierarchical clustering was performed using Ward's minimum variance method. Overall survival (OS) was defined as the interval from the date of blood sampling to the date of death from any cause. Survival rates were estimated using the Kaplan–Meier method and compared using the log-rank test. All statistical analyses were performed using JMP PRO software (JMP version 17.0.0; SAS Institute, Cary, NC).

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

S.N., Y.K., T.H., and R.Y. designed the overall experiments and had unrestricted access to all data. S.N. and Y.K. performed statistical analyses and drafted the manuscript. S.N., Y.K., T.T., T.S., K.Y., K.M., K.Y., K.T., T.M., and K.N. collected patient samples. H.E. and Y.D. provided critical reading of the manuscript and insightful comments. All authors read and approved the final draft of the manuscript and take full responsibility for its content.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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