Aberrant gene methylation in the peritoneal fluid is a risk factor predicting peritoneal recurrence in gastric cancer

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AIM: To investigate whether gene methylation in the peritoneal fluid (PF) predicts peritoneal recurrence in gastric cancer patients.

METHODS: The gene methylation of CHFR (checkpoint with forkhead and ring finger domains), p16, RUNX3 (runt-related transcription factor 3), E-cadherin, hMLH1 (mutL homolog 1), ABCG2 (ATP-binding cassette, subfamily G, member 2) and BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein 3) were analyzed in 80 specimens of PF by quantitative methylation-specific polymerase chain reaction (PCR). Eighty patients were divided into 3 groups; Group A (n = 35): the depth of cancer invasion was less than the muscularis propria; Group B (n = 31): the depth of cancer invasion was beyond the muscularis propria. Both group A and B were diagnosed as no cancer cells in peritoneal cytology and histology; Group C (n = 14): disseminated nodule was histologically diagnosed or cancer cells were cytologically defined in the peritoneal cavity.

RESULTS: The positive rates of methylation in CHFR, E-cadherin and BNIP3 were significantly different among the 3 groups and increased in order of group A, B and C (0%, 0% and 21% in CHFR, P < 0.05; 20%, 45% and 50% in E-cadherin, P < 0.05; 26%, 35% and 71% in BNIP3, P < 0.05). In addition, the multigene methylation rate among CHFR, E-cadherin and BNIP3 was correlated with group A, B and C (9%, 19% and 57%, P < 0.001). Moreover, the prognosis was analyzed in group B, excluding 3 patients who underwent a non-curative resection. Two of the 5 patients with multigene methylation showed peritoneal recurrence after surgery, while those without or with a single gene methylation did not experience recurrence (P < 0.05).

CONCLUSION: This study suggested that gene methylation in the PF could detect occult neoplastic cells in the peritoneum and might be a risk factor for peritoneal metastasis.

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Key words: Ascites; Dissemination; Gastric cancer; Methylation; Peritoneal fluid; Quantitative methylation-specific polymerase chain reaction

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INTRODUCTION

Peritoneal metastasis is the most frequent event in recurrent gastric cancers, and occurs in 34% of patients with recurrences, even after curative resection of the primary tumor[1-3]. In addition, peritoneal metastasis shows resistance to various chemotherapeutic drugs and causes massive ascites and intestinal obstruction. In Japan, cytological examination of peritoneal washes using Papanicolaou staining is commonly performed during surgery to detect peritoneal metastasis. However, peritoneal metastasis sometimes occurs even in cases that show a negative cytological examination. The efficacy of immunocytochemistry[4,14], tumor markers[5,16] and reverse transcriptase polymerase chain reaction (PCR) analysis of carcinoembryonic antigen (CEA), and cytokeratin (CK) mRNA20,21 in the peritoneal washes has been examined.

Epigenetic gene silencing through DNA methylation occurs in various cancers. DNA methylation occurs in the CpG rich promoters of tumor suppressor genes, DNA repair and cell cycle checkpoint genes, resulting in suppressed gene expression[13,14]. Numerous studies have investigated gene methylation to assess the correlation with carcinogenesis and tumor progression in various cancers[15-17]. Recently, several reports have demonstrated aberrant gene methylation detected in salivary rinses[18], pleural effusion[19], peritoneal fluid (PF)[20,21], lymph node[22,23], breast ductal fluid[24], bile[25], pancreatic juice[25], urine[25], stool[25], serum and plasma[20,21,24] from patients with various tumors and suggested the feasibility of methylation analysis in the evaluation of occult neoplastic cells or micrometastasis.

The present study investigated whether DNA methylation using PF is a possible marker for determining gastric cancer micrometastasis to the peritoneum. The DNA methylation levels of 7 genes; CHFR (checkpoint with forhead and ring finger domains), p16 (cyclin-dependent kinase inhibitor 2A), RUNX3 (runt-related transcription factor 3), E-cadherin, hMLH1 (mutL homolog 1), ABCG2 (ATP-binding cassette, sub-family G, member 2), BNP3 (BCL2/adenovirus E1B 19 kDa interacting protein 3) in 80 PF specimens were analyzed by quantitative methylation-specific polymerase chain reaction (q-MSP). Furthermore, quantitative reverse transcriptase-PCR (qRT-PCR) of CEA and CK19 mRNA was examined using the same samples and the results were compared with that of q-MSP. The goal of this study was to clarify whether gene methylation in PF is feasible for determining micrometastasis to the peritoneum in gastric cancer.

MATERIALS AND METHODS

Ethics
The study protocol was approved by the Ethics Committee of Saga University Faculty of Medicine. Informed consent was obtained from all the patients before collection of the samples.

Patients and sample collection
Peritoneal lavage fluid was obtained from 80 patients who underwent surgery at the Department of Surgery, Saga University Hospital from May 2007 to August 2008. A total volume of 200 mL of normal saline was poured into Douglas’s pouch and the left subphrenic space. One hundred milliliter of PF was examined by conventional cytological diagnosis with Papanicolaou staining. The remaining PF was centrifuged at 1200 g for 10 min and the pelleted cells were stored at −80°C until the extraction of genomic DNA and RNA. A gastrectomy was subsequently performed in 72 patients. A bypass operation or exploratory laparotomy was carried out in the remaining 8 patients due to either peritoneal dissemination or cytologically positive cancer cells. The histological type, depth of tumor invasion and clinical stage were determined on the basis of the criteria of the Japanese Classification of Gastric Carcinoma guidelines[13]. The 80 patients were further divided into 3 groups: Group A (n = 35); the depth of cancer invasion was less than the muscularis propria [tumor invasion of mucosa and/or muscularis mucosa (M) or submucosa (SM), tumor involved the muscularis propria (MP)]; Group B (n = 31); the depth of cancer invasion was beyond the muscularis propria [tumor involved the subserosa (SS), tumor penetrated the serosa (SE), tumor invasion of adjacent structures (SI)]; Group C (n = 14); a peritoneal metastasis was histologically diagnosed [P (+)] or cancer cells were present on peritoneal cytology [CY (+)]. No peritoneal metastasis [P (-)] and benign/indeterminate cells on peritoneal cytology [CY (-)] were confirmed at surgery in the 66 patients in group A and group B. CY (+) or P (+) was simultaneously diagnosed at surgery in 12 of 14 patients in group C. In the remaining 2 patients, cancerous ascites were collected at the recurrence. The methylation analysis was performed using specimens obtained from all 80 patients. The mRNA analysis was done using 63 samples, because high quality RNA could not be extracted from specimens from the remaining 17 cases.

DNA extraction, sodium bisulfite modification and q-MSP
The genomic DNA was isolated from cell pellets from the abdominal fluid using an EZ1 DNA tissue kit (Qiagen, Hilden, Germany). Bisulfite modification was carried out using the EpiTet® Bisulfite Kit (Qiagen, Hilden, Germany) with 1500 ng of genomic DNA. Bisulfite-treated DNA was amplified by EpiTet® Whole Bisulfiteome Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The 80 DNA samples with bisulfite modification were quantitatively analyzed for the methylation levels of 8 genes (CHFR, p16, RUNX3, E-cadherin, hMLH1, ABCG2, BNP3 and β-actin, an internal marker). For q-MSP, a 2 μL aliquot was amplified by PCR using a primer set along with the Taqman probe specific for methylated sequences. A q-MSP (Methylight) was carried out with the Light-Cycler™ instrument system (Roche, Mannheim, Germany) using the LightCycler® TaqMan® Master (Roche, Mannheim, Germany) according to a previous report[22]. The primer sequences are shown in Table 1[33-37]. After a denaturing step at 95°C
for 10 min, PCR amplification was performed with 45 cycles of 15 s denaturing at 95°C, 5 s annealing at 60°C and a 10 s extension at 72°C. These experiments were carried out in triplicate and the mean value was then calculated. CpGenome Universally Methylated DNA (Chemicon, Temecula, CA, USA) was used as a positive control for methylation, and CpGenome Universal Unmethylated DNA (Chemicon) was used as a negative control. The quantified value of DNA methylation of a target gene was normalized by β-actin.

RNA extraction, conversion to cDNA and qRT-PCR
Total RNA was extracted from the 80 cell pellets using the ISOGEN kit (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Samples of RNA (100 ng) were converted into cDNA and amplified using the QuantiTect® Whole Transcriptome Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In order to quantitatively estimate the expression level of CEA and CK19 mRNA, qRT-PCR was performed on a Light-Cycler™ instrument system (Roche, Mannheim, Germany) using LightCycler® TaqMan® Master (Roche, Mannheim, Germany) according to the manufacturer's instructions. The primer sequences are shown in Table 2. After denaturing at 95°C for 10 min, qRT-PCR amplification was performed with 45 cycles of 15 s denaturing at 95°C, 5 s annealing at 60°C and a 10 s extension at 72°C. These experiments were all carried out in triplicate and the mean value was then calculated. The quantified value of mRNA expression of a target gene was normalized by β-actin.

Comparison of gene methylation between cancer tissue and peritoneal fluid
The conventional qualitative MSP was analyzed using several cancer tissue specimens. Genomic DNA was isolated from the tissue and bisulfite treatment was carried out as described above. The methylation status in the tissue samples was determined by MSP. Amplification was performed using Takara ExTaq Hot Start Version (Takara, Shiga, Japan) according to the manufacturer’s instructions. The primer se-
quences are shown in Table 1 and PCR was performed in an iCycler (Bio-Rad, Hercules, CA, USA) under the following conditions. After heating at 96°C for 3 min, 35 cycles at 96°C for 30 s, 60°C for 30 s, 72°C for 30 s and 72°C for 5 min. The PCR products were separated on 1.5% agarose gel, stained with ethidium bromide, and were then observed under ultraviolet light.

**Statistical analysis**
A cut-off value for distinguishing methylation status was determined using a receiver-operator characteristic (ROC) curve, which was obtained by comparing methylation values between group A and C. The cut-off value of CEA and CK19 mRNA expression was also determined by a ROC curve as described above. Differences in the frequencies were analyzed by the χ² test, while also applying Fisher’s exact test. A statistical analysis was carried out using the SPSS 1.5 Jstatistical software package for Windows (SPSS Japan Inc.). A P value of less than 0.05 was considered to be statistically significant.

**RESULTS**
The clinicopathological characteristics of the patients in this study are summarized in Table 3. The 80 patients included 28 (35.0%) females and 52 (65.0%) males with a mean age of 65.9 years (range, 39-88 years). The number of patients in group A, B and C was 35 (43.8%), 31 (38.8%) and 14 (17.5%) cases, respectively.

**DNA hypermethylation of peritoneal fluids**
Initially, q-MSP of CHFR, p16, RUNX3, E-cadherin, hMLH1, ABCG2 and BNIP3 was examined in 80 PFs. Figure 1 shows the methylation level of each gene in the 3 groups. The 80 cases were then divided into 2 groups, a methylation positive and a methylation negative group as described in the Materials & Methods section. Table 4 shows that the methylation status of CHFR, E-cadherin and BNIP3 were significantly different among the 3 groups (P < 0.05). In these 3 genes, the positive rate of methylation increased in order of group A, B and C. The correlation of multigene methylation among CHFR, E-cadherin and BNIP3 in the 3 groups was further analyzed. In group A and B, 3 of 35 cases (9%) and 6 of 31 cases (19%) showed multigene methylation in 2 or more genes, respectively (Table 5). In contrast, 8 of 14 cases (57%) were methylation positive in 2 or more genes in group C. The results showed a significant relationship between methylation status of more than 2 genes and the 3 groups (P < 0.001).

**mRNA expression of CEA and CK19 in peritoneal fluids**
The expression of CEA and CK19 mRNAs in the 63 PFs were quantitatively analyzed by qRT-PCR to detect gastric cancer cells in the peritoneal washes. Figure 2 shows the expression level of CEA and CK19 mRNAs in the 3 groups. The mRNA level was further divided into negative and positive groups and compared among the 3 groups. The results showed that CK19, but not CEA, was significantly correlated with the 3 groups (Table 6, P < 0.05, P = 0.352). The positive rate of CK19 increased in order of group A (25%), B (46%) and C (73%).

**Comparison of gene methylation between the cancer tissue and ascites fluid**
The gene methylation of CHFR, E-cadherin and BNIP3 was examined in the primary or metastatic cancer tissue and was compared with those in the corresponding PF.

### Table 3 Clinicopathological factors of the patients (n = 80)

| Clinicopathological factors | n (%) |
|----------------------------|-------|
| Gender                     |       |
| Female                     | 28 (35.0) |
| Male                       | 52 (65.0) |
| Age (yr)                   | 65.9 ± 10.8 |
| Range                      | 39-88 |
| Histological type          |       |
| tub                        | 36 (45.0) |
| por/sig/muc                | 44 (55.0) |
| Classification             |       |
| Group A: M-MP with CY(-) and P(-) | 35 (43.8) |
| Group B: SS-SI with CY(-) and P(-) | 31 (38.8) |
| Group C: CY(+) or P(+)     | 14 (17.5) |

| Clinical stage at the sample collection | n (%) |
|----------------------------------------|-------|
| I                                      | 37 (46.3) |
| II                                     | 8 (10.0) |
| III                                    | 18 (22.5) |
| IV                                     | 15 (18.8) |
| Peritoneal recurrence                  | 2 (2.5) |

| DNA methylation status                | Group A: M-MP with CY(-) and P(-) | Group B: SS-SI with CY(-) and P(-) | Group C: CY(+) or P(+) | P-value |
|---------------------------------------|------------------------------------|------------------------------------|------------------------|---------|
| CHFR                                  | 35 (100)                           | 31 (100)                           | 11 (79)                | < 0.05  |
| p16                                   | 0 (0)                              | 0 (0)                              | 3 (21)                 | 0.821   |
| RUNX3                                 | 14 (40)                            | 14 (45)                            | 5 (36)                 | 0.304   |
| E-cadherin                            | 21 (60)                            | 17 (55)                            | 9 (64)                 |         |
| BNIP3                                 | 19 (54)                            | 11 (35)                            | 6 (43)                 | < 0.05  |
| ABCG2                                 | 16 (46)                            | 20 (65)                            | 8 (57)                 | 0.244   |
| hMLH1                                 | 28 (80)                            | 17 (55)                            | 7 (50)                 | 0.861   |
| BNIP3                                 | 13 (43)                            | 16 (52)                            | 7 (50)                 | < 0.05  |
| E-cadherin                            | 12 (34)                            | 10 (32)                            | 8 (57)                 |         |
| ABCG2                                 | 23 (66)                            | 21 (68)                            | 6 (43)                 |         |
| hMLH1                                 | 26 (74)                            | 20 (65)                            | 4 (29)                 |         |
| CHFR                                  | 9 (26)                             | 11 (35)                            | 10 (71)                |         |
in group C. Nine of 14 cases were analyzed because the tissues were not obtained in remaining 5 cases. The same methylation pattern was present in the cancer tissues and ascites in 100% of $\text{CHFR}$, 88.9% of $\text{E-cadherin}$ and 77.8% of $\text{BNIP3}$ (Table 7).

**Relationship between multigene methylation or mRNA expression and peritoneal recurrence**

The prognosis of patients in group A or B was followed for at least 8 mo after surgery. None of the 35 patients in group A had cancer recurrence (data not shown). The prognosis of only 28 of 31 patients in group B was followed because the remaining 3 patients were excluded from this analysis based on the results of a non-curative resection. In 28 of the patients in group B, 2 of the 5 patients with multigene methylation showed peritoneal recurrence after surgery, while patients without or with a single gene methylation did not experience recurrent cancer (Table 8). There was a significant correlation between peritoneal recurrence and multigene methylation in group B (Table 8, $P < 0.05$). Peritoneal recurrence was observed in only 1 of 10 cases that were CK19 positive, however statistical significance was not observed (Table 8, $P = 0.943$).

**DISCUSSION**

Postoperative recurrence of gastric cancer usually occurs in the peritoneum, lymph node and liver\(^1\). Peritoneal metastasis occurs most frequently and is highly resistant to various chemotherapies, which leads to a poor prognosis in gastric cancer patients. Peritoneal recurrence has been reported to depend on the depth of invasion, as 97.2% of recurrences occur beyond the MP. In addition, peritoneal recurrence was demonstrated in 34.9% of SS cases, 46.7% of SE cases and 60.0% of SI cases\(^2\).
Another study reported that 50%-60% of gastric cancer patients with serosal invasion after a curative resection eventually developed peritoneal metastasis. Furthermore, the average survival after peritoneal recurrence is 4.9 mo. Therefore, the detection of micrometastasis in peritoneal lavage is essential, not only to make an accurate diagnosis, but also to start chemotherapy before the metastatic nodule is grossly formed in the peritoneum. The introduction of molecular technology such as RT-PCR of cancer specific genes has addressed the detection of micrometastasis in the LN, ascites, bone marrow and peripheral blood in gastrointestinal cancer. Various types of mRNA, such as CEA, CK19, and CK20 have been analyzed by RT-PCR and used as molecular markers in detecting micrometastasis in gastrointestinal cancer. Several studies have reported that the positive expression of mRNA in PF shows a significant correlation with peritoneal recurrence and survival.

Recently, an analysis of cancer specific gene methylation has been utilized to detect micrometastasis in salivary rinses for head and neck cancer patients, pleural effusion for lung cancer and malignant mesothelioma, ducal fluid for breast cancer, ascites for ovarian cancer and colorectal cancer, bile for gallbladder cancer, pancreatic juice for pancreatic cancer, urine for prostate cancer, stool for colorectal cancer and serum. However, few studies have addressed gene methylation for the detection of micrometastasis to PF in gastrointestinal cancer.

The present study analyzed the promoter methylation of cancer related genes in 80 PFs. CHFR, p16, RUNX3, E-cadherin, hMLH1, ABCG2 and BNIP3 were chosen for the methylation analysis, because the frequent methylation of these 7 genes has been reported in several malignancies including gastric cancer. Peritoneal recurrence has been reported to depend on the depth of invasion. Therefore, 80 samples from gastric cancer patients were classified into 3 groups [Group A: cancer invasion was restricted in M, Group B: cancer invasion deeper than MP, Group C: CY(+)+ or P(+)] and correlated with the gene methylation. As a result, q-MSP analysis using the 80 PFs demonstrated that the methylation status of CHFR, E-cadherin and BNIP3 were significantly increased depending on the depth of cancer invasion. In contrast, the methylation status of the other genes was not significantly changed among the 3 groups (Table 4). These results indicate that the increasing value of the methylation of CHFR, E-cadherin and BNIP3 from group A to group C was possibly derived from the metastatic cancer cells in the peritoneum. On the other hand, a q-MSP analysis might detect methylation from normal cells in the peritoneum at the basal level in p16, RUNX3, hMLH1 and ABCG2 genes. Based on these findings, CHFR, E-cadherin and BNIP3 methylation was thus suggested to be preserved during cancer invasion, finally resulting in the occurrence of peritoneal seeding. Therefore, the methylation in more than 2 genes was compared among the 3 genes in each group. The results showed that there was a significant difference between multigene methylation and the 3 groups (Table 5). Eight of 14 patients (57%) in group C carried the multigene methylation while only 3 of 35 (9%) patients in group A exhibited multigene methylation. On the other hand, a qRT-PCR analysis examined the expression of CEA and CK19 mRNA in 63 samples (Table 6). Unexpectedly, CEA was not correlated with the classification even in group C with the highest positive rate. However, CK19 was significantly increased depending...
on the depth of cancer invasion, CY and P classification. It was desirable that gene methylation should be detected in up to 100% of samples in group C with CY1 or P1. However, only 21% of gene methylation was observed in CHFR, 46% of E-cadherin, 71% of BNIP3 and 57% of more than 2 genes were methylated in group C, indicating that gene methylation did not universally occur in all cancer cells. Thus, it is important to improve the sensitivity of multigene methylation analysis using PFs by increasing the number of genes that are specifically methylated in cancer cells. To clarify whether the methylation status of the PF originated from the cancer tissue, the methylation status of the primary or metastatic tissue in group C was compared with the methylation in the PF. The methylation status in the primary tumor was highly preserved in the PF (Table 7), thus suggesting that the methylation of the 3 genes assessed in the PF was derived from the primary tumor.

This study finally evaluated whether multigene methylation predicts peritoneal recurrence after surgery. In 21 patients in group B, peritoneal recurrence was found in 2 of 5 patients (40%) carrying multigene methylation. On the other hand, recurrence occurred in only 1 of 10 patients (10%) with positive CK19. There was a significant association between peritoneal recurrence and multigene methylation, but not CK19 (Table 8). These results suggested that multigene methylation may be a risk factor for peritoneal metastasis in the patients in group B even though the metastasis was not detected during surgery. An RT-PCR method using epithelial markers is critical in the diagnosis of micrometastasis. However, these methods only diagnose the presence of cancer cells. A methylation analysis that diagnosed micrometastasis in PFs would provide more information not only concerning the existence of cancer cells but also carcinogenesis, tumor progression and chemosensitivity, based on information on methylation status.

In conclusion, the present study investigated the methylation status in PF by both q-MSp and qRT-PCR analyses. The multigene methylation of CHFR, E-cadherin and BNIP in PF revealed the clinical feasibility of detecting occult neoplastic cells in the peritoneum. A methylation analysis along with a cytological examination might increase the positive detection of cancer cells in PF.

**Innovations and breakthroughs**

Few studies have addressed gene methylation for the detection of micrometastasis to peritoneal fluid in gastrointestinal cancer. The authors’ results indicate that gene methylation in the peritoneal fluid could detect occult neoplastic cells in the peritoneum and might be a risk factor for peritoneal metastasis.

**Applications**

The development of this system may improve the accurate diagnosis of peritoneal dissemination and improve the prognosis of gastric cancer patients.

**Terminology**

DNA methylation is an epigenetic modification in humans, and changes in methylation patterns play an important role in carcinogenesis, cancer progression and chemosensitivity.

**Peer review**

This paper is interesting and written well.

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