Correlation between Serum DNA Methylation and Prognosis in Gastric Cancer Patients

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Abstract. *Background: Gastric carcinogenesis is thought to involve multiple genetic and epigenetic changes. The relationships between the promoter methylation status of relevant genes in the serum and outcomes in patients undergoing curative gastrectomy for cancer were investigated. Materials and Methods: Pre-operative serum samples obtained from 97 gastric cancer patients, who underwent radical gastrectomy, were subjected to methylation-specific polymerase chain reaction (MSP) assays for the p16, E-cadherin and retinoic acid receptor beta (RARβ) genes. Results: Promoter hypermethylation of p16, E-cadherin and the RARβ gene was detected in sera from 18 (19%), 24 (25%) and 24 patients (25%), respectively. Altogether, 47 patients (48%) showed hypermethylation of at least one gene analyzed. Survival curves differed significantly between groups defined by the methylation status of E-cadherin ($p<0.05$), but not those defined by p16 or RARβ ($p=0.77$ and 0.19, respectively). Conclusion: Serum MSP assays can provide not only diagnostic, but also prognostic information in gastric cancer.*

Despite many clinicopathological studies and its importance as a frequent cause of cancer mortality worldwide (1), gastric cancer is incompletely understood. Its development and progression generally are thought to represent a multi-step process involving a variety of genetic and epigenetic changes, suggesting that new molecular approaches to diagnosis and treatment could improve survival in gastric cancer (2).

In previous reports, we demonstrated the effectiveness of two polymerase chain reaction (PCR)-based screening assays in examining serum samples for evidence of gastric cancer. In the carcinoembryonic antigen (CEA)-specific reverse transcription-PCR (RT-PCR) assay, a positive result indicates the presence of circulating tumor cells (3); the methylation-specific PCR (MSP) assay differentiates between methylated and unmethylated promoter regions of tumor-suppressor genes (4-6). Promoter hypermethylation indicates down-regulation or silencing of those genes, thus detecting the presence of tumor-derived DNA fragments. We demonstrated that both PCR assays can serve as serum tests comparable to those for conventional tumor markers, such as CEA and carbohydrate antigen (CA) 19-9, with a possible advantage of the MSP assay for p16, E-cadherin and retinoic acid receptor beta (RARβ) genes over the RT-PCR assay or conventional tumor marker assays (4-6).

In the present study, the relationships between serum methylation status and long-term patient survival after curative gastrectomy for cancer were analyzed and the potential clinical implications of the serum MSP assay, not only as a diagnostic modality, but also as a prognostic tool in gastric cancer were considered.

Materials and Methods

One hundred and nine gastric cancer patients, who underwent radical gastrectomy, were enrolled in this retrospective study. Peripheral blood samples were obtained from a catheter inserted into a peripheral artery before surgery. The resected gastric cancer specimens were fixed in buffered formalin and embedded in paraffin for pathological examination using standard methods. The macroscopic and microscopic classifications of tumors were based on the General Rules for Gastric Cancer Study formulated by the Japanese Research Society for Gastric Cancer (JCGC) (7). Of these specimens, 37 gastric cancer tissue samples were used for DNA extract experiments.

The MSP assay was performed as previously described (4-6, 8). In brief, serum-derived DNA was treated with sodium bisulfate using the CpGenome DNA modification kit (Intergen, New York, NY, USA). The modified DNA was then subjected to MSP. The primer sequence for the methylated and unmethylated p16, E-cadherin and RARβ gene promoter regions were described previously (8-10). PCR was performed using specific primers in 25 μl of a mixture containing 1 x PCR buffer, 0.5-μM concentration of each primer, 0.5 mM dNTP, 1 unit of Taq polymerase and the modified DNA as template.

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Key Words: Promoter hypermethylation, E-cadherin, serum assays, prognosis, gastric cancer.

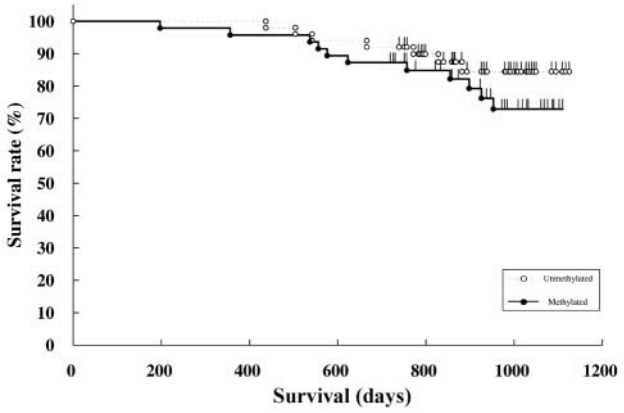

Figure 1. *Survival curves for patients with and without promoter hypermethylation affecting at least one of the three genes analyzed. The two groups showed no significant difference in survival.*

PCR conditions were as follows: initial denaturation at 95˚C for 10 min; then 40 cycles of 95˚C for 30 sec denaturation, 57˚C for 30 sec annealing and 72˚C for 30 sec extension. The final extension was for 10 min at 72˚C. The PCR product was electrophoresed on a 2% agarose gel together with a size marker, stained with ethidium bromide and visualized under ultraviolet illumination. Control methylation-positive and -negative DNA were amplified for each reaction.

During regular visits to our out-patient clinic, patients were followed-up by chest roentegenography, ultrasonography, endoscopy, computed tomography (CT), and conventional tumor markers such as CEA and CA19-9. No patient was lost to follow-up. Cumulative survival rates were calculated by the Kaplan-Meier method. Statistical analyses were performed by a log rank test (for survival curves) and a Chi-square test (for the relationship between the MSP results and clinicopathological features). A p value less than 0.05 was considered to indicate significance.

Results

Six patients who underwent non-curative operations were excluded from prognostic analysis. Two patients who died of post-operative complications and four who died of disease unrelated to gastric cancer were also excluded. The remaining 97 patients included 51 in JCGC stage I, 14 in stage II, 26 in stage III and six in stage IV. Promoter hypermethylation of the p16 gene was detected in serum from 18 patients (19%), of the E-cadherin gene in 24 (25%) and of the RARβ gene in 24 (25%). In all, 47 patients (48%) showed hypermethylation of at least one gene analyzed. Unmethylated promoter regions of all three genes were additionally detected by MSP in all patients, reflecting the presence of some non-neoplastic cells in serum samples.

The follow-up intervals during the study period ranged from 719 to 1125 days (median, 952). To date, tumor recurrence has been observed in 23 patients, while 19 of these patients died of recurrent disease.

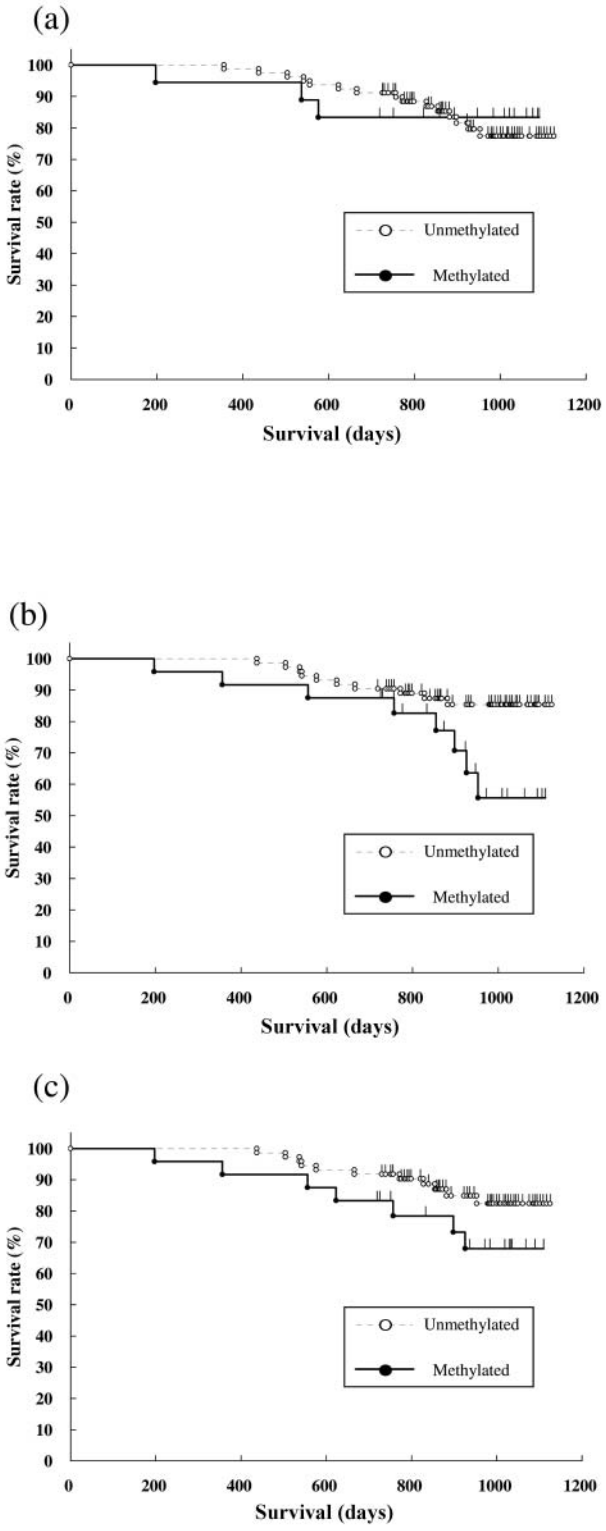

Figure 2. *Survival curves for patients grouped according to the methylation status of p16 (a), E-cadherin (b) and RAR-β (c). Survival differed significantly between the two groups defined by the methylation status of E-cadherin, but not between groups categorized by either p16 or RAR-β. methylation status.*

Table I. *Methylation status of E-cadherin gene in serum and clincopathological features of primary gastric cancer (n=97).*

	E-cadherin		
	M	U	*P* value
Age			
(years)	64	64.5	0.8563
Gender			
Male	16	56	
Female	8	17	0.329
Macroscopic form			
Localized	11	29	
Diffuse	13	44	0.598
Size			
(mm)	51.6	54.8	0.1787
Histology			
Diff.	8	45	
Undiff.	16	28	0.0157
Lymph nodes			
Negative	10	43	
Positive	14	30	0.1412
Stage			
I, II	12	53	
III, IV	12	20	0.041

Diff, differentiated adenocarcinoma; Undiff, undifferentiated adenocarcinoma; M, methylated; U, unmethylated.

Overall, no significant difference in survival was evident between patients with methylation of at least one gene and those without, although the methylation group showed a slightly lower 3-year survival rate (Figure 1; 73% *vs.* 81%, $p=0.38$). The survival curves for patients grouped according to the methylation status of specific genes were of greater interest (Figure 2). Patients with hypermethylation of E-cadherin in the serum showed a poorer 3-year survival rate than those without hypermethylation (56% *vs.* 83%, $p<0.05$). On the other hand, differences in survival were not significant between groups defined by the methylation status of either p16 or RARβ ($p=0.77$ and $p=0.19$, respectively).

The relationships between the methylation status of the E-cadherin gene in serum and the clinicopathological features of the primary tumors are indicated in Table I. As patients with hypermethylation of the E-cadherin promoter were more likely to have advanced and histologically undifferentiated gastric cancers, the prognostic value of the serum test was further examined in a subgroup analysis including only patients with E-cadherin gene hypermethylation in the primary tumor tissues. The survival rate of patients with hypermethylation of E-cadherin in serum also tended to be lower than that of those without in the subgroup analysis, although the difference was not significant ($p=0.15$) (Figure 3).

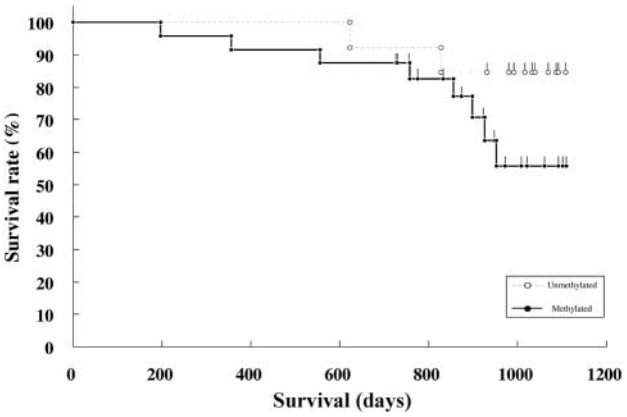

Figure 3. *Subgroup analysis including only those patients with hypermethylation of E-cadherin in the primary tumor tissues. The survival rate of patients with hypermethylation of E-cadherin in the serum tended to be lower than that of those without.*

Discussion

The hypermethylation of CpG islands within the promoter region has been established as an important epigenetic mechanism for suppression of gene expression. Methylation inactivating tumor-suppressor genes, such as E-cadherin, p15, p16, hMLH, APC, RARβ, DLC-1 and CDH4 have been implicated in the development and progression of gastric cancer (9-13). An increase in free DNA fragments released into the circulation from necrotic and apoptotic tumor cells was reported in cancer patients (14-16); several recent studies accordingly addressed the clinical usefulness of performing MSP assays in serum samples as a new molecular diagnostic screening test for clinically occult gastric cancer (17, 18).

We also reported that the MSP assay could provide diagnostic markers for gastric cancer (4-6). Apart from this initial diagnostic application, we currently examined the prognostic value of the serum MSP assay in gastric cancer patients.

The hypermethylation of the E-cadherin promoter in serum was found to be a significant prognostic factor in a survival analysis of all patients. However, an imbalance in characteristics between the methylated and unmethylated patient groups might be directly responsible for the survival difference, since E-cadherin gene hypermethylation in serum has been linked with anatomically advanced stages and poor histological differentiation, which are adverse prognostic factors (11). In the present study, patients with E-cadherin gene hypermethylation in their serum samples were indeed somewhat more likely to have advanced disease and histologically undifferentiated gastric cancers, although those differences fell short of significance. We, therefore, further examined the prognostic value of the serum test in a subgroup survival analysis including only those patients with

a primary tumor showing hypermethylation of the E-cadherin gene. While statistical significance was lost in this subgroup analysis, patients with hypermethylation of E-cadherin in the serum still tended to show poorer survival than those without hypermethylation. The results suggest a possible prognostic value of the serum MSP assay in gastric cancer patients in addition to a diagnostic value. The small number of patients in this analysis might be responsible for the lack of statistical significance. Lager studies are needed to better define prognostic implications.

The molecular mechanisms underlying the prognostic effect of the methylation status in serum are incompletely known. Prerequisites for a positive MSP result include hypermethylation of genes in the primary tumor and release of tumor-specific DNA fragments into the circulation. These fragments are thought to arise from necrotic and apoptotic tumor cells. On the other hand, several recent reports have demonstrated the presence of circulating tumor cells in peripheral blood samples from patients with gastric cancer (3, 19), such circulating cells may be another origin of hypermethylated DNA fragments in cancer patients. Positive results in the MSP assay in terms of E-cadherin might thus occur more frequently in patients with hematogenous dissemination of cancer cells.

In conclusion, the E-cadherin gene promoter methylation status in serum might serve as a prognostic marker in patients with gastric cancer, in addition to the diagnostic applications of the assay. Demonstration of a circulating hypermethylated promoter may allow us to predict which patients will require more aggressive post-operative chemotherapy.

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