Detection of *p16* promoter hypermethylation in serum of gastric cancer patients

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For the early detection of tumor-related aberrant DNA in the serum of patients, we examined promoter hypermethylation of the *p16* gene using methylation-specific PCR (MSP) in paired tumor and serum samples of 60 gastric cancer patients. Aberrant *p16* methylation was found in 23 of 60 (38%) primary gastric cancers, but in none of the corresponding gastric mucosae. Of these 23 patients, 6 (26%) exhibited the same alteration in their serum DNA. As a control, we screened for aberrant methylation in the serum DNA of 37 patients with gastric cancers whose corresponding tumor DNA had no methylation in the *p16* promoter. We also screened for methylation in the serum DNA of 16 non-cancer individuals. No methylation was found in serum DNA of these control groups. Our results suggest that *p16* methylation would be a good marker for the detection of tumor DNA in the serum of primary gastric cancer patients. (Cancer Sci 2003; 94: 418–420)

Gastric cancer is one of the most common malignancies worldwide.[1)] Though the prognosis of this cancer has improved in recent years, many patients still die from it and, early detection is critical for curative resection.

Previous studies have proposed that enriched circulating DNA can be found in the serum of cancer patients.[2, 3)] On the basis of these studies, many attempts have been made to achieve early detection of tumor-related aberrant DNA in the serum of patients with various malignancies.[4–6)]

Epigenetic changes may also be detected in the serum of cancer patients. In particular, we as well as others have detected *p16* hypermethylation in the serum of patients with many kinds of malignancies.[7–10)]

Accumulating evidence indicates that gastric cancer is the result of various genetic and epigenetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell-cycle regulators, and cell adhesion molecules.[11)] DNA hypermethylation has been particularly well studied, and has been found in the CpG islands of several genes. In gastric cancer, the inactivation of *hMLH1*, *MGMT*, *TIMP-3* and *p16* by promoter hypermethylation has been demonstrated.[12–15)] In particular, the *p16* tumor suppressor gene is frequently inactivated by promoter hypermethylation. The frequency of *p16* promoter hypermethylation was reported to be about 40% in gastric cancer,[16, 17)] suggesting that the detection of *p16* hypermethylation in serum could be one of the more reliable diagnostic and prognostic markers for gastric cancer.

To determine whether *p16* hypermethylation is a potential diagnostic marker, we have examined promoter hypermethylation of the *p16* gene using methylation-specific PCR (MSP) in paired tumor and serum samples of primary gastric cancer patients.

Materials and Methods

Sample collection and DNA preparation. Tumor samples were obtained at the time of surgery from 60 patients with primary gastric carcinoma at Nagoya University Hospital, Nagoya. Sixty corresponding serum samples were obtained prior to surgery. Sixteen serum samples from non-cancer individuals were also collected. Written informed consent, as required by the institutional review board, was obtained from all patients. Tumor and serum samples were immediately frozen and stored at −80°C until DNA was extracted. The samples were digested with proteinase K, and DNA was prepared as described previously.[18)]

Bisulfite modification. DNA samples were modified with sodium bisulfite and the methylation status of CpG islands of the *p16* gene was determined by MSP as previously described.[7)] Briefly, 1 μg of DNA was denatured with NaOH and modified with sodium bisulfite. DNA samples were then purified using Wizard purification resin (Promega Corp., Madison, WI), again treated with NaOH, precipitated with ethanol, and resuspended in water.

MSP. The methylation status of *p16* was determined by MSP. The modified DNA samples were amplified with primers specific for both methylated and unmethylated sequences. The primer sequences were described previously.[7)] The PCR amplification of modified tumor samples consisted of 1 cycle of 95°C for 5 min, 1 cycle of 78°C for 10 min, 30 cycles of 95°C for 30 s, 68°C for 1 min, and 72°C for 1 min; then 1 cycle of 70°C for 10 min. The PCR amplification of modified serum samples consisted of 1 cycle of 95°C for 5 min, 1 cycle of 78°C for 10 min, 32 cycles of 95°C for 30 s, 69°C for 1 min, and 72°C for 1 min; and then 1 cycle of 70°C for 10 min. DNAs from L132 (embryonic lung cell line) and H1299 (lung cancer cell line) were used as positive controls for unmethylated and methylated alleles, respectively. Controls without DNA were performed for each set of PCR. Ten microliters of each PCR product was directly loaded onto non-denaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. Each MSP was repeated at least three times.

Statistical analysis. We evaluated the correlation between the *p16* methylation status of serum samples and their clinicopathological characteristics. Statistical significance was evaluated by means of Fisher's exact test.

Results

We first examined the methylation status of *p16* promoter in tumors and corresponding normal tissues using the MSP technique. Twenty-three of 60 primary tumors (38%) exhibited aberrant *p16* promoter hypermethylation while no corresponding gastric mucosae exhibited it. This frequency is consistent with that described previously in other reports,[15–17)] indicating that *p16* methylation may be a good marker to detect gastric cancer DNA in serum because of its high methylation rate in tumors. Subsequently, we examined promoter hypermethylation in the paired serum DNA of patients with a *p16* alteration in their primary tumor. Six of 23 patients (26%) exhibited the same alteration in their serum DNA. Representative results of

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Fig. 1. Representive MSP of *p16* promoter hypermethylation in primary tumors (T) and the corresponding serum samples (S) from primary (cases 3 and 18) gastric cancers. A visible PCR product in lanes U indicates the presence of unmethylated genes; a visible product in lanes M indicates the presence of methylated genes in all cases. In each case, modified DNA from L132 and H1299 were used as positive controls for unmethylated and methylated alleles, respectively.

MSP for *p16* promoter are shown in Fig. 1. As a control, we screened for aberrant methylation in the serum DNA of 37 patients with gastric cancers whose corresponding tumor DNA had no methylation in *p16* promoter. We also screened for methylation in the serum DNA of 16 non-cancer individuals. No methylation was found in serum DNA of these control groups.

After completion of MSP analysis in all specimens, clinicopathological data were correlated with the molecular analysis (Table 1). We found no association of overall aberrant methylation in the tumor and serum DNA with the histologic type, depth of invasion, stage of the disease, presence of lymph node metastasis, liver metastasis, peritoneal dissemination, lymphatic invasion, or venous invasion (Table 2). Interestingly, abnormal methylation was found in the serum of patients at all clinical

Table 1. Clinicopathological features and *p16* methylation in serum DNA from gastric cancer patients with *p16* methylation in tumor DNA

Case no.	Age (yrs)	Sex	*p16* methylation in serum	Lauren's classification	TNM classification				Peritoneal metastasis	Liver metastasis	Lymphatic invasion	Venous invasion
					pT	pN	pM	Stage				
1	85	M	+	Diffuse type	T2	N3	M0	4	–	–	–	+
2	73	M	–	Diffuse type	T1	N0	M1	4	–	+	+	+
3	57	M	+	Diffuse type	T3	N1	M0	3b	–	–	–	+
4	66	M	–	Intestinal type	T1	N0	M0	1a	–	–	–	–
5	65	M	+	Intestinal type	T2	N0	M0	1b	–	–	–	+
6	62	M	–	Intestinal type	T2	N1	M0	2	–	–	–	+
7	51	M	–	Diffuse type	T2	N1	M0	2	–	–	–	+
8	55	M	–	Intestinal type	T1	N0	M0	1a	–	–	–	–
9	63	F	–	Intestinal type	T3	N1	M1	4	–	+	+	+
10	53	M	–	Diffuse type	T1	N0	M0	1a	–	–	–	–
11	69	M	–	Diffuse type	T1	N0	M0	1a	–	–	–	+
12	72	F	–	Intestinal type	T3	N2	M1	4	+	–	+	+
13	60	F	–	Diffuse type	T2	N1	M0	2	–	–	–	–
14	50	F	–	Diffuse type	T3	N3	M1	4	+	–	–	+
15	75	F	–	Intestinal type	T2	N2	M0	2	–	–	–	–
16	70	M	–	Diffuse type	T2	N1	M0	2	–	–	–	+
17	59	M	+	Diffuse type	T2	N2	M0	3a	–	–	–	+
18	47	M	+	Intestinal type	T1	N0	M0	1a	–	–	+	+
19	66	M	–	Diffuse type	T2	N0	M0	1b	–	–	–	+
20	66	M	–	Intestinal type	T2	N1	M0	2	–	–	–	+
21	45	F	–	Diffuse type	T2	N0	M0	1b	–	–	–	+
22	54	M	–	Diffuse type	T2	N0	M0	1b	–	–	–	+
23	68	M	+	Diffuse type	T3	N3	M0	4	–	–	Unknown	+

Table 2. Correlation between clinical characteristics of gastric cancer patients with tumor *p16* methylation and serum *p16* methylation status

	No. cases of serum *p16* methylation status				No. cases of serum *p16* methylation status		
	Positive	Negative	*P* value[1)]		Positive	Negative	*P* value
Histological type				Liver metastasis			
Intestinal type	2	7	>0.999	Negative	6	15	>0.999
Diffuse type	4	10		Positive	0	2	
Depth of invasion				Lymphatic invasion			
Mucosa, submucosa	1	5	>0.999	Negative	0	5	0.273
Beyond submucosa	5	12		Positive	6	12	
Lymph node metastasis				Venous invasion			
Negative	2	8	0.66	Negative	4	14	>0.999
Positive	4	9		Positive	1	3	
Peritoneal metastasis				Stage			
Negative	6	15	>0.999	I & II	2	12	0.162
Positive	0	2		III & IV	4	5	

1) Fisher's exact test.

stages, suggesting that gastric cancer could be detected from its early stages using the MSP technique.

Discussion

In previous studies, tumor-related aberrant DNA has been identified in the serum of cancer patients.[4–6] In particular, hypermethylation of the *p16* tumor suppressor gene has been studied in various malignancies such as esophageal cancer, non-small cell lung cancer, colorectal cancer, liver cancer, and head and neck cancer.[7–10, 19] The MSP technique is useful for the detection of promoter hypermethylation in these cancers, because of its high sensitivity and specificity. No abnormal methylation was found in serum DNA if this alteration was not present in the primary tumor. Moreover, MSP has sufficient sensitivity to detect even scanty tumor DNA in serum; it detected nearly 1 methylated gene copy/1000 unmethylated copies in dilution experiments.[8]

Several studies have been made on the detection of gastric cancer cells in peripheral blood using reverse transcription-PCR (RT-PCR). These studies have focused on the detection of several tumor-specific genes such as *CEA* and *hTERT*.[20, 21] Compared to this RT-PCR approach, the DNA-based approach in this study appears to have advantages. For example, DNA extracted from tumors and serum can be easily amplified by the PCR technique. Furthermore, aberrant DNA methylation represents a chemically and biologically stable tumor-specific marker that can be readily detected, independently of the level of gene expression. However, the DNA-based method is available only for tumors with DNA alterations.

Thus, we found that the serum detection of early-stage gastric cancer is feasible, although there was no correlation between *p16* methylation status and clinicopathological data. Indeed, several studies have shown that *p16* promoter hypermethylation is an early and frequent event in the initial stage of stomach carcinogenesis.[16, 17] These findings suggest that *p16* hypermethylation could be a new marker for early detection of gastric cancer.

In this modest study, we could not detect any association between the existence of tumor DNA in serum and the clinicopathological data. This result may have been due to sampling bias involving differences in tumor cell density in the blood, because some investigators have noted that tumor cells are intermittently shed into peripheral blood.[22, 23] Additional studies are needed to determine the clinical relevance of identifying specific genetic alterations in the serum of gastric cancer patients as markers of the prognosis and for monitoring of the disease.

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