Reprimo as a Potential Biomarker for Early Detection in Gastric Cancer

Carolina Bernal,[1] Francisco Aguayo,[2] Cynthia Villarroel,[1,2] Macarena Vargas,[1,2] Ignacio Díaz,[1] Francisco J. Ossandon,[1] Eudocia Santibáñez,[1] Mariana Palma,[3] Edmundo Aravena,[3] Carlos Barrientos,[3,4] and Alejandro H. Corvalan[1,2]

Abstract

Purpose: Gastric cancer is a curable disease if diagnosed at early stage. However, most cases are diagnosed at advanced stage because of the lack of screening programs. Therefore, the identification of plasma biomarkers for early detection is necessary.

Experimental Design: To search for these biomarkers, we evaluated the DNA methylation patterns of 24 genes by Methylation-specific PCR in primary tissues from 32 retrospectively collected gastric cancer cases (testing group). Correlation between methylation and gene expression was evaluated in the MKN-45 cell line after treatment with 5-aza-2′-deoxycytidine. The most frequently hypermethylated genes were next evaluated in primary tissues and plasma samples from 43 prospectively collected gastric cancer cases as well as plasma samples from 31 asymptomatic age- and gender-matched controls (validation group).

Results: In the testing group, 11 genes were hypermethylated in at least 50% of cases (*APC, SHP1, E-cadherin, ER, Reprimo, SEMA3B, 3OST2, p14, p15, DAPK*, and *p16*). Eight genes (*BRCA1, p73, RARβ, hMLH1, RIZI, RUNX3, MGMT*, and *TIMP3*) were statistically associated with a particular variant of gastric cancer, the signet-ring cell type ($P = 0.03$). Seven genes (*APC, SHP1, E-cadherin, ER, Reprimo, SEMA3B*, and *3OST2*) were next evaluated in the validation group. We confirm the high frequency of methylation in primary tumors for all seven genes. However, only *APC* and *Reprimo* were frequently methylated in pair plasma samples. In asymptomatic controls, only *Reprimo* was infrequently methylated in comparison with plasma from gastric cancer cases ($P < 0.001$).

Conclusion: Our results identified specific methylation profile associated to signet-ring cell-type histology and aberrant hypermethylation of *Reprimo* as a potential biomarker for early detection of gastric cancer.

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death in the world (1). In spite of the improvements in the treatment, the worse prognosis of gastric cancer is correlated with tumor invasion (2). When the disease is confined to the mucosa or submucosa layers of the stomach (early gastric cancer), the 5-year survival rate is 95%. In contrast, when extended to the muscularis propia or serosa (advanced gastric cancer), the survival rate is <10% to 20% (3). Because most cases of gastric cancer are asymptomatic until advanced stage, the diagnosis of early gastric cancer is difficult (3). In recent years, particular combinations of genetic alterations (i.e., gene amplification and point mutations) in cancer-related genes have been implicated in the pathogenesis of this tumor (4). However, these alterations varies according to histologic subtypes indicating distinct carcinogenetic pathways of gastric cancer (5). Thus, a direct application to early diagnosis of gastric cancer is precluded. Therefore, new molecular markers for early diagnosis of gastric cancer are needed.

Epigenetics has emerged as one of the most exciting frontiers in the study of human carcinogenesis. Epigenetic processes control the packaging and function of the human genome and contribute to normal development and disease (6). This new field promises novel insights in the pathogenesis of cancer because its potential to detect quantitative alterations and multiplex modifications of regulatory sequences outside of genes (7). The first documented epigenetic alteration in gastric cancer was the promoter hypermethylation of the DNA mismatch repair genes (*hMSH2* and *hMLH1*; ref. 8). Subsequently, several other specific tumor suppressor genes have

Authors' Affiliations: [1]Department of Anatomic Pathology and [2]Laboratory of Molecular Pathology and Epidemiology, Centro Investigaciones Medicas, Pontificia Universidad Católica de Chile, [3]Instituto Chileno Japonés de Enfermedades Digestivas Hospital Clínico San Borja Arriaran, and [4]Department of Internal Medicine, Campus Centro, Universidad de Chile, Santiago, Chile

Received 10/4/07; revised 2/20/08; accepted 3/12/08.

Grant support: Chilean government grants FONDECYT 1030130 and FONIS.SA06I20019, and FONDECYT1080563.

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Note: This work has been partially presented at 3rd Annual Gastrointestinal Oncology Conference, September 21-23, 2006, Arlington, Virginia.

Requests for reprints: Alejandro H. Corvalan, Department of Pathology, University of Texas, M. D. Anderson Cancer Center, Houston, TX 77030. Phone: 713-563-9202; Fax: 713-792-0309; E-mail: acorvala@mdanderson.org.

doi:10.1158/1078-0432.CCR-07-4522

been described as inactivated by hypermethylation in gastric cancer (5). However, and although recent reports explore gene methylation of specific genes (9–11), a comprehensive DNA methylation profile in gastric cancer has not been carried out to date. Here, to find genes with aberrant hypermethylation that might be useful for early diagnosis, we used a candidate gene approach through searching CpG island in promoter region in 24 candidate genes in 32 retrospectively collected gastric cancer cases. To evaluate the significance of the most relevant methylated genes for early detection, the seven most frequently methylated genes were evaluated in 43 prospectively collected cases from which paired tumor and plasma samples were available as well as in plasma samples from 31 asymptomatic age- and gender-matched controls. We found that aberrant methylation of *Reprimo* might be a potential biomarker for early detection of gastric cancer.

Materials and Methods

Clinical samples and cell line. To identified the DNA methylation patterns of 24 genes, we used a testing-validation approach (12). As a testing set, we selected 32 surgically resected gastrectomy specimens with diagnoses of gastric cancer according to the WHO (13). Testing set was composed by 21 males (65.6%) with an age average of 65 years old, 11 (34.3%) of these tumors were located in the cardia, 7 (21.8%) in the middle third, and 14 (43.7%) in the antrum. Four (12.5%) tumors were at early stage and 22 (68.8%) were lymph node positive. Thirteen (40.6%) tumors were signet-ring cell histology according to the WHO (14). As a validation set, 43 prospectively collected endoscopic biopsies and plasma samples from gastric cancer patients with similar characteristics to that of the testing set were obtained together with plasma samples from 31 asymptomatic age- and gender-matched controls. All cases were immediately snap-frozen at the time of surgery or endoscopic procedure. None of cases had family history of gastric cancer. This study was approved by the Institutional Review Board at Hospital San Borja-Arriaran and Pontificia Universidad Catolica de Chile. The cell line MKN45 was used to confirm the association between CpG island hypermethylation and RNA expression of selected genes.

DNA extraction. Five, 15-μm cryostat sections with representative areas of gastric cancer were cut and placed into a 0.5 mL tube for DNA extraction. DNA extraction was done in 100 μL extraction solution [1 mol/L Tris (pH 8.0), 50 mmol/L EDTA, 0.5% and Tween 20] with 1 mg/mL Proteinase K (Sigma) for 12 h at 55°C. Proteinase K was inactivated by boiling at 100°C for 10 min and DNA was purified by phenol-chloroform extraction and ethanol precipitation according to standard protocols. DNA concentration was determined by spectroscopy using 1 OD260 for 50 μg/mL. We chose plasma as a source for DNA analysis based on previous reports indicating that plasma is superior than serum for DNA methylation detection (15).

Methylation assays. DNA was treated with sodium bisulfite as described previously (16). Briefly, 1 μg of genomic DNA was denatured by incubation with 0.2 mol/L NaOH for 10 min at 37°C. Hydroquinone (10 mmol/L; 30 μL; Sigma) and 3 mol/L sodium bisulfite (pH 5.0; 520 μL; Sigma) were added, and the solution was incubated at 50°C for 16 h. Treated DNA was purified by use of Wizard DNA Purification System (Promega Corp.), desulfonated with 0.3 mol/L NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was stored at -80°C until used. The methylation status of 24 genes was determined by Methylation-specific PCR (16). These genes were chosen because they are tumor suppressors genes, methylation at these CpG sites is associated with gene silencing, they cover six essential alterations in cell physiology that collectively dictate malignant growth (self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis), and they had previously been described as undergoing hypermethylation in other tumor types (16–33). Gene names, gene location, function, and reference for methodology for each gene selected in this study is summarized in Table 1. Only cases with positive unmethylated bands were considered informative for methylation status in this study. Therefore, our rate of informative cases ranges from 6 to 32. Negative control samples without DNA were included for each set of PCR.

Reverse transcription-PCR and demethylating agent 5-aza-2′-deoxycytidine. For all 24 genes selected for analysis, an association between hypermethylation and gene silencing has been previously established (16–33). However, we confirmed this association for *SHP1*, *APC*, *Reprimo*, *FHIT*, *E-cadherin*, and *SEMA3B* by using reverse transcription-PCR in the cell line MKN45, a poorly differentiated gastric cancer cell line from mongoloid female origin. Total RNA was extracted using the Qiagen RNeasy System (Qiagen), according to the manufacturer's recommendations. RNA concentration was determined by measuring absorbance at 260 nm, and quality was verified by the integrity of 28S and 18S rRNA after ethidium bromide staining of total RNA samples subjected to 0.8% agarose gel electrophoresis. Total cDNA was synthesized with Moloney Murine Leukemia Virus reverse transcriptase (ThermoScript RT; Invitrogen). Reverse transcription-PCR was done using 1 μg of total cellular RNA to generate cDNA. Primers sequences have been previously established (29, 31, 32, 34–36). Reverse transcription-PCR for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene served as a control for RNA loading. Ten microliters of each PCR reaction were directly loaded onto 2% agarose gels and separated by electrophoresis; the gels were stained with ethidium bromide and visualized under UV illumination. To restore silenced gene expression of methylated genes, we treated a cell line for 72 h with the demethylating agent 5-aza-2′-deoxycytidine (Sigma) at a concentration of 2 μmol/L, as described previously (29).

Data analysis. Frequencies of methylation were compared using χ^2 test or Fisher exact tests. For all tests, probability values of $P < 0.05$ were regarded as statistically significant.

Results

DNA methylation patterns of the CpG Island of multiple genes. The complete spectrum of DNA methylation patterns of 24 genes observed in 32 retrospectively collected gastric cancer cases is shown in Fig. 1A. Twenty-three (96%) genes showed promoter methylation in at least 3 or more of the tested cases, whereas 11 genes were hypermethylated in at least 50% of cases (*APC*, *SHP1*, *E-cadherin*, *ER*, *Reprimo*, *SEMA3B*, *3OST2*, *p14*, *p15*, *DAPK*, and *p16*). Two of these 11 genes (*SHP1* and *SEMA3B*) have not been reported previously methylated in gastric cancer. No promoter methylation was evident for *GSTp1* gene in any of the tested cases. Representative examples of the DNA methylation analysis are shown in Fig. 1B.

DNA methylation patterns and loss of RNA expression in MKN-45 cell line. To establish the association between DNA methylation patterns and gene silencing in gastric cancer, we determined mRNA expression of 5 genes that were methylated in at least 50% of cases (*APC*, *SHP1*, *E-cadherin*, *Reprimo*, and *SEMA3B*). To this purpose, we treated the cell line MKN45 with the demethylating agent 5-aza-2-deoxycitidine. As shown in Fig. 2, the gene expression reactivation was associated with the addition of the demethylating drug.

Clinicopathologic associations. We explore the relationship among clinical and pathologic characteristics of cases and DNA methylation patterns in the testing group cases. Methylation of eight genes (*BRCA1*, *p73*, *RARβ*, *hMLH1*, *RIZI*, *RUNX3*, *MGMT*, and *TIMP3*) was statistically associated with a particular variant

Table 1. Summary data of genes tested for aberrant promoter hypermethylation in gastric cancer

Gene abbreviation	Gene name	Location	Function	Reference
3OST2	Heparan sulfate (glucosamine)	16p12	Angiogenesis	(15)
APC	Adenomatous polyposis coli gene	5q21	Signal transduction	(16)
BLU	Homologous to the MTG/ETO family of transcription factors	3p21.3	Cell cycle regulation	(14)
BRCA1	Breast cancer 1 gene	17q2	DNA repair	(17)
COX2	Cyclooxygenase 2	1q25	Angiogenesis	(18)
DAPK	Death-associated protein kinase	9q34	Evasion of programmed cell death	(19)
CDH1	E-cadherin	16q22	Tissue invasion and metastasis	(19)
ER	Estrogen Receptor	6q25.1	DNA binding, activation transcription	(20)
FHIT	Fragile histidine triad	3p14.2	Evasion of programmed cell death	(14)
GSTp1	Homologous to the MTG/ETO family of transcription factors	3p21.3	Cell cycle regulation	(21)
hMLH1	Human homologues of the MutL gene of bacteria	3p21.3	DNA repair (Mismatch repair genes)	(22)
MGMT	O-6-methylguanine-DNA methyltransferase	10q26	DNA repair	(23)
p14	Cyclin-dependent kinase inhibitor 2B	9p21	Cell cycle regulation	(16)
p15	Cyclin-dependent kinase inhibitor 2B	9p21	Cell cycle regulation	(24)
p16	Cyclin-dependent kinase inhibitor 2A	9p21	Cell cycle regulation	(16)
p73	TP73	1p36	Angiogenesis	(19)
PTEN	phosphatase and tensin homologue	10q23	Signal transduction	(25)
RAR-β	Retinoic acid receptor β 2 gene	3p24	DNA binding, activation transcription	(26)
Reprimo	TP53 dependent G_2 arrest mediator candidate	2q23.	Cell cycle regulation	(27)
RIZ1	Rb-interacting zinc finger gene 1	1p36	Methyltransferase superfamily	(19)
RUNX3	runt-related transcription factor 3	1p36	Signal transduction (TGF-β pathway)	(28)
SEMA3B	Semaphorin 3B	3p21.3	Evasion of programmed cell death	(29)
SHP1	Hematopoietic cell-specific protein-tyrosine phosphatase SHPTP1	12p13	Signal Transduction (JAK-STAT pathway)	(30)
TIMP3	Tissue inhibitor of metalloproteinase-3	22q12	Tissue invasion and metastasis	(31)

of gastric cancer, the signet-ring cell type ($P = 0.03$ by Fisher's exact test). These data suggest that at molecular level, the signet-ring cell type should be considered a distinct subtype of gastric cancer. No other clinical or pathologic associations were found either by multiple- or single-gene analysis.

DNA methylation patterns of the CpG island in prospectively collected gastric cancer cases and asymptomatic controls. Next, to evaluate the significance of the DNA methylation patterns of the CpG island for clinical biomarkers for early diagnosis of gastric cancer, the seven most frequently hypermethylated genes in the testing group (*APC*, *SHP1*, *E-cadherin*, *ER*, *Reprimo*, *SEMA3B*, and *3OST2*) were examined in 43 prospectively collected gastric cancer cases from which pair tumor gastric biopsies and plasma samples were available. In addition, plasma from 31 asymptomatic age- and gender-matched controls were also prospectively collected and examined for

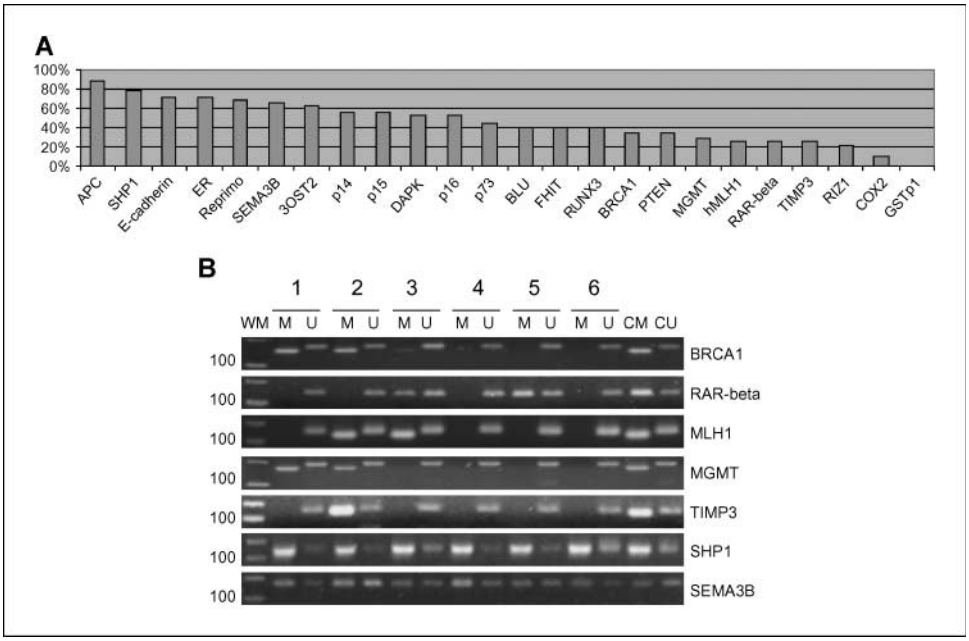

Fig. 1. Methylation-specific PCR analysis of 32 retrospectivelly collected gastric cancer cases. *A,* histogram representing the percentage of tumors showing methylation for the 24 genes as indicated. *B,* illustrative methylation-specific PCR results for seven of the genes studied in 6 cases (*lanes 1-6*). *WM,* weight marker; *M,* PCR product with primers specific for methylated DNA; *U,* PCR product with primers for unmethylated DNA; *CM,* MKN-45 *in vitro* methylated MKN-45 cell line (positive control for methylated genes). *CU,* peripheral blood lymphocytes DNA (used as a control of unmethylated genes).

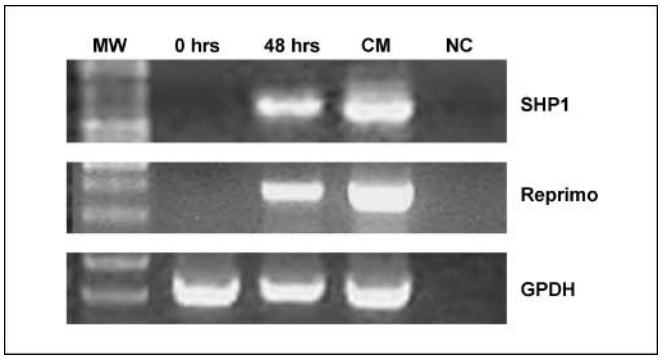

Fig. 2. Representative examples of RT-PCR for (*A*) *SHP1* and (*B*) *Reprimo* expression in gastric cancer MKN45 cell line before (0 h) and after (48 h) treatment with 5-Aza-CdR. Expression of the housekeeping gene *GAPDH* was run as a control for RNA integrity. cDNA was synthesized with (+) or without (-) reverse transcriptase. *CM,* MKN-45 *in vitro* methylated MKN-45 cell line (positives control for methylated genes); *NC,* negative control (water blank).

these genes. As shown in Fig. 3A, only methylation of *Reprimo* was identified in 97.7% (42 of 43) and 95.3% (41 of 43) of tumor and plasma from gastric cancer patients, respectively. However, among asymptomatic controls, methylation of *Reprimo* was identified in only 9.7% (3 of 31) of cases tested. These differences were statistically significant ($P < 0.00001$ by Fisher's exact test). Although methylation of *APC* was frequently methylated in tumor and pair plasma samples from gastric cancer patients, no differences were observed with pair plasma from asymptomatic controls. Representative examples of these analyses are shown in Fig. 3B-D. The other five genes (*SHP1*, *E-cadherin*, *ER*, *SEMA3B*, and *3OST2*), although frequently methylated in tumor samples were less frequently methylated in pair plasma samples.

Discussion

We and others are currently attempting to define the DNA methylation patterns of each type of human cancer (16, 21, 37–42). In gastric cancer, the first documented epigenetic alteration was the promoter hypermethylation of the DNA mistmatch repair genes (*hMSH2* and *hMLH1*; refs. 8, 43). Although several other genes have been described inactivated by epigenetics (5, 9–11, 18, 44–46), a comprehensive methylation profiling in gastric cancer has not been done up to date. Our approach using 24 genes led us to identify specific genes (*BRCA1*, *p73*, *RARβ*, *hMLH1*, *RIZI*, *RUNX3*, *MGMT*, and *TIMP3*) associated with a particular variant of gastric cancer, the signet-ring cell type. It has been suggested that signet-ring cell type is epidemiologically, clinico-pathologic, and molecularly a distinct subtype of gastric cancer (47, 48). Thus, our finding not only identified the methylation profile of this emerging variant of gastric cancer but also gives support to the hypothesis that the hypermethylation of the CpG island does not occur randomly but by specific selection process targeting key tumor suppressor genes (41). Our candidate gene approach also led us to identify a two genes (*SHP1* and *SEMA3B*) significative methylated in gastric cancer. *SHP1* (*Hematopoietic cell-specific protein-tyrosinephosphatase*) is a member of JAK-STAT pathway and located in 12p13. This gene has been described frequently inactivated by methylation in leukemia and lymphomas (49) and more recently in gallbladder carcinoma (21). *SEMA3B*,

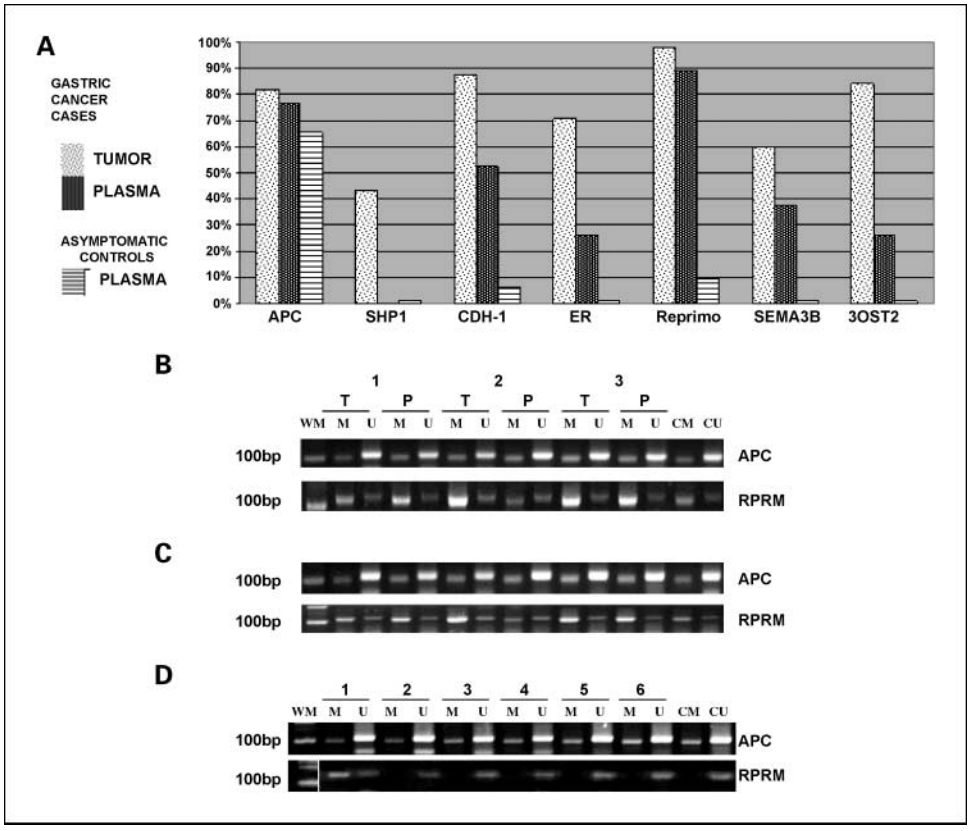

Fig. 3. Methylation-specific PCR analysis of 43 prospectively collected gastric cancer cases and 31 asymptomatic age- and gender-matched controls. *A,* histogram representing the percentage of tumors and pair samples showing methylation for the 7 genes as indicated. *B* to *D,* illustrative methylation-specific PCR results for two (APC and Reprimo) of the seven genes in tubular/papillary and poorly differentiated types (*B*), mucinous/signet-ring cell type (*C*) and asymptomatic controls. *D, WM,* weight marker, *M,* PCR product with primers specific for methylated DNA; *U,* PCR product with primers for unmethylated DNA; *CM,* MKN-45 *in vitro* methylated MKN-45 cell line (positive control for methylated genes). *CU,* peripheral blood lymphocytes DNA (used as a control of unmethylated genes).

a member of the 3p21.3 tumor suppressor cluster, has been also described inactivated by methylation in several neoplasms such as liver, gallbladder, lung, and ovary (16). To our knowledge, this is the first report on *SHP1* and *SEMA3B* methylation in gastric cancer.

Several studies have addressed the diagnostic utility of epigenetic biomarkers in detection of human cancer (7). Methylation abnormalities have been detected in blood or sputum of patients with lung cancer, in serum or plasma of head and neck cancers, in ductal lavage fluid of breast cancer, and in urine from patients with prostate and bladder cancer (7). To explore the diagnostic utility of epigenetic biomarkers in the detection of gastric cancer, we evaluated the most frequently hypermethylated genes from the testing set (*APC*, *SHP1*, *E-cadherin*, *ER*, *Reprimo*, *SEMA3B*, and *3OST2*) in an independent prospectively collected validation set. In this validation set, we confirm the high frequency of methylation among primary tissues. However, only two (*APC* and *Reprimo*) were frequently methylated (>70%) in pair plasma samples. When these genes were evaluated among plasma samples from asymptomatic controls, only *Reprimo* was significantly less frequently methylated than the others. Taken together, our data are consistent with previous reports in which *Reprimo* has been found frequently methylated in several cancers but rarely in nonmalignant tissues (29). However, our results in plasma samples are the first to indicate *Reprimo* as a potential biomarker for early detection of gastric cancer. *Reprimo* is a downstream mediator of p53-induced G_2 cell cycle arrest (50). When overexpressed, *Reprimo* induces cell cycle arrest at the G_2 phase, suggesting that has tumor suppressor function (50). Because functional abrogation of the p53 tumor suppressor gene and its downstream mediators, such as *14-3-3-σ*, *Reprimo* is central to the development of human cancers.

Our findings of *APC* cannot substantiate the results of Leung et al. (51) who found only 17% of methylation of *APC* in serum of patients with gastric cancer. This discrepancy may partly be due to differences in the pathogenesis of gastric cancer between South America and Hong Kong, or methodologies used (Methylight versus Methylation-specific PCR). On the other hand, our findings of methylation of CDH1 in plasma of gastric cancer cases were similar to Lee et al. (52), the first to study the feasibility of detecting aberrant methylation in serum of gastric cancer patients.

The prognosis of gastric cancer in prognosis is correlated with tumor invasion (2, 3). Prospective studies using photofluorographic methods has shown 2-fold decrease in gastric cancer mortality (relative risk, 0.52; 95% confidence interval, 0.36-0.74) between screened and nonscreened subjects (53). However, the implementation of such massive screening program in asymptomatic population is expensive. This scenario indicates the necessity of develop methodologies for early detection of gastric cancer. The term "serologic biopsy" is a noninvasive alternative method for massive detection of gastric cancer. The serologic biopsy includes the detection of pepsinogen and gastrin 17 for the identification of gastric atrophy, the precursor lesion of gastric cancer (54). Here, we describe a novel gene, *Reprimo*, which displayed high frequency of methylation in primary tumors and pair plasma samples in gastric cancer cases but low frequency in plasma samples from asymptomatic controls. Thus, *Reprimo* should be considered to be included in the serologic biopsy to directly identified gastric cancer.

In summary, our results provide additional information on the significance of epigenetic modification in gastric cancer and delineate distinct methylation profiles of histologic variants of gastric cancer. Our findings are relevant from clinical perspective because we found that promoter hypermethylation of *Reprimo* might be a potential candidate for early detection of gastric cancer. Further research will be necessary to validated the potential clinical effect of these results.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Parkin DM. International variation. Oncogene 2004; 23:6329–40.
2. Rivera F, Vega-Villegas ME, Lopez-Brea MF. Chemotherapy of advanced gastric cancer. Cancer Treat Rev 2007;33:315–24.
3. Nomura S, Kaminishi M. Surgical treatment of early gastric cancer. Dig Surg 2007;24:96–100.
4. Yasui W, Oue N, Aung PP, Matsumura S, Shutoh M, Nakayama H. Molecular-pathological prognostic factors of gastric cancer: a review. Gastric Cancer 2005; 8:86–94.
5. Tamura G. Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric cancer. World J Gastroenterol 2006;12: 192–8.
6. Callinan PA, Feinberg AP. The emerging science of epigenomics. Hum Mol Genet 2006;15 Suppl 1: R95–101.
7. Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. Nat Rev Cancer 2005;5:223–31.
8. Fleisher AS, Esteller M, Wang S, et al. Hypermethylation of the hMLH1 gene promoter in human gastric cancers with microsatellite instability. Cancer Res 1999;59:1090–5.
9. Kang GH, Lee S, Kim JS, Jung HY. Profile of aberrant CpG island methylation along the multistep pathway of gastric carcinogenesis. Lab Invest 2003;83: 635–41.
10. Kim HC, Kim JC, Roh SA, et al. Aberrant CpG island methylation in early-onset sporadic gastric carcinoma. J Cancer Res Clin Oncol 2005;131:733–40.
11. Oue N, Mitani Y, Motoshita J, et al. Accumulation of DNA methylation is associated with tumor stage in gastric cancer. Cancer 2006;106:1250–9.
12. Retamales E, Rodriguez L, Guzman L, et al. Analytical detection of immunoglobulin heavy chain gene rearrangements in gastric lymphoid infiltrates by peak area analysis of the melting curve in the LightCycler System. J Mol Diagn 2007;9:351–7.
13. Fenoglio-Preiser C, Carneiro F, Correa P, et al. Gastric Carcinoma. In: Hamilton HB, Aaltonen L, editors. Pathology and Genetics of Tumours of the Digestive System. Lyon: IARC Press; 2000. p. 37–68.
14. Fenoglio-Preiser C, Carneiro F, Correa P, et al. Gastric Carcinoma. In: Hamilton HB, Aaltonen L, editors. Pathology and Genetics of Tumours of the Digestive System. Lyon: IARC Press; 2000.
15. Jung M, Klotzek S, Lewandowski M, Fleischhacker M, Jung K. Changes in concentration of DNA in serum and plasma during storage of blood samples. Clin Chem 2003;49:1028–9.
16. Riquelme E, Tang M, Baez S, et al. Frequent epigenetic inactivation of chromosome 3p candidate tumor suppressor genes in gallbladder carcinoma. Cancer Lett 2007;250:100–6.
17. Miyamoto K, Asada K, Fukutomi T, et al. Methylation-associated silencing of heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2 (3-OST-2) in human breast, colon, lung and pancreatic cancers. Oncogene 2003;22:274–80.
18. Sarbia M, Geddert H, Klump B, Kiel S, Iskender E, Gabbert HE. Hypermethylation of tumor suppressor genes (p16INK4A, p14ARF and APC) in adenocarcinomas of the upper gastrointestinal tract. Int J Cancer 2004;111:224–8.
19. Chan KY, Ozcelik H, Cheung AN, Ngan HY, Khoo US. Epigenetic factors controlling the BRCA1 and BRCA2 genes in sporadic ovarian cancer. Cancer Res 2002;62:4151–6.
20. Xu XL, Yu J, Zhang HY, et al. Methylation profile of the promoter CpG islands of 31 genes that may contribute to colorectal carcinogenesis. World J Gastroenterol 2004;10:3441–54.
21. Takahashi T, Shivapurkar N, Riquelme E, et al. Aberrant promoter hypermethylation of multiple genes in gallbladder carcinoma and chronic cholecystitis. Clin Cancer Res 2004;10:6126–33.
22. Mori T, Martinez SR, O'Day SJ, et al. Estrogen receptor-α methylation predicts melanoma progression. Cancer Res 2006;66:6692–8.

23. Kim J, Lee HS, Bae SI, Lee YM, Kim WH. Silencing and CpG island methylation of GSTP1 is rare in ordinary gastric carcinomas but common in Epstein-Barr virus-associated gastric carcinomas. Anticancer Res 2005;25:4013–9.

24. Kawaguchi K, Oda Y, Saito T, et al. DNA hypermethylation status of multiple genes in soft tissue sarcomas. Mod Pathol 2006;19:106–14.

25. Kim HC, Roh SA, Ga IH, Kim JS, Yu CS, Kim JC. CpG island methylation as an early event during adenoma progression in carcinogenesis of sporadic colorectal cancer. J Gastroenterol Hepatol 2005;20:1920–6.

26. Liu Z, Wang LE, Wang L, et al. Methylation and messenger RNA expression of p15INK4b but not p16INK4a are independent risk factors for ovarian cancer. Clin Cancer Res 2005;11:4968–76.

27. Sato K, Tamura G, Tsuchiya T, et al. Analysis of genetic and epigenetic alterations of the PTEN gene in gastric cancer. Virchows Arch 2002;440:160–5.

28. Virmani AK, Rathi A, Zochbauer-Muller S, et al. Promoter methylation and silencing of the retinoic acid receptor-β gene in lung carcinomas. J Natl Cancer Inst 2000;92:1303–7.

29. Takahashi T, Suzuki M, Shigematsu H, et al. Aberrant methylation of Reprimo in human malignancies. Int J Cancer 2005;115:503–10.

30. Li QL, Ito K, Sakakura C, et al. Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell 2002;109:113–24.

31. Kuroki T, Trapasso F, Yendamuri S, et al. Allelic loss on chromosome 3p21.3 and promoter hypermethylation of semaphorin 3B in non-small cell lung cancer. Cancer Res 2003;63:3352–5.

32. Oka T, Ouchida M, Koyama M, et al. Gene silencing of the tyrosine phosphatase SHP1 gene by aberrant methylation in leukemias/lymphomas. Cancer Res 2002;62:6390–4.

33. Wild A, Ramaswamy A, Langer P, et al. Frequent methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene in pancreatic endocrine tumors. J Clin Endocrinol Metab 2003;88:1367–73.

34. Deng G, Song GA, Pong E, Sleisenger M, Kim YS. Promoter methylation inhibits APC gene expression by causing changes in chromatin conformation and interfering with the binding of transcription factor CCAAT-binding factor. Cancer Res 2004;64:2692–8.

35. Iwai M, Kiyoi H, Ozeki K, et al. Expression and methylation status of the FHIT gene in acute myeloid leukemia and myelodysplastic syndrome. Leukemia 2005;19:1367–75.

36. Takeno S, Noguchi T, Fumoto S, Kimura Y, Shibata T, Kawahara K. E-cadherin expression in patients with esophageal squamous cell carcinoma: promoter hypermethylation, Snail overexpression, and clinicopathologic implications. Am J Clin Pathol 2004;122:78–84.

37. Shames DS, Girard L, Gao B, et al. A genome-wide screen for promoter methylation in lung cancer identifies novel methylation markers for multiple malignancies. PLoS Med 2006;3:e486.

38. Shames DS, Minna JD, Gazdar AF. Methods for detecting DNA methylation in tumors: From bench to bedside. Cancer Lett 2007;251:187–98.

39. Plass C, Smiraglia DJ. Genome-wide analysis of DNA methylation changes in human malignancies. Curr Top Microbiol Immunol 2006;310:179–98.

40. Esteller M. Cancer epigenetics: DNA methylation and chromatin alterations in human cancer. Adv Exp Med Biol 2003;532:39–49.

41. Parrella P, Poeta ML, Gallo AP, et al. Nonrandom distribution of aberrant promoter methylation of cancer-related genes in sporadic breast tumors. Clin Cancer Res 2004;10:5349–54.

42. Alaminos M, Davalos V, Cheung N-KV, Gerald WL, Esteller M. Clustering of gene hypermethylation associated with clinical risk groups in neuroblastoma. J Natl Cancer Inst 2004;96:1208–19.

43. Fleisher AS, Esteller M, Tamura G, et al. Hypermethylation of the hMLH1 gene promoter is associated with microsatellite instability in early human gastric neoplasia. Oncogene 2001;20:329–35.

44. Leung WK, Yu J, Ng EK, et al. Concurrent hypermethylation of multiple tumor-related genes in gastric carcinoma and adjacent normal tissues. Cancer 2001;91:2294–301.

45. Tamura G, Yin J, Wang S, et al. E-Cadherin gene promoter hypermethylation in primary human gastric carcinomas. J Natl Cancer Inst 2000;92:569–73.

46. To KF, Leung WK, Lee TL, et al. Promoter hypermethylation of tumor-related genes in gastric intestinal metaplasia of patients with and without gastric cancer. Int J Cancer 2002;102:623–8.

47. Kim DY, Park YK, Joo JK, et al. Clinicopathological characteristics of signet ring cell carcinoma of the stomach. ANZ J Surg 2004;74:1060–4.

48. Chang YT, Wu MS, Chang CJ, Huang PH, Hsu SM, Lin JT. Preferential loss of Fhit expression in signet-ring cell and Krukenberg subtypes of gastric cancer. Lab Invest 2002;82:1201–8.

49. Hayslip J, Montero A. Tumor suppressor gene methylation in follicular lymphoma: a comprehensive review. Mol Cancer 2006;5:44.

50. Ohki R, Nemoto J, Murasawa H, et al. Reprimo, a new candidate mediator of the p53-mediated cell cycle arrest at the G2 phase. J Biol Chem 2000;275:22627–30.

51. Leung WK, To KF, Chu ES, et al. Potential diagnostic and prognostic values of detecting promoter hypermethylation in the serum of patients with gastric cancer. Br J Cancer 2005;92:2190–4.

52. Lee TL, Leung WK, Chan MW, et al. Detection of gene promoter hypermethylation in the tumor and serum of patients with gastric carcinoma. Clin Cancer Res 2002;8:1761–6.

53. Miyamoto A, Kuriyama S, Nishino Y, et al. Lower risk of death from gastric cancer among participants of gastric cancer screening in Japan: a population-based cohort study. Prev Med 2007;44:12–9.

54. Rollan A, Ferreccio C, Gederlini A, Serrano C, Torres J, Harris P. Non-invasive diagnosis of gastric mucosal atrophy in an asymptomatic population with high prevalence of gastric cancer. World J Gastroenterol 2006;12:7172–8.