Reprimo as a Potential Biomarker for Early Detection in Gastric Cancer

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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, RIZ1, 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
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 range 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 (14–33). However, we confirmed this association for SHPI, 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 χ² 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, SHPI, E-cadherin, ER, Reprimo, SEMA3B, 30ST2, p14, p15, DAPK, and p16). Two of these 11 genes (SHPI 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, SHPI, E-cadherin, Reprimo, and SEMA3B). To this purpose, we treated the cell line MKN45 with the demethylating agent 5-aza-2-deoxycytidine. 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
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

<table>
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<tr>
<th>Gene abbreviation</th>
<th>Gene name</th>
<th>Location</th>
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<td>COX2</td>
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**Imaging, Diagnosis, Prognosis**

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

**Fig. 1.** Methylation-specific PCR analysis of 32 retrospectively 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, peripheral blood lymphocytes DNA (used as a control of unmethylated genes).
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 mismatch 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 ($BRCA1$, $APC$, $RIZ1$, $RUNX3$, $MCMT$, 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-tyrosine phosphatase) 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,
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 OST2) 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 G2 cell cycle arrest (50). When overexpressed, Reprimo induces cell cycle arrest at the G2 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-ε, 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 Methylight-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


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