Detection of Gene Promoter Hypermethylation in the Tumor and Serum of Patients with Gastric Carcinoma


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Abstract

Purpose: Aberrant promoter methylation, an alternative mechanism for gene silencing, is frequently detected in gastric cancer. We studied the feasibility of detecting aberrant methylation in serum of gastric cancer patients.

Experimental Design: Patients (54) with gastric adenocarcinoma were studied. The tumor and the paired serum were examined for aberrant methylation in DAP-kinase, E-cadherin, GSTP1, p15, and p16 by methylation-specific PCR. Serum from 30 age-matched noncancer patients was used as control.

Results: Promoter methylation in DAP-kinase, E-cadherin, GSTP1, p15, and p16 were detected in 70.3, 75.9, 18.5, 68.5, and 66.7% of primary tumor. In serum of gastric cancer patients, methylation in DAP-kinase, E-cadherin, GSTP1, p15, and p16 were detected in 48.1, 57.4, 14.8, 55.6, and 51.9%, respectively. None of the control serum showed aberrant methylation. Aberrant methylation in serum DNA was all accompanied with methylation in the corresponding tumor samples. In general, >60% of serum from cancers with aberrant methylation demonstrated these epigenetic alterations.

Conclusion: Our findings suggest that aberrant promoter methylation in serum can be detected in a substantial proportion of gastric cancer patients, and this strategy should be evaluated in the screening and surveillance of gastric cancer.

Introduction

Gastric cancer is only second to lung cancer as the most common cause of cancer death worldwide (1). Despite the advances in management of this disease, the prognosis remains dismal. One of the main factors limiting the improved survival of these patients is related to the late presentation of this malignancy. Although early gastric cancers are typically associated with no or minimal symptoms, the detection of these potentially curable lesions is difficult. As yet, screening for gastric cancer may not be cost effective in most countries and is not widely practiced except in Japan (2, 3). To complicate the issue, the best screening strategy remains poorly defined. Although endoscopy is considered to be the most sensitive tool in screening for gastric cancer, the cost and risk associated with endoscopy is considerable. Additionally, patient’s acceptability needs to be taken into account. Thus, the need of a reliable noninvasive test, preferably a blood test, for screening and diagnostic purposes is obvious.

The presence of tumor DNA in serum has been reported >2 decades ago (4). Early studies have demonstrated the presence of K-ras and p53 mutations in the serum of patients with colorectal, pancreas, and breast cancer (5–7). More recently, the presence of gene promoter hypermethylation in serum DNA has also been demonstrated in patients with cancers of lung, head and neck, liver, and breast (7–10). Under most circumstances, promoter hypermethylation usually results in transcriptional silencing of genes. If tumor suppressor genes are involved, it may result in inactivation of that particular gene. Gastric cancer frequently demonstrated promoter hypermethylation in various genes, including the p15, p16, hMLH1, and E-cadherin (11–16). However, it remains unknown whether these alterations can be detected in serum DNA. In the present study, we sought to determine the feasibility and the clinical correlations of detecting gene promoter hypermethylation in the serum of patients with gastric adenocarcinoma.

Materials and Methods

Patients. Fifty-four consecutive patients with confirmed gastric adenocarcinoma were examined. All patients had tumor and corresponding serum samples available for analysis. Primary gastric cancer tissues were obtained during endoscopic examination because some patients were not operated attributable to advanced disease or comorbid illnesses. Tumors were verified histologically to contain gastric carcinoma and subtyped into intestinal, diffuse, or mixed type, as suggested by Lauren (17). Gastric cancers were staged according to the fifth edition of the TNM staging system (18). In patients with locally advanced or metastatic disease on presentation that did not undergo surgery, staging was based on clinical and radiological methods.

Blood samples were obtained at the time of diagnosis before surgery or other forms of treatment. Serum was separated...
It was then followed by 40 cycles of amplification at 95 °C for 30 s, the specific annealing temperature for 45 s, and 72 °C for 1 min containing 1 × PCR buffer II (Perkin-Elmer, Boston, MA), 2 mM MgCl₂, 0.25 mM dideoxynucleotide triphosphate, 1 μM each primer, and 1 unit of AmpliTaq Gold polymerase (Perkin-Elmer) at 95 °C for 10 min. It was then followed by 40 cycles of amplification at 95 °C for 30 s, the specific annealing temperature for 45 s, and 72°C for 1 min.

DNA Extraction from Tissue and Serum Samples. Fifteen paraffin sections, each 5-mm thick, of gastric tissue samples were used for DNA extraction. The areas rich in gastric tumor cells were selected and carefully microdissected from the slides. Gastric tissues were retrieved by using xylene and alcohol. Serum was isolated by centrifugation at 3000 g until further processing. Genomic DNA from microdissected tissue and serum samples was isolated by the high pure PCR template preparation kit (Boehringer Mannheim Corp., Indianapolis, IN).

Bisulfite Treatment and Methylation-specific PCR. Aberrant DNA methylation in the CpG Island of the genes was determined by chemical modification of genomic DNA with sodium bisulfite and followed by methylation-specific PCR. The bisulfite modification procedure was carried out by the Intergen CpGenome DNA modification kit (Intergen, Purchase, NY). In brief, 1 μg of genomic DNA was denatured by sodium hydroxide and then chemically modified by sodium bisulfite for 20 h. The unmethylated cytosine was converted to uracil, whereas methylated cytosine remains unchanged. The modified DNA was recovered by ethanol precipitation and resuspended in PCR-grade water.

The primer sequences were based on previous reports (8, 19) and are listed in Table 1. Two μl of bisulfite-modified DNA were amplified in a total volume of 25 μl containing 1 × PCR buffer II (Perkin-Elmer, Boston, MA), 2 mM MgCl₂, 0.25 mM dideoxynucleotide triphosphate, 1 μM each primer, and 1 unit of AmpliTaq Gold polymerase (Perkin-Elmer) at 95 °C for 10 min. It was then followed by 40 cycles of amplification at 95 °C for 30 s, the specific annealing temperature for 45 s, and 72°C for 1 min. In vitro methylated DNA (Intergen) was used as a positive control for methylation, and water was used as a negative control. Ten μl of PCR product were loaded on 10% nondenaturing polyacrylamide gel stained with ethidium bromide and visualized under UV illumination. Sample was scored as methylated when there was a clearly visible band on the gel with the methylated primers (Fig. 1). Electrophoresis results were interpreted by two independent investigators, and in case of discrepancy, the opinion of a third investigator was sought. All experiments were repeated to ensure the reproducibility of the results.

**Statistical Analyses.** Categorical data were analyzed by χ² or Fisher’s exact test, and numerical data were analyzed by Student’s t test. Correlation between methylation statuses of different genes was analyzed by Spearman Correlation Coefficient. A P < 0.05 was considered to be of statistical significance.

**Results**

**Patients and Tumor Characteristics.** Fifty-four consecutive gastric adenocarcinoma were examined. The mean age of these patients was 62.6 years (range 32–84 years). There were 43 (79.6%) distal and 11 (20.4%) proximal cancers. For histological types, 24 (44.4%) were intestinal type, 22 (40.7%) were diffuse type, and 8 (14.8%) were of mixed type. There were only two early (T1) gastric cancers. Forty of them had undergone gastrectomy, whereas 14 were considered inoperable because of advanced clinical staging or comorbid illnesses. The mean age of the noncancer control patients was 60.1 years (range 30–88 years).

**Gene Promoter Hypermethylation in Primary Gastric Carcinoma.** All primary tumors showed aberrant methylation in at least one of the five tumor-related genes. The frequency of aberrant methylation of an individual gene was listed in Table 2. Thirty-seven (68.5%) gastric cancers displayed hypermethylation in three or more genes, but less in GSTP1.

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**Table 1** Primer sequences used in methylation-specific PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence</th>
<th>Tm (°C)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAP-kinase</td>
<td>Unmethylated: 5'-GGAGGATAGTTGAGTTAATTGT-3' (sense)</td>
<td>60</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Methylated: 5'-CAGATCCTTCGAAACAGA-3' (antisense)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTP1</td>
<td>Unmethylated: 5'-TATATTAGGGTTATTGTGTT-3' (sense)</td>
<td>60</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Methylated: 5'-CGCTCTTTTGGTTATTGTGTT-3' (antisense)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Unmethylated: 5'-TGGAGGAGTGGGGCGGATCGC-3 (antisense)</td>
<td>53</td>
<td>19</td>
</tr>
<tr>
<td>p15</td>
<td>Unmethylated: 5'-TTCGGGGTGTAGCGCTGTC-3 (antisense)</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methylated: 5'-TTCGGGGTGTAGCGCTGTC-3 (antisense)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16</td>
<td>Unmethylated: 5'-TTATATTAGGGTTATTGTGTT-3 (sense)</td>
<td>60</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Methylated: 5'-TTATATTAGGGTTATTGTGTT-3 (sense)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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References

- Categorical data were analyzed by χ² or Fisher’s exact test, and numerical data were analyzed by Student’s t test. Correlation between methylation statuses of different genes was analyzed by Spearman Correlation Coefficient. A P < 0.05 was considered to be of statistical significance.

- Patients and Tumor Characteristics. Fifty-four consecutive gastric adenocarcinoma were examined. The mean age of these patients was 62.6 years (range 32–84 years). There were 43 (79.6%) distal and 11 (20.4%) proximal cancers. For histological types, 24 (44.4%) were intestinal type, 22 (40.7%) were diffuse type, and 8 (14.8%) were of mixed type. There were only two early (T1) gastric cancers. Forty of them had undergone gastrectomy, whereas 14 were considered inoperable because of advanced clinical staging or comorbid illnesses. The mean age of the noncancer control patients was 60.1 years (range 30–88 years).

- Gene Promoter Hypermethylation in Primary Gastric Carcinoma. All primary tumors showed aberrant methylation in at least one of the five tumor-related genes. The frequency of aberrant methylation of an individual gene was listed in Table 2. Thirty-seven (68.5%) gastric cancers displayed hypermethylation in three or more genes (≥60%) genes (Fig. 2). Hypermethylation was frequently (>60%) detected in p15, p16, DAP-kinase, and E-cadherin but less in GSTP1.
Concordant methylation in tumors was detected in \( p15 \) and \( E\text{-cadherin} \) \((r = 0.364, \ P = 0.007)\). Otherwise, there was no association between methylation statuses of other genes in the primary tumors.

As shown in Table 3, aberrant methylation in primary gastric tumors had no correlation with patients’ demographic data, including age and gender, the location, histological subtypes, and staging of the tumor.

Epigenetic Alterations in Serum DNA from Gastric Cancer Patients. Aberrant methylation in one or more tumor-related genes was detected in the serum of 45 (83.3%) gastric cancer patients (Table 2 and Fig. 2). On the other hand, none of the 30 control serum samples had hypermethylated DNA sequences detected. For all serum samples with aberrant methylation, the paired tumor samples also showed methylation in the corresponding genes.

**Fig. 1** Representative gel electrophoresis pictures demonstrating aberrant methylation in \( E\text{-cadherin} (A) \), \( D\text{AP-kinase} (B) \), \( GSTP1 (C) \), \( p15 (D) \), and \( p16 (E) \) for primary gastric cancer (top row) and the corresponding serum DNA (middle row). There was no hypermethylation in the control serum (bottom row). \( M \) and \( U \), the methylated and unmethylated primers, respectively. \( \text{IVD, in vitro methylated DNA (positive control). Water was used as negative control for each amplification.} \)
Methylation in DAP-kinase, E-cadherin, p15, and p16 was detected frequently in the serum, as well as in the corresponding tumor. Overall, >68% of patients with hypermethylation in the tumor DNA demonstrated abnormal methylation in the corresponding serum DNA [DAP-kinase = 26 of 38 (68.4%), E-cadherin = 31 of 41 (75.6%), GSTP1 = 8 of 10 (80%), p15 = 30 of 37 (81.1%), and p16 = 28 of 36 (77.8%)].

As shown in Fig. 2, 26 (48.1%) serum samples showed concurrent methylation in three or more tumor genes. The presence of hypermethylated DNA sequences in serum did not appear to have any correlation with patients’ demographic data or characteristics of the primary cancer (Table 4). There was no association between clinical staging and the presence of methylation in DAP-kinase, E-cadherin, p15, and p16 in serum DNA. However, unmethylated GSTP1 was more common in cancer with more advanced clinical staging (P = 0.03).

Discussion

Promoter hypermethylation of tumor-related genes play a pivotal role in tumorigenesis. If tumor suppressor genes are affected, it results usually in transcriptional silencing and, hence, inactivation of that gene. It may then confer growth advantages to these cells that favor cancer development. In this study, we have studied 54 consecutive gastric cancer patients that have undergone endoscopic examination in our hospital. In contrast to the use of gastrectomy specimens that would exclude patients with advanced clinical staging, the use of endoscopic biopsies include cancers with all clinical staging and, hence, minimize the bias in selecting early stage cancer only. In this study, >65% of primary tumors demonstrated hypermethylation in at least one of the following genes, including p15, p16, DAP-kinase, and E-cadherin. Additionally, we have extended the detection of promoter hypermethylation from tumor DNA to serum DNA. Unlike tumor samples, serum is usually more accessible for sampling and analysis. We noticed that a substantial proportion of gastric cancer patients had promoter methylation detectable in primary gastric tumor, as well as in the serum. Generally speaking, >65% of patients with aberrant methylation in primary tumors also exhibited hypermethylation in serum DNA. It is noteworthy that detection of promoter methylation in serum is a specific event: (a) aberrant methylation was not detected in any of the 30 control serum; in accordance with this finding, our previous study showed that aberrant methylation was not detected in gastric biopsy obtained from gastritis patients (16); and (b) the same methylation profiles were found in the serum and the corresponding tumor; in addition, aberrant methylation was not detected in the serum of gastric cancer patients without methylation in the corresponding tumor.

Hypermethylation of E-cadherin, DAP-kinase, and p16 was detected frequently in gastric cancer (11–16). On the other hand, there is no published data on the role of p15 hypermethylation in gastric cancer. Recently, we have demonstrated p15 hypermethylation in 73% of gastric cancer and 80% of cancer cell lines, suggesting the potential importance of p15 in gastric carcinogenesis (16). Interestingly, in the present study, there was concordant hypermethylation of p15 and E-cadherin in primary gastric cancer. The real significance of this correlation remains elusive. Given the fact that hypermethylation in p15 and E-cadherin are both common in gastric cancer, this association could be because of chance alone and should be interpreted with cautious. Whether there is any genuine association or interaction between p15 and E-cadherin in gastric cancer deserves further evaluation.

The exact mechanism how tumor DNA gets into systemic circulation and serum remains unclear for the moment. Intuitively, the presence of promoter methylation in serum should be associated with more advanced tumor (i.e., tumor with lymph node or systemic metastasis). However, we failed to show any correlation between the detection of aberrant methylation in DAP-kinase, E-cadherin, p15, and p16 in serum with lymph node metastasis or clinical staging. Previous studies on other cancers, including lung, liver, esophageal, and head and neck cancers, also failed to demonstrate any correlation between the detection of promoter methylation in serum and tumor staging (8–10, 20). Surprisingly, the presence of unmethylated GSTP1 in serum was found to be associated with gastric cancer with more advanced clinical staging. However, there is no published data to support the association between methylation in GSTP1 and gastric cancer staging. Additionally, similar association cannot be demonstrated in the primary tumors. Therefore, we believed that this borderline association could be just a random event and may not have any clinical significance.

Because gastric cancer is the second most common cause of cancer-related mortality in the world, the need for a more reliable noninvasive screening test is obvious. This is particularly relevant in Asian countries with high gastric cancer incidences. Past attempts in identifying potential serum markers for gastric cancer relied on the detection of Helicobacter pylori.
antibody, CagA antibody, and/or assay of pepsinogen level (21, 22). These serum markers, however, lack sensitivity and specificity. To this end, the detection of aberrant methylation in serum DNA may offer a new approach in the noninvasive diagnosis of gastric cancer. This method is highly specific and correlates with tumor methylation status. Apart from screening of gastric cancer, it would be interesting to see whether the detection of aberrant methylation in the serum can be used in the monitoring of disease progress after curative surgery. If methylated DNA disappears shortly in serum after curative surgery, the reappearance of these markers may suggest recurrence of disease that may require more intensive screening and aggressive treatment. Additional studies are necessary to elucidate the role of detecting aberrant methylation in serum as a tool for screening and surveillance of gastric cancer.

References


Table 3 Clinicopathological characteristics of gastric cancer patients with aberrant methylation in primary tumors

<table>
<thead>
<tr>
<th>DAP</th>
<th>E-cad</th>
<th>GSTP1</th>
<th>p16</th>
<th>p15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
</tr>
<tr>
<td>No.</td>
<td>38</td>
<td>16</td>
<td>41</td>
<td>13</td>
</tr>
<tr>
<td>Age</td>
<td>63.4</td>
<td>60.8</td>
<td>0.53</td>
<td>63.5</td>
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<tr>
<td>Male</td>
<td>26</td>
<td>10</td>
<td>0.76</td>
<td>28</td>
</tr>
<tr>
<td>Intestinal type</td>
<td>17</td>
<td>7</td>
<td>1.0</td>
<td>20</td>
</tr>
<tr>
<td>Distal cancer</td>
<td>30</td>
<td>13</td>
<td>1.0</td>
<td>32</td>
</tr>
<tr>
<td>Early cancer</td>
<td>0</td>
<td>2</td>
<td>0.07</td>
<td>1</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>31</td>
<td>11</td>
<td>1.0</td>
<td>32</td>
</tr>
</tbody>
</table>

* Comparison of age was made by Student’s t test, and all other comparisons were by the χ² test or Fisher’s exact test.
* M, methylated; U, unmethylated.
* Comparison was made between stage I–II and stage III–IV because of the small number of cases. Data for clinical staging were missing in two cases (n = 52).

Table 4 Clinicopathological characteristics of gastric cancer patients with hypermethylation in serum DNA

<table>
<thead>
<tr>
<th>DAP-kinase</th>
<th>E-cadherin</th>
<th>GSTP1</th>
<th>p16</th>
<th>p15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
</tr>
<tr>
<td>No.</td>
<td>26</td>
<td>28</td>
<td>31</td>
<td>23</td>
</tr>
<tr>
<td>Age</td>
<td>65.8</td>
<td>59.6</td>
<td>0.09</td>
<td>65.0</td>
</tr>
<tr>
<td>Male</td>
<td>18</td>
<td>18</td>
<td>0.77</td>
<td>21</td>
</tr>
<tr>
<td>Intestinal type</td>
<td>11</td>
<td>13</td>
<td>0.79</td>
<td>16</td>
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<td>Distal cancer</td>
<td>22</td>
<td>21</td>
<td>0.51</td>
<td>26</td>
</tr>
<tr>
<td>Early cancer</td>
<td>0</td>
<td>2</td>
<td>0.49</td>
<td>1</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>22</td>
<td>20</td>
<td>0.73</td>
<td>24</td>
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</table>

* Comparison of age was made by Student’s t test, and all other comparisons were by the χ² test or Fisher’s exact test.
* M, methylated; U, unmethylated.
* Comparison was made between stage I–II and stage III–IV because of the small number of cases. Data for clinical staging were missing in two cases (n = 52).
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