Urinary ADAM12 and MMP-9/NGAL Complex Detect the Presence of Gastric Cancer

Takaya Shimura1,2, Adelle Dagher1, Monisha Sachdev1, Masahide Ebi3, Tamaki Yamada4, Tomonori Yamada5, Takashi Joh3, and Marsha A. Moses1,2

Abstract

Although the early diagnosis of gastric cancer provides the opportunity for curative endoscopic resection, comprehensive screening endoscopy would be invasive and expensive. To date, there is a complete absence of clinically useful gastric cancer biomarkers. With the goal of discovering noninvasive biomarkers for the early diagnosis of gastric cancer, we have conducted a case–control study using urine samples from individuals with gastric cancer versus healthy control samples. Of the enrolled 106 patients from September, 2012 to April, 2013, a cohort of 70 patients composed of 35 patients with gastric cancer and 35 age- and sex-matched healthy controls was analyzed. The gastric cancer group was composed of stage IA of 62.9% (22/35). The urinary levels of MMP-9/NGAL complex (uMMP-9/NGAL) and ADAM12 (uADAM12) were significantly higher in the gastric cancer group compared with the healthy control group as determined by monospecific ELISAs (uMMP-9/NGAL: median, 85 pg/mL vs. 0 pg/mL; P = 0.020; uADAM12: median, 3.35 ng/mL vs. 1.44 ng/mL; P < 0.001). Multivariate analysis demonstrated that both uMMP-9/NGAL and uADAM12 were significant, independent diagnostic biomarkers for gastric cancer. Moreover, MMP-9/NGAL activity was significantly elevated as determined by gelatin zymography. The combination of uMMP-9/NGAL with uADAM12 distinguished between control samples and gastric cancer samples with an AUC of 0.825 (P < 0.001) in an ROC analysis. Significantly, immunohistochemical analyses demonstrated a high coexpression of MMP-9 and NGAL (P < 0.001) and a high expression of ADAM12 (P < 0.001) in gastric cancer tissues compared with adjacent normal tissues (N = 35). In summary, uMMP-9/NGAL and uADAM12 are potential noninvasive biomarkers for gastric cancer, including early-stage disease. Cancer Prev Res; 8(3); 240–8. ©2015 AACR.

Introduction

Gastric cancer is the fourth most common malignancy and the second leading cause of cancer-related deaths in the world (1). The standard treatment of gastric cancer is endoscopic resection for mucosal cancer (T1a), surgical resection for deep submucosal cancer (T1b) and resectable advanced cancer, and palliative chemotherapy for unresectable advanced and metastatic cancer (2). The 5-year survival rate for patients with gastric cancer is greater than 90% for stage IA, in contrast with 15% for stage IV with metastasis (3). It is clear that early diagnosis is critically important to achieve life-saving therapy for this disease.

Laparotomy such as partial or total gastrectomy is a useful, and currently indispensable, method of treating many gastric cancers; however, some patients suffer from post-gastrectomy syndromes, including anemia after surgery despite the surgery being therapeutically successful for gastric cancer. Moreover, patients who have experienced partial or complete stomach resection often experience a significant decline in physical strength due to their considerably decreased food intake. In contrast, endoscopic treatment can completely preserve the stomach, thereby enabling maintenance of a better quality of life. In addition, endoscopic resection without laparotomy does not result in intraabdominal adhesions and scarring of abdominal skin. Were gastric cancer to be diagnosed at the T1a stage, even a large tumor could be endoscopically treated using new endoscopic developments such as endoscopic submucosal dissection (4) in which complication rate is significantly less than gastrectomy (5). The early detection of gastric cancer is, therefore, clinically necessary to both achieve a cure as well as to prevent a significant decline in the quality of life for patients with this disease.

Because the incidence of gastric cancer is especially high in Eastern Asian countries, including Japan (6), serious attempts have been made to develop screening systems for gastric cancer in Japan. Photofluorography using barium is currently the most widespread and most recommended gastric cancer screening method used in Japan. This test demonstrates 57% to 60% sensitivity and 81% to 92% specificity (7). Most case–control studies show a 40% to 60% decrease in gastric cancer–related mortality with photofluorography, however, the effects were not consistently observed in the prospective studies (6). Moreover, it
is difficult for photofluorography to detect early gastric cancer, and photofluorography is not in prevalent use in other countries due to technical difficulties, risk of radiation exposure, and other reasons. Endoscopy is also used as a screening tool for gastric cancer and the detection rate of gastric cancer via endoscopy has been reported to be approximately 2.7 to 4.6 times higher than that of photofluorography (8). However, this technique is highly variable, completely dependent on the skill of the endoscopist, and is not currently recommended as a screening method because it requires significant staffing, and is not cost-effective (7). It is also highly invasive. A serum pepsinogen test and a Helicobacter pylori (H. pylori) antibody have been used as a serologic test for gastric cancer screening. Atrophic gastritis through H. pylori infection is one of the major causes of gastric cancer (9). Serum pepsinogen I and pepsinogen I/II ratio have been recently used as a relatively new screening test for gastric cancer with 40% to 80% sensitivity and <80% specificity (7). Testing for the presence of H. pylori antibody may also be useful in identifying the high-risk group; however, the exclusive screening for only active infection of H. pylori is inadequate due to the fact that H. pylori cannot remain in a stomach with severe atrophic gastritis. For these and other reasons, serum pepsinogen and H. pylori tests are not recommended as population-based screening tools. A recent study using serum trefoil factor family 3 (TFF3; ref. 10) has not yet been used in the clinical setting. The serum tumor markers CEA and CA19-9 have sometimes been used in clinical practice; however, their utilization has not been recommended for diagnosis of gastric cancer because of their very low sensitivity for early stage (<20%) and advanced stage (20%–50%) disease (11). In fact, the latest meta-analysis, which analyzed results of more than 5,000 patients with gastric cancer in 46 studies, also reported that the sensitivity of CEA and CA19-9 was only 24.0% and 27.0%, respectively, and 13.7% and 9.0% for stage I gastric cancer, respectively (12). Moreover, these values are not elevated in the serum of mucosal atrophic gastritis (TG1a) at all. Taken together, there are currently no clinically used biomarkers for the early detection of gastric cancer.

Materials and Methods

Patients and study design

All samples were prospectively collected from September, 2012 to April, 2013, at three Japanese institutions participating in the present study (Nagoya City University Hospital, Japanese Red Cross Nagoya Daini Hospital, and Okazaki Public Health Center). Patients who met all of the following inclusion criteria were enrolled in this study: between the age of 20 and 90 years; histologically confirmed adenocarcinoma by biopsy for the gastric cancer group; no treatment before study enrollment for the gastric cancer group, and no neoplasms of any type for the healthy control group. Patients with a previous history of neoplasms of any type and/or with duplicated neoplasms were excluded from enrollment in this study. The healthy control cohort was composed of individuals who were asymptomatic and who had no evidence of neoplasms at their annual checkup. The present study included age-matched and sex-matched healthy control samples among recruited whole cohorts.

To assure the accuracy and comprehensiveness of reporting of this case–control biomarker study, the present study complied with the REMARK guideline (13) and the STROBE statement (14). The study protocol was approved by the ethics committee at each institution and was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008). All patients provided their written, informed consent before study entry.

Samples and definition

All urine samples were collected before any treatment for gastric cancer, immediately frozen and stored at −80°C until assay, as previously reported by us (15). Tissue samples of gastric cancer were obtained from primary tumors at the time of initial surgical and endoscopic resection or biopsy in patients without resection and fixed in formalin and embedded in paraffin (4). Clinical stages were determined by final pathologic diagnosis after resection according to the seventh edition of the Union for International Cancer Control (UICC) tumor–node–metastasis (TNM) classification (16). H. pylori status was analyzed using the serum anti-H. pylori IgG antibody, the 13C-urea breath test and the RAPIDRUN test (Otsuka Pharmaceutical Co., Ltd.), which is designed to detect anti-H. pylori IgG in urine (17). Patients who were formerly positive for H. pylori, but whose infection was successfully eradicated were also included in a positive group of H. pylori. Performance status (PS) was graded according to the Eastern Cooperative Oncology Group (18).

Protein array analysis

Because angiogenesis is required for cancer development and progression (19) and changes in the microvasculature have been described in very early stage of gastric cancer (20), we hypothesized that angiogenic factors would be potentially useful biomarkers of the early detection of gastric cancer. To screen for urinary proteins in the urine of patients with early gastric cancer, human angiogenesis antibody arrays, which include 43 angiogenic factors were used (RayBiotech, Inc.) according to the manufacturer’s instructions. We randomly selected two healthy controls and 2 patients with early gastric cancer who were completely matched by age and sex, and these four urine samples were incubated with antibody-coated array membranes at the same time at 4°C for overnight. To determine the relative expression level of gastric cancer samples composed to healthy control, the signal intensities of each protein were quantified by densitometry.

Substrate gel electrophoresis

The presence and activity of urinary matrix metalloproteinases (MMP) was determined using substrate gel electrophoresis (zymography) as previously reported by us (15, 21). Each urine sample was mixed with buffer composed of 4% SDS, 0.15 mol/L Tris (pH 6.8), 20% glycerol, and 0.5% (w/v) bromophenol blue, and then applied to a 10% SDS–acrylamide gel containing 0.1% (w/v) gelatin (Bio-Rad). Proteins were separated using a mini gel apparatus at 200V/gel for 50 minutes. After electrophoresis, gels were placed into a 2.5% Triton X-100 solution with gentle shaking for 30 minutes at room temperature and then incubated in substrate buffer [50 mmol/L Tris–HCl (pH 8), 5 mmol/L CaCl2 and 0.02% NaN3], with gentle shaking overnight, at 37°C. On the next day, gels were stained for 30 minutes in 0.5% Coomassie Blue R-250 dissolved in a 1:3.6 solution of acetic acid, isopropyl alcohol, and water, and then detained in a 1:3.6 solution of acetic acid, ethanol, and water. Urinary MMPs were assessed by an independent and blinded evaluator.

Enzyme-linked immunosorbent assays

We measured the urinary protein concentration of each of the proteins of interest via monospecific ELISAs according to the
manufacturer's instructions as we have previously reported (22). Quantikine ELISA kits (R&D Systems, Inc.) were used for IL8, MMP-2, MMP-9, neutrophil gelatinase-associated lipolalin (NGAL), and MMP-9/NGAL complex, a disintegrin and metalloprotease 12 (ADAM12), and the human platelet endothelial cell adhesion molecule-1 (PECAM-1) ELISA Kit for quantification of PECAM-1 (RayBiotech, Inc.). All measurements were performed in a blinded manner.

Immunohistochemistry

Immunohistochemical single and double staining was performed using serial sections of each sample as follows. Consecutive sections (5-µm thick) were deparaffinized and dehydrated through a graded series of xylenes and ethanols. After inhibiting endogenous peroxidase activity by 3% hydrogen peroxide (DAKO) for 5 minutes, blocking of non-inhibiting endogenous peroxidase activity by 3% hydrogen peroxide (DAKO) for 20 minutes according to the manufacturer's instruction.

For single staining, each section was incubated with primary antibodies, murine anti-human MMP-9 (4 µg/mL, Chemicon International, Inc.), rabbit anti-human ADAM12 (1.3 µg/mL, Proteintech Group, Inc.), or rat anti-human NGAL (10 µg/mL, R&D Systems, Inc.) for 1 hour. Samples were then incubated with an anti-mouse polymer-horseradish peroxidase secondary antibody (DAKO) for MMP-9, and an anti-rabbit polymer-horseradish peroxidase secondary antibody (DAKO) for ADAM12, whereas an anti-rabbit polymer-alkaline phosphatase secondary antibody (DAKO) was used as a secondary antibody after incubation with biotinylated, affinity-associate- ed rabbit anti-rat immunoglobulins for NGAL. Finally, immune complexes were visualized by incubation in DAB (DAKO) for MMP-9 and ADAM12 and Vulcan Fast Red (Biocare Medical, Concord) for NGAL. Hence, expression of MMP-9 and ADAM12 stained brown and NGAL expression stained red. Mayer's hematoxylin was used for nuclear counterstaining.

For double staining, the same primary antibodies for MMP-9 and NGAL were simultaneously incubated as in single staining, followed by each secondary antibody. Finally, the same chromogen systems as single staining were used. Colocalization of both brown and red colors was considered to be positive immunostaining for MMP-9/NGAL complex.

All immunostained specimens were assessed by an independent gastroenterologist trained in gastrointestinal pathology who was blinded to all clinical information. The staining intensity was classified as negative (0), weak (1+), moderate (2+), or strong (3+), and the extent of the staining was defined as the percentage of positive staining areas scored on a scale of 0 to 4 as follows: 0, 0%; 1, 1% to 10%; 2, 11% to 30%; 3, 31% to 50%; 4, ≥51%, which was slightly modified from the original methods of other tumors (23, 24). The sum of the staining-intensity and staining-extent scores was used as the final staining score.

Statistical analyses

The primary end point of this study was to identify urinary proteins that can discriminate between individuals who are healthy controls and those with gastric cancer. For the purpose of this study, 35 patients from each group were necessary to ensure two-sided, 5% significance level, a power of 80%, and effect size of 0.7. The sample size of 90 patients, including 45 patients in each group, was ensured, allowing for about a 20% of unmatched rate in each group.

Quantitative variables were described with median and inter-quartile range (IQR) and analyzed using the Mann–Whitney U test. Other data were analyzed using the $\chi^2$ test or the Fisher exact probability test, as appropriate. An ROC curve analysis was used to calculate AUC for each biomarker and the representative value was shown as AUC value with 95% confidence interval (CI). The true-positive fractions and the false-positive fractions for diagnosis of gastric cancer were calculated for every cutoff level, and the cutoff level was determined by selecting the farthest point from the baseline using ROC analysis. A logistic regression model was used to estimate the OR with 95% CI. The estimated coefficients of a logistic regression model were used to construct a composite score, which was used to calculate the AUC for the combination of biomarkers. A two-tailed $P$ value of less than 0.05 was considered statistically significant.

Results

Patients

In total, 106 patients were enrolled from September, 2012 to April, 2013 at three Japanese institutions, consisting of 61 patients with healthy control and 45 patients with gastric cancer. One patient with a previous cancer history in the healthy control group and one with colon cancer in the gastric cancer group were excluded. A total of 70 patients (35 patients in each group) were ultimately used by matching age and sex in the present study (Fig. 1). The characteristics of the present study are shown in Table 1. The median age was 63 years (IQR, 53–67 years) in the healthy control group and 65 years (56–68 years) in the gastric cancer group. The healthy control group was comprised of 26 males and 9 females and the gastric cancer group of 28 males and 7 females. Median serum creatinine was 0.80 mg/dL in both groups. Baseline clinical characteristics, including age, gender, PS, and serum creatinine were comparable and no significant differences were noted between the two groups.

The detail breakdown of gastric cancer group is also shown in Table 1. Median tumor size was 30 mm (IQR, 15–62 mm), and histologic type was 27 in intestinal type and 8 in diffuse type. Among 35 gastric cancers, 22 lesions (62.9%) were stage I, four lesions (11.4%) were stage II, five lesions (14.3%) were stage III, and four lesions (11.4%) were stage IV. All 22 stage I samples were stage IA without lymph node metastasis, composed of 12 T1a and 10 T1b.

Urinary protein array analyses

We used an angiogenesis protein array analysis to identify potential candidate biomarkers involved in, or related to, neovascularization. As shown in Supplementary Fig. S1A, the expression levels of MMP-9, IL8, and PECAM-1 were increased in the urine of patients with gastric cancer, compared with healthy controls (Supplementary Fig. S1B).

Substrate gel electrophoresis

We next assessed the activity of MMP-9, which exhibited the most significant difference between gastric cancer and healthy control samples in the protein array, on all the urine samples using gelatin zymography. The activity of MMP-2, MMP-9, and MMP-9/NGAL complex was detected in the samples.
Representative images of healthy control and gastric cancer urines are shown in Fig. 2A. As shown in Fig. 2B, the positive rate of MMP-9/NGAL complex in zymography was significantly higher in gastric cancers than in healthy controls (48.6% vs. 25.7%; \(P = 0.048\)). In contrast, no significant differences were noted for the positive rate of MMP-2 (40.0% vs. 22.9%; \(P = 0.122\)) and MMP-9 (62.9% vs. 40.0%; \(P = 0.056\)) between the two groups.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Characteristics</th>
<th>Healthy control</th>
<th>Gastric cancer</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y), median (IQR)</td>
<td>65 (33–67)</td>
<td>65 (56–68)</td>
<td>0.249a</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>26</td>
<td>28</td>
<td>0.569b</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>9</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>0</td>
<td>35</td>
<td>33</td>
<td>0.493c</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Serum Cr (mg/dL), median (IQR)</td>
<td>0.80 (0.70–0.90)</td>
<td>0.80 (0.70–0.85)</td>
<td>0.410a</td>
<td></td>
</tr>
<tr>
<td>Tumor size (mm), median (IQR)</td>
<td>30 (15–62)</td>
<td></td>
<td></td>
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<tr>
<td>Histologic type, (N) (%)</td>
<td></td>
<td></td>
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<tr>
<td>Intestinal</td>
<td>27 (77.1)</td>
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<tr>
<td>Diffuse</td>
<td>8 (22.9)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T stage, (N) (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T1a</td>
<td>12 (34.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1b</td>
<td>10 (28.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>2 (5.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>1 (2.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4a</td>
<td>8 (22.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4b</td>
<td>2 (5.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage, (N) (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>22 (62.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>4 (11.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>5 (14.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>4 (11.4)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Resection, (N) (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoscopy</td>
<td>18 (51.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrectomy</td>
<td>13 (37.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonresection</td>
<td>4 (11.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** Clinical stage is according to the seventh edition of UICC-TNM classification.

**Abbreviation:** Cr, creatinine.

*The Mann–Whitney U test.

*The \(\chi^2\) test.

*The Fisher exact probability test.

Enzyme-linked immunosorbent assays

On the basis of results from the angiogenesis array analyses and zymography, we next determined the urinary protein concentrations of the following six factors (IL8, PECAM-1, MMP-2, MMP-9, NGAL, and MMP-9/NGAL complex) using quantitative monospecific ELISAs. We also measured the urinary level of ADAM12, a metalloproteinase, which is highly expressed in several cancer tissues (25) and which we have detected in the urine of patients with breast cancer and its level correlates with the urinary level of MMP-9 (26). It has also been detected the urine of patients with bladder cancer as well (27). The concentration of these urinary proteins in both healthy control and gastric cancer groups are shown in Table 2. Urinary levels of MMP-9/NGAL complex (uMMP-9/NGAL) and ADAM12 (uADAM12) were significantly higher in the gastric cancer cohort compared with healthy controls (uMMP-9/NGAL: median, 85 pg/mL vs. 0 pg/mL, \(P = 0.020\); uADAM12: median, 3.35 ng/mL vs. 1.44 ng/mL, \(P < 0.001\)). Urinary levels of uMMP-9/NGAL and uADAM12, normalized to total urinary protein were also significantly higher in the gastric cancer cohort compared with healthy controls (uMMP-9/NGAL: median, 1.72 pg/μg protein vs. 0 pg/μg protein, \(P = 0.044\); uADAM12: median, 70.4 pg/μg protein vs. 33.0 pg/μg protein, \(P = 0.002\)). In contrast, no significant differences between the two groups were detected for the other five proteins (IL8, PECAM-1, MMP-2, MMP-9, and NGAL).

Current or previous infections with *H. pylori* are well-known risk factors. Because positive ratios for *H. pylori* were significantly higher in the gastric cancer group compared with the healthy controls (74.3% vs. 45.7%, \(P = 0.015\)), we performed multivariate analysis using three significant variables (*H. pylori*, MMP-9/NGAL, and ADAM12) on univariate analysis. Multivariate analysis demonstrated that uMMP-9/NGAL (OR, 6.71; 95% CI, 1.96–23.3; \(P = 0.002\)) and uADAM12 (OR, 15.4; 95% CI, 2.76–83.3; \(P = 0.002\)) were significant, independent diagnostic biomarkers for gastric cancer (Table 3A) emphasizing the important utility of these urinary biomarkers. Moreover, uMMP-9/NGAL and uADAM12 significantly distinguished between the healthy control and gastric cancer groups in ROC analyses (uMMP-9/NGAL: AUC, 0.657; 95% CI, 0.527–0.789; \(P = 0.024\); uADAM12: AUC, 0.757; 95% CI, 0.642–0.871; \(P < 0.001\)). When the cutoff values were determined as ≥20.0 pg/mL in uMMP-9/NGAL and ≥1.30 ng/mL in uADAM12, gastric cancer could be detected with 77.1%...
Urinary levels of each protein

Table 2.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Healthy control median (IQR)</th>
<th>Gastric cancer median (IQR)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILB (pg/mL)</td>
<td>10.30 (11.5–25.45)</td>
<td>16.10 (8.90–36.00)</td>
<td>0.111</td>
</tr>
<tr>
<td>PECAM-1 (ng/mL)</td>
<td>6.21 (4.33–7.73)</td>
<td>5.83 (4.86–6.23)</td>
<td>0.449</td>
</tr>
<tr>
<td>MMP-2 (ng/mL)</td>
<td>4.97 (3.56–6.69)</td>
<td>3.58 (4.44–6.20)</td>
<td>0.893</td>
</tr>
<tr>
<td>MMP-9 (ng/mL)</td>
<td>0.65 (0.22–1.95)</td>
<td>0.80 (0.31–1.82)</td>
<td>0.293</td>
</tr>
<tr>
<td>NGAL (ng/mL)</td>
<td>6.72 (4.69–8.72)</td>
<td>7.67 (5.11–12.87)</td>
<td>0.220</td>
</tr>
<tr>
<td>MMP-9/NGAL (pg/mL)</td>
<td>0 (0–213)</td>
<td>85 (45–224)</td>
<td>0.002</td>
</tr>
<tr>
<td>ADAM12 (ng/mL)</td>
<td>1.44 (0.81–2.30)</td>
<td>3.35 (1.88–5.19)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Gelatinase activity at approximately 125 kDa corresponds to MMP-9/NGAL complex, at approximately 92 and 80 kDa to MMP-9, and at approximately 72 and 65 kDa to MMP-2.

Table 3A. Multivariate analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. pylori (+)</td>
<td>2.54 (0.76–8.55)</td>
<td>0.131</td>
</tr>
<tr>
<td>MMP-9/NGAL (&gt;20.0 pg/mL)</td>
<td>6.71 (1.96–23.3)</td>
<td>0.002</td>
</tr>
<tr>
<td>ADAM12 (&gt;130 ng/mL)</td>
<td>15.4 (2.76–83.3)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 3B. AUC of MMP-9/NGAL complex and ADAM12

<table>
<thead>
<tr>
<th>Protein</th>
<th>AUC (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9/NGAL</td>
<td>0.657 (0.527–0.789)</td>
<td>0.024</td>
</tr>
<tr>
<td>ADAM12</td>
<td>0.757 (0.642–0.875)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP-9/NGAL + ADAM12</td>
<td>0.825 (0.724–0.926)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Discussion

Using a variety of biochemical approaches, we have identified uADAM12 and uMMP-9/NGAL as noninvasive biomarkers of early-stage gastric cancer. The ADAMs, a family of MMP-related metalloproteinases, are associated with cell adhesion, cell signaling, and proteolytic processing of a number of transmembrane proteins and play crucial roles in cancer progression and metastasis. ADAM12 is a member of this family and contributes to cancer progression and invasion through cleavage of various membrane-bound proteins and high expression of ADAM12 has been reported in several cancer tissues such as breast (30), lung (31) and others. We have previously reported that ADAM12 promotes growth and metastasis of breast cancer (32) as well as breast cancer risk (26) and that urinary levels of ADAM12 are predictive of disease status and stage in patients with breast cancer (33). In addition, we have demonstrated that ADAM12-mediated HB-EGF shedding plays a critical role in gastric cancer development in a preclinical model (34). Although there have been two studies of the presence of ADAM12 in gastric cancer (35) and esophageal adenocarcinoma tissues (36) in which protein or mRNA of ADAM12 was highly expressed, there have been no reports to date of the presence of ADAM12 in the urine or serum of patients with gastric cancer.

Immunohistochemical single and double staining of MMP-9 and NGAL using different chromogens were performed to investigate the colocalization of MMP-9 and NGAL in cancer and adjacent normal tissues (Fig. 3A). Immunoreactive MMP-9 was detected in some normal epithelial cells, whereas MMP-9 immunoreactivity was detected in most of the cancer epithelial cells. Immunoreactive NGAL was detected in a small number of normal stromal cells, in contrast with most of the cancer epithelial and some stromal cells in which it was very strong. Importantly, colocalization of MMP-9 and NGAL was observed only in cancer tissues, but not in adjacent normal tissues, in gastric cancer with high uMMP-9/NGAL. Interestingly, colocalization of MMP-9 and NGAL was never observed in either cancer or normal tissue in gastric cancer in the absence of uMMP-9/NGAL being present in the urine (Supplementary Fig. S2).

Immunoreactive ADAM12 was strongly detected in most of the cancer tissues, while being weakly expressed in some stromal cells of normal tissues (Fig. 3B). Moreover, immunostaining scores for ADAM12 and coexpression of MMP-9 and NGAL were significantly higher in cancer tissues compared with normal tissues.

Sensitivity and 82.9% specificity. This combination of uMMP-9/NGAL and uADAM12 demonstrated a higher accuracy in distinguishing between patients with gastric cancer and healthy controls than each single biomarker alone (AUC, 0.825; 95% CI, 0.724–0.926; P < 0.001; Table 3B). No significant differences were noted for either urinary proteins between stage I and stage II–IV [uMMP-9/NGAL: median, 75 pg/mL (IQR, 0–245) vs. 106 pg/mL (IQR, 66–195), P = 0.472; uADAM12: median, 3.58 ng/mL (IQR, 2.44–5.05) vs. 4.05 ng/mL (IQR, 1.47–5.94), P = 0.827].

Immunohistochemistry

Using a variety of biochemical approaches, we have identified uADAM12 and uMMP-9/NGAL as noninvasive biomarkers of early-stage gastric cancer. The ADAMs, a family of MMP-related metalloproteinases, are associated with cell adhesion, cell signaling, and proteolytic processing of a number of transmembrane proteins and play crucial roles in cancer progression and metastasis. ADAM12 is a member of this family and contributes to cancer progression and invasion through cleavage of various membrane-bound proteins and high expression of ADAM12 has been reported in several cancer tissues such as breast (30), lung (31) and others. We have previously reported that ADAM12 promotes growth and metastasis of breast cancer (32) as well as breast cancer risk (26) and that urinary levels of ADAM12 are predictive of disease status and stage in patients with breast cancer (33). In addition, we have demonstrated that ADAM12-mediated HB-EGF shedding plays a critical role in gastric cancer development in a preclinical model (34). Although there have been two studies of the presence of ADAM12 in gastric cancer (35) and esophageal adenocarcinoma tissues (36) in which protein or mRNA of ADAM12 was highly expressed, there have been no reports to date of the presence of ADAM12 in the urine or serum of patients with gastric cancer.

Table 3A. Multivariate analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. pylori (+)</td>
<td>2.54 (0.76–8.55)</td>
<td>0.131</td>
</tr>
<tr>
<td>MMP-9/NGAL (&gt;20.0 pg/mL)</td>
<td>6.71 (1.96–23.3)</td>
<td>0.002</td>
</tr>
<tr>
<td>ADAM12 (&gt;130 ng/mL)</td>
<td>15.4 (2.76–83.3)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 3B. AUC of MMP-9/NGAL complex and ADAM12

<table>
<thead>
<tr>
<th>Protein</th>
<th>AUC (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9/NGAL</td>
<td>0.657 (0.527–0.789)</td>
<td>0.024</td>
</tr>
<tr>
<td>ADAM12</td>
<td>0.757 (0.642–0.875)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP-9/NGAL + ADAM12</td>
<td>0.825 (0.724–0.926)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
MMPs, which can be produced by both tumor and stromal cells, are a multigene family of metal-dependent degradative enzymes that are required for extracellular matrix remodeling (37–39). The overexpression of MMP-9 (gelatinase B) has been demonstrated in cancer tissues of many organs, including breast (40), pancreas (41), stomach (42), and others. NGAL, or lipocalin

Figure 3.
Immunohistochemical analyses of MMP-9, NGAL, and ADAM12. A, representative immunohistochemical images of MMP-9 and NGAL. These images are a representative case of single and double staining for MMP-9 and NGAL in cancer and adjacent normal tissues of early gastric cancer (stage IA). The quantity of uMMP-9/NGAL in this case was 1225 pg/mL. With MMP-9 stained brown and NGAL stained red, MMP-9/NGAL complex was recognized as colocalization of MMP-9 (brown) and NGAL (red). MMP-9 (brown) was positive in cancer epithelial cells, whereas NGAL (red) was positive in most of tumor epithelial cells and some stromal cells. Colocalization of MMP-9 and NGAL was observed in most of the tumor epithelial cells. MMP-9 (brown) was weakly detected in some normal epithelial cells, whereas NGAL (red) was detected in a small number of normal stromal cells. No colocalization of MMP-9 and NGAL was observed in the normal tissue. B, representative immunohistochemical images of ADAM12. These images are a representative case of ADAM12 staining in cancer and adjacent normal tissues of early gastric cancer (stage IA). The quantity of uADAM12 in this case was 3.81 ng/mL. ADAM12 (brown) diffusely and strongly expressed in cancer tissues, whereas weak to moderate immunoreactivity to ADAM12 was detected in normal stromal tissues. C, quantitative analysis of immunohistochemistry. Staining scores were calculated in cancer and adjacent normal tissues of all gastric cancers. Data were analyzed using the Mann–Whitney U test.
2, is a member of the lipocalin protein family and was originally identified as a 25-kDa protein forming a complex with MMP-9 in human neutrophils (43). NGAL has been reported to be involved in many processes, including the immune response (44), the progression and metastasis of breast cancer (45, 46), and colorectal cancer (47). We have also previously reported other regulatory roles of NGAL, including its protection of the enzyme activity of MMP-9 from autodegradation through the binding to MMP-9 (48, 49). In the present study, the MMP-9/NGAL complex was present at significantly higher levels in the urine of patients with gastric cancer compared with age- and sex-matched healthy controls and was identified as a significant biomarker for gastric cancer. Our finding is supported by a study demonstrating increased levels of MMP-9/NGAL complex in human gastric cancer tissues (50). This report studied 81 gastric cancer tissues and showed that the protein concentration of MMP-9/NGAL complex was significantly higher in homogenates of gastric cancer tissues than in those of adjacent normal mucosa. However, the urine of patients with gastric cancer was not studied, and only 28% of samples in this previous report were stage I samples. The present study is the first to report that uADAM12 and uMMP-9/NGAL, when multiplexed, have clinical potential as diagnostic biomarkers of gastric cancer.

The majority of this study population (62.9%) is at stage IA. Importantly, most of these stage IA patients (81.8%), who also represent 51.4% of all patients with gastric cancer, are at a very early stage of gastric cancer that could be treated by endoscopy. In the present study, uADAM12 and uMMP-9/NGAL discriminated between patients with gastric cancer and healthy controls. ADAM12 and MMP-9/NGAL were also significantly expressed in the cancer tissues compared with normal tissues, consistent with previous reports (35, 50). Importantly, no colocalization of MMP-9 and NGAL was found in both cancer and normal tissues in patients with cancer with a lack of uMMP-9/NGAL. Moreover, we and other groups have previously shown that urinary levels of metalloproteinases correlate tumor presence and status in other type of tumors (15), and that urinary levels of these proteins decrease after resection and successful treatment (22, 51). Taken together, these results suggest that ADAM12 and MMP-9/NGAL complex are already present at early phases of gastric cancer and urinary level of those proteins reflect their expression in gastric cancer. We previously showed that NGAL protects the enzymatic activity of MMP-9 and facilitates angiogenesis and tumor growth in a preclinical model of breast cancer (49). MMP-9 has also been reported to induce the angiogenic switch in a carcinogenic model of pancreatic islets, which is a critical requirement for the acquisition of the angiogenic phenotype in early cancer development (52). Also worth noting is the fact that the mRNA of ADAM12 has been previously detected in cancer tissues of early-stage lung cancer (31). On the basis of these previous reports and our current results, ADAM12 and MMP-9/NGAL complex appear to be promising sentinels of the presence of gastric cancer.

Using this cohort of samples, which included a high population of very early-stage gastric cancers, the present study demonstrated AUCs of 0.757 for uADAM12 and 0.657 for uMMP-9/NGAL, and by multiplexing uADAM12 and uMMP-9/NGAL, the accuracy of these two biomarkers in predicting the presence of gastric cancer rose to a significant AUC of 0.825. uADAM12 and uMMP-9/NGAL may enable gastric cancer to be noninvasively diagnosed because diagnostic power with 77.1% sensitivity and 82.9% specificity is superior to currently used methods such as photofluorography and serum pepsinogen.

This study has two potential limitations. First, this case-control report with a relatively small sample size is a proof-of-principle study. It is important to note that this study was enriched in very early-stage gastric cancer, and there currently do not exist any biomarkers that can identify early-stage gastric cancer. The fact that these potential gastric cancer diagnostics are noninvasive is a significant advantage given their low cost and ease of sample acquisition. Second, this study exclusively consists of samples from a Japanese population. However, the Japanese population is eminently suitable for the study of early detection of gastric cancer because the discovery rate of early-stage gastric cancer is relatively higher in Japan than that of other countries (3, 53). In the future, we plan to conduct a large prospective cohort study in Japan to verify the efficacy of uADAM12 and uMMP-9/NGAL for detection of gastric cancer.

In conclusion, uADAM12 and uMMP-9/NGAL represent noninvasive biomarkers for the early detection of gastric cancer with the potential to save lives and improve the quality of life for patients with gastric cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed by the authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Shimura, M.A. Moses
Writing, review, and/or revision of the manuscript: T. Shimura, M.A. Moses
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Shimura, A. Dagher, M. Sachdev, M.A. Moses
Study supervision: T. Joh, M.A. Moses

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References


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