Panel of autoantibodies against multiple tumor-associated antigens for detecting gastric cancer

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Gastric cancer is the second leading cause of cancer death in the world, and effective diagnosis is extremely important for good outcome. We assessed the diagnostic potential of an autoantibody panel that may provide a novel tool for the early detection of gastric cancer. We analyzed data from patients with gastric cancer and normal controls in test and validation cohorts. Autoantibody levels were measured against a panel of six tumor-associated antigens (TAAs) by ELISA: p53, heat shock protein 70, HCC-22-5, peroxiredoxin VI, KM-HN-1, and p90 TAA. We assessed serum autoantibodies in 100 participants in the test cohort. The validation cohort comprised 248 participants. Autoantibodies to at least one of the six antigens showed a sensitivity/specificity of 49.0% (95% confidence interval [CI], 39.2–58.8%)/92.4% (95% CI, 87.2–97.6%), and 52.0% (95% CI, 42.2–61.8%)/90.5% (95% CI, 84.8–95.3%) in the test and validation cohorts, respectively. In the validation cohort, no significant differences were seen when patients were subdivided based on age, sex, depth of tumor invasion, lymph node metastasis, distant metastasis, peritoneal dissemination, or TNM stage. Patients who were positive for more than two antibodies in the panel tended to have a worse prognosis than those who were positive for one or no antibody. Measurement of autoantibody response to multiple TAAs in an optimized panel assay to discriminate patients with early stage gastric cancer from normal controls may aid in the early detection of gastric cancer.

Although the incidence of gastric cancer has declined in recent years, it is still the fourth most common cancer in the world and the second leading cause of cancer-related deaths worldwide.1) More than 950 000 new cases occur each year. An estimated 720 000 patients died from gastric cancer in 2012. More than 70% of cases occur in developing countries.2) Patients with advanced stage gastric cancer have an extremely poor survival rates.3) To date, diagnosis of gastric cancer has been based on clinical symptoms together with techniques such as endoscopy and barium meal test; however, these methods have certain drawbacks in the detection of gastric cancer. In addition, serum tumor markers, such as CEA, CA19-9, and CA72-4 also have limited sensitivity and specificity for gastric cancer screening.4) Furthermore, because of the lack of expression of these markers in the early stages of cancer, their serum levels are not sufficiently high for early detection. Therefore, there is a need for novel, reliable, non-invasive biomarkers of gastric cancer.

The immune system recognizes tumor cells even in early stages of cancer5,6 including a mutated version of the p53 tumor suppressor protein that is overexpressed in gastric cancer.7) Although serum p53 antibodies have been detected even in the early stages of tumors, the positive rate for stage I tumors is <10%.8–10) To overcome this problem, subsequent studies have provided better sensitivity in the diagnosis of cancer by screening for multiple autoantibodies against a panel of TAAs.11–20)

The panel of six antigens selected in this study includes a well-recognized TAA, p53, which is mutated in a large number of cancers. This antigen was the first described to elicit autoantibodies in cancer.21) Such anti-p53 antibodies can, in some cases, be detected before the detection of cancer using other methods.22,23) Heat shock protein 70 is possibly the most intriguing because it is a stress response protein involved in various cell processes, such as folding and assembly of newly synthesized proteins as well as inhibition of apoptosis through the caspase-dependent pathway.24) Overexpression of HSP70 leads to increased resistance to apoptosis-inducing agents, such as tumor necrosis factor-α and doxorubicin,25) and can promote the growth and metastatic potential of tumors in rodent models.26) Moreover, autoantibodies against HSP70 have been identified in esophageal squamous cell carcinoma.27) Both purified HCC-22-5 and HCC-22-5 fusion proteins have an immune response to serum antibodies of HCC. Anti-HCC-22-5 antibody is not found in sera of patients with...
gastroenterological disease or lung cancer or in sera of healthy individuals, but it is found in sera of patients with HCC as well as those with other liver diseases. Peroxiredoxin (Prx) VI is a member of the Prx gene family. Peroxiredoxins are ubiquitous enzymes, such as antioxidant enzymes, that control intracellular levels of H2O2 by catalyzing its reduction to water. These proteins are stress inducible and associated with cell-signaling pathways. They also participate in cellular antioxidant defense by inducing cell proliferation and protecting cells from undergoing apoptosis. KM-HN-1 was identified in the serum of a patient with squamous cell carcinoma of the head and neck by means of serologic identification of antigens by recombinant expression cloning and a testis cDNA expression library. The aberrant expression of the KM-HN-1 gene in a broad spectrum of human neoplasms characterizes KM-HN-1 as a cancer antigen. A cancerous inhibitor of protein phosphatase 2A, p90, was cloned using a cDNA expression library from patients with HCC. It has been reported as an endogenous inhibitor of the phosphatase activity of protein phosphatase 2A, which extends the half-life of oncogenic protein c-Myc and promotes cell survival by regulating protein kinase B dephosphorylation.

Here we provide a novel hypothesis regarding the efficiency of a panel consisting of six antigens to help discriminate gastric cancer patients from healthy controls. Using an optimal combination of the six markers determined above, we assayed 173 samples that included 73 control samples and validated the outcome with 248 independent samples.

Materials and Methods

Ethical approval. Informed patient consent was obtained, and the study was approved by the Ethics Committee of Chiba Cancer Center (no. 21-26; Chiba, Japan) and Toho University School of Medicine (nos. 22-112 and 22-047; Tokyo, Japan).

Collection of serum samples. Serum samples were obtained from BioBank (Tokyo, Japan), and collected at the Department of Gastroenterological Surgery, Chiba Cancer Center, according to established standard procedures and stored at −80°C until use.

Gastric cancer was defined on the basis of gastroscopy and confirmed with histopathology. Tumor stage was clinically determined with gastroscopy and computed tomography and was defined according to the seventh edition of the American Joint Committee on Cancer Staging Manual. Healthy controls in the test cohort were without any previous malignant disease.

The cohorts analyzed for this retrospective study were characterized as follows. Autoantibody test cohort: (i) 100 patients with gastric cancer, whose serum samples were obtained from BioBank Japan; and (ii) 79 healthy controls. Autoantibody validation cohort: (i) 248 patients with gastric cancer, whose serum samples were collected at Chiba Cancer Center; and (ii) 74 healthy controls.

Purification of recombinant TAAs. For the expression and purification of recombinant protein, full-length cDNA of the TAAs p53 (GenBank accession number: AB082923), HCC-22-5 (NM 004683), HSP70 (NM 004134), PrxVI (NM 004905), KM-HN-1 (NM152775), and p90 (AF334474) were amplified by polymerase chain reaction. The amplified gene was inserted into a plasmid expressed as tag. These recombinant proteins were expressed in Escherichia coli BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA, USA) and then dissolved in PBS. The TAA extract was applied to Ni Sepharose 6 Fast Flow (GE Healthcare, Little Chalfont, UK), and the column was washed with 50 mM imidazole in PBS. Purified TAA recombinant proteins were eluted with 200 mM imidazole in PBS. The expression and purity of the recombinant proteins were examined with 12.5% SDS-PAGE. DNA sequencing analysis confirmed that the correct gene was inserted into the constructed plasmid.

Detection of serum antibodies and other conventional tumor markers. Serum samples from patients and healthy controls were analyzed by ELISA, as previously described. Briefly, purified recombinant proteins were coated onto 96-well microtiter plates (Maxisorp; Nunc, Rochester, NY, USA). Tumor-associated antigens were diluted in PBS to final concentrations of 0.5–5.0 μg/mL and added to the plates (100 μL/well), which were then incubated overnight at 4°C; PBS was used as control. After two washes with PBS, the proteins were blocked with 200 μL PBS containing 1% BSA and 5% sucrose at room temperature for 3 h. All human serum samples were diluted (1:100) in PBS containing 0.15% Tween 20, 1% casein, and 0.2 mg/mL E. coli extract. Then, 100 μL diluted serum was added to each TAA- or PBS-coated well and incubated at room temperature for 20 g for 60 min. After washing with PBS containing 0.05% Tween-20 (PBST) four times, 100 μL HRP-conjugated antihuman IgG (1:5000; MBL, Nagoya, Japan) diluted in 20 mM HEPES, 135 mM NaCl, 1% BSA, and 0.1% hydroxyphenylacetic acid was added to each well as a secondary antibody and incubated at room temperature at 20 g for 60 min. The wells were washed four times with PBST buffer, and autoantibodies were detected by addition of 100 μL 3,3′5,5′-...
controls, and to identify correlations of individual and combined antibody assay positivity with clinical parameters. The correlation between overall survival and autoantibody status was calculated using the log–rank test, and the results are presented as curves determined using the Kaplan–Meier method. In all tests, we considered $P$-values of <0.05 (two-sided) to indicate statistical significance.

**Results**

**Autoantibodies in gastric cancer.** In total, 421 participants were recruited, 179 in the test cohort and 322 in the validation cohort. The presence of autoantibodies to all TAAAs in both cohorts is shown for one concentration of antigen in the scatter plots in Figure 1. All six TAAAs were clearly elevated in serum samples from patients with gastric cancer compared with serum from healthy controls. The levels of autoantibodies to individual antigens in patients with gastric cancer and healthy controls are shown in Table 1 and Figure 2. The levels of autoantibodies to all of these antigens were significantly different between patients with gastric cancer and healthy controls. Using all six antibodies provided an enhanced panel sensitivity of 49.0% (95% CI, 39.2–58.8%) in the test and validation cohorts, respectively. The importance of autoantibody responses to individual antigens in the panel assay varied. Results in the validation cohort using the cut-off values for individual autoantibodies of the test cohort were similar to the results in the test cohort (Table 2).

**Clinicopathological features and autoantibody status in patients with gastric cancer.** Patient samples in the validation cohort were obtained at Chiba Cancer Center. The demographics of the patients and the clinicopathological characteristics of their tumors are shown in Table 3. Significantly more male than female patients were autoantibody panel positive ($P = 0.025$). No other patient characteristics were significantly related to autoantibody panel status (Table 4). In 28.7% of the autoantibody-positive individuals in panel 1 of 6, autoantibodies were raised to a second antigen in samples from patients with gastric cancer in the validation cohort. We assessed the correlations between clinicopathological features and the positive number of antigens (Table 5). None of the features were found to be significantly related to the positive number of autoantibodies (1 or ≥2).

**Autoantibody panel for early detection.** To verify the diagnostic power of this six-autoantibody panel, we further assessed its sensitivity for the detection of gastric cancer. We
first compared its sensitivity with the sensitivities of the traditional tumor markers CEA and CA19-9. In the validation cohort, the sensitivities of CEA and CA19-9 were 18.1% and 14.1%, respectively, whereas the sensitivity of the six-autoantibody panel was 52.0%, and there was a significant difference between the sensitivities of the panel and traditional tumor markers (Fig. 3). The sensitivity of the panel was significantly higher than that of combinations, including CEA and CA19-9. Furthermore, the sensitivity of the six-autoantibody panel plus CEA and CA19-9 was significantly higher than that of the six-autoantibody panel alone.

To assess the usefulness of the panel in the clinical setting, we examined the sensitivity of the panel for detecting gastric cancers with various clinical features. We found that the sensitivity of the panel did not differ between tumors that differed in any of the features that we assessed in this study: depth of tumor invasion (T1 or ≥ T2), lymph node metastasis (+ or –), distant metastasis (+ or –), peritoneal dissemination (+ or –), TNM stage (I or ≥ II), or pathological type (such as tubular adenocarcinoma, signet ring cell carcinoma, and papillary adenocarcinoma). In contrast, with conventional tumor markers, even with the combination of CEA and CA19-9, the sensitivity of the marker for detecting gastric cancers significantly differed in tumors with different clinical features. In brief, conventional tumor markers could not detect gastric cancer in the early stages (Fig. 4).

**Prognostic role of autoantibodies in patients with gastric cancer.** We evaluated the 3-year survival rates of the autoantibody-positive and -negative groups and found no differences between them (Fig. 5a).
We also divided the patients into two groups; the group with patients positive for two or more autoantibodies had a worse prognosis than that of the other group with patients positive for no or one antibody (Fig. 5b).

**Discussion**

Diagnosis of gastric cancer in the early stages is a problem because of the lack of specific symptoms. Carcinoembryonic antigen, CA19-9, CA-50, and other tumor markers are currently used in the diagnosis of gastric cancer in clinical practice. These markers lack high sensitivity and specificity, particularly for early stage gastric cancer. Recently, multiple molecular biomarkers have been explored and reported to have potential efficacy as diagnostic and prognostic tools in gastric cancer. However, their use is still limited, and they need further validation to be used as markers of gastric cancer.

The production of autoantibodies reflects greater immunologic reactivity in patients with cancer and enhanced immune surveillance for cancer cells. Autoantibodies to TAAs have recently received attention as potential biomarkers of cancer because they can be easily measured in serum obtained with

### Table 4. Patient details of panel positive in validation cohort

| Positive | + | P |
|----------|---|---|
| Number (%) | 119 (48.0) | 129 (52.0) |
| Gender, n (%) | Male 79 (43.6) | 102 (56.4) | 0.025 |
| | Female 40 (59.7) | 27 (40.3) |
| Mean age ± SD, years | 66.3 ± 10.9 | 68.5 ± 9.3 |
| Age range, years | 38-89 | 37-87 |
| Depth of tumor invasion, n (%) | T1 63 (25.4) | 74 (29.8) | 0.421 |
| | T2 16 (6.5) | 16 (6.5) |
| | T3 19 (7.7) | 12 (4.8) |
| | T4 21 (8.5) | 27 (10.9) |
| Lymph node metastasis, n (%) | Positive 40 (16.1) | 39 (15.7) | 0.730 |
| | Negative 60 (24.2) | 65 (26.2) |
| | Unknown 19 (7.7) | 25 (10.1) |
| Distant metastasis, n (%) | Positive 20 (8.0) | 21 (8.5) | 0.705 |
| | Negative 99 (40.0) | 108 (43.5) |
| | Unknown 0 (0.0) | 0 (0.0) |
| Peritoneal dissemination, n (%) | Positive 16 (6.5) | 15 (6.0) | 0.972 |
| | Negative 103 (41.5) | 114 (46.0) |
| | Unknown 0 (0.0) | 0 (0.0) |
| TNM stage, n (%) | I 70 (28.2) | 85 (34.3) | 0.727 |
| | II 7 (2.8) | 1 (0.4) |
| | III 14 (5.6) | 14 (5.6) |
| | IV 28 (11.3) | 29 (11.7) |
| | Unknown 0 (0.0) | 0 (0.0) |
| CEA, n (%) | Positive 17 (6.8) | 28 (11.3) | 0.130 |
| | Negative 102 (41.1) | 101 (40.7) |
| CA19-9, n (%) | Positive 15 (6.0) | 20 (8.0) | 0.512 |
| | Negative 104 (41.9) | 109 (44.0) |

CA, carbohydrate antigen; CEA, carcinoembryonic antigen.

### Table 5. Gastric cancer patients positive for one or multiple autoantibodies in validation cohort

| Number of antigen positive | 1 | ≥2 | P |
|---------------------------|---|----|---|
| Number (%) | 92 (71.3) | 37 (28.7) |
| Gender, n (%) | Male 69 (53.5) | 33 (25.6) | 0.073 |
| | Female 23 (17.8) | 4 (3.1) |
| Mean age ± SD, years | 68.5 ± 9.3 | 66.7 ± 13.5 |
| Age range, years | 37-87 | 36-85 |
| Depth of tumor invasion, n (%) | T1 51 (39.5) | 23 (17.8) | 0.310 |
| | T2 15 (11.6) | 1 (0.8) |
| | T3 9 (7.0) | 3 (2.3) |
| | T4 17 (13.2) | 10 (7.8) |
| Lymph node metastasis, n (%) | Positive 28 (21.7) | 11 (8.5) | 0.796 |
| | Negative 48 (37.2) | 17 (13.2) |
| | Unknown 16 (12.4) | 9 (7.0) |
| Distant metastasis, n (%) | Positive 14 (10.9) | 7 (5.4) | 0.969 |
| | Negative 78 (60.5) | 30 (23.3) |
| | Unknown 0 (0.0) | 0 (0.0) |
| Peritoneal dissemination, n (%) | Positive 8 (6.2) | 7 (5.4) | 0.410 |
| | Negative 84 (65.1) | 30 (23.3) |
| | Unknown 0 (0.0) | 0 (0.0) |
| TNM stage, n (%) | I 62 (48.1) | 23 (17.8) | 0.759 |
| | II 0 (0.0) | 1 (0.8) |
| | III 12 (9.3) | 2 (1.6) |
| | IV 18 (14.0) | 11 (8.5) |
| | Unknown 0 (0.0) | 0 (0.0) |
| CEA, n (%) | Positive 19 (14.7) | 9 (7.0) | 0.825 |
| | Negative 73 (56.6) | 28 (21.7) |
| CA19-9, n (%) | Positive 16 (12.4) | 4 (3.10) | 0.513 |
| | Negative 76 (58.9) | 33 (25.6) |

CA, carbohydrate antigen; CEA, carcinoembryonic antigen.
Fig. 4. Sensitivity for tumors with various clinical features in patients with gastric cancer. (a) Depth of tumor invasion. (b) Lymph node metastasis. (c) Distant metastasis. (d) Peritoneal dissemination. (e) TNM stage. (f) Pathological type. *$P < 0.05$; **$P < 0.001$. CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen.

Fig. 5. Overall survival curves of gastric cancer patients with different autoantibody status. (a) Autoantibody-positive and -negative groups. (b) Group with ≥2 positive autoantibodies and group with one autoantibody positive or negative.
minimally invasive blood sampling. Serum TAA levels are believed to increase even in very early stages of cancer.\(^\text{[30]}\)

We previously reported that several TAAs, including p53, tumor associated calcium signal transducer (TROP2), tripartite motif containing 21 (TRIM21), glucose transporter 1 (GLUT-1), myomegalin, and New York esophageal squamous cell carcinoma 1 (NY-ESO-1), are useful to identify gastrointestinal malignant diseases.\(^{[6,40,41]}\) In the present study, we showed that the detection of autoantibodies to a panel of specific TAAs could have strong diagnostic power in patients with gastric cancer compared with the use of conventional tumor markers such as CEA and CA19-9. Five TAAs that we chose in this study were reported to be potentially useful to detect cancer with high efficiency, even when only a single biomarker is used.\(^{[21–23,27,28,31]}\)

Consistent with these earlier data, our data on the individual sensitivities and specificities of all TAAs used in this study were enough to warrant further exploration. Serum p53 antibodies had the highest sensitivity among TAAs in our panel: 15.0% in the test cohort and 16.5% in the validation cohort. This sensitivity was almost equal to that of CEA alone (18.1%) and might surpass the sensitivity of CA19-9 alone (14.1%) in this study. However, although all six of the antigens examined in our study have the potential to detect gastric cancer, similar to CEA and CA19-9, none except p53-Ab had a high degree of diagnostic efficiency.

On the basis of these results, it is apparent that a multiple-autoantibody panel approach may improve the sensitivity associated with a single biomarker.\(^{[20]}\) In our cases, a six-autoantibody panel had sensitivities of 49.0% and 52.0% in the test and validation cohorts, respectively. Moreover, autoantibodies statuses were not associated with traditional tumor markers, such as CEA and CA19-9, statuses. A combination of a multiple-autoantibody panel with these traditional tumor markers might have an additive effect on sensitivity. Furthermore, our multiple-autoantibody panel can detect gastric cancer even as early as stage I. Surprisingly, the efficacy of the panel for detecting gastric cancer was not affected by any of the clinical features of the tumors that we assessed in this study: depth of tumor invasion (T1 or ≥T2), lymph node metastasis (+ or –), distant metastasis (+ or –), peritoneal dissemination (+ or –), or TNM stage (I or ≥II). Our data show that the levels of the traditional tumor markers CEA and CA19-9 were elevated in advanced stages of cancer under most situations. This should mean that our panel of six TAAs helps distinguish patients with early-stage gastric cancer from healthy controls.

It remains unclear whether autoantibody status affects the prognosis of cancer. Previous reports showed that increasing serum levels of p53-Ab indicated a poor prognosis in patients with colorectal cancer.\(^{[42,43]}\) On the contrary, Suppiah et al.\(^{[44]}\) reported that p53-Ab was not related to the prognosis of colorectal cancer in long-term follow-up. In our present series, although there was a difference that was not statistically significant, the autoantibody-positive group had poorer survival than the autoantibody-negative group. Although the prognostic impacts of other antigens have not been precisely evaluated, except for p53, expression of HSP70 was associated with reduced survival in patients with esophageal cancer.\(^{[45]}\) Serum HSP70 autoantibody reaction might reflect this biological effect. Moreover, upregulation of p90 is reported in a wide variety of malignant tumors.\(^{[46–49]}\) Overexpression of p90 could be associated with worse prognosis and might serve as a prognostic marker in numerous human cancers. These findings might support our hypothesis that a positive ratio of TAAs was positively related to poor prognosis. Positive numbers of autoantibodies may correlate with poor prognosis in patients with gastric cancer in our study. Further examination and long-term follow-up will be needed to clarify this question.

In summary, this relatively large cohort study reports the clinical usefulness of a panel of six TAAs to diagnose gastric cancer, especially in its early stages. A peripheral blood test for autoantibodies is non-invasive and has the advantages of lower cost and absence of side-effects compared with invasive diagnostic methods.

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Abbreviations

| CA                | carbohydrate antigen                        |
|------------------|--------------------------------------------|
| CEA               | carcinoembryonic antigen                   |
| HCC               | hepatocellular carcinoma                   |
| HSP               | heat shock protein                         |
| Ptx VI            | peroxiredoxin VI                           |
| TAA               | tumor-associated antigen                   |

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