# Tests for Serum Levels of Trefoil Factor Family Proteins Can Improve Gastric Cancer Screening

SUSUMU AIKOU,* YASUKAZU OHMOTO,‡ TOSHIAKI GUNJI,§ NOBUYUKI MATSUHASHI,§ HIROSHI OHTSU,‖ HIRONA MIURA,* KENSUKE KUBOTA,¶ YUKINORI YAMAGATA,* YASUYUKI SETO,* ATSUSHI NAKAJIMA,¶ JAMES R. GOLDENRING,# MICHIO KAMINISHI,** and SACHIYO NOMURA*

*Departments of *Gastrointestinal Surgery, Graduate School of Medicine, and ‖Clinical Trial Data Management, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; ‡Otsuka Pharmaceutical Company, Limited, Tokyo, Japan; §NTT Kanto Central Hospital, Tokyo, Japan; ¶Department of Gastroenterology, School of Medicine, Yokohama City University, Yokohama, Japan; #Nashville VA Medical Center and the Departments of Surgery and Cell and Developmental Biology, Epithelial Biology Center, Vanderbilt University School of Medicine, Nashville, Tennessee; and **Showa General Hospital, Tokyo, Japan*

**BACKGROUND & AIMS:** Improving methods for early detection of gastric cancer could reduce mortality. Measurements of serum pepsinogen levels have been used for screening in Japan without satisfactory levels of sensitivity or specificity. Trefoil factor family (TFF) proteins (TFF1, TFF2, and TFF3) are small and stable molecules secreted by the mammalian gastrointestinal tract. Foveolar hyperplasia, spasmolytic polypeptide (TFF2)-expressing metaplasia, and intestinal metaplasia are histologic changes observed in patients with atrophic gastritis; they express TFF1, TFF2, and TFF3, respectively. We investigated whether serum levels of TFF can be used as markers for gastric cancer screening. **METHODS:** Serum was collected from 183 patients with gastric cancer and 280 healthy individuals without cancer. Serum levels of anti–*Helicobacter pylori* immunoglobulin G, pepsinogen I, pepsinogen II, TFF1, TFF2, and TFF3 were measured by enzyme-linked immunosorbent assay and associated with gastric cancer. **RESULTS:** Using a cutoff of 3.6 ng/mL, the level of TFF3 was significantly increased in serum samples from patients with cancer (odds ratio, 18.1; 95% confidence interval, 11.2–29.2); using this test, patients with cancer were identified with 80.9% sensitivity and 81.0% specificity. The test for TFF3 had a significantly higher odds ratio than that for pepsinogen. A test for the combination of TFF3 and pepsinogen had better results than the test for only pepsinogen. **CONCLUSIONS: Serum levels of TFF3 are a better marker of gastric cancer than pepsinogen; a test for the combined levels of serum pepsinogen and TFF3 could improve gastric cancer screening.**

*Keywords:* Stomach Cancer; Prevention; *H pylori*; Diagnostic; Blood Test.

Gastric cancer is the second leading cause of cancer-related death in the world.[1] Operative procedures for gastric cancer have been improved and many anticancer drugs have been developed; however, the curability of gastric cancer greatly depends on the stage of the disease. An early detection program for gastric cancer is conducted in Japan for people older than 40 years using a barium meal or endoscopy.[2] Even with this program, approximately one-half of the cases of gastric cancer found in Japan are in an advanced stage. One of the reasons for this failure is the invasive nature of these screening examinations, leading to avoidance of necessary testing.[3] Biomarkers that can be analyzed in blood samples to detect gastric cancer have the possibility to reduce the number of advanced cases of gastric cancer.

Trefoil factors (TFFs) are small (12–22 kilodaltons) and stable molecules secreted by the mammalian gastrointestinal tract.[4–7] They were named due to the existence of a common 3-loop structure, which makes the peptides extremely stable toward proteolytic digestion as well as acid and heat degradation. TFFs constitute a family of 3 peptides (TFF1, TFF2, and TFF3) that are widely expressed in a tissue-specific manner in the gastrointestinal tract. TFF1 is expressed in surface mucous cells in the gastric mucosa; TFF2 is expressed in mucus neck cells of the gastric fundus, deep antral gland cells, and Brunner's gland in duodenum; and TFF3 is expressed in the goblet cells of the small and large intestine.[4–7]

Gastric cancer arises following chronic *Helicobacter pylori* infection through chronic atrophic gastritis.[8] Mucosal histologic changes in chronic atrophic gastritis include oxyntic atrophy accompanied by foveolar hyperplasia, spasmolytic polypeptide (TFF2)-expressing metaplasia (SPEM), and intestinal metaplasia in later stages.[9–20] Foveolar hyperplasia is elongation of the gastric pit composed of foveolar surface mucous cells, originally expressing TFF1. SPEM is an antral phenotype lineage characterized by TFF2-positive cells in the gastric fundus.[21–29] SPEM is frequently observed in the gastric mucosa surrounding gastric cancer, and TFF2 is positive in 58% of cases of early gastric cancer.[18,19] Intestinal metaplasia is characterized by intestinal phenotype cells arising in gastric mucosa and is believed to be a precancerous lesion of intestinal type of gastric cancer. TFF3 is expressed in the goblet cells of the small and large intestine as well as intestinal metaplasia in the stomach.

*Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristic; SPEM, spasmolytic polypeptide (TFF2)-expressing metaplasia; TFF, trefoil factor family.


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These characteristics of TFFs prompted us to analyze whether serum TFFs can be biomarkers of gastric cancer. In this study, serum levels of TFFs in patients with gastric cancer and health check volunteers were determined and the possibility of serum levels of TFFs as biomarkers of the gastric cancer was analyzed. The results indicate that measurement of TFF3 levels in the serum may represent an improved method for detection of gastric cancer.

## Patients and Methods

### *Subjects*

The patient group consisted of 183 patients with gastric cancer treated at the Department of Gastrointestinal Surgery of University of Tokyo Hospital from February 2006 to September 2008. We obtained blood samples from the 183 patients before treatment. The patients were stratified into bearing early cancer or advanced cancer, histologic types (differentiated and undifferentiated), number, depth, size of the tumor, lymph node metastasis, and clinical stage. The control group consisted of 280 healthy male and female blood donors who received a health check at NTT Kanto Central Hospital from September 2006 to November 2006. For the validation of the results, a second cohort of patients, consisting of 59 patients with gastric cancer treated at the Department of Gastrointestinal Surgery of University of Tokyo Hospital from August 2009 to March 2010, were analyzed for serum TFF3 and pepsinogen levels. The age-matched control group consisted of 45 healthy male and female blood donors who received a health check at NTT Kanto Central Hospital from January 2011 to April 2011 and were compared with 15 patients in their 30s to 60s in the second cohort. The group of patients with pancreatic cancer consisted of 8 patients with untreated pancreatic cancer admitted to Yokohama City University Hospital from March 2011 to April 2011. The patient group of pre– and post–bariatric surgery consisted of 20 patients who received Roux-en-Y bypass at Vanderbilt University Medical Center. Serum samples were collected for research under an institutional review board–approved protocol.

This study was approved by the Institutional Review Board of the University of Tokyo Hospital. Written informed consent was obtained from participants in accordance with the Declaration of Helsinki and its later revision.

### *Construction of Human TFF1, TFF2, and TFF3 Expression Plasmids and Expression and Purification of Recombinant Human TFF1, TFF2, and TFF3*

These methods are shown in Supplementary Materials and Methods.

### *Immunoassays for TFFs*

Serum TFF1, TFF2, and TFF3 levels were measured by enzyme-linked immunosorbent assay (ELISA). Antisera were prepared from rabbits immunized with human TFFs. Validation of the ELISA system and precise methods are described in Supplementary Figures 2–4 and Supplementary Materials and Methods. Sensitivities of TFFs were 7 pg/mL for TFF1, 30 pg/mL for TFF2, and 30 pg/mL for TFF3. Each TFF antibody reacted specifically and showed no cross-reactivity for the other TFFs.

### *Immunoassays for Anti–H pylori Immunoglobulin G, Pepsinogen I, and Pepsinogen II*

Serum anti–*H pylori* immunoglobulin (Ig) G, pepsinogen I, and pepsinogen II levels were measured by ELISA. Anti–*H pylori* IgG level was measured with the *Helicobacter pylori* IgG ELISA Kit (Biohit Plc, Helsinki, Finland) to define *H pylori* infectious status in this study. Pepsinogen I level was measured with the Pepsinogen I ELISA Kit (Biohit Plc), and pepsinogen II level was measured with the Pepsinogen II ELISA Kit (Biohit Plc). Each sample was analyzed in duplicate. The *H pylori* infection status was diagnosed to be positive when the IgG level was greater than 9.9 U/mL.

### *Immunohistochemistry*

The methods of TFF immunohistochemistry for normal gastric mucosa and atrophic gastritis are shown in Supplementary Materials and Methods.

### *Statistical Analysis*

All statistical analyses were performed using JMP7 software (SAS Institute, Inc, Cary, NC). The mean of variables was compared between 2 groups by a *t* test. The receiver operating characteristic (ROC) curve for each evaluation was used to extract the corresponding cutoff point, which can be used to discriminate different gastric status. For that purpose, the area under each ROC curve was used to measure the discriminatory ability of the model. The resulting value of the cutoff point for each evaluation was applied to the determination of the sensitivity, specificity, and odds ratio. Consequently, 95% confidence intervals were calculated. The values were determined as the median. A 2-sided *P* value of less than .05 was considered statistically significant.

## Results

### *Patients and Donor Characteristics*

Supplementary Table 1 shows the backgrounds of the patients and the controls. The average age of patients in the cancer group was 66.0 ± 10.7 years, and that of the controls was 50.1 ± 9.9 years. The ratio of male to female patients in the cancer group was 124/59, and that of the controls was 238/31. The positive rate of *H pylori* in the cancer group was 62.3%, and that of the controls was 34.9%. The patients in the cancer group were older, and there was a higher number of female patients and patients with *H pylori* infection than in the control group. The ratio of advanced gastric cancer to early gastric cancer was 107/76. The distribution of histologic types of gastric cancer with differentiated type versus undifferentiated type was 86/97. In the follow-up period, 7 of the 183 patients were found to have cancer in other organs: gallbladder (1), colon (2), prostate (2), and lung (2).

### *Immunohistochemistry*

Immunohistochemistry of TFFs for gastric mucosa is shown in Supplementary Figure 5. Foveolar cells were positive for TFF1 (Supplementary Figure 5*A*), and these TFF1-positive cells were expanded in atrophic gastritis, making foveolar hyperplasia in both fundus and