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

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Abstract

Background & Aims—Improving methods for early detection of gastric cancer could reduce mortality. Measurements of serum pepsinogen 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, stable molecules secreted by the mammalian gastrointestinal tract. Foveolar hyperplasia, spasmolytic polypeptide (TFF2)-expressing metaplasia (SPEM), and intestinal metaplasia are histological 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.

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Conflict of interest

Yasukazu Ohmoto is an employee of Otsuka Pharmaceutical Company, which is making anti-cancer drugs.

Author Contributions

Susumu Aikou did immunostaining, ELISA measuring, and acquisition of data. Yasukazu Ohmoto made up the ELISA system. Toshiaki Gunji and Nobuyuki Matsuhashi took informed consent and collected blood samples, and analyzed the data. Hiroshi Ohtsu statistically analyzed the data. Hirona Miura measured the serum TFF3 level of second cohort for revised manuscript. Kensuke Kubota and Atsushi Nakajima collected pancreas cancer patients' serum and measured TFF3 of them. Yukinori Yamagata helped Susumu Aikou for his staining and measuring. Yasuyuki Seto and Michio Kaminishi supervised the study. Naji N. Abumrad collected the obesity patient sera under an IRB approved protocol and analyzed the data. James R Goldenring designed the study and reviewed the manuscript. Sachiyo Nomura designed the study, conducted the study, interpreted the data and drafted the manuscript.

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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 ELISA and associated with gastric cancer.

Results—Using a cut-off of 3.6 ng/ml, the level of TFF3 was significantly increased in serum samples from cancer patients (odds ratio of 18.1; 95% confidence interval, 11.2–29.2); using this test cancer patients 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.

Conclusion—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

Introduction

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 anti-cancer 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 over 40 years old using Barium meal or endoscopy.² Even with this program, around half of the gastric cancer found in Japan is still in advanced stage. One of the reasons of 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 kD) 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 towards proteolytic digestion as well as acid and heat degradation. TFFs constitute a family of three 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* (*H. pylori*) infection through chronic atrophic gastritis.⁸ Mucosal histological changes in chronic atrophic gastritis include oxyntic atrophy accompanied by foveolar hyperplasia, spasmodic 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% early gastric cancer.^{18, 19} Intestinal metaplasia is characterized by intestinal phenotype cells arising in gastric mucosa and is thought to be a precancerous lesion of intestinal type of gastric cancer. TFF3 is expressed in the goblet cells of small and large intestine, as well as intestinal metaplasia in stomach.

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 gastric cancer patients 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.

Materials 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, histological 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. 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 compared to 15 patients aged 30s–60s in the second cohort. The pancreas cancer patients group consisted of 8 patients with untreated pancreas 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 IRB-approved protocol.

This study was approved by the IRB 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, TFF3 expression plasmids, and Expression and purification of recombinant human TFF1, TFF2, TFF3

These methods are shown in Supplemental methods.

Immunoassays for TFFs

Serum TFF1, TFF2 and TFF3 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 written in Supplemental Figures 2–4 and Supplemental 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-*Helicobacter Pylori* IgG, Pepsinogen I, and Pepsinogen II

Serum anti-*Helicobacter Pylori* IgG, Pepsinogen I, Pepsinogen II were measured by enzyme-linked immunosorbent assay (ELISA). Anti-*Helicobacter Pylori* IgG was measured with the *Helicobacter pylori* IgG ELISA kit (Biohit Plc., Helsinki, Finland) to define *H. pylori* infectious status in this study. Pepsinogen I was measured with the Pepsinogen I ELISA kit (Biohit Plc.). Pepsinogen II 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 was more than 9.9 U/ml.

Immunohistochemistry

The methods of TFFs immunohistochemistry for normal gastric mucosa and atrophic gastritis are shown in Supplemental Methods.

Statistical analysis

All statistical analyses were performed using JMP7 software (SAS Institute, Cary, NC, USA). The mean of variables was compared between two groups by a t-test. The receiving operating characteristics (ROC) curve for each evaluation was used to extract the corresponding cut-off 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 cut-off 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 two-sided *P* value of less than 0.05 was considered statistically significant.

Results

Patients and Donor characteristics

Supplemental Table 1 shows backgrounds of the patients and the controls. The average age of patients in the cancer group was 66.0 ± 10.7 and that of the controls was 50.1 ± 9.9 . The ratio of male: female 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 showed higher number of females and patients with *H. pylori* infection than the control group. The ratio of advanced gastric cancer to early gastric cancer was 107:76. The distribution of histological types of gastric cancer with differentiated type versus undifferentiated type was 86:97. In the follow up period, 7 patients among 183 were found to have cancer in other organs: gall bladder (1), colon (2), prostate (2), and lung (2).

Immunohistochemistry

Immunohistochemistry of TFFs for gastric mucosa is shown in Supplemental Fig. 5. Foveolar cells were positive for TFF1, (Supplemental Fig. 5A) and these TFF1 positive cells were expanded in atrophic gastritis, making foveolar hyperplasia in both fundus and antrum (Supplemental Fig. 5B). Mucous neck cells in fundic glands (Supplemental Fig. 5C) and mucous cells in antral glands were positive for TFF2 in normal mucosa. SPEN, arising in the fundic mucosa with atrophic gastritis, was much more strongly positive for TFF2 (Supplemental Fig. 5D). Normal gastric mucosa was essentially negative for TFF3 (Supplemental Fig. 5E); however, goblet cells in intestinal metaplasia were strongly positive for TFF3 (Supplemental Fig. 5F).

Serum TFF levels

Fig. 1 shows the serum TFF1, TFF2 and TFF3 levels in control group patients subgrouped by presence of *H. pylori* infection and in gastric cancer patients. *H. pylori* infection status was diagnosed by serum anti-*H. pylori* IgG level.

In the control group without *H. pylori* infection, the serum TFF1 level was 0.57 ± 0.29 (median 0.51, range 0.18–3.10) ng/ml. The serum TFF1 level in the control group with *H. pylori* infection was 2.51 ± 1.52 (median 2.43, range 0.36 – 6.94) ng/ml and was significantly higher than in the group without infection. The serum TFF1 level in patients with gastric cancer was 3.35 ± 3.06 (median 2.37, range 0.31 – 19.1) ng/ml and was also significantly higher than in the control group without *H. pylori* infection. However, the

TFF1 level in cancer patients was not significantly higher than in the control group with *H. pylori* infection. The serum TFF2 level in the control group without *H. pylori* infection was 2.88 ± 1.04 (median 2.7, range 0.53 – 7.2) ng/ml. The TFF2 level in the control group with *H. pylori* infection was 5.15 ± 2.41 (median 4.83, range 1.51–14.5) ng/ml and was significantly higher than without infection. The serum TFF2 level in patients with gastric cancer was 8.79 ± 16.2 (median 6.36, range 0.78–210) ng/ml and was also significantly higher than both control groups. The serum TFF3 level in the control group without *H. pylori* infection was 2.72 ± 0.80 (median 2.56, range 1.22–5.30) ng/ml. TFF3 level in the control group with *H. pylori* infection was 3.05 ± 1.10 (median 2.79, range 1.40–6.25) ng/ml and was significantly higher than in control patients without infection. The serum TFF3 level in the patients with gastric cancer was 6.44 ± 6.19 (median 5.02, range 1.85–74.4) ng/ml and was significantly higher than in both control groups. The serum TFF3 levels in 7 patients who developed a second cancer in the follow-up period were not significantly different from other patients.

Serum levels of TFFs as markers of *H. pylori* infection

To test the diagnostic accuracy of serum TFFs for identifying *H. pylori* infection, receiver operating characteristic (ROC) analysis was done. Fig. 2A shows ROC curves for serum TFF levels to distinguish patients with *H. pylori* infection defined by the serum anti-*H. pylori* IgG level. The area under the curve for TFF1 was 0.95. Using a cutoff of 1.0 ng/ml, the odds ratio for serum TFF1 level was 141.3 (56.4–356.0); the sensitivity was 87.7%, specificity was 95.6%. Thus, the TFF1 level in serum was well-correlated with *H. pylori* infection. ROC curves for TFF2 and TFF3 did not show strong sensitivity or specificity for *H. pylori* infection.

Serum TFF levels as predictors of Gastric cancer

To test the diagnostic accuracy of serum TFFs and pepsinogen I/II ratio for identifying gastric cancer, ROC analyses were done. Pepsinogen testing is already used for screening of gastric cancer in Japan.³ In pepsinogen testing, serum pepsinogen I < 70 ng/ml and serum pepsinogen I/II ratio < 3 was defined as positive. Fig. 2B shows ROC curves for the ability of serum levels of TFFs and Pepsinogen I/II ratio to distinguish patients with gastric cancer. The area under the curve for TFF3, TFF1, TFF2, and PG1/2 was 0.89, 0.83, 0.79, and 0.78, respectively. ROC curves indicated a higher observed accuracy for TFF3 and TFF1 compared with pepsinogen I/II ratio. ROC was also analyzed for gastric cancer patients and the health check patients without cancer, separated into *H. pylori* negative and positive groups. Fig. 2C shows ROC curves for *H. pylori* negative people. All the curves had larger area under the curves compared to Fig. 2B, but TFF3 and TFF1 were slightly better markers than TFF2 and pepsinogen I/II. The ROCs for *H. pylori* positive people (Fig. 2D) showed much smaller area under the curves for TFF1 and pepsinogen I/II, indicating that they are less robust markers for gastric cancer. TFF2 also showed a smaller area under the curve, compared to the TFF2 ROC for all of the analyzed subjects. However, TFF3 still remained with a large area under the curve, suggesting that it represented the strongest marker for detecting gastric cancer patients.

Fig. 3 shows sensitivity, specificity and odds ratio for TFFs, the pepsinogen test and anti-*H. pylori* IgG for detecting gastric cancer using a cutoff of 1.0 ng/ml of serum TFF1, 4.0 ng/ml of serum TFF2 and 3.6 ng/ml of serum TFF3. The odds ratio for serum TFF1 level was 18.1 (10.5–31.0), the sensitivity was 89.6%, and the specificity was 67.7%. The odds ratio for serum TFF3 level was 18.1 (11.2–29.2), the sensitivity was 80.9%, and the specificity was 81.0%. TFF1 and TFF3 showed significantly higher odds ratios than the pepsinogen test. As the mean age in the cancer patients group was higher than in the control group, serum TFF3 in each age group was compared (Supplemental Table 2). High serum TFF3 showed a high

odds ratio even after patients and control individuals were subgrouped by age. For validation of high serum TFF3 in gastric cancer patients, we analyzed second cohort of 15 patients, aged 30s–60s, compared to 45 age matched controls (Supplemental Figure 6). Serum TFF3 in cancer patients was significantly higher than age matched control people ($p<0.001$).

The serum TFF3 level as a coordinate marker with Pepsinogen I/II

Serum pepsinogen testing is presently used for gastric cancer screening in Japan. We analyzed the usefulness of TFF3 determination together with pepsinogen testing. The number of gastric cancer patients with positive and negative results for both tests in this study is shown in Table 1. When the cut-off value for the Pepsinogen test is defined as positive for serum pepsinogen I < 70 ng/ml and serum pepsinogen I/II ratio < 3 , 85 out of 182 patients were not detected as bearing gastric cancer with screening. However, if the serum TFF3 testing is added to the screening, 69 patients of these 85 patients, who were not identified by pepsinogen testing, can be picked up for the second examination. On the other hand, 8 out of 182 patients were not detected by TFF3 testing, but were detected by pepsinogen testing.

For the validation of the study, we analyzed second cohort of 59 gastric cancer patients with serum TFF3 and pepsinogen testing (Table 1). The pepsinogen testing could not detect 27 out of 59 patients, however, with adding serum TFF3, 26 patients out of these 27 patients could be detected.

The ROC curves for Pepsinogen I/II, TFF3, and the combination testing are shown in Fig. 4. Adding serum TFF3 testing to pepsinogen testing attained a higher sensitivity in the identification of patients with gastric cancer. The PPV and the NPV of Pepsinogen I/II is 0.608 and 0.874, respectively and those of TFF3 is 0.709 and 0.895, respectively. The PPV and the NPV of the combination of Pepsinogen I/II and TFF3 is 0.828 and 0.878, respectively, and the combination statistically significantly improved tumor detection compared to Pepsinogen I/II or TFF3 alone, however, adding serum TFF1 did not improve TFF3 testing.

The serum levels of TFFs and histological types of gastric cancer

To test the influence of gastric cancer on serum TFF levels, the levels of TFFs in serum were compared with the histological types of gastric cancer. Serum TFF1 and TFF2 levels of differentiated gastric cancer patients were significantly lower than in patients with undifferentiated gastric cancer. (TFF1: $p=0.018$, TFF2: $p=0.016$) The serum TFF3 level did not differ in patients with gastric cancer of differentiated type and undifferentiated type. ($p=0.312$) (Fig. 5) There was no significant difference in serum TFF levels in any other pathological status of gastric cancer such as the number, depth, size, lymph node metastasis and the clinical stage (data not shown).

The serum TFF levels before and after gastric resection

To evaluate the origin of elevated serum TFF levels, serum TFF levels before and after resection of gastric cancer were compared. In 46 patients with gastric cancer, we compared serum TFF1, TFF2 and TFF3 levels before operation and one week after operation. Nine patients underwent total gastrectomy, 32 patients underwent distal gastrectomy and 5 patients underwent proximal gastrectomy.

Fig. 6 shows the distributions of serum TFFs level before and after gastrectomy. One week after operation, the mean values of serum TFF1 and TFF2 levels were decreased to less than half the level before operation. Surprisingly, there was no significant change in serum TFF3 level before and after operation. As patients are still under acute inflammation state one

week after operation, we measured serum TFFs more than 3 months after operation and confirmed that serum TFF1 and TFF2 are kept low and serum TFF3 remained high even long after operation (Supplemental Figure 7). To validate the possibility of raised TFF3 after operation by alteration of the physiological food stream, serum TFF3 in patients pre- and post-bariatric surgery with Roux-en Y bypass was measured. Serum TFF3 level of pre-operation was 7.13 ± 8.18 ng/ml and of post-operation was 5.32 ± 2.04 ng/ml. The patients showed higher TFF3 levels than healthy controls, however, no up-regulation after operation was observed. To confirm the possibility of serum TFF3 elevation in cancer bearing states, serum TFF3 of pancreatic cancer patients was measured. Serum TFF3 in pancreatic cancer patients was 17.1 ± 13.0 ng/ml (6.5–43.4 ng/ml) and was much higher than control individuals.

Discussion

Gastric cancer still remains the second most common cause of cancer death worldwide and good screening markers are needed for reducing cancer related death and improving medical economy. The pepsinogen test is used for screening of gastric cancer in Japan.³ The sensitivity of the serum pepsinogen test is reported to range from 40% to 80%, and the specificity ranges from 70% to 80% compared with the results of endoscopy at the same time.^{3, 30} Several studies reported that the sensitivity of the serum pepsinogen test is higher than that of radiography.³¹ In our cohort, sensitivity of the pepsinogen test was 44.8%, specificity was 87.4% and odds ratio was 5.6, showing low sensitivity and high specificity. On the other hand, TFF1 and TFF3 showed significantly higher odds ratios (18.1 for both) than pepsinogen test. The best marker candidate, TFF3, showed sensitivity of 80.9% and specificity of 81.0%. TFFs are reported to have a tendency to increase slightly according to age.³² In our cohort, the gastric cancer patients are older than non-cancer bearing health check patients, and this age difference may make TFFs better markers than pepsinogen testing. To validate the utility of TFF3 as a gastric cancer screening marker, a second cohort of patients were compared to age-matched controls, and serum TFF3 was confirmed to be higher in the gastric cancer patient group.

Pepsinogen testing is presently employed as the major non-invasive method for gastric cancer screening in Japan.^{33–35} However, 85 (47%) of the gastric cancer patients in our cohort were negative for pepsinogen testing. Adding TFF3 testing to the screening program, more than 80% of these mis-screened patients can be picked up for the follow-up examination. On the other hand, 27 (15%) out of 182 patients were not detected by TFF3 testing, but 8 out of 27 patients were detected by pepsinogen testing. In ROC analysis, addition of pepsinogen with TFF3 provides an extra benefit of statistical significance. The PPV of the combined test is higher than each single test, so in the future, studies assessing both markers as a possible combined test would be reasonable, and TFF3 with pepsinogen should become the new standard of care. In the future, a TFF3 ELISA system with a sensitive monoclonal antibody would be needed.

In the *H. pylori* negative people, all the markers, TFFs and the pepsinogen test, showed large areas under the ROC curves for detecting gastric cancer. However, in the *H. pylori* positive people, only the TFF3 showed a good ROC curve, and the pepsinogen test performed poorly as a marker of gastric cancer. In this study, *H. pylori* infection status was defined by serum anti-*H. pylori* IgG level. Anti-*H. pylori* IgG level is reduced when atrophic gastritis spans most of the fundic area of the stomach after long time infection of *H. pylori*.^{36, 37} The anti-*H. pylori* IgG negative people may include both originally *H. pylori* negative people and people with far advanced atrophic gastritis. People who have never been infected with *H. pylori* have no atrophic gastric mucosa and a very low risk of gastric cancer. The mechanism of both the pepsinogen test and TFFs is based on histological change in the background of

the gastric mucosa from atrophic gastritis. The cohort of *H. pylori* negative people in this study may include gastric cancer patients with far advanced atrophic gastritis and people without cancer who have never been infected with *H. pylori*. The *H. pylori* positive cohort includes gastric cancer patients and non-cancer bearing people with the same severity of atrophic gastritis and cancer detection is difficult in this cohort using atrophic gastritis related markers. However, TFF3 is a good marker in both *H. pylori* negative and positive subjects.

The depth, number, stage and lymph node metastasis of gastric cancer did not affect serum TFF levels. This suggests that gastric cancer itself did not affect serum TFF levels, but rather reflected the total gastric mucosal status. On the other hand, serum TFF1 and TFF2 levels in patients with differentiated gastric cancer were lower than with undifferentiated gastric cancer. Differentiated gastric cancer was reported to arise from more advanced atrophic gastritis compared to undifferentiated gastric cancer.^{38, 39} In our previous observation of progress of atrophic gastritis with long time *H. pylori* infection, the first histological change was foveolar hyperplasia expressing TFF1, then SPEM expressing TFF2 emerges, and finally SPEM is replaced by intestinal metaplasia.²⁰ The lower TFF1 and TFF2 levels of patients with differentiated gastric cancer may therefore reflect replacement of foveolar hyperplasia and SPEM with intestinal metaplasia. On the other hand, foveolar hyperplasia expressing TFF1 was the first histological change after *H. pylori* infection and serum TFF1 level reflected *H. pylori* infection well. TFF1 and TFF2 are reported to be down-regulated in gastric cancer compared to the surrounding gastric mucosa, and TFF3 was reported to be down-regulated in 16.7% of gastric cancers and up-regulated in 41.7% of gastric cancers.⁴⁰ While these studies likely compared metaplastic mucosa to cancer, rather than normal mucosa to cancer, they do suggest that gastric cancer itself is less likely to be the sole origin of raised serum TFFs.

To confirm gastric origin of TFFs, we measured pre- and post-operative serum TFF levels in gastric cancer patients. Serum TFF1 and TFF2 decreased dramatically after gastric resection, confirming the gastric origin of these peptides. Kim et al. has reported that TFF1 is up-regulated in metastatic gastric cancer compared to primary cancer, and there is a possibility that serum TFF1 could be a marker of recurrence after resection.⁴¹ However, serum TFF3 level was not changed after gastric resection. We examined serum TFF3 as a gastric cancer marker because TFF3 was secreted from gastric intestinal metaplasia, a gastric cancer associated lesion.⁴² TFF3 is produced in goblet cells throughout the intestine and colon, while it is not produced in the normal gastric mucosa. The area of the intestine and colon is far larger than that of intestinal metaplasia in stomach. Nevertheless, we expected that TFF3 from gastric intestinal metaplasia would increase serum levels due to a more leaky epithelium than that in the intestine and colon, predominantly due to damage and inflammation from the gastritis. Thus, while TFF3 appears to represent a good marker for gastric cancer, the origin of the high serum TFF3 levels remains unclear, and it cannot be a marker for gastric cancer recurrence after resection. There is a possibility that operative damage and/or stress due to changes in the route of the foods passage switched the origin of serum TFF3 level from gastric intestinal metaplasia to the intestine.^{43, 44} To evaluate this possibility, we measured pre- and post- operation serum from patients who received Roux-en Y bypass for bariatric surgery. However there was almost no change of serum TFF3 levels and thus these possibilities are less likely. Otherwise, serum TFF3 level may reflect some systemic cancer potential.⁴⁴ Serum TFF3 level in pancreatic cancer patients was significantly higher than in control individuals, and this supports the latter possibility. Therefore, elevated serum TFF3 levels may not be specific for gastric cancer. People found to have high serum TFF3 should receive further examination, not only by upper gastrointestinal endoscopy, but also other modalities. Further experiments will be needed to identify the precise origin of high serum TFF3 levels in post-gastrectomy patients.

In summary, this study has demonstrated that the serum level of TFF3 can be an effective marker of gastric cancer. Combination of TFF3 measurement with pepsinogen I/II testing may provide a screening modality with increased sensitivity. Prospective studies compared with the results of endoscopy are needed to confirm the predictive utility of TFF3 in gastric cancer and gastritis. In the future, addition of other markers may provide increased specificity and sensitivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Serum TFFs levels were measured by ELISA. Serum TFFs levels in gastric cancer patients are significantly higher than in control group without *H. pylori* infection. Serum TFFs levels in control group with *H. pylori* infection are also significantly higher than in control group without *H. pylori* infection. Serum TFF2 and TFF3 levels in gastric cancer patients are also higher than in control group with *H. pylori* infection.

Fig. 2.

ROC curves for serum TFFs level to distinguish people with *H. pylori* infection. The area under curve of TFF1, TFF2 and TFF3 are 0.952, 0.811 and 0.587. TFF1 correlates very well with *H. pylori* infectious status. (A) ROC curves for serum TFFs level to distinguish patients with gastric cancer. The area under curve of TFF1, TFF2, TFF3 and pepsinogen I/II ratio are 0.835, 0.735, 0.886 and 0.756 for all the analyzed patients. The PPV of TFF1, TFF2, TFF3 and pepsinogen I/II ratio are 0.648, 0.665, 0.709, and 0.608. The NPV of them are 0.918, 0.810, 0.895, and 0.874. (B) For *H. pylori* negative patients, all the markers shows good ROC curves. The PPV of TFF1, TFF2, TFF3 and pepsinogen I/II ratio are 0.919, 0.843,

0.784, and 0.814. The NPV of them are 0.934, 0.865, 0.935 and 0.886. (C) However, for *H. pylori* positive patients, only TFF3 shows good ROC curve. The PPV of TFF1, TFF2, TFF3 and pepsinogen I/II ratio are 0.618, 0.812, 0.776, and 0.606. The NPV of them are 0.68, 0.587, 0.805, and 0.509. (D)

	sensitivity	specificity	odds ratio	1	10	100
TFF1 cutoff; 1.0ng/ml	89.6	67.7	18.1(10.5-31.0)		●	
TFF2 cutoff; 4.0ng/ml	77.6	71.4	8.63(5.58-13.4)		●	
TFF3 cutoff; 3.6ng/ml	80.9	81.0	18.1(11.2-29.2)		●	
Pepsinogen test	44.8	87.4	5.61(3.53-8.91)		●	
Anti-HP IgG	62.3	65.1	3.06(2.08-4.54)		●	

Fig. 3. Sensitivity, specificity and odds ratio of TFFs, pepsinogen test and anti-*H. pylori* IgG for detecting gastric cancer. TFF1 and TFF3 show significantly higher odds ratio than pepsinogen test.

Fig. 4.
The ROC curves of PGI/II, TFF3, and TFF3+PGI/II are shown. TFF3+PGI/II can provide the best gastric cancer screening marker among them.

Fig. 5.

The serum TFF1 and TFF2 of the patients with undifferentiated cancer was significantly higher than the patients with differentiated cancer (TFF1: $p=0.018$, TFF2: $p=0.016$). The serum TFF3 levels of the patients with differentiated gastric cancer and with undifferentiated gastric cancer did not differ significantly ($p=0.312$).

Fig. 6. Serum TFF levels before and after gastrectomy. Serum TFF1 and TFF2 levels were significantly decreased after operation for gastric cancer. There was no significant change in serum TFF3 level between before and after operation.

Table 1

Gastric cancer patients evaluation with Pepsinogen I/II and TFF3

	TFF3		Total	Second Cohort	TFF3		Total
	-	+			-	+	
PG I/II	-	16 18.82%	69 81.18%	85	-	1 3.7%	26 96.3%
	+	8 8.25%	89 91.75%	97	+	2 6.25%	30 93.75%
Total	27	155	182		3	56	59