Mol Cell Toxicol (2010) 6:261-269
DOI 10.1007/s13273-010-0036-z

ORIGINAL PAPER

# Decreased expression of TFF2 and gastric carcinogenesis

**Kwang Hwa Jung[1,*], Pum Joon Kim[2,*], Jeong Kyu Kim[1], Ji Heon Noh[1], Hyun Jin Bae[1], Jung Woo Eun[1], Hong Jian Xie[1], Jin Mei Shan[3], Win Yin Ping[3], Won Sang Park[1], Jung Young Lee[1] & Suk Woo Nam[1]**

Received: 26 April 2010 / Accepted: 20 May 2010


**Abstract** The trefoil factor family (TFF) of peptides, which are protease-resistant and have a strong affinity for mucins, play an important role in gastrointestinal mucosal protection and restitution. Prior studies have indicated that dysregulation of TFF2 was associated with cell migration, resistance to apoptosis, and gastric cancer invasion; however, the underlying mechanism associated with these actions remains unclear. Thus, to investigate the relationship between TFF2 and carcinogenesis in gastric cancer, TFF2 expression was analyzed by Western blot analysis in nine selected gastric cancer tissues and immunohistochemical staining was performed in paraffin-embedded samples from 157 gastric cancers. A reduced TFF2 expression was observed by Western blot analysis in the gastric cancer tissues. However, there was no significant difference in the TFF2 expression according to clinical and pathological parameters of the gastric cancers. To investigate the biological role of TFF2 in the development and progression of gastric cancer, a TFF2 expression plasmid was constructed for *in vitro* experiments of function. Introduction of TFF2 cDNA into a gastric cancer cell line did not affect tumor cell growth, cell migration or invasion. In conclusion, down-regulation of TFF2 in gastric cancer cells and restoration of TFF2 did not affect the tumorigenic potential of gastric cancer cells *in vitro*. The loss of TFF2 expression might be an early event of the multi-step process of gastrocarcinogenesis and may play a limited role in the mucosal protection of the normal gastric physiology.

**Keywords** TFF2 expression, Gastric cancer, Cell growth, Motility, Invasion

[1]Department of Pathology, Microdissection Genomics Research Center, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea
[2]Department of Cardiology, College of Medicine, The Catholic University of Korea, Seoul, Korea
[3]Department of Pathology, First Hospital Jilin University, China
*These authors contributed equally to this work
Correspondence and requests for materials should be addressed to S. W. Nam (✉swnam@catholic.ac.kr)

Gastric cancer is a highly prevalent cancer in Asia and is one of the leading causes of cancer deaths worldwide. Although, over the last few years, many attempts have been made to better define the biological processes involved in the development of gastric cancer with the aim of improving the understanding of the carcinogenesis of gastric cancer as well as improving early diagnosis and treatment, the molecular mechanisms underlying gastric carcinogenesis remain the subject of intensive investigation[1,2].

The trefoil factors are a family of polypeptides secreted by mucous cells in the gastrointestinal tract and are important for gastrointestinal mucosal protection and repair[3]. The trefoil factor family (TFF) is a family of secreted proteins, characterized by the presence of at least 40- amino acid residues with three conserved intramolecular disulfide bonds in a compact triple loop structure[4-6]. This protein family consists of three members: TFF1 (formerly pS2 or pNR2) expressed by surface mucous cells of the stomach[7], TFF2 (formerly spasmolytic polypeptide, SP) found in mucous neck cells and Brunner's glands of the duodenum[8] and TFF3 (formerly intestinal trefoil factor, ITF) expressed by goblet cells of the intestine and colon[9]. TFF1 and TFF3 have a single trefoil motif, and there is evidence that their active forms are homodimers[10-12]. Whereas, TFF2 has two trefoil motifs with a glutathione-sensitive extra SS bond joining the C and N-terminals[13].

TFFs are rapidly up-regulated in the setting of gas-

trointestinal damage or injury, where they are involved in mucosal protection and epithelial repair[4,14-17]. Generally, TFFs are not found to be proliferative stimuli[12,18-20]. TFFs may influence the promotion of cell motility/migration and cell survival by their anti-apoptotic effects[11,18,21]. In general, TFF1 functions as a tumor suppressor gene associated with gastric cancer, whereas TFF2 and TFF3 have been thought to promote tumorigenesis by increasing cell invasion and metastasis[22,23]. Although, the regulatory mechanisms of TFFs are not fully understood, expression of TFFs is dysregulated during the progression of invasive cancer cells[24,25].

TFF2 is involved in epithelial restitution, cytoprotection and wound healing in the gastrointestinal tract by stimulating cell migration and inhibiting apoptosis[14,26,27]. However, a recent study reported that TFF2 is frequently up-regulated in preneoplastic lesions[28]. In addition, overexpression of TFF2 in gastric carcinoma has been reported to be associated with cancer invasion, metastasis, and a poor prognosis[22,29,30]. Based on these studies, TFF2 appears to play a role both in normal mucosal cytoprotection and in pathological tumor development in the gastric environment. However, the role of TFF2 in the setting of preneoplasia and neoplasia remains unclear.

Therefore, to investigate whether aberrant TFF2 expression is involved in the development and progression of gastric cancers, TFF2 expression was analyzed by Western blot analysis and immunohistochemical staining of gastric cancer tissues. In addition, to explore the biological role of TFF2 in gastric cancer, recombinant TFF2 was generated and *in vitro* tumorigenesis experiments performed.

**Table 1.** Relationship between the expression of TFF2 protein and the clinical/pathological parameters.

| Parameter | TFF2 expression | | *P*-value |
|---|---|---|---|
| | + | – | |
| Lauren † | | | 0.810 |
| Intestinal | 28 | 22 | |
| Diffuse | 40 | 39 | |
| Mixed | 14 | 14 | |
| Lymph node metastasis † | | | 0.106 |
| 0 | 0 | 4 | |
| 1-5 | 25 | 22 | |
| ≥6 | 57 | 49 | |
| Site † | | | 0.686 |
| Upper | 9 | 6 | |
| Middle | 30 | 25 | |
| Lower | 43 | 44 | |
| Size † | | | 0.779 |
| <6.5 cm | 51 | 45 | |
| ≥6.5 cm | 31 | 30 | |
| Total | 82 | 75 | |

† Chi-Square test, $P>0.05$

### Aberrant expression of TFF2 in gastric cancers

Previous studies have demonstrated reduced expression of TFF2 in gastric cancer by immunohistochemical staining[31]. Thus, to confirm the aberrant expression of TFF2 in gastric cancers, the expression of TFF2 in tissue microarrays (TMA) containing 157 gastric cancers was studied. The results of the immunohistochemical staining of TMA with TFF2 antibodies are summarized in Table 1. Representative images of the immunostaining are shown in Figure 1. Moderate-to-strong immunopositive results for TFF2 were clearly observed in the cell membrane and in the cytoplasm of the gastric mucosal epithelial cells and gastric cancer cells. Immunonegative or weakly expressed TFF2 was detected in 75 (47.8%) out of the 157 gastric cancers. Moderate-to-strong immunopositive results for TFF2 were detected in 82 (52.2%) out of 157 cases. Among these were: 28 (56.0%), 40 (50.6%) and 14 (50.0%) of the 50 intestinal-type, 79 diffuse-type and 28 mixed-type gastric cancer samples, respectively. Statistically, there was no significant relationship between TFF2 protein expression and the clinical/pathological parameters, including tumor location, tumor size, and lymph node metastasis (Chi-Square test, $P>0.05$; Table 1). We next examined the expression level of TFF2 in nine selected gastric cancer tissues and compared the results to the corresponding adjacent non-cancerous gastric epithelial mucosa; the samples were obtained from patients that had surgical resection of gastric cancer. As shown in Figure 2A, six out of nine samples had detectable TFF2 antibodies; among these cases, five had decreased expression of TFF2 compared to the corresponding non-cancerous tissues. The endogenous expression levels of TFF2 were next examined in the gastric cancer cell lines. As expected, most of the tested gastric cancer cell lines showed no expression of TFF2 or only barely detectable TFF2 antibodies; with the exception of SUN-688 by Western blot analysis. Although previous studies reported that basal expression of TFF2 was not detectable in human gastric cancer cell lines[32,33], the experimental conditions used for this study detected TFF2.

### In vitro experimental tumorigenesis of recombinant TFF2

To investigate the biological consequences of aberrant expression of TFF2 in gastric cancer, full-length human TFF2 cDNA was constructed as described in the materials and methods. To validate the endogenous expression of TFF2, pcDNA3.1-TFF2 was trans-

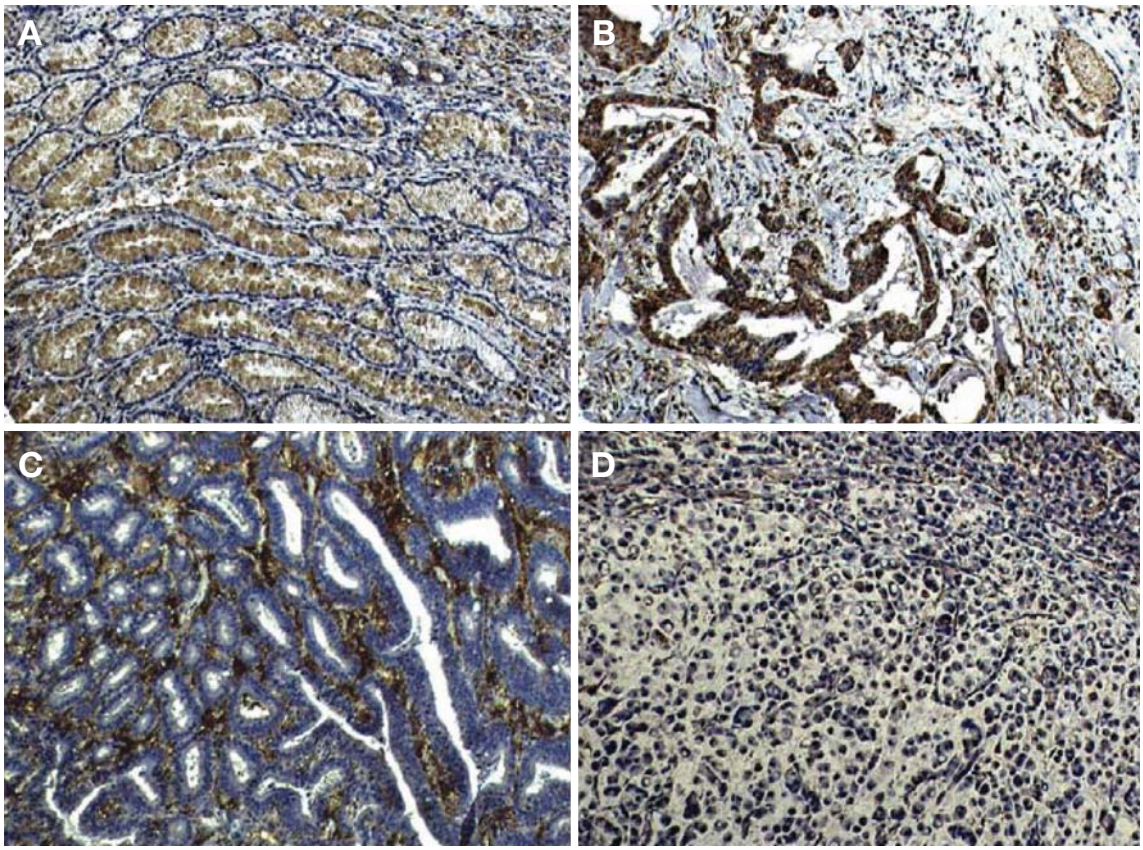


**Figure 1.** Representative photomicrographs of immunohistochemical analysis of gastric tissues demonstrating expression of TFF2. (A) An example of normal gastric mucosa showing positive staining for TFF2 expression. (B) An example of gastric cancer showing negative staining against TFF2 antibody. (C and D) Examples of intestinal and diffuse types of gastric cancer with negative TFF2 immunostaining. All representative photomicrographs show ×200 magnification.

fected to COS-1 cells. High expression of TFF2 was detected by RT-PCR and Western blot analysis (Figure 3A); the same result was confirmed in the SNU-484 gastric cancer cells (Figure 3B). Because TFF2 is a secretary protein[34,35], we then tried to detect TFF2 in culture medium and purified it from conditioned media. As shown in Figure 3C, after purification using lectin affinity chromatography with concanavalin A-agarose, recombinant TFF2 (rTFF2) was detected in the conditioned media of both the COS-1 and SNU-484 cells.

Previous reports on breast and colorectal cancer cells showed that TFF2 had weak proliferative effects[36], and promoted cell survival though the inhibition of apoptosis in the breast and colorectal cancer cells[37]. The over-expression of TFF2 in gastric cancer cell proliferation was investigated in this study. As shown in Figure 4A, when a TFF2 expression plasmid was introduced into the SNU-484 cells, it did not appear to affect cell growth; a similar result was observed for the AGS cells (Figure 4B). Previous studies reported that TFFs may influence the promotion of cell motility/migration. Therefore, the cell migratory effects of purified rTFF2 using the Boyden chamber assay were investigated. As shown in Figure 4C, over-expression of TFF2 in the AGS cells did not enhance the cell migratory response to fetal bovine serum (FBS) as a chemoattractant. In addition, when AGS cells were stimulated by rTFF2, tumor cell migration was not induced compared to the 5% FBS used as a positive control (Figure 4D). To confirm the motile activity of rTFF2, pcDNA3.1-TFF2 transfected cells were then placed in the bottom chamber as a chemoattractant as described in a previous report[41]. As shown in Figure 4E, although it appeared that pcDNA3.1-TFF2 transfected cells secreted some factors that stimulated cell migration, it did not increase the migratory response to the ASG cells compared to the mock-transfectant (AGS-M). These results suggest that TFF2 has a minimum effect on cell proliferation and promotion of cell motility/migration in gastric cancer cells under the experimental conditions used in this study.

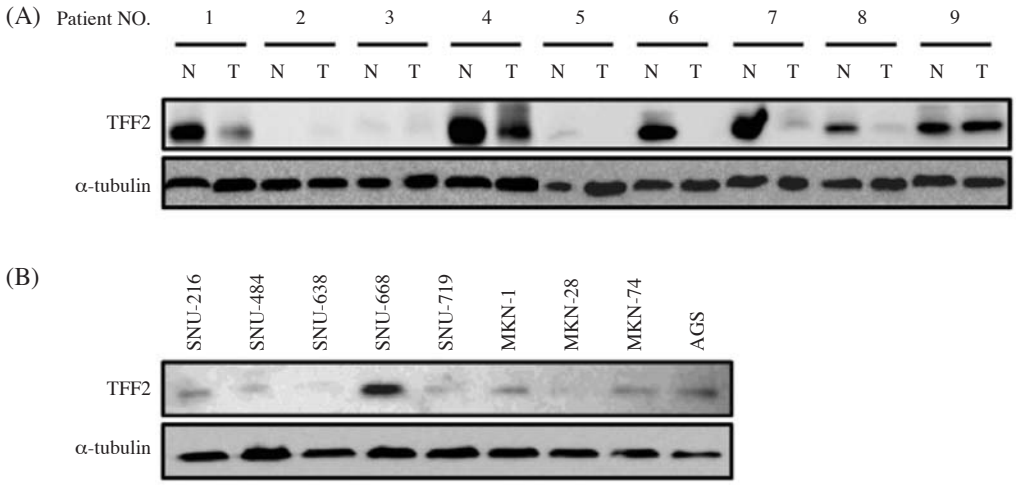


**Figure 2.** Expression of TFF2 in gastric cancer tissues and cell lines. (A) The protein expression of TFF2 was determined by Western blot analysis. (B) Endogenous expression of TFF2 in human gastric cancer cell lines was determined by Western blot analysis. The α-tubulin was used as a protein loading control.

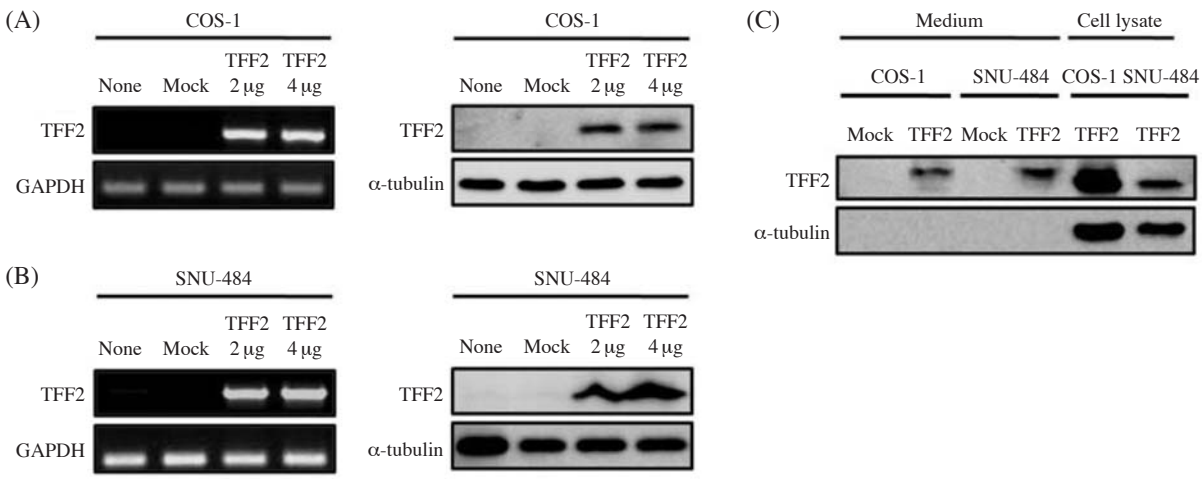


**Figure 3.** Expression of recombinant TFF2. (A) TFF2 mRNA and protein expression from COS-1 cells transfected by TFF2 expression vectors (pcDNA3.1-TFF2). The COS-1 cells were transfected with none (None), empty vector (Mock), 2 μg and 4 μg of pcDNA3.1-TFF2 expression vectors. GAPDH and α-tubulin were used as a loading control. (B) TFF2 mRNA and protein expression from SNU-484 cells transfected by TFF2 expression vectors. SNU-484 cells were transfected with none (None), empty vector (Mock), 2 μg and 4 μg of pcDNA3.1-TFF2 expression vectors. GAPDH and α-tubulin were used as loading controls. (C) Western blot analysis from conditioned media of pcDNA3.1-TFF2 transfected SNU-484 and COS-1 cells.

## Discussion

Gastric carcinoma occurs with a high incidence in Asia, including Korea, and is one of the leading causes of cancer death worldwide. However, the molecular mechanisms underlying the carcinogenesis associated with the development of gastric cancer remains unclear. TFFs are widely expressed in the mucosa of endoblasts and the gastrointestinal tract; TFF2 expression is primarily localized to the stomach[38]. In previously published experiments using either rats or mice, TFF2 was shown to be constitutively expressed in the gastrointestinal mucosa within specialized epithelial cells and was markedly upregulated in response to inflammation, injury, and restitution, as demonstrated by the immunohistochemisty of tissue sections[3,4,14-17]. Although several biological roles of TFFs have been elucidated, the mechanisms of action, of these small peptides, remain largely unknown. TFFs have a number of beneficial effects in mucosal damage or injury

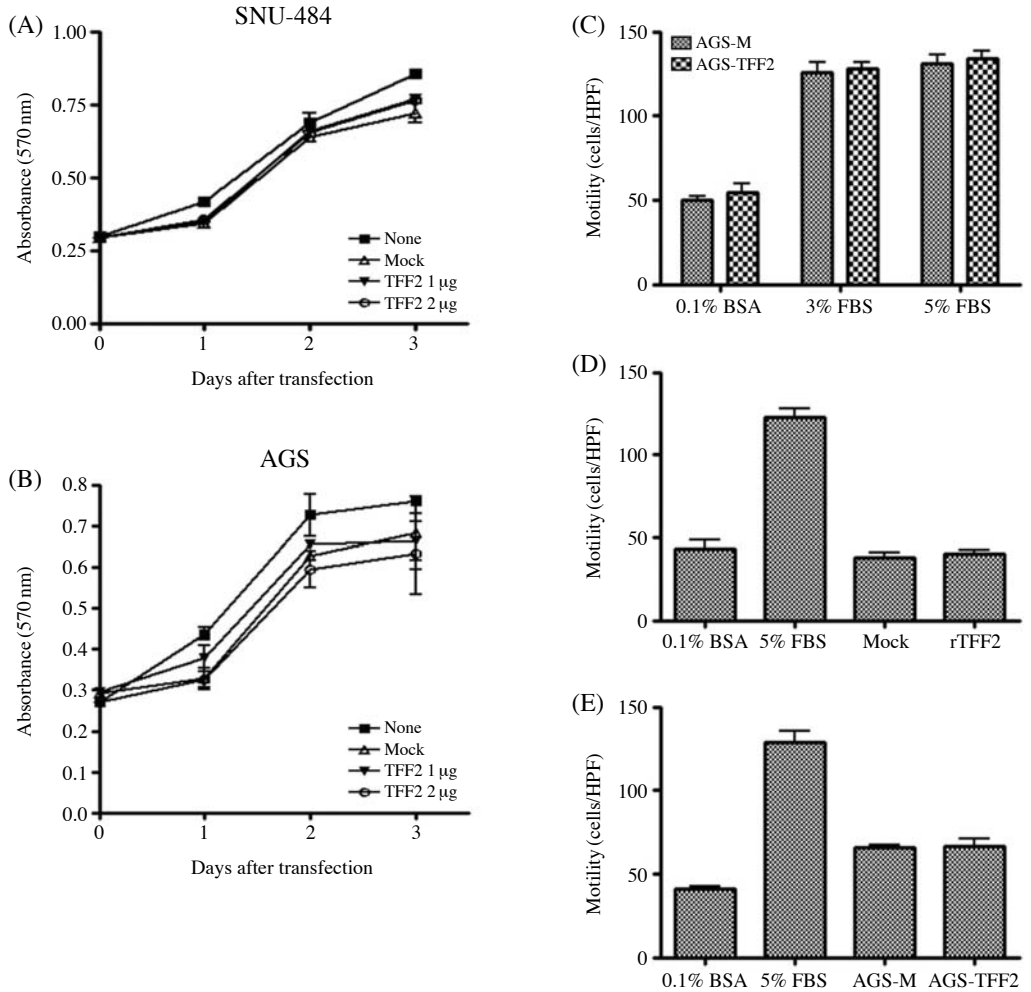


**Figure 4.** Effects of TFF2 over-expression on tumor cell growth and motility of human gastric cancer cell lines. (A & B) SNU-484 and AGS cells were transfected with none (None), empty vector (Mock), 1 μg and 2 μg of pcDNA3.1-TFF2 expression vectors. The relative growth rate was determined by the MTT assay at each indicated time (0, 1, 2, 3 days). The data are presented as the mean ± standard error for three experiments. (C) Cell motility of TFF2 expressing tumor cells. The motility assay of AGS cells with transfected empty plasmid vector (AGS-M) or TFF2 plasmid expression vector (AGS-TFF2) was assessed by Boyden chamber assay using FBS as chemoattractant. (D) Effect of rTFF2 on cell motility. Approximately 100-fold concentrated conditioned media from AGS cells, which were transfected with TFF2 plasmid expression vector (rTFF2) or empty plasmid vector (Mock). (E) Effect of cell migration of TFF2 expressing cells. Cells that were transfected with TFF2 vector (AGS-TFF2) or empty vector (AGS-M) as chemoattractants with 0.1% bovine serum albumin placed in the bottom chamber. AGS cells were then placed in the upper chamber, and incubated for 16 h at 37°C in a 5% $CO_2$ humidified incubator. Cell migration experiments were performed in triplicate under a light microscope by counting the cells in five randomly chosen, high power fields.

and contribute to epithelial restitution. In addition, the possible effects on proliferation, apoptosis, migration/motility and invasion during carcinogenesis have also been suggested. Therefore, TFFs may play a role in injured gastrointestinal mucosa as well as in carcinogenesis of gastric cancer.

In this study, the aberrant TFF2 expression in gastric cancer tissues was studied in addition to whether abnormal expression of TFF2 was associated with gastric carcinogenesis. The immunohistochemical experiments showed that down-regulated TFF2 expression was observed in 47.8% of 157 gastric cancers, and down-regulated TFF2 expression levels were also confirmed in human gastric cancer tissues (Figure 2). However, TFF2 expression was not significantly associated with clinical/pathological parameters, including tumor location, tumor size and lymph node metastasis in cases with gastric cancer (Table 1). A previous study has reported similar results for TFF2[31]. Therefore, it is likely that the same regulation of this gene

occurs at different locations and in different histological types. Other studies have reported that TFF2 influences cell proliferation and inhibits apoptosis in breast and colorectal cancer cell lines[37,39]. By contrast, the results of this study showed that tumor cell growth and migration were not influenced after restoration of TFF2 expression.

In conclusion, the expression of TFF2 in gastric cancer was observed. However, this aberrant regulation of TFF2 may not play a significant role in gastric cancer development and progression. The TFF2-positive rate in the gastric mucosa adjacent to the cancer areas was higher than that of the gastric cancer cells, and may represent an early phase of carcinogenesis. Further studies are required to understand not only the pathogenesis of gastric cancer, but also the mechanisms involved in TFF2 expression in gastric epithelial cells.

## Materials & Methods

### Tissue samples and tissue microarray

Nine frozen gastric cancer tissue samples and their corresponding normal background gastric tissue samples from nine patients were evaluated in this study. Frozen tissues were ground to a very fine powder in liquid nitrogen, and then were preserved for molecular testing. For the tissue microarray (TMA) studies, a total of 157 gastric cancer specimens (50 intestinal-type, 79 diffuse-type and 28 mixed-type) from formalin fixed, paraffin-embedded gastric cancer samples were obtained from the archives of the Department of Pathology at our institution. Two and one tissue cores, from the cancer and normal tissue, were taken and placed in a new recipient paraffin block using a commercially available microarray instrument (Beecher Instruments, Micro-Array Technologies, Silver Spring, MD), according to established methods[40]. Approval was obtained from the institutional review board of the Catholic University of Korea, College of Medicine and the Jilin University of China, College of Medicine. Informed consent was provided by every patient according to the Declaration of Helsinki. There was no evidence of familial cancer in any of the patients.

### Immunohistochemistry analysis of TFF2

To investigate the level of TFF2 protein in the gastric cancers, immunohistochemical staining was performed with monoclonal antibodies against TFF2 (1 : 100, Biovision, Mountain View, CA) on the tissue microarray gastric cancer samples. Prior to the immunostaining, TMA slides were deparaffinized and hydrated through a graded ethanol series to deionized the water. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide-methanol buffer. Antigens were retrieved by boiling the slides in a steamer with sodium citrate buffer (pH 6.0) for 20 min. After incubation with monoclonal antibodies against TFF2, overnight at 4˚C, detection was carried out using biotinylated goat anti-rabbit antibodies (1 : 200; Sigma, ST. Louis, MO), followed by incubation with the peroxidase-linked avidin-biotin complex. Diaminobenzidine (DAB) was used as the chromogen, and the slides were then lightly counterstained with Mayer's hematoxylin. As a negative control, the slides were treated with non-immune serum instead of the primary antibody.

### Evaluation of immunohistochemical staining

Scoring of the TMA was performed independently by two pathologists. In the event of disagreement, the two reached a consensus by jointly re-evaluating the TMA using a multi-head microscope. When the number of immunostained cells were less than 10%, we considered the case negative for staining.

### Cell culture

The human gastric cancer cell line AGS and monkey kidney fibroblast cell line COS-1 were purchased from the American Type Culture Collection (ATCC; Manassas, VA). The human gastric cancer cell lines: SNU-216, SNU-484, SNU-638, SNU-668, SNU-719, MKN-1, MKN-28 and MKN-74 were purchased from the Korean Cell Line Bank (KCLB, Korea). The cells were maintained in RPMI 1640 or DMEM medium supplemented with 10% fetal bovine serum (Sigma, St Louis, MO) and 1 mg/mL of penicillin/streptomycin (Invitrogen, Carlsbad, CA).

### Plasmid construction and DNA transfection

This plasmid was used as a template for PCR to construct the following expression vector. A cDNA of the full-length human TFF2 form was prepared by digestion with HindIII- EcoRI restriction enzymes and ligated into an indentically cleaved pcDNA 3.1 (Invitrogen, Carlsbad, CA) vector. Lipofectamine 2000 was used for all transfections, following the manufacturer's recommended protocol (Invitrogen, Carlsbad, CA). Concurrent transfections with an appropriate empty vector served as the controls.

### Purification of TFF2

SNU-484, AGS and COS-1 cells were transfected with pcDNA 3.1/TFF2 expression vectors, and then the

cells were maintained for 48 h in serum-free RPMI 1640 or DMEM supplemented with 0.1% bovine serum albumin (conditioned media). The empty vector transfected SNU-484, AGS and COS-1 cells served as negative controls. The conditioned medium from each cell line was collected, washed with storage buffer (50 mM Tris, 20% ethylene glycol, pH 7.5), and concentrated 200-fold in an Amicon Ultra-15 centrifuge filter unit (Millipore, Billerica, MA). For selected experiments, the media was then partially purified by lectin affinity chromatography[41] with concanavalin A-agarose (Vector Laboratories, Burlingame, CA). Approximately 0.5 mL of each concentrated conditioned medium was washed with binding buffer (50 mM Tris, 0.1 M NaCl, 0.01 M $CaCl_2$, 20% ethylene glycol, pH 7.2), added to 6 mL of additional binding buffer and then to 2 mL of packed concanavalin- A agarose beads. The sample was allowed to bind at 4˚C on a rocking platform for 2-3 h. After incubation, the beads were washed with an additional 15 mL of binding buffer, and then incubated with elution buffer (50 mM Tris, 0.1 M NaCl, 0.01 M $CaCl_2$, 20% ethylene glycol, 500 mM a-D-mannopyranoside, pH 7.2) on the rocking platform overnight at room temperature. Each eluted sample was washed with storage buffer, and concentrated to a volume of 0.25 mL (a 200-fold concentration from the original conditioned medium). Aliquots were tested by Western blot analysis or cell motility assays.

**Reverse transcription-PCR**

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) and quality control was performed using RNA 6000 Nano chips on an Agilent 2001 Bioanalyzer (Agilent Technologies, Germany). Then, 1 μg RNA was used for the cDNA synthesis reaction with the RNA PCR Core Kit (Roche, Branchburg, NJ). cDNA was used per reverse transcription-PCR (RT-PCR) reaction. RT-PCR program was 95˚C 30 s, 63˚C 30 s, 72˚C 30 s for 30 cycles. RT-PCR primer sequences are as follows: TFF2 forward 5′-CGCGGATT CATGGGACGGCGAGACGCCCAG, reverse 5′-CC GGAATTCTTAGTAATGGCAGTCTTCCACAG; GAPDH forward 5′-ACCAGGTGGTCTCCTCTG AC, reverse 5′-TGCTGTAGCCAAATTCGTTG.

**Western blot analysis**

Whole-cell extracts were prepared with radio-immunoprecipitation assay (RIPA) lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mmol/L Phenylmethane-sulfonylfluoride containing protease inhibitors, Roche, Mannheim, Germany). Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL) and absorbance of the protein samples were read at 570 nm with the VICTOR3™ Multilabel Plate Reader (PerkinElmer Inc, Boston, MA). RIPA lysates containing 10 μg or 15 μg of protein were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond™-P, Buckinghamshire, UK) and kept in 5% skim milk (BD Biosciences, Sparks, MD) in TBS solution containing 0.05% Tween-20 (Usb Corporation, Cleveland, OH) overnight to protect against non-specific binding. The membranes were incubated with each of the primary antibodies and HRP-conjugated secondary antibodies (Pierce, Rockford, IL). The ECL plus Western blotting detection system (GE healthcare, Buckinghamshire, UK) was used to detect immobilized specific antigens conjugated to Horseradish Peroxidase (HRP) labeled antibodies. TFF2 and α-tubulin antibodies were purchased from Biovision and Santa Cruz (Delaware, CA). The membrane was exposed to LAS 3000 (Fuji Photo Film Co. LTD, Japan).

**Cell proliferation assay**

For the cell proliferation assay, the SNU-484 and AGS cells were seeded in a 24-well culture plate at a density of $4 \times 10^4$ cells per well with RPMI 1640 medium containing 10% FBS and maintained for 16-18 h. Four hours after the pcDNA 3.1/TFF2 transfection, RPMI 1640 medium with 10% FBS was replaced in each of the 24-wells in the culture plate and the cells were maintained at 37˚C in a 5% $CO_2$ humidified incubator. To evaluate cell proliferation, the cells were incubated with 5 mg/mL of the yellow {3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide; Sigma, St. Louis, MO} solution for the indicated time (0, 1, 2, 3 days). Two hours after incubation, dark blue formazan products were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) and the absorbance of cells were determined in a VICTOR3™ Multilabel plate reader with a test wavelength of 570 nm (PerkinElmer Inc, Boston, MA).

**Cell motility assay**

Motility assays of AGS cells were performed in 48-well modified Boyden chambers (Neuroprobe, Cabin John, MD) as described previously[42]. The cells were harvested using a trypsin/EDTA solution (Invitrogen, Carlsbad, CA) and resuspended in RPMI 1640. The bottom wells were filled with varying concentrations of FBS, purified recombinant TFF2 and TFF2 transfected cells as chemoattractants. The upper and lower chambers were separated by gelatin-coated polyvinyl-pyrrolidine-free polycarbonate filters with 8 μm

pores (Neuroprobe, Cabin John, MD). The cells ($5 \times 10^4$ or $1 \times 10^5$) were placed into each upper well, and then the chamber was incubated for 4 h or overnight at 37°C in a 5% $CO_2$ humidified incubator. The membranes were fixed and stained with Diff-Quik (Sysmex, Japan) and mounted on a glass slide. Cell migration experiments were performed in triplicate and quantified under the light microscope by counting the cells in five randomly chosen, high-powered fields.

**Acknowledgements** This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0072504) and by a grant of the Korea Healthcare technology R & D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A084930).

## References

1. Bulitta, C. J. *et al*. Autoinduction of the trefoil factor 2 (TFF2) promoter requires an upstream cis-acting element. *Biochem Biophys Res Commun* **293**:366-374 (2002).
2. Matsumoto, Y. *et al*. Helicobacter pylori infection triggers aberrant expression of activation-induced cytidine deaminase in gastric epithelium. *Nat Med* **13**:470-476 (2007).
3. Maeda, K. *et al*. Vascular endothelial growth factor expression in preoperative biopsy specimens correlates with disease recurrence in patients with early gastric carcinoma. *Cancer* **86**:566-571 (1999).
4. Alison, M. R. *et al*. Experimental ulceration leads to sequential expression of spasmolytic polypeptide, intestinal trefoil factor, epidermal growth factor and transforming growth factor alpha mRNAs in rat stomach. *J Pathol* **175**:405-414 (1995).
5. Taupin, D. & Podolsky, D. K. Trefoil factors: initiators of mucosal healing. *Nat Rev Mol Cell Biol* **4**:721-732 (2003).
6. Thim, L. Trefoil peptides: from structure to function. *Cell Mol Life Sci* **53**:888-903 (1997).
7. Poulsom, R. Trefoil peptides. *Baillieres Clin Gastroenterol* **10**:113-134 (1996).
8. Rio, M. C. *et al*. Breast cancer-associated pS2 protein: synthesis and secretion by normal stomach mucosa. *Science* **241**:705-708 (1988).
9. Tomasetto, C. *et al*. hSP, the domain-duplicated homolog of pS2 protein, is co-expressed with pS2 in stomach but not in breast carcinoma. *EMBO J* **9**:407-414 (1990).
10. Suemori, S., Lynch-Devaney, K. & Podolsky, D. K. Identification and characterization of rat intestinal trefoil factor: tissue- and cell-specific member of the trefoil protein family. *Proc Natl Acad Sci USA* **88**: 11017-11021 (1991).
11. Chadwick, M. P., Westley, B. R. & May, F. E. Homodimerization and hetero-oligomerization of the single-domain trefoil protein pNR-2/pS2 through cysteine 58. *Biochem J* **327(Pt 1)**:117-123 (1997).
12. Kinoshita, K., Taupin, D. R., Itoh, H. & Podolsky, D. K. Distinct pathways of cell migration and antiapoptotic response to epithelial injury: structure-function analysis of human intestinal trefoil factor. *Mol Cell Biol* **20**:4680-4690 (2000).
13. Marchbank, T. *et al*. Dimerization of human pS2 (TFF1) plays a key role in its protective/healing effects. *J Pathol* **185**:153-158 (1998).
14. Otto, W. R. *et al*. Effects of pancreatic spasmolytic Polypeptide (PSP) on epithelial cell function. *Eur J Biochem* **235**:64-72 (1996).
15. Hanby, A. M. *et al*. Spasmolytic polypeptide is a major antral peptide: distribution of the trefoil peptides human spasmolytic polypeptide and pS2 in the stomach. *Gastroenterology* **105**:1110-1116 (1993).
16. Podolsky, D. K. *et al*. Identification of human intestinal trefoil factor. Goblet cell-specific expression of a peptide targeted for apical secretion. *J Biol Chem* **268**: 6694-702 (1993).
17. Oertel, M. *et al*. Trefoil factor family-peptides promote migration of human bronchial epithelial cells: synergistic effect with epidermal growth factor. *Am J Respir Cell Mol Biol* **25**:418-424 (2001).
18. Playford, R. J. *et al*. Human spasmolytic polypeptide is a cytoprotective agent that stimulates cell migration. *Gastroenterology* **108**:108-116 (1995).
19. Bossenmeyer-Pourie, C. *et al*. The trefoil factor 1 participates in gastrointestinal cell differentiation by delaying G1-S phase transition and reducing apoptosis. *J Cell Biol* **157**:761-770 (2002).
20. Calnan, D. P. *et al*. The trefoil peptide TFF1 inhibits the growth of the human gastric adenocarcinoma cell line AGS. *J Pathol* **188**:312-317 (1999).
21. Marchbank, T. *et al*. Effect of ectopic expression of rat trefoil factor family 3 (intestinal trefoil factor) in the jejunum of transgenic mice. *J Biol Chem* **276**: 24088-24096 (2001).
22. Taupin, D. R., Kinoshita, K. & Podolsky, D. K. Intestinal trefoil factor confers colonic epithelial resistance to apoptosis. *Proc Natl Acad Sci USA* **97**:799-804 (2000).
23. Lefebvre, O. *et al*. Gastric mucosa abnormalities and tumorigenesis in mice lacking the pS2 trefoil protein. *Science* **274**:259-262 (1996).
24. Tebbutt, N. C. *et al*. Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice. *Nat Med* **8**:1089-1097 (2002).
25. Hippo, Y. *et al*. Differential gene expression profiles of scirrhous gastric cancer cells with high metastatic potential to peritoneum or lymph nodes. *Cancer Res* **61**:889-895 (2001).
26. Missiaglia, E. *et al*. Analysis of gene expression in cancer cell lines identifies candidate markers for pan-

creatic tumorigenesis and metastasis. *Int J Cancer* **112**:100-112 (2004).
27. Cook, G. A., Familari, M., Thim, L. & Giraud, A. S. The trefoil peptides TFF2 and TFF3 are expressed in rat lymphoid tissues and participate in the immune response. *FEBS Lett* **456**:155-159 (1999).
28. Farrell, J. J. *et al*. TFF2/SP-deficient mice show decreased gastric proliferation, increased acid secretion, and increased susceptibility to NSAID injury. *J Clin Invest* **109**:193-204 (2002).
29. Houghton, J. *et al*. Gastric cancer originating from bone marrow-derived cells. *Science* **306**:1568-1571 (2004).
30. Dhar, D. K. *et al*. Expression of cytoplasmic TFF2 is a marker of tumor metastasis and negative prognostic factor in gastric cancer. *Lab Invest* **83**:1343-1352 (2003).
31. Emami, S. *et al*. Trefoil factor family (TFF) peptides and cancer progression. *Peptides* **25**:885-898 (2004).
32. Shi, S. Q., Cai, J. T. & Yang, J. M. Expression of trefoil factors 1 and 2 in precancerous condition and gastric cancer. *World J Gastroenterol* **12**:3119-3122 (2006).
33. Chi, A. L., Lim, S. & Wang, T. C. Characterization of a CCAAT-enhancer element of trefoil factor family 2 (TFF2) promoter in MCF-7 cells. *Peptides* **25**:839-847 (2004).
34. Vestergaard, E. M. *et al*. Immunoassays of human trefoil factors 1 and 2: measured on serum from patients with inflammatory bowel disease. *Scand J Clin Lab Invest* **64**:146-156 (2004).
35. Dubeykovskaya, Z., Dubeykovskiy, A., Solal-Cohen, J. & Wang, T. C. Secreted trefoil factor 2 activates the CXCR4 receptor in epithelial and lymphocytic cancer cell lines. *J Biol Chem* **284**:3650-3662 (2009).
36. Hoosein, N. M., Thim, L., Jorgensen, K. H. & Brattain, M. G. Growth stimulatory effect of pancreatic spasmolytic polypeptide on cultured colon and breast tumor cells. *FEBS Lett* **247**:303-306 (1989).
37. Siu, L. S. *et al*. TFF2 (trefoil family factor2) inhibits apoptosis in breast and colorectal cancer cell lines. *Peptides* **25**:855-863 (2004).
38. Otto, W. R. & Patel, K. Trefoil factor family (TFF)-domain peptides in the mouse: embryonic gastrointestinal expression and wounding response. *Anat Embryol (Berl)* **199**:499-508 (1999).
39. Lalani, E. N. *et al*. Trefoil factor-2, human spasmolytic polypeptide, promotes branching morphogenesis in MCF-7 cells. *Lab Invest* **79**:537-546 (1999).
40. Kononen, J. *et al*. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* **4**:844-847 (1998).
41. Nam, S. W. *et al*. A sensitive screening assay for secreted motility-stimulating factors. *Cell Motil Cytoskeleton* **46**:279-842 (2000).
42. Song, J. *et al*. Autotaxin (lysoPLD/NPP2) protects fibroblasts from apoptosis through its enzymatic product, lysophosphatidic acid, utilizing albumin-bound substrate. *Biochem Biophys Res Commun* **337**:967-975 (2005).