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# Upregulation of Vanilloid Receptor-1 in Functional Dyspepsia With or Without *Helicobacter pylori* Infection

Yoon Jin Choi, MD, Nayoung Kim, MD, Jinjoo Kim, MD, Dong Ho Lee, MD,  
Ji Hyun Park, MSc, and Hyun Chae Jung, MD

**Abstract:** The etiological basis of functional dyspepsia (FD) is incompletely understood. The aim of this study was to evaluate the involvement of nociceptor-related genes and *Helicobacter pylori* (HP) in the pathogenesis of FD. The expression of nociceptor-related genes was measured in gastric cell lines that were co-cultured with HP. FD patients (n = 117) and controls (n = 55) were enrolled from a tertiary hospital gastroenterology clinic. Expression of the genes nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), and transient receptor potential cation channel subfamily V member 1 (TRPV1) in the gastric mucosa were detected by reverse transcription polymerase chain reaction (RT-PCR), and immunohistochemical staining of TRPV1 was analyzed. These measurements were repeated after 1 year. TRPV1, GDNF, and NGF expression was elevated in gastric cell lines co-cultured with HP. TRPV1 immunostaining was stronger in HP-positive than HP-negative subjects. The FD group showed higher expression levels of TRPV1, GDNF, and NGF and increased TRPV1 immunostaining compared with those of the control group (all  $P < 0.05$ ). Among 61 subjects who were followed up at 1 year, controls with successful HP eradication and patients whose symptoms had improved both showed significant reductions in the expression of TRPV1 and NGF (all  $P < 0.05$ ) compared with controls without HP eradication and patients whose symptoms had not improved, respectively. The expression of NGF, GDNF, and TRPV1 may be associated with the pathogenesis of FD. Since HP infection may induce the increased expression of these genes, anti-HP therapy could be beneficial for HP-positive patients with FD.

(*Medicine* 95(19):e3410)

**Abbreviations:** EPS = epigastric pain syndrome, FD = functional dyspepsia, GDNF = glial cell-line derived neurotrophic factor, GI = gastrointestinal, GPA = global patient assessment, HP = *Helicobacter pylori*, IBS = irritable bowel syndrome, NERD = non-erosive reflux disease, NGF = nerve growth factor, PDS = postprandial distress syndrome, PPI = proton-pump inhibitor, qPCR = quantitative polymerase chain reaction, TRP = transient receptor potential, TRPV1 = transient receptor potential cation channel subfamily V member 1.

## INTRODUCTION

Functional dyspepsia (FD) is one of the most common gastrointestinal (GI) disorders and has a prevalence of up to 30%<sup>1</sup>; however, its pathogenesis remains unclear. The Rome III classification categorized FD into 2 subtypes: postprandial distress syndrome (PDS) and epigastric pain syndrome (EPS).<sup>2–5</sup>

Transient receptor potential (TRP) channel subunits are expressed in neurons and cells in the alimentary tract.<sup>6</sup> They play important roles in taste, mechanosensation, pain, GI motility, and secretion.<sup>6</sup> Among the 20 TRP channels characterized so far, TRP cation channel subfamily V member 1 (TRPV1) is believed to be an important integrator of the transmission and modulation of pain (nociception).<sup>7</sup> TRPV1 is a chemoreceptor that is stimulated by various spice-derived phytochemicals, including capsaicin (red pepper), piperine (black pepper), gingerol (ginger), and allyl isothiocyanate (mustard/wasabi).<sup>8</sup> It is also activated by low pH (<6), heat, and inflammatory mediators such as serotonin, bradykinin, and Adenosine triphosphate.<sup>9</sup> TRPV1 is predominantly expressed in neurons, but has also been identified in the mucosa of several GI tract regions, such as the esophagus,<sup>10,11</sup> small intestine,<sup>12</sup> and stomach.<sup>13–16</sup>

Since patients with dyspepsia frequently manifest a low pain threshold to chemical challenge,<sup>17,18</sup> any part of the GI tract that detects and transmits pain might be augmented in FD. Hence, the upregulation of TRPV1 has been proposed to be associated with visceral hypersensitivity in FD.

Evidence has accumulated indicating that activation of TRPV1 causes pain and hyperalgesia in the human alimentary tract. Oral ingestion or administration of capsaicin into the human proximal jejunum has been reported to evoke epigastric pain, heartburn, and a sensation of warmth.<sup>19,20</sup> Infusion of a capsaicin-containing chili sauce into the gastric fundus was found to enhance the mechano-sensitivity of the proximal stomach.<sup>21</sup> In addition, esophageal instillation of capsaicin induced symptoms of epigastric burning in a dose-dependent fashion.<sup>22</sup> Concordantly with these observations, upregulation of TRPV1 has been reported in patients with rectal hypersensitivity and non-erosive reflux disease (NERD).<sup>23,24</sup>

Gastric sensations are transmitted to the central nervous system by unmyelinated C fibers or thin myelinated Aδ fibers.

Editor: Zarko Babic.

Received: February 4, 2016; revised: March 17, 2016; accepted: March 18, 2016.

From the Department of Internal Medicine and Seoul National University Bundang Hospital, Seongnam, Gyeonggi-do (YJC, NK, DHL); and Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul (NK, JK, DHL, JHP, HCJ), South Korea.

Correspondence: Nayoung Kim, Department of Internal Medicine, Seoul National University Bundang Hospital, 82, Gumi-ro 173 beon-gil, Bundang-gu, Seongnam, Gyeonggi-do, 463–707, South Korea (e-mail: nayoungkim49@empas.com).

This work was supported by a National Research Foundation of Korea (NRF) grant for the Global Core Research Center (GCRC) funded by the Korean government (MSIP) (No. 2011–0030001).

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent was obtained from all individual participants included in the study.

The authors declare that they have no conflict of interest.

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ISSN: 0025-7974

DOI: 10.1097/MD.00000000000003410

These polymodal C fibers are regulated by several neurotrophic factors, such as nerve growth factor (NGF) or glial cell line-derived neurotrophic factor (GDNF).<sup>25</sup> Neurotrophic factors are important for supporting the survival of neurons in both the central and peripheral nervous system and are also involved in pain signaling. In particular, NGF is upregulated during inflammation,<sup>26</sup> and plays a proalgesic role.<sup>27</sup> GDNF belongs to the transforming growth factor- $\beta$  superfamily and its overexpression has also been reported in irritable bowel syndrome (IBS) patients.<sup>28</sup>

Studies evaluating the potential relationship between TRPV1 expression in the stomach and developing FD have been very limited to date. Furthermore, there have been very few reports evaluating the relationship between TRPV1 and HP infection, which is a major cause of gastric mucosal inflammation. Against this background, we hypothesized that the expression of TRPV1 is elevated in the gastric mucosa of FD patients together with that of GDNF and/or NGF, and that such changes are associated with certain dyspeptic symptoms. In addition to testing this hypothesis, we also aimed to evaluate the effects of HP infection and eradication on the expression of nociceptor-related genes.

## METHODS

### Study Population

From February 2011 to June 2013, subjects with dyspeptic symptoms and healthy controls recruited from a cancer-screening program were enrolled at the Seoul National University Bundang Hospital. All participants underwent upper GI endoscopy. Under the supervision of well-trained interviewers and after giving informed written consent, all participants completed a questionnaire that was modified from Talley's bowel disease questionnaire and validated in Korea.<sup>29,30</sup> The questionnaire met the Rome III criteria. Severities of dyspeptic symptoms were scored using a 5-point Likert scale as follows: 0: none, 1: mild, 2: moderate, 3: severe, and 4: very severe. Patients were divided into 3 FD subtypes based on their symptoms: PDS, EPS, and mixed.<sup>31</sup>

Patients with peptic ulcer, reflux esophagitis, or typical IBS; concomitant systemic diseases such as diabetes mellitus or any malignancy; major depression, psychosis, or eating disorders; and daily users of NSAID or anticoagulants were excluded. Symptomatic subjects whose symptoms did not fulfill the Rome III criteria were also excluded.

A total of 55 control subjects and 117 patients with dyspeptic symptoms (PDS:  $n=46$ , EPS:  $n=33$ , mixed:  $n=38$ ) were analyzed. To evaluate whether changes in the expression of nociceptor-related genes correlate with variations in dyspeptic symptoms, we investigated the mRNA expression of *TRPV1*, *GDNF*, and *NGF* in gastric biopsy specimens at baseline and 1 year after the patients were enrolled. In addition, the expression levels of these nociceptor-related genes before and after HP eradication were compared. The study protocol was approved by the ethics committee at Seoul National University Bundang Hospital (B-1101-119-010).

### *Helicobacter pylori* Strains and Cell Culture

*Helicobacter pylori* (HP) ATCC 43504 (O4 strain; *cagA*<sup>+</sup>, *vacA*<sup>+</sup>) was purchased from American Type Culture Collection (ATCC; Manassas, VA) and G27 strain (*cagA*<sup>+</sup>, *vacA*<sup>+</sup> [s1, m1]) was obtained from Professor S. Falkow (Stanford University, Stanford, CA). Bacteria were cultured under microaerophilic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub>) at 37°C. The effect

of HP infection on the expression of *TRPV1*, *GDNF*, and *NGF* in human gastric cell lines was investigated. Human gastric adenocarcinoma (AGS) cells (ATCC CRL 1739) were purchased from ATCC and grown in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin solution at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Cells were seeded at a density of  $2 \times 10^5$  cells/well in 6-well plates and  $2 \times 10^6$  cells/well in 10 cm dishes. After the cells reached 70% to 80% confluence, *H pylori* were re-suspended in RPMI 1640 and co-cultured with the cells at a multiplicity of infection of 200 after overnight serum starvation. Het-1A (ATCC CRL-2692),<sup>11</sup> MKN45 (KCLB 80103), and Colo205 (KCLB 10222) cells were purchased from ATCC and Korean Cell Line Bank (KCLB; Seoul, South Korea).

### Gastric Mucosa Specimens and *H pylori* Evaluation

During endoscopy, 2 biopsy specimens were taken for histology and determining of HP infection status.<sup>32</sup> One biopsy was taken from the gastric antrum and the other from the gastric body. Two additional biopsies were obtained from the greater curvature of the high body and were used for measuring the expression of *TRPV1*, *GDNF*, and *NGF* and preparing paraffin blocks.

The baseline HP infection status was determined based on modified Giemsa staining, culture, and rapid urease test (CLO test; Delta West, Bentley, Australia). If one of these invasive HP tests was positive, the patient was diagnosed with HP infection. As for the histologic features, the degree of inflammatory cell infiltration, atrophy, and intestinal metaplasia was recorded using the updated Sydney scoring system (0: none, 1: slight, 2: moderate, and 3: marked).<sup>33</sup>

### Measurement of TRPV1, GDNF, and NGF mRNA Expression in Cells and Tissues

After 1, 6, 12, and 24 hours of cell co-culture, the medium was removed and cells were washed with phosphate-buffered saline. Total RNA was isolated from cells and tissue biopsy specimens using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time quantitative polymerase chain reaction (qPCR) for mRNA expression was performed using SYBR Green probes and an ABI 7500 thermal cycler (Applied Biosystems). The following primers were used, based on a previous study. *TRPV1* forward, 5'-GAGTTTCAGGCAGACACTGGAA-3'; *TRPV1* reverse, 5'-CTATCTCGAGCACTTGCCTCT-3'; *GDNF* forward, 5'-CTTGGGTCTGGGCTATGAAAC-3'; *GDNF* reverse, 5'-CAAAGGC-GATGGGTCTGC-3'; *NGF* forward, 5'-AGCAAGCGGTCAT-CATCC-3'; and *NGF* reverse, 5'-GTGGCGGTGGTCTTATCC-3'. *GAPDH* was used as an endogenous reference. The amplification protocol consisted of an initial denaturation step at 95°C for 10 seconds, followed by 40 cycles of denaturation for 5 seconds at 95°C and annealing/extension for 33 seconds at 60°C. Relative expression levels were normalized by dividing the target gene Ct values by the endogenous *GAPDH* Ct values.

### Western Blot Analysis

After 24 hours of co-culture, cells were washed twice with phosphate-buffered saline and then lysed with radioimmuno-precipitation assay buffer (Cell Signaling Technology, Beverly, MA), and bicinchoninic acid protein assay was performed to

determine the protein concentration of the lysates (Pierce, Rockford, IL). Cell extracts (30 µg protein) were subjected to 10% (v/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the separated proteins were transferred to polyvinylidene difluoride membranes. After blocking the non-specific binding sites with non-fat dry milk, the membranes were incubated with anti-TRPV1 antibody (1:2000; H00007442-M01; Abnova, Taiwan) at 4 °C overnight, then blots were incubated with secondary antibody (goat-anti mouse antibody, 1:4000; Santa Cruz Biotechnology, Dallas, TX). Detection was achieved using enhanced chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK).

### Immunohistochemistry

Immunostaining of TRPV1 was performed using core tissue biopsies (2 mm in diameter) obtained from paraffin-embedded gastric mucosa. Test procedures included a human control slide for immunohistochemistry analysis (SuperBio-Chips Laboratories, Seoul, Korea). A primary antibody against TRPV1 (1:100 dilution; ab63083; Abcam, Cambridge, UK) was used. For each tissue section, the intensity of immunohistochemical staining was scored as 0, no staining; 1+, faint/barely perceptible partial staining; 2+, weak to moderate staining; or 3+, strong staining. A composite measure of staining area and intensity was then calculated by multiplying the intensity by the positively stained area (%) in the epithelial glands. The resulting score ranged from 0 to 300. Each sample was scored by a blinded reviewer.

### Medication for Dyspepsia

HP eradication was recommended for HP-positive patients. Some HP-positive control subjects with a family history of gastric cancer also underwent HP eradication. As the first-line anti-HP therapy, proton-pump inhibitor (PPI)-based triple therapy for 7 days was prescribed. At least 4 weeks after the completion of the therapy,<sup>13</sup> C-urea breath test (UBI-Tkit; Otsuka Pharmaceutical, Tokushima, Japan) was performed. If the first line therapy failed, bismuth-based quadruple therapy was given for 14 days. HP-negative FD patients or HP-positive patients who refused the anti-HP therapy received symptomatic management with PPI, H<sub>2</sub>-antagonists, or prokinetics.

### Evaluation of Symptom Response and Follow-Up

Every 2 to 3 months after enrollment, the severity of the dyspeptic symptoms of participants was assessed using a global patient assessment (GPA) score.<sup>35</sup> The GPA score was used to assess whether symptoms remained the same, improved, or deteriorated over time compared with the measurements made at baseline. Patients were asked: “How were your overall dyspeptic symptoms during the past 2–3 months, compared with your symptoms at enrollment in the study?” Responses were scored on a 7-point Likert scale (1: extremely improved, 2: improved, 3: slightly improved, 4: not changed, 5: slightly aggravated, 6: aggravated, and 7: extremely aggravated).<sup>35</sup> Participants received a second upper GI endoscopy assessment at 1 year after enrollment. Those who had agreed to mucosal biopsy were included in the follow-up mRNA analysis. At the 1-year follow-up evaluation, participants who consistently answered “1” or “2” were defined as responders, while patients who answered “4”, “5”, “6”, or “7” were assigned to the non-responders group. Participants who answered “3” were assigned to a separate, unclear response group. The

schematic diagram for follow-up was summarized in Supplementary Digital Content 2, <http://links.lww.com/MD/A946>.

### Statistical Analysis

Categorical variables were analyzed by chi-square test and parametric variables were analyzed by Student *t*-test or 1-way analysis of variance (ANOVA) followed by Scheffe test. Spearman correlation test was used to evaluate potential correlations between nociceptor gene expression and dyspeptic symptoms. Changes in values over time were compared with paired *t*-test or Wilcoxon rank sum test. SPSS Statistics version 20.0 (IBM, Armonk, NY) was used. All statistical tests were 2-sided, and a value of *P* < 0.05 was considered to be statistically significant.

## RESULTS

### Identification of TRPV1 Expression in Gastric Cell Lines

To confirm the presence of *TRPV1* mRNA in gastric cells, quantitative reverse transcription polymerase chain reaction was performed (Supplementary Digital Content 1, <http://links.lww.com/MD/A946>). AGS and MKN45 cells showed 1.1-fold and 1.3-fold higher expression of *TRPV1* mRNA than HET-1A, respectively.<sup>11</sup> A 37.18-kDa band was detected using a TRPV1 monoclonal antibody, confirming the presence of TRPV1 receptors in these gastric epithelial cell lines (Supplementary Digital Content 1, <http://links.lww.com/MD/A946>).

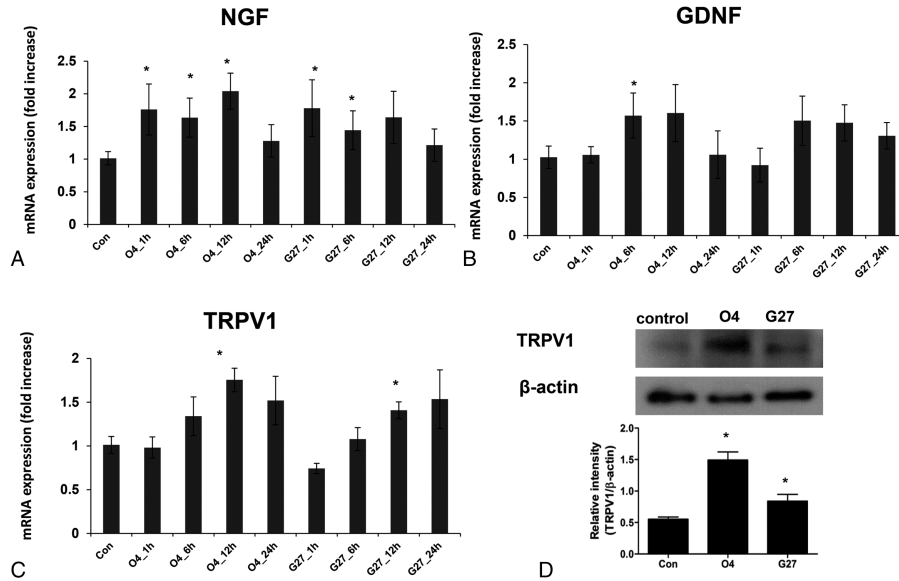
### Upregulation of NGF, GDNF, and TRPV1 in Gastric Cell Lines after *H pylori* Infection

We next examined whether HP infection upregulates the expression of *NGF*, *GDNF*, and *TRPV1* mRNAs. As shown in Figure 1, AGS cells were cultured with the 43504 (O4) and G27 strains of HP for 1, 6, 12, and 24 hours. *NGF* expression quickly increased and was maintained during the infections. For example, 12 hours after infection by HP O4 or G27, the AGS cells still showed almost the same level of *NGF* expression as they did at 1 and 6 hours. AGS cells incubated with HP G27 did not show a significant change in *NGF* expression at 12 hours (Figure 1A). *GDNF* expression started to increase at 6 and 12 hours, but it was significantly increased only at 6 hours after incubation with HP O4 (Figure 1B). *TRPV1* expression was significantly elevated 12 hours after infection with HP O4 or G27 (Figure 1C). Western blot analysis demonstrated a significant increase in the abundance of TRPV1 in AGS cells that were co-incubated with HP O4 or G27 (Figure 1D).

### General Characteristics of Subjects

Based on our *in vitro* results, we addressed the possibility that TRPV1 may be associated with FD in human. Baseline characteristics of 55 control subjects and 117 FD patients are shown in Table 1. When comparing the controls and FD patients, never-smokers were more common in controls (*P* = 0.016). Otherwise, age, body mass index, alcohol consumption, regular spicy food ingestion, current HP infection status, degree of neutrophil/monocyte infiltration, atrophic gastritis, and intestinal metaplasia were not significantly different between the controls and FD patients. When these variables were compared with controls and the 3 FD subtype groups, there were no significant differences in the aforementioned variables, except for smoking; that is, a higher proportion of patients with EPS smoked, compared with the control subjects.





**FIGURE 1.** Expression of *NGF*, *GDNF*, and *TRPV1* in AGS cell lines co-cultured with *H pylori* 43504 and G27 strains. (A–C) mRNA expression of (A) *NGF*, (B) *GDNF*, and (C) *TRPV1*. (D) Western blot data of *TRPV1* protein abundance after 24 hours of co-culture. Results are expressed as the mean ± standard error of 3 experiments. \**P* < 0.05, significantly different from the control non-HP-infected group at 1 hour after the infection. *GDNF* = glial cell-line derived neurotrophic factor, *NGF* = nerve growth factor, *TRPV1* = transient receptor potential vanilloid receptor 1.

### Comparison of NGF, GDNF, and TRPV1 Expression in the Functional Dyspepsia and Control Groups

Next, we evaluated the expression of *NGF*, *GDNF*, and *TRPV1* mRNAs as possible etiological factors for FD. When control subjects and the overall FD group were compared, the FD group showed significantly higher expression of *NGF*, *GDNF*, and *TRPV1*, compared with that of the control group (Table 2). After subgroup analyses, the PDS, EPS, and mixed groups showed a higher expression of *NGF* (Figure 2A), *GDNF*

(Figure 2B), and *TRPV1* (Figure 2C) mRNAs than that of the control group. However, the expression of *NGF* mRNA in the PDS group did not reach statistical significance (Table 2 and Figure 2A).

We observed *TRPV1* immunoreactivity in the base or neck region of gastric mucosa (Figure 3). Immunoreactivity of *TRPV1* in the gastric mucosa was compared between patients with FD and control subjects. The mucosa from FD patients showed significantly stronger staining than that of control subjects (Table 2). The specimens from control subjects showed

**TABLE 1.** Baseline Characteristics in 55 Healthy Controls and 117 FD Patients

Variables	Control (n = 55)	FD (n = 117)	PDS (n = 46)	EPS (n = 33)	Mixed (n = 38)	<i>P</i> FD*	<i>P</i> PDS*	<i>P</i> EPS*	<i>P</i> Mixed*
Mean age, year (SD)	52.3 (10.7)	52.4 (12.1)	51.9 (12.9)	53.5 (10.9)	52.0 (12.1)	0.946	0.999	0.972	0.999
Men, n (%)	23 (41.8)	39 (33.3)	16 (34.8)	11 (33.3)	12 (31.6)	0.280	0.472	0.429	0.316
Mean BMI, kg/m <sup>2</sup> (SD)	23.5 (2.8)	22.8 (2.9)	23.2 (2.9)	23.1 (2.9)	22.1 (2.9)	0.160	0.973	0.933	0.174
Smoker (current/ex), n (%)	5 (9.1)	29 (21.4)	10 (21.7)	11 (33.3)	8 (21.1)	<b>0.016</b>	0.194	<b>0.004</b>	0.102
Alcohol, n (%)	17 (30.9)	25 (21.4)	9 (19.6)	5 (15.2)	11 (28.9)	0.174	0.075	0.098	0.839
Spicy food, n (%)	30 (75.0)	63 (59.3)	29 (69.0)	17 (77.3)	17 (63.0)	0.503	0.549	0.842	0.291
Current HP (+), n (%)	30 (54.5)	49 (41.9)	17 (37.0)	15 (45.5)	17 (44.7)	0.120	0.078	0.409	0.352
Neutrophil infiltration <sup>†</sup>	1.0 (0.1)	0.7 (0.1)	0.6 (0.1)	0.8 (0.2)	0.7 (0.1)	0.077	0.270	0.890	0.676
Monocyte infiltration <sup>†</sup>	1.6 (0.1)	1.4 (0.1)	1.4 (0.1)	1.5 (0.1)	1.4 (0.1)	0.062	0.395	0.861	0.350
Glandular atrophy <sup>†</sup>	0.4 (0.1)	0.3 (0.1)	0.3 (0.1)	0.4 (0.1)	0.3 (0.1)	0.539	0.890	0.998	0.986
Intestinal metaplasia <sup>†</sup>	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.728	0.999	0.986	0.974

Continuous variables comparisons conducted with ANOVA, otherwise by Pearson chi-square test.

BMI = body mass index, EPS = epigastric pain syndrome, FD = functional dyspepsia, HP = *H pylori*, PDS = postprandial distress syndrome, SD = standard deviation, s.e. = standard error. Bold style indicates the statistical significance.

\*Compared with control.

<sup>†</sup>By Sydney score, presented as mean (s.e.).



**TABLE 2.** Relative mRNA Expression of NGF, GDNF, and TRPV1 and TRPV1 Immunohistochemistry

Group	NGF		GDNF		TRPV1		TRPV1 IHC	
	Mean (SE)	<i>P</i> *	Mean (SE)	<i>P</i> *	Mean (SE)	<i>P</i> *	Mean (SE)	<i>P</i> *
Control (n = 55)	1.1 (0.1)		1.2 (0.1)		1.1 (0.1)		47.4 (7.3)	
FD (n = 117)	2.0 (0.2)	<b>&lt;0.001</b>	2.1 (0.1)	<b>&lt;0.001</b>	1.6 (0.1)	<b>&lt;0.001</b>	88.1 (7.1)	<b>0.011</b>
PDS (n = 46)	2.1 (0.4)	0.117	2.0 (0.2)	<b>0.009</b>	1.5 ± 0.2	<b>0.037</b>	128.5 (8.7)	<b>&lt;0.001</b>
EPS (n = 33)	1.8 (0.2)	<b>0.017</b>	2.0 (0.2)	<b>0.001</b>	1.6 (0.1)	<b>0.017</b>	123.9 (10.4)	<b>&lt;0.001</b>
Mixed (n = 38)	2.0 (0.3)	<b>0.025</b>	2.2 (0.3)	<b>0.009</b>	1.8 (0.2)	<b>0.014</b>	14.3 (9.2)	<b>&lt;0.001</b>
HP infection								
Current HP (-) (n = 93)	1.6 (0.2)	0.335	1.9 (0.2)	0.378	1.5 (0.1)	0.648	67.7 (7.4)	<b>0.022</b>
Current HP (+) (n = 79)	1.8 (0.2)		1.7 (0.2)		1.4 (0.1)		94.4 (9.2)	
HP-negative								
HP (-) control (n = 25)	1.1 (0.1)	<b>0.006</b>	1.1 (0.1)	<b>&lt;0.001</b>	1.0 (0.1)	<b>0.003</b>	54.7 (9.3)	0.241
HP (-) FD (n = 66)	1.8 (0.2)		2.2 (0.2)		1.7 (0.1)		69.9 (8.8)	
HP-positive								
HP (+) control (n = 30)	1.2 (0.1)	<b>0.003</b>	1.2 (0.1)	<b>0.008</b>	1.1 (0.1)	<b>0.004</b>	38.7 (10.7)	<b>&lt;0.001</b>
HP (+) FD (n = 51)	2.2 (0.3)		1.9 (0.2)		1.6 (0.2)		122.2 (10.1)	

EPS = epigastric pain syndrome, FD = functional dyspepsia, GDNF = glial cell-line derived neurotrophic factor, HP = *H. pylori*, IHC = immunohistochemistry, NGF = nerve growth factor, PDS = postprandial distress syndrome, SE = standard error, TRPV1 = transient receptor potential vanilloid receptor 1.

Bold style indicates the statistical significance.

\*Comparisons made by ANOVA following Scheffe test.

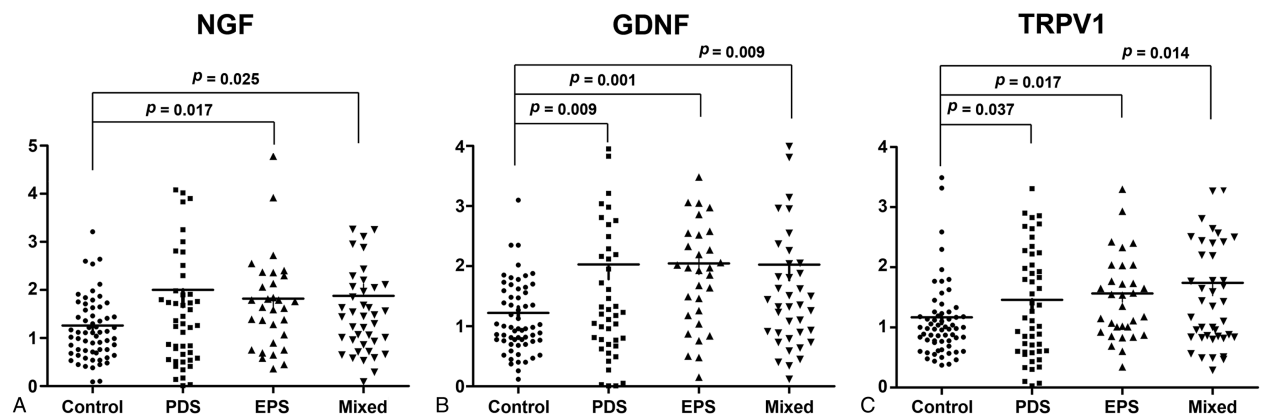
weaker TRPV1 immunoreactivity compared with that of specimens from the PDS, EPS, and mixed groups (Table 2).

**Association Between Expression of Nociceptor-Related Genes and Dyspeptic Symptoms**

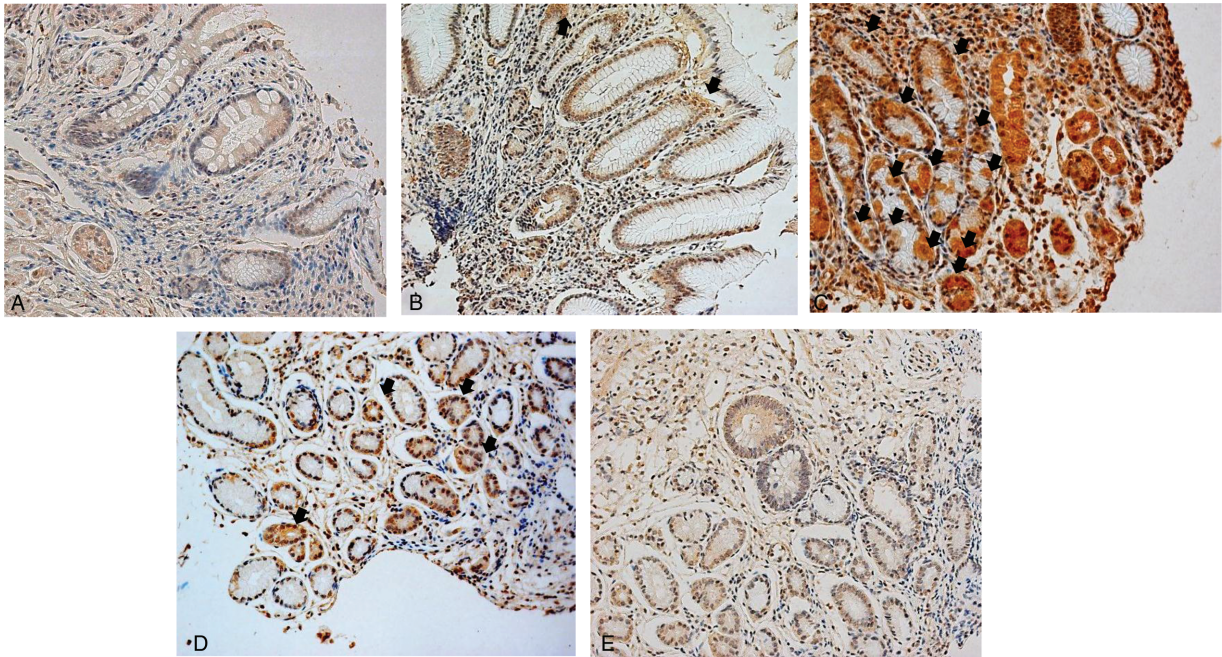
Spearman rank correlation tests were performed to evaluate whether the expression of nociceptor-related genes was associated with specific dyspeptic symptoms including early satiety, postprandial fullness, or epigastric pain/burning. For the epigastric pain/burning and postprandial fullness scores, the expression of the 3 nociceptor-related genes showed weak but significant correlations with the symptom score (epigastric

pain/burning: *NGF*:  $r = 0.291$ ; *GDNF*:  $r = 0.316$ ; and *TRPV1*:  $r = 0.288$ , all  $P < 0.001$ ) (postprandial fullness scores: *NGF*:  $r = 0.156$ ,  $P = 0.042$ ; *GDNF*:  $r = 0.185$ ,  $P = 0.016$ ; and *TRPV1*:  $r = 0.160$ ,  $P = 0.037$ ). However, the early satiety score was not significantly associated with the expression of any of the 3 genes (*NGF*:  $r = 0.115$ ,  $P = 0.136$ ; *GDNF*:  $r = 0.111$ ,  $P = 0.149$ ; and *TRPV1*:  $r = 0.104$ ,  $P = 0.176$ ).

Expression of *TRPV1* was strongly correlated with that of *NGF* and *GDNF* ( $r = 0.769$ ,  $P < 0.001$  and  $r = 0.768$ ,  $P < 0.001$ , respectively). Furthermore, the expression levels of *NGF* and *GDNF* were also positively correlated with each other ( $r = 0.664$ ,  $P < 0.001$ ).



**FIGURE 2.** Comparison of gastric *NGF*, *GDNF*, and *TRPV1* expression in control and FD subgroups. (A) *NGF* expression in the EPS and mixed groups was higher than that in control ( $P = 0.017$ ,  $P = 0.025$ ). (B) *GDNF* expression in the PDS, EPS, and mixed groups was higher than in control ( $P = 0.009$ ,  $P = 0.001$ , and  $P = 0.009$ , respectively). (C) *TRPV1* expression was higher in the PDS, EPS, and mixed groups than in the control group ( $P = 0.037$ ,  $P = 0.017$ , and  $P = 0.014$ , respectively). Comparisons were made by analysis of variance followed by Scheffe test. EPS = epigastric pain syndrome, FD = functional dyspepsia, GDNF = glial cell-line derived neurotrophic factor, NGF = nerve growth factor, PDS = postprandial distress syndrome, TRPV1 = transient receptor potential vanilloid receptor 1.



**FIGURE 3.** Representative images of TRPV1 immunostaining of human gastric mucosa. TRPV1 staining of (A) *H pylori*-negative healthy control, (B) *H pylori*-positive healthy control, (C) *H pylori*-positive epigastric pain syndrome patient, and (D) *H pylori*-positive postprandial distress syndrome patient. (E) The same patient shown in (D), 1-year after *H pylori* eradication. Cytoplasm and cell junctions were stained (arrow) ( $\times 20$  magnification). TRPV1 = transient receptor potential vanilloid receptor 1.

### Expression of NGF, GDNF, and TRPV1 in Relation to *H pylori* Infection Status in Human Gastric Mucosa

In order to evaluate the possible effects of HP infection on the human gastric expression of *NGF*, *GDNF*, and *TRPV1*, we analyzed the expression of these genes in gastric mucosa according to HP infection status. There were no significant differences in nociceptor-related gene expression between HP-positive and HP-negative subjects (Table 2). Higher expression of *NGF*, *GDNF*, and *TRPV1* was observed in FD compared with controls in both the HP-negative and HP-positive groups (Table 2). In terms of TRPV1 immunohistochemistry, HP-positive subjects showed stronger immunostaining intensity than HP-negative subjects did (Table 2) (Figure 3A and B). While there was no significant difference in TRPV1 immunostaining between FD patients and control subjects in the HP-negative group, the FD group showed a significantly increased immunostaining intensity compared with that of control subjects in the HP-positive group (Table 2) (Figure 3B and C).

### Changes in NGF, GDNF, and TRPV1 Expression According to the Alteration of Dyspeptic Symptoms at the 1-Year Follow-Up Evaluation

We performed re-biopsy and repeated symptom assessments 1 year after the first assessment. The expression of *NGF*, *GDNF*, and *TRPV1* was evaluated in 44 FD patients and 17 healthy control subjects (Table 3). The mean duration of follow-up was 12.1 months. The FD patients consisted of 5 PDS, 16 EPS, and 23 mixed patients. Of the 44 followed-up FD patients, only 23 patients answered that their symptoms were either

“extremely improved” or “improved” upon assessment. The 3 symptom response groups (control, symptom improved, and non-improved groups) were compared (Table 3). There were no differences in age, sex, distribution of FD subtypes, current HP positivity, or follow-up duration. Baseline epigastric burning/pain score was higher in the symptom improved group than in the other groups ( $P = 0.018$ ), but postprandial fullness and early satiation scores were not significantly different among groups (Table 3). Among the 11, 16, and 9 HP-positive subjects in each symptom response group, HP eradication was achieved in 10, 14, and 9 patients, respectively. Among the 23 HP-eradicated FD patients, 14 (60.9%) reported that their symptoms had improved. Ten (71.4%) of these patients received only anti-HP therapy, while all of the patients in the non-improved group maintained empirical treatment after anti-HP therapy ( $P < 0.001$ ).

Approximately 1 year after enrollment, changes in 3 representative dyspeptic symptoms were analyzed (Table 3). While the patients who reported symptomatic improvements showed a significant reduction in all 3 symptoms, the group without symptomatic improvement displayed an increase in epigastric burning/pain symptoms (Table 3). HP was eradicated in 10 of 11 controls. None of the healthy controls presented dyspeptic symptoms during the follow-up period.

The expression of *NGF* and *TRPV1* significantly decreased in the control and symptom-improved groups, while their expression in the non-improved FD group did not change significantly (Table 3). *GDNF* expression did not change significantly during the follow-up period in any of the groups.

At the 1-year follow-up evaluation, the symptom-improved group showed a reduced intensity of TRPV1

**TABLE 3.** Dyspeptic Symptoms and Expression of Nociceptor-Related Genes at the Baseline and 1-Year Follow-Up

Variables	Control (n = 17)			Symptom Improved (n = 23)			Not-Improved (n = 21)				
	Before	After	P*	Before	After	P*	Before	After	P*	P†	
Mean age, year (SD)	52.1 (10.3)			56.6 (11.8)			54.1 (10.4)				0.401
Men, n (%)	10 (58.8)			9 (39.1)			10 (47.6)				0.468
PDS:EPS:Mixed, n	(–)			2:11:10			3:5:13				0.252
Current HP (+), n (%)	11 (64.7)	1		16 (69.6)	2		9 (42.9)	0		0.169	
Anti-HP therapy, n (successful eradication)‡	11 (10)			15 (14)			9 (9)				0.251
Mean symptom score (SE)											
Fullness	0.0 (0.0)	0.1 (0.1)	0.332	1.8 (0.3)	0.3 (0.1)	<0.001	1.7 (0.2)	2.0 (0.3)	0.166	0.966	
Early satiation	0.0 (0.1)	0.0 (0.0)	0.999	1.3 (0.3)	0.1 (0.1)	0.002	1.0 (0.2)	1.1 (0.2)	0.257	0.532	
Epigastric burning/pain	0.1 (0.1)	0.1 (0.1)	0.999	2.9 (0.2)	0.6 (0.2)	<0.001	2.0 (0.2)	2.6 (0.3)	0.008	0.001	
Gene expression (SE)											
NGF mRNA	1.1 (0.1)	0.9 (0.1)	0.030	1.9 (0.4)	1.0 (0.1)	<0.001	1.4 (0.2)	1.6 (0.3)	0.217	0.054	
GDNF mRNA	3.3 (0.5)	3.3 (0.5)	0.878	2.8 (0.7)	2.1 (0.3)	0.207	2.7 (0.5)	2.8 (0.5)	0.828	0.580	
TRPV1 mRNA	1.1 (0.1)	0.9 (0.1)	0.068	1.6 (0.3)	0.6 (0.1)	<0.001	1.1 (0.1)	1.4 ± 0.2	0.689	0.135	
TRPV1IHC	30.4 (13.5)	29.2 (12.1)	0.498	101.5 (13.7)	60.8 (12.2)	0.028	114.8 (28.6)	75.5 (18.5)	0.507	0.001	

Mean duration of follow-up was 12.1 months.

EPS = epigastric pain syndrome, F/U = follow-up, GDNF = glial cell-line derived neurotrophic factor, HP = *H pylori*, IHC = immunohistochemistry, IM = intestinal metaplasia, NGF = nerve growth factor, PDS = postprandial distress syndrome, SD = standard deviation, SE = standard error, TRPV1 = transient receptor potential vanilloid receptor 1.

Bold style indicates the statistical significance.

\*Wilcoxon-rank sum test was used.

†Among the initial values of the control, symptom-improved and not improved groups.

‡Eleven, 16 and 9 subjects in each group were *H pylori*-positive at the base line, and 11, 15, 9 received anti-*H pylori* therapy. Among them, 10, 14, and 9 achieved *H pylori* eradication at the 1-year follow-up.

immunostaining compared with that at baseline, while the healthy controls and non-improved group did not present significant changes (Table 3).

### Expression of NGF, GDNF, and TRPV1 after HP Eradication at the 1-Year Follow-Up Evaluation

To evaluate the effects of HP eradication on the expression of nociceptor-related genes and the reported severity of symptoms, the relative expression levels of *NGF*, *GDNF*, and *TRPV1* were compared based on changes in HP status and FD symptoms (Table 4). There was a significant improvement of gastritis in all of the HP-eradicated groups.

The HP-eradicated healthy control group showed a reduction in the expression of *NGF* and *TRPV1*, but the change in the expression of the former gene did not reach statistical significance. However, the HP-negative control group did not present any changes in the expression of the 3 nociceptor-related genes.

In the symptom-improved groups, the expression of *NGF*, *GDNF*, and *TRPV1* was significantly decreased in both the HP-eradicated and HP-negative groups, although the change in *GDNF* expression in symptom-improved patients who underwent HP eradication did not reach statistical significance. Unlike the symptom-improved group, there was no significant change in the expression of the 3 nociceptor-related genes or the gastritis score.

The intensity of TRPV1 immunostaining was compared before and after HP eradication. The HP-eradicated group showed a decreased immunostaining score, compared with that at baseline (Figure 3D and E).

### Responses to Treatment by the Patients With Functional Dyspepsia

The responses of the FD patients to different treatments are illustrated in Figure 4. Among the 49 HP-positive FD patients, 37 received anti-HP therapy. At the 1-year follow-up evaluation, HP eradication had been achieved for 27 patients, but 8 patients failed to achieve HP eradication due to low compliance, adverse effects, or resistance. The remaining 2 patients were lost during follow-up. As a result, 18 (66.7%) of 27 patients with successful HP eradication showed improved dyspeptic symptoms at a higher rate compared with the patients who failed or refused HP eradication (18/27 vs. 2/19,  $P < 0.001$ ). The median duration for the improvement of symptoms after the completion of anti-HP therapy was 6.3 months (1.4–14.2 months). Among the 18 symptom-improved patients in whom HP was eradicated, 13 (72.2%) maintained their improved status without any supportive drugs for a median of 15.1 months (4.5–38.1 months). The other 5 symptom-improved patients maintained the improvements for around 6 to 9 months after the eradication, but they needed supplementary prokinetics and PPI thereafter and answered “extremely improved or improved since enrollment” at the 1-year follow-up evaluation. On the other hand, the 68 HP-negative FD patients received supportive care with PPI, H<sub>2</sub>-antagonists, or prokinetics. Excluding 13 patients who could not be followed up, 25 (45.5%) of 55 patients reported an improvement of their symptoms at a median of 12.1 months after enrollment.

### DISCUSSION

Our study showed that the expression of *NGF*, *GDNF*, and *TRPV1* in FD patients was higher than in the control group. The



**TABLE 4.** Change of Nociceptor-Related Gene Expressions in Different Symptom Groups According to the Existence of Successful *H pylori* Eradication

Variables	HP-Eradicated Group						HP-Negative Group											
	Control (n = 10)		Symptom Improved (n = 14)		Non-Improved (n = 9)		Control (n = 6)		Symptom Improved (n = 7)		Non-Improved (n = 12)							
	Before	After	P*	Before	After	P*	Before	After	P*	Before	After	P*						
mRNA expression, mean (SE)																		
NGF	1.0 (0.1)	0.7 (0.2)	0.139	1.5 (0.7)	0.9 (0.6)	<b>0.013</b>	1.7 (0.4)	1.9 (0.5)	0.859	1.3 (0.2)	1.2 (0.2)	0.310	2.6 (1.0)	0.8 (0.1)	<b>0.015</b>	1.2 (0.3)	1.3 (0.4)	0.695
GDNF	3.5 (0.7)	3.4 (0.6)	0.878	1.6 (0.4)	1.3 (0.4)	0.372	2.9 (0.9)	2.8 (0.7)	0.441	3.0 (0.6)	3.1 (0.8)	0.499	4.6 (1.5)	2.2 (0.6)	<b>0.021</b>	2.5 (0.6)	2.7 (0.7)	0.433
TRPV1	1.0 (0.1)	0.7 (0.0)	<b>0.037</b>	1.4 (0.2)	0.7 (0.1)	<b>0.016</b>	1.3 (0.2)	1.6 (0.3)	0.374	1.3 (0.2)	1.2 (0.2)	0.463	2.1 (0.6)	0.7 (0.1)	<b>0.008</b>	1.1 (0.1)	1.2 (0.3)	0.433
Antral/corporal histology (SE) <sup>†</sup>																		
Neutrophil	2.0 (0.3)	0.2 (0.1)	<b>0.007</b>	2.1 (0.2)	0.4 (0.2)	<b>0.003</b>	0.9 (0.3)	0.0	<b>0.046</b>	0.4 (0.3)	0.4 (0.3)	0.999	0.7 (0.4)	0.2 (0.2)	0.102	0.3 (0.1)	0.1 (0.1)	0.157
Monocyte	2.0 (0.1)	1.0 (0.3)	<b>0.015</b>	1.9 (0.2)	1.2 (0.2)	<b>0.013</b>	1.8 (0.2)	1.2 (0.1)	<b>0.059</b>	1.3 (0.2)	1.4 (0.2)	0.317	1.3 (0.2)	1.3 (0.2)	0.999	1.0 (0.0)	1.0 (0.0)	0.999
Atrophy	0.5 (0.3)	0.0	0.157	0.4 (0.2)	0.1 (0.1)	0.157	0.3 (0.3)	0.0	0.317	0.0	0.2 (0.2)	0.317	0.1 (0.1)	0.3 (0.2)	0.317	0.0	0.0	0.999
IM	0.5 (0.3)	0.9 (0.3)	0.157	0.5 (0.2)	0.4 (0.2)	0.783	0.9 (0.5)	0.6 (0.3)	0.461	0.0	0.1 (0.1)	0.137	0.1 (0.1)	0.3 (0.3)	0.317	0.0	0.2 (0.2)	0.317

GDNF = glial cell-line derived neurotrophic factor, HP = *H pylori*, IM = intestinal metaplasia, NGF = nerve growth factor, SE = standard error, TRPV1 = transient receptor potential vanilloid receptor 1. Bold style indicates the statistical significance.

\*By Wilcoxon rank sum test.

<sup>†</sup>By Sydney score.

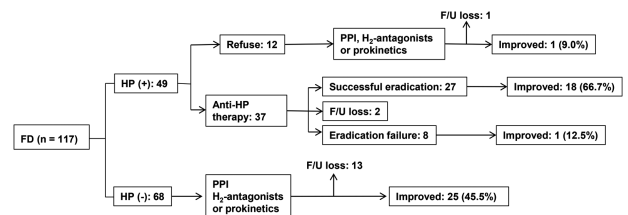
severities of epigastric pain/burning and postprandial fullness significantly correlated with the expression of these 3 nociceptor-related genes. These results imply that *TRPV1* and neurotrophic factors play an important role in the pathogenesis of FD, epigastric pain/burning, and fullness after meals. Moreover, since the in vitro data showed that infection with HP induced *NGF*, *GDNF*, and *TRPV1* in human gastric cell lines, the present study provides a clue as to the possible mechanism of HP-related dyspepsia.

Similar to the results reported here, previous studies have also indicated that *TRPV1* was upregulated in other functional GI diseases including IBS,<sup>36</sup> NERD, and erosive reflux disease.<sup>24,37</sup>

We also demonstrated that the gastric epithelium contains *TRPV1* receptors, as has previously been shown in gastric cell lines and human gastric tissue. This confirms that *TRPV1* receptors are present not only in the neuronal structures of the submucosa,<sup>23,27</sup> but also in the epithelial cells, in agreement with previous data.<sup>11,13,15,16</sup> *TRPV1* immunoreactivity was previously demonstrated in parietal cells<sup>15</sup> and gastrin cells<sup>16</sup> of the human gastric antrum and body, and in small nerves in the mucosa.

The precise role of HP infection in FD is still under debate,<sup>38,39</sup> although there are several studies supporting the therapeutic effect of HP eradication on FD.<sup>40,41</sup> Patients with active inflammatory bowel disease<sup>42</sup> or erosive reflux disease<sup>34</sup> showed enhanced *TRPV1* expression in the inflamed tissues, suggesting that inflammation can trigger overexpression of *TRPV1*. Moreover, since inflammation induces *NGF* expression and *NGF* protein directly activates the *TRPV1* receptor,<sup>43</sup> HP, which is a leading cause of gastric inflammation, could be involved in promoting FD development via *TRPV1* upregulation. In vitro experiments with AGS cells in the present study demonstrated that HP induced the overexpression of *NGF*, *GDNF*, and *TRPV1*, supporting the abovementioned hypothesis. However, the association between HP and FD in human is not straightforward. There were no significant differences in gastric *TRPV1*, *GDNF*, or *NGF* expression among the FD groups according to their HP infection status, although the *TRPV1* immunostaining score was increased in the HP-positive subjects, compared with the HP-negative group. The proportion of HP-positive cases did not differ between the control and FD groups. Together, these data indicate that multiple factors are involved in FD development.

Nonetheless, at the 1-year follow-up evaluation, not only symptom-improved patients but also healthy controls showed reductions in *NGF* and *TRPV1* expression, suggesting that HP eradication could resolve some of the inflammation-induced changes in nociceptor-related gene expression. Although it has not so far been possible to predict which FD patients will show



**FIGURE 4.** Summary of the responses to different treatments by 117 patients with functional dyspepsia based on the global patient assessment score. FD=functional dyspepsia, F/U=follow-up, HP = *H pylori*, PPI = proton-pump inhibitor.

reductions in their dyspeptic symptoms after anti-HP therapy, the findings of this study support the usefulness of anti-HP therapy for some subsets of HP-positive dyspepsia patients.<sup>44</sup>

Although IBS could be associated with inflammation-induced *TRPV1* upregulation,<sup>36</sup> inflammation does not explain all instances of *TRPV1* overexpression or the generation of functional GI disorder symptoms. When comparing these genes even in the HP-negative subjects, *TRPV1* and neurotrophic factors were upregulated in the FD group compared with the control group (Table 2). In contrast, at the 1-year follow-up evaluation of the empirically treated category, which was HP-negative, the 3 nociceptor-related genes were reduced in the symptom-improved patients, but not in the non-improved patients. This implies that *TRPV1* expression can be closely associated with dyspeptic symptoms, even when HP-associated inflammation is absent. In line with our results, some previous studies have reported *TRPV1* overexpression in GI diseases without overt inflammation, such as in NERD<sup>24</sup> and idiopathic rectal or fecal urgency.<sup>23</sup> The cause of the increased *TRPV1* expression in HP-negative FD patients might be attributed to central and peripheral factors leading to increased NGF levels. Interestingly, NGF levels have been shown to alter with psychosocial influences<sup>45</sup> and to increase with stress.<sup>46</sup> Furthermore, chronic stress induces hypersensitivity in the absence of inflammation,<sup>47</sup> while acid secretion may also drive *TRPV1* overexpression.<sup>48</sup> Consequently, HP infection could play a role in the overexpression of inflammation-induced neurotrophic factors and *TRPV1*, but cannot be considered an exclusive factor of FD.

Unlike *NGF*, details about the role of *GDNF* in the GI system are very limited, although some studies have suggested its importance in GI sensory transduction together with *TRPV1*.<sup>34</sup> The present study showed that the expression of *GDNF*, along with that of *NGF* and *TRPV1*, was higher in FD patients than in control subjects. However, its reduction was not as prominent as that of *NGF* or *TRPV1* in subjects whose dyspepsia symptoms were improved or in HP-eradicated subjects. There were no significant changes in *GDNF* expression at the 1-year follow-up evaluation across all symptom groups in HP-eradicated subjects, in comparison with baseline levels. This result is not consistent with the assumption that the expression pattern of neurotrophic factors would be similar to that of *TRPV1*. The reason for this discordance is unclear, but *NGF* expression appeared to be more related to the early phase of inflammation, while *GDNF* showed a delayed response compared with *NGF*.<sup>49</sup> In the present study, there were differences among the 3 nociceptor-related genes in the timing of their elevated expression. *NGF* expression was elevated first, followed by that of *GDNF* and *TRPV1*. The elevation of *GDNF* mRNA was only sustained for a relatively short time. Further study of these 2 neurotrophic factors is needed.

There are several limitations to the present study. Since this is not a clinical trial, but rather an observational study, a few patients for whom HP eradication was achieved took protonic inhibitors. This makes it difficult to isolate the effect of HP eradication on the dyspeptic symptoms. The sample size for the re-biopsy of patients was not large, since a majority of the patients rejected it. Whether increased *TRPV1* expression is the cause of dyspeptic symptoms and the significance of the *TRPV1*-stained mucosa remain to be clarified.

Nonetheless, the present study is the first to have demonstrated upregulation of *TRPV1* and the expression of NGF genes in FD. The association of these molecular changes with

FD suggests that their further study is warranted, since they may represent etiological factors. The present study also provided *in vitro* HP infection data and human results after HP eradication. These findings suggested that HP-induced inflammation and the accompanying elevation of *TRPV1* mRNA and protein may be involved in the pathogenesis of HP-related dyspepsia, and indicated that anti-HP therapy could be useful for this disease category.

## ACKNOWLEDGMENTS

The authors are indebted to J. Patrick Barron, Professor Emeritus, Tokyo Medical University and Adjunct Professor, Seoul National University Bundang Hospital for his pro bono editing of this manuscript. In addition, the authors thank the Division of Statistics in Medical Research Collaborating Center at Seoul National University Bundang Hospital for statistical analyses.

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